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β-Lactamase production and antimicrobial susceptibility of subgingival bacteria from refractory periodontitis

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This study assessed the extent of β-lactamase-producing bacteria in subgingival plaque samples obtained from 25 patients with refractory marginal periodontitis in the USA. β-Lactamase-positive isolates were characterized using commercial diagnostic kits and partial sequencing of the 16S rRNA gene. The susceptibilities to different antimicrobial agents were tested and, in addition, the isolates were screened for the presence of extended spectrum β-lactamases (ESBLs). β-lactamase-producing bacteria were detected in 18 (72%) patients. The most prominent β-lactamase-producing organisms belonged to the anaerobic genus *Prevotella*. Other enzyme-producing anaerobic strains were *Fusobacterium nucleatum, Propionibacterium acnes* and *Peptostreptococcus* sp. Facultative bacteria, such as *Burkholderia* spp., *Ralstonia pickettii*, *Capnocytophaga* spp., *Bacillus* spp., *Staphylococcus* spp. and *Neisseria* sp., were also detected among the enzyme-producers. Minimum inhibitory concentrations (MICs) of ampicillin and amoxicillin were in the range 1.5–256 μg/ml and 4–256 μg/ml, respectively, for the isolates of the *Prevotella* species. All *Prevotella* isolates were susceptible to amoxicillin/clavulanate and metronidazole, but they showed variable resistance to tetracyclines. Two of the *Prevotella* isolates had high MICs of cefotaxime and ceftazidime. ESBL activity was not detected in any of the β-lactamase-producing isolates by the Etest method. Thus, our study demonstrated a wide variety of β-lactamase-producing bacteria that may play a role in refractory periodontal disease.

Key words: antimicrobial resistance; β-lactamase; refractory periodontitis

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Systemic antimicrobial therapy is often used to eradicate periodontal infections. The most commonly prescribed antimicrobials in the USA are the β-lactam antibiotics (5). One important mechanism of bacterial resistance towards penicillin is the production of β-lactamases. Several investigators have reported β-lactamase production in periodontal isolates of *Prevotella* spp. (3, 6–8, 13, 15, 18, 29, 30). Significant resistance has also been detected in other periodontal pathogens, such as *Fusobacterium* spp., *Capnocytophaga* spp., and in oral superinfecting Enterobacteriaceae and staphylococci (9, 10, 13, 16, 20, 21, 25). Since periodontal infections are polymicrobial, the emergence of β-lactamase-producing bacteria may protect nonproducing bacteria of subgingival plaques against the β-lactam antibiotics, leading to therapeutic failure or disease recurrence. Linkage of several classes of antibiotic resistance on conjugative elements and gene transfer between different species have been demonstrated in oral *Prevotella* (12, 31). Thus, the *Prevotella* group may function as an antibiotic resistance gene reservoir and influence the success rate of antibiotic therapy in the oral cavity and elsewhere in the body. The purpose of this study was to assess the prevalence, variety and susceptibility patterns of β-lactamase-producing bacteria in the subgingival microflora from patients with refractory marginal periodontitis in the USA. The results were compared to those obtained from a similar study performed in Norway (13).

Material and methods

Description of the study material

Bacterial samples from 25 patients, 25–76 years of age, with refractory periodontitis were examined in this study. None of the patients responded to the conventional treatment consisting of scaling and root planing, with or without periodontal surgery, followed by a documented maintenance periodontal program. Four patients (Nos. 7, 8, 11, and 12) (Table 1) had received amoxicillin as an adjunct to conventional treatment within the 3 months prior to sampling. The other patients had not received any antibiotics in the past 3 months. Following informed consent, subgingival bacterial samples were collected from periodontal sites exhibiting active destruction; probing depth >5 mm, radiographic evidence of bone loss, and bleeding on probing. After first removing any visible supragingival plaque, subgingival plaque samples were collected with sterile endodontic paper points (#504, Henry Schein, Melville, NY). Up to two sterile paper points were inserted apically into the periodontal pocket until resistance was encountered, moved gently across the horizontal axis of the tooth, left in place for 10 s, removed and placed into a 1.0 ml aliquot of sterile Amies transport medium (2) supplemented with 1% gelatin in a 2 ml snap-top tube. This transport system is adequate for the transport and recovery of fastidious oral anaerobic bacteria, including *Tannerella forsythia* and *Porphyromonas gingivalis*, in similar samples received from various centers within the USA. Once collected, the samples were refrigerated until shipped by overnight courier, either on the same day or the following day to the microbiology laboratory in Oslo, Norway, where they were plated within 3–5 days after collection.

Sample processing and culture

In the laboratory, the sealed tubes with the microbiological samples were agitated for 10 s on a whirly mixer (Labinco, Breda, the Netherlands). Three serial 10-fold dilutions of the transport fluid medium were made in one-quarter strength prereduced anaerobically sterilized (PRAS) Ringer's solution supplemented with 0.05% L-cysteine free base (Sigma, St. Louis, MO). A VPI Anaerobic Culture System (Bellco, Vineland, NJ) was used to flush the tubes continuously with an anaerobic gas mixture (90% N_2, 5% H_2, 5% CO_2) during seeding. Each dilution, while kept in the Anaerobic Culture System, was pipetted out in volumes of 0.1 ml onto nonselective trypticase soy agar plates supplemented with 5% defibrinated human blood, hemin (5 mg/ml) and menadione (0.05 mg/ml). In addition, selective agar plates (Wolinella medium, CVE medium, clindamycin blood agar, TSBV agar, mitis salivarius agar, mannitol salt agar, MacConkey agar and trypticase soy agar plates supplemented with 5% defibrinated human blood, hemin [5 mg/ml] and N-acetyl muramic acid [10 mg/ml]) were inoculated with 0.1 ml of undiluted sample for recovery of periodontal pathogens. For isolation of β-lactamase-producing organisms relatively resistant to amoxicillin, blood agar plates were supplemented with 3 μg/ml amoxicillin. Nonselective and selective agar plates were incubated anaerobically (90% N_2, 5% H_2, 5% CO_2) at 37°C for up to 14 days in anaerobic jars (Anoxomat, WS9000, Mart, the Netherlands). All morphotypes of bacteria on plates were subcultured and then tested for β-lactamase production.

Testing of β-lactamase activity

β-Lactamase production was assessed using chromogenic nitrocefin-impregnated disks (BBL™ DrySlide™ Nitrocefin, Becton Dickinson, Sparks, MA) (30). β-Lactamase-positive and -negative strains of *Staphylococcus aureus*, provided by the Microbiology Laboratory at the National Hospital, Oslo, were always included as controls. β-Lactamase-positive bacteria were preserved in 1.5 ml Todd-Hewitt solution at −70°C and further characterized.

Identification of β-lactamase-producing isolates

Preliminary identification of pure cultures was based on aerotolerance, colony and cell morphology, colony pigmentation and gram-staining of cells. Enzymatic/biochemical profiling relied on commercial diagnostic kits designed for identification of a number of different microorganisms (API, bioMérieux, Marcy-l'Etoile, France). The preparation and incubation of the kits were carried out according to the manufacturer's recommendations. Reading of the kits occurred automatically in an ATB reader (API, bioMérieux). The results of the reactions, transferred into a numerical code, were treated in a database system for identification (API Plus, bioMérieux).

To confirm species identification, partial sequencing of the 16S rRNA gene was performed. A loopful of colonies was suspended in an Eppendorf tube with 100 μl TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8.0). The suspension was boiled for 10 min, cooled on ice, and centrifuged at 13,000 *g* for 5 min at 4°C. The supernatant was used as a template. DNA (1 μl of supernatant) was amplified in a reaction mixture consisting of 5 μl of 10 × polymerase chain reaction (PCR) buffer, 4 μl of 10 μM total deoxynucleotide triphosphates, 1 μl of 10 μM primer PA and PD (MWG-Biotech, GmbH, Ebersberg, Germany) (28), 37.75 μl of distilled H_2O, and 0.25 μl of Amplitaq DNA polymerase. PCR conditions included 30 cycles of denaturation at 95°C for 40 s, annealing at 58°C for 1 min, and extension at 72°C for 40 s. DNA amplicons (7 μl) were purified in a mixture containing 1 μl exonuclease and 1 μl shrimp alkaline phosphatase, and incubated for 15 min at 37°C and 15 min at 80°C, as recommended by the manufacturer (Amersham Biosciences, Cleveland, OH). The purified product was sequenced on both strands using the primers PB and PC (MWG-Biotech, GmbH) (28) and the Big-Dye Terminator mix (Applied Biosystems, Foster City, USA), according to the manufacturer's instruction. The elongation products were then applied into Long-Ranger gel 5.0% (Cambrex, Rockland, ME), and the DNA sequences were read automatically with an ABI Prism 377 DNA sequencer and entered into the AUTO ASSEMBLER™ software vs. 2.1 program (Applied Biosystems). The sequences were analyzed with the Blast 2.1 program from the GenBank Online Service.

Antimicrobial susceptibility testing

In vitro antimicrobial susceptibility to ampicillin, amoxicillin, amoxicillin/clavulanic acid, tetracycline, minocycline, doxycycline, clindamycin, metronidazole, cefotaxime and ceftazidime was assessed using the Etest (AB Biodisk, Solna, Sweden). Etest ESBLs (strips of cefotaxime-cefotaxime plus clavulanic acid and strips of ceftazidime-ceftazidime plus clavulanic acid) were used to confirm the presence of clavulanic inhibitable extended-spectrum β-lactamase enzymes. For anaerobes and fastidious bacteria, Brucella agar plates supplemented with 5% defibrinated sheep blood, hemin (5 mg/l) and vitamin K_1 (1 mg/l) were streaked with a McFarland standard no. 1 inoculum in Brucella broth using oxygen-free cotton-tipped swabs. For facultative organisms, PDM agar plates were streaked with a McFarland

Table 1. Prevalence and identity of β-lactamase-producing bacterial isolates

Patient No.	β-lactamasec +/or –	Identity	
		16S rRNA (% homology)	API (% reliability)
1	+	*Burkholderia (Pseudomonas)* sp. (99)	*Acinetobacter/Pseudomonas* sp. (98)
2	–		
3	–		
4	+	*Prevotella* sp. (97)	*Prevotella* sp. (unacceptable profile)
5	+	*Bacillus licheniformis* (100)	*B. licheniformis* (100)
6	+	*Fusobacterium nucleatum* ssp. *polymorphum* (100)	*F. nucleatum* (62)
7	+	*Prevotella buccae* (100)	*P. buccae* (100)
8	+	*Capnocytophaga gingivalis* (100)	*Capnocytophaga* sp. (100)
		Prevotella melaninogenica (99)	*Prevotella denticola* (74)
9	+	*Prevotella* sp. oral strain B31FD (98)	ND*
10	–		
11	+	*C. gingivalis* (100)	*Capnocytophaga* sp. (100)
12	+	*Ralstonia (Pseudomonas) pickettii* (100)	*Burkholderia* sp. (*Pseudomonas*) 'SAP II' (100)
		Stenotrophomonas (*Pseudomonas*) *maltophilia* (97)	*Acinetobacter/Pseudomonas* sp. (100)
13	–		
14	+	*B. licheniformis* (100)	*B. licheniformis* (100)
15	+	*Bacillus* sp. (100)	*B. cereus* (100)
16	+	*C. gingivalis* (100)	*Capnocytophaga* sp. (100)
17	–		
18	–		
19	–		
20	+	*Prevotella oris* (100)	*P. buccae* (84)
		Propionibacterium acnes (100)	*P. acnes* (100)
		Prevotella sp. (99)	*P. buccae* (98)
21	+	*Staphylococcus epidermidis* (100)	*S. epidermidis* (50)
		Capnocytophaga sp. clone BM058 (100)	*Capnocytophaga* sp. (100)
		Prevotella loescheii (99)	*P. loescheii* (38)
22	+	*S. epidermidis* (100)	*S. epidermidis* (98)
		Neisseria sp. (99)	*Neisseria* sp. (100)
23	+	*Prevotella* sp. PUS9.180 (99)	*P. loescheii* (85)
		Prevotella sp. (97)	*P. buccae* (100)
		Peptostreptococcus sp. (99)	ND
		Prevotella sp. oral strain B31FD (99)	*P. loescheii* (85)
		P. buccae (100)	*P. buccae* (99)
24	+	*C. gingivalis* (100)	*Capnocytophaga* sp. (100)
25	+	*Prevotella nigrescens* (100)	*Prevotella intermedia* (99)

*ND, no data provided; lost during subcultivation.

standard no. 0.5 inoculum suspended in 0.85% NaCl. The following strains, provided by the Norwegian Institute of Public Health, Oslo, were used for quality control: *Bacteroides fragilis* ATCC 25285, *Escherichia coli* ATCC 35218, *E. coli* ATCC 25922, *S. aureus* ATCC 29213 and *S. aureus* ATCC 43300 and an ESBL-producing strain of *Klebsiella pneumoniae*.

Results

At least one isolate with β-lactamase activity was detected in 18 (72%) of the 25 patients with refractory periodontitis, and a total of 29 β-lactamase-producing bacterial morphotypes were found in the subgingival samples from these patients. Two to five β-lactamase-producing strains were isolated from six patients. The β-lactamase-positive isolates identified are listed in Table 1. The most prominent β-lactamase-producing species were anaerobic gram-negative rods that belonged to the genus *Prevotella* (41%). More than one species of *Prevotella* was found in samples from two patients: no. 20, *Prevotella oris* and a strain of another species of *Prevotella,* no. 23, three different strains of *Prevotella* not assigned to a species, and a strain of *Prevotella buccae*. When comparing the API system and partial sequencing of the 16S rRNA gene, accordance at the species level was seen in nine of the 29 (27%) identified strains: *P. buccae* (two out of 12 *Prevotella* strains isolated), *Prevotella loescheii* (one out of 12 *Prevotella* strains isolated), *Fusobacterium nucleatum* (one strain isolated), *Propionibacterium acnes* (one strain isolated), *Bacillus licheniformis* (two out of three *Bacillus* strains isolated), and *Staphylococcus epidermidis* (two strains isolated). The API system attempted to identify *Prevotella* at species level for several of the strains that could not be distinguished by using partial sequencing of the 16S rRNA gene. Identification of the facultative β-lactamase producers correlated well when comparing the two identification methods, although none of the *Capnocytophaga* spp. could be identified at species level with the API system. Identification at genus level gave identical results using API and partial 16S rRNA gene sequencing in all the β-lactamase-producing bacterial isolates.

Minimum inhibitory concentrations (MICs) of ampicillin and amoxicillin, determined by Etest, were 1.5–256 μg/ml and 4–256 μg/ml, respectively, for the *Prevotella* species (Table 2). MICs of 0.016–0.50 μg/ml were observed for amoxicillin/clavulanate. Three of the *Prevotella* isolates presented with high MICs of ampicillin and amoxicillin (24–256 μg/ml) and cefotaxime and ceftazidime (256 μg/ml). According to the interpretative categories of NCCLS (19), these isolates were also resistant or intermediately resistant to the tetracyclines (6–16 μg/ml). Among the non-β-lactam antibiotics, all the *Prevotella* strains tested

Table 2. Susceptibilities to antimicrobial agents, as determined by the Etest method

Identity (16S rRNA)	Patient No.	MIC (μg/ml) of antibiotics[a]									
		Ampicillin	Amoxicillin	Amoxicillin-Clavulanic acid	Tetracycline	Minocycline	Doxycycline	Clindamycin	Metronidazole	Cefotaxime	Ceftazidime
Prevotella sp.	4	256/R	256/R	0.125/S	16/R	6/I	16/R	0.016/S	0.016/S	256/R	256[d]
P. buccae	7	12/R	16/R	0.125/S	0.19/S	0.032/S	0.064/S	0.016/S	0.19/S	4/S	12[d]
P. melaninogenica	8	64/R	256/R	0.50/S	0.38/S	0.032/S	0.125/S	0.016/S	0.25/S	12/S	1.5[d]
Prevotella sp. oral strain B31FD	9	ND[b]									
P. oris	20	4/R	12/R	0.064/S	4/S	1/S	1.5/S	0.016/S	0.016/S	16/S	8[d]
Prevotella sp.	20	256/R	256/R	0.19/S	16/R	16/R	12/I	0.016/S	0.023/S	256/R	256[d]
P. loescheii	21	1.5/R	4/R	0.032/S	0.023/S	0.016/S	0.016/S	0.016/S	0.19/S	0.125/S	0.19[d]
Prevotella sp. PUS9.180	23	12/R	16/R	0.016/S	16/R	12/1	6/1	0.016/S	0.016/S	3/S	0.75[d]
Prevotella sp.	23	8/R	24/R	0.047/S	0.032/S	0.023/S	0.032/S	0.016/S	0.023/S	4/S	16[d]
Prevotella sp. oral strain B31FD	23	24/R	64/R	0.047/S	12/I	4/S	4/S	0.016/S	0.016/S	256/R	256[d]
P. buccae	23	8/R	16/R	0.032/S	0.032/S	0.016/S	0.023/S	0.016/S	0.016/S	3/S	0.75[d]
P. nigrescens	25	ND[c]									
F. nucleatum	6	256/R	256/R	0.125/S	0.50/S	0.19/S	0.25/S	0.023/S	0.016/S	0.032/S	0.50[d]
P. acnes	20	ND[c]									
Peptostreptococcus sp.	23	ND[c]									
C. gingivalis	8	0.75[d]	2[d]	0.064[d]	0.25[d]	0.047[d]	0.064[d]	0.016[d]	8[d]	3[d]	1.5[d]
C. gingivalis	11	256[d]	256[d]	0.75[d]	0.25[d]	0.064[d]	0.125[d]	0.016[d]	256/R	256[d]	256[d]
C. gingivalis	16	1.5[d]	4[d]	0.094[d]	0.125[d]	0.032[d]	0.094[d]	0.016[d]	256/R	8[d]	4[d]
Capnocytophaga sp.	21	0.75[d]	2[d]	0.064[d]	0.125[d]	0.023[d]	0.125[d]	0.016[d]	16[d]	0.125[d]	0.047[d]
Capnocytophaga sp.	24	0.5[d]	1[d]	0.032[d]	1.5[d]	0.38[d]	1.5[d]	0.016[d]	256/R	6[d]	4[d]
Burkholderia sp.	1	4[d]	8[d]	8[d]	32/R	4/S	8/I	256[d]	256/R	0.064/S	0.094/S
Burkholderia sp.	12	4[d]	8[d]	8[d]	24/R	6/I	8/I	256[d]	256/R	0.094/S	0.064/S
R. pickettii	12	24[d]	256[d]	64[d]	2/S	1/S	1.5/S	256[d]	256/R	1.5/S	24/S
B. licheniformis	5	256[d]	256[d]	8[d]	0.38[d]	0.047[d]	0.50[d]	256[d]	256/R	256[d]	256[d]
B. licheniformis	14	256[d]	256[d]	6[d]	0.75[d]	0.064[d]	0.38[d]	16[d]	256R	6[d]	256[d]
Bacillus sp.	15	256[d]	256[d]	256[d]	2[d]	1.5[d]	0.75[d]	0.38[d]	256/R	256[d]	256[d]
S. epidermidis	21	0.50/R	0.75/R	0.064/S	0.125/S	0.094/S	0.25/S	0.064/S	256/R	0.25/S	2/S
S. epidermidis	22	0.75/R	0.75/R	0.094/S	0.38/S	0.125/S	0.38/S	0.125/S	256/R	0.38/S	2/S
Neisseria sp.	22	16[d]	64[d]	0.25[d]	0.38[d]	0.38[d]	0.75[d]	6[d]	256/R	0.032[d]	0.032[d]

[a]R, resistant; I, intermediate; S, susceptible.
[b]ND, no data provided; fastidious bacterium.
[c]ND, no data provided; lost during subcultivation.
[d]No available interpretative categories.

were susceptible to clindamycin and metronidazole. *F. nucleatum* was resistant to ampicillin and amoxicillin and susceptible to all other antimicrobial agents tested. All five *Capnocytophaga* strains had ampicillin and amoxicillin MICs >0.5 μg/ml. One isolate presented with high MICs (256 μg/ml) of ampicillin, amoxicillin, cefotaxime and ceftazidime. These *Capnocytophaga* isolates were all susceptible to the tetracyclines and clindamycin. The β-lactamase-producing strain of a *Neisseria* sp. presented with high MICs of ampicillin and amoxicillin; 16 and 64 μg/ml, respectively. Two of the facultative non-spore-forming gram-negative rods (*Burkholderia* spp.) showed resistance to tetracycline (MICs 24–32 μg/ml). Otherwise, these bacteria were susceptible to amoxicillin combined with clavulanic acid and to the cephalosporins. The two strains of *S. epidermidis* had MICs >0.5 μg/ml for the penicillins. Both strains were susceptible to all other agents tested. High MICs (256 μg/ml) were observed for the *Bacillus* isolates towards the β-lactams and cephalosporins; one of the strains had an MIC of 256 μg/ml for the amoxicillin/clavulanate combination. No ESBLs could be demonstrated by the method used in this study.

Discussion

The results of this investigation revealed that a high proportion (72%) of the patients with refractory periodontitis in the USA harbored β-lactamase-producing bacteria in their subgingival plaque. Previous studies in the USA reported prevalences of 76% and 64% in untreated periodontitis patients and maintenance patients, respectively (17, 33). The prevalence of bacteria producing β-lactamase was similar to that found in a study of 25 patients with refractory periodontitis in Norway (68%) (13). High prevalences of β-lactamase-producing bacteria have also been reported in patients suffering from chronic periodontitis (15, 17) and rapidly progressive periodontitis (8). In Norway, limited data on antibiotic prescribing practices among periodontists are available, but it is likely that the average weekly prescription frequency of antibiotics for the American and Norwegian dentist is fairly similar (24). More β-lactamase-producing strains were recovered in the present study (29 strains) than in the Norwegian study (24 strains) and the variety of species was also greater in the samples from the USA. The *Prevotella* isolates recovered in the American and Norwegian materials varied. In the American material, *Prevotella* strains that could not be identified at species level dominated (50%), whereas in the Norwegian material, β-lactamase-producing *Prevotella melaninogenica* and *P. buccae* were the most frequently isolated bacteria. β-lactamase-producing *Capnocytophaga* spp., *F. nucleatum*, *P. acnes* sp., *Peptostreptococcus* sp., *Burkholderia* spp. and *Ralstonia pickettii* were recovered only in the American material; *Serratia* sp., *Erwinia* sp. and *E. coli* were isolated only from the Norwegian samples. This might be related to differences in previous antibiotic treatment of these patient groups. In the Norwegian material none of the patients that harbored β-lactamase-producing bacteria

had received antibiotic treatment the past 3 months, whereas four of the patients in the American material (patient Nos. 7, 8, 11 and 12) harboring the enzyme-producing strains had received antibiotics within the last 3 months prior to sampling.

The importance of the *Prevotella* species as β-lactamase producers is emphasized in this study. Most investigations of β-lactamase production in subgingival bacteria have focused on strictly anaerobic gram-negative bacteria and have found *Prevotella* to be the most frequently involved genus (13, 15, 17, 21, 30, 33). In the present study, five β-lactamase-producing *Capnocytophaga* species were recovered. The β-lactamase production was associated with high-level resistance to amoxicillin, ampicillin, cefotaxime and ceftazidime in one of the cases (MICs of 256 μg/ml), and this patient had received antibiotic treatment in the last 3 months. Kinder et al. (16) isolated seven penicillin- resistant β-lactamase-producing *Capnocytophaga* isolates in the subgingival flora of patients with adult periodontitis, and Nyfors et al. (21) found two β-lactamase-positive isolates of *Capnocytophaga ochracea* in saliva of infants with documented exposure to antibiotics. The present study, as well as our previous study from Norway (13), demonstrated that a range of β-lactamase-producing facultative bacteria are present in refractory periodontal disease. This could be important to the pathogenesis of this disease since major nosocomial pathogens have emerged concomitantly with development of resistance to β-lactams.

Identification largely based upon the determination of a diverse set of phenotypic characters may be influenced by cultivation conditions. In the present study, API identification showed limited concordance with that based on partial sequencing of the 16S rRNA gene when identifying the *Prevotella* species. Whereas API identification showed reliability percentages of 38–100%, partial sequencing of the 16S rRNA gene resulted in 97–100% homology with partial 16S rRNA gene sequences in the GenBank database, and represents a more accurate way of identifying bacterial species. However, identification of the facultative β-lactamase producers was concordant by the two methods, at least at the genus level.

All β-lactamase-producing *Prevotella* species isolated in the present study had amoxicillin and ampicillin MICs >0.5 mg/ml which, according to NCCLS breakpoints (19), confirms the presence of a β-lactamase. In accordance with previous findings (1, 13, 14, 34), 100% susceptibility to amoxicillin/clavulanate and metronidazole was observed in the *Prevotella* isolates, but variable resistance to tetracycline was found. The tetracyclines achieve levels in the gingival fluid that are two to five times higher than blood levels (4, 11) and have been used frequently in the past to treat periodontal disease (24, 26). Patients diagnosed as having refractory periodontitis often present with a history of tetracycline therapy and a microflora that is relatively resistant to tetracyclines (22, 23, 32). According to the latest published NCCLS breakpoints (19), three of the *Prevotella* species isolated in our study were resistant to cefotaxime and ceftazidime (MIC of 256 μg/ml). The use of oral cephalosporins in the treatment of upper respiratory tract infections may contribute to the selection of cephalosporin-resistant oral *Prevotella* (27). Higher, but not significantly higher, MIC values towards ampicillin and amoxicillin were observed for several of the β-lactamase-producing *Prevotella* spp. in the USA as compared to those recovered from the material in Norway (data not shown).

In summary, β-lactamase production was detected in a wide variety of bacteria from the subgingival flora of refractory periodontitis patients in the USA. The prevalence (72%) and variety of β-lactamase-producing bacteria in the USA was comparable to that found in a similar material from Norway. If the resistant bacteria represent potential pathogens, the periodontal disease may not be resolved by β-lactam antibiotic treatment and may even be aggravated. Microbiological screening and susceptibility testing are therefore of major importance if antibiotics are indicated in the treatment of periodontal diseases. Bacteria in a subgingival biofilm are not always easily accessible to antimicrobials. Resistance mechanisms such as modifying enzymes, efflux pumps, and target mutations are therefore not sufficient to explain most cases of antibiotic-resistant biofilm infections. Methods are now being developed to determine the biofilm inhibitory concentration (BIC) as a supplement to the traditionally used minimum inhibitory concentration (MIC) based on planktonically growing bacteria. Although this might be more appropriate, there is at the moment no generally accepted standardized method by which such a concentration can be determined. Thus, before initiation of antimicrobial therapy, an effort must have been made to mechanically (scaling and root planing) remove and disrupt the subgingival biofilm.

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