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ORIGINAL ARTICLE

Resistance to β-lactams and distribution of β-lactam resistance genes in subgingival microbiota from Spanish patients with periodontitis

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Abstract

Objectives The aim of this study was to analyze the distribution of β-lactamase genes and the multidrug resistance profiles in β-lactam-resistant subgingival bacteria from patients with periodontitis.

Materials and methods Subgingival samples were obtained from 130 Spanish patients with generalized periodontitis stage III or IV. Samples were grown on agar plates with amoxicillin or cefotaxime and incubated in anaerobic and microaerophilic conditions. Isolates were identified to the species level by the sequencing of their 16S rRNA gene. A screening for the following β-lactamase genes was performed by the polymerase chain reaction (PCR) technique: bla_{TEM}, bla_{SHV}, $bla_{CTX\text{-}M}$, bla_{CfxA}, bla_{CepA}, bla_{CblA}, and bla_{ampC}. Additionally, multidrug resistance to tetracycline, chloramphenicol, streptomycin, erythromycin, and kanamycin was assessed, growing the isolates on agar plates with breakpoint concentrations of each antimicrobial.

Results β-lactam-resistant isolates were found in 83% of the patients. Seven hundred and thirty-seven isolates from 35 different genera were obtained, with *Prevotella* and *Streptococcus* being the most identified genera. bla_{CfxA} was the gene most detected, being observed in 24.8% of the isolates, followed by bla_{TEM} (12.9%). Most of the isolates (81.3%) were multidrug-resistant.

Conclusions This study shows that β-lactam resistance is widespread among Spanish patients with periodontitis. Furthermore, it suggests that the subgingival commensal microbiota might be a reservoir of multidrug resistance and β-lactamase genes.

Clinical relevance Most of the samples yielded β-lactam-resistant isolates, and 4 different groups of *bla* genes were detected among the isolates. Most of the isolates were also multidrug-resistant. The results show that, although β-lactams may still be effective, their future might be hindered by the presence of β-lactam-resistant bacteria and the presence of transferable *bla* genes.

Keywords Periodontitis · Antibiotic resistance · β-Lactams · Multidrug resistance

Introduction

Periodontitis is a complex infectious disease caused by a dysbiosis of the subgingival biofilm and a disproportionate response of the host's immune system [1]. Supra- and subgingival debridement along with motivation and oral hygiene instructions are the standard treatment for periodontitis, and antimicrobials can be used as adjunctive therapy if patient's conditions apply [2–4]. The microbial etiology of inflammatory periodontal diseases provides the rationale for the use of antimicrobial medication in periodontal therapy. Antibiotics may be specially indicated for periodontal patients 36 years of age or younger with periodontitis stage II or for patients with attachment or radiographic bone loss at more than two nonadjacent sites [5]. The most used antimicrobials in periodontitis are the β-lactams, particularly amoxicillin (AMX) which can be administered together with metronidazole [2]. It has been described that those bacteria resistant to β-lactams may also be resistant to other antibiotics such as tetracyclines, aminoglycosides, and chloramphenicol [6–8]. The most important resistance mechanism to β-lactam antibiotics are the β-lactamases, enzymes that break the β-lactam ring and inactivate the antimicrobial [9]. Extended spectrum

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β-lactamases (ESBLs) can hydrolyze a wide array of β-lactams such as penicillins, cephalosporins, and monobactams while inhibited by β-lactamase inhibitors such as clavulanic acid [8, 10, 11]. ESBLs were first detected in the 1980s in a *Klebsiella pneumoniae* isolate, and now these are found in both enteric and non-enteric microorganisms. More than 230 ESBLs have been described, including TEM-type, SHV-type, CTX-M-type, OXA-type, and KPC-type [11]. In the oral environment, bla_{TEM} have been described as the most prevalent ESBLs, although there are only a few studies that have screened for these genes [12–15]. Other β-lactamases found in the oral biofilm include bla_{CfxA}, $bla_{CepA/CblA}$, and bla_{ampC}, suggesting that the mouth might be a reservoir for β-lactamase genes. In fact, the oral environment, being a transit place for bacteria entering the digestive tract and harboring more than 700 bacterial species that grow to form a biofilm, offers an excellent opportunity for horizontal gene transfer to occur [16, 17].

Spain is one of the European countries with the highest consumption of antibiotics, which has been suggested to be linked with the increase of antibiotic resistance [18]. Little is known about the distribution of β-lactamase genes in the subgingival microbiota of Spanish patients with periodontitis. For this reason, the aim of the current study was to screen for β-lactamase genes in the AMX- and cefotaxime-resistant subgingival microbiota isolated from 130 patients with periodontitis. Additionally, these isolates were tested for their multidrug resistance (MDR) to chloramphenicol, tetracycline, kanamycin, erythromycin, and streptomycin.

Materials and methods

Patients involved in the study

Subgingival samples were taken consecutively from 130 patients, recruited between 2016 and 2017, and diagnosed with generalized severe chronic periodontitis or generalized aggressive periodontitis according to the 1999 classification [19]. Nowadays, this diagnosis corresponds to generalized periodontitis stage III or IV, according to the new classification [20]. Stages were assessed according to the interproximal attachment loss. Grading was established considering the coefficient radiographic bone loss and patients age, adding the status of the systemic conditions such as smoking and diabetes. The samples were obtained from patients that attended at the Department of Periodontology of the Universitat Internacional de Catalunya (UIC) (Barcelona, Spain). All patients were supervised by the same clinician (CM), who also took the microbial samples. The study was previously approved by the Ethics Committee of the UIC (Study number: ODO-2014-01) and complied with the principles of the Declaration of Helsinki. Additionally, all the participants signed an Institutional Review Board approved informed consent form. None of the patients had taken antibiotics at least 3 months prior to the sampling.

Clinical evaluation

The following clinical parameters were collected from a whole-mouth evaluation at baseline: probing pocket depth (PPD), clinical attachment level (CAL), full mouth plaque index (FMPI), full mouth bleeding on probing (FMBP), and mobility and furcation involvement. The PPD was measured at 6 sites per tooth (mesiobuccal, mid-buccal, distobuccal, mesiolingual, mid-lingual, and distolingual) as the distance in millimeters from the free gingival margin to the base of the probeable pocket using a handheld periodontal probe (PCP-UNC 15; Hu-Friedy Mfg. Co., Chicago, IL, USA). The CAL was measured at 6 sites per tooth from the cemento-enamel junction (CEJ) or from the base of the dental restoration or prosthesis to the bottom of the pocket. The plaque index (PI) was recorded according to the criteria described by Silness and Löe [21]. Bleeding on probing (BOP) was determined as being present or absent (±) within 30 s after probing of the aforementioned 6 sites per tooth. Only the parameters of PPD, CAL, PI, BOP, and mobility of the selected teeth, those with deepest PPD of each quadrant, were evaluated in the study.

Sample collection

Subgingival microbial samples were taken from the deepest periodontal pocket of each quadrant. Each area was isolated with cotton rolls, the supragingival plaque deposits were carefully removed with curettes, and subgingival microbial samples were obtained by inserting two sterile paper points in each subgingival pocket and keeping them in place for 20 s. Samples from each patient were pooled in a vial containing 1.5 ml of cold sterilized reduced transport medium without ethylenediaminetetraacetic acid (EDTA) [22] and sent to the microbiology laboratory at 4 °C for processing within the same day.

Microbial culture and bacterial selection

Subgingival biofilm samples were dispersed by vortex for 45 s. Serial tenfold dilutions were plated on blood agar (blood agar base no. 2; Oxoid Ltd., Basingstoke, UK) containing 5% of horse blood, hemin (5 mg/l), menadione (1 mg/l), and on the same media with 2 μg/ml of cefotaxime (CTX) or 8 μg/ml of AMX (Sigma Aldrich, St. Louis, MO, USA). Both concentrations were selected based on the breakpoint concentrations recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and the Clinical and Laboratory Standards Institute (CLSI) [23, 24]. Given that

the oral microbiota harbors many different bacterial genera and that many of these are not mentioned in either the EUCAST or the CLSI guidelines, these concentrations were chosen based on taxonomic relatedness to bacteria of the oral environment, using the higher concentration of antibiotics when in doubt. Therefore, bacteria that grew on plates with antibiotics were considered to be resistant. In order to obtain a wider array of the subgingival microbiota, plates were incubated at 37 °C under microaerophilic (5% CO_2) and anaerobic (10% H_2, 10% CO_2, and 80% N_2) conditions for 48–72 h. Resistant colonies were isolated according to their morphology (two of each morphology), replated to obtain pure cultures, and preserved at − 80 °C in a 30% sterilized glycerol solution.

DNA isolation and 16S rRNA gene sequencing

Genomic DNA extraction was performed on each isolate using the ATP™ Genomic DNA mini Kit (ATP Biotech Inc., Taipei City, Taiwan) following the manufacturer's instructions. Once extracted, DNA was visualized in a 0.7% agarose gel with ethidium bromide and quantified using a Nanodrop 2000C UV-vis spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA).

Isolates were identified to a species level, through 16S rRNA gene sequencing [25]. Zero point five (0.5) micromolar of primers 27F and 1544R (Table 1) were added to the polymerase chain reaction (PCR) mix with 30–100 ng of DNA, 1X PCR buffer, 1X dNTPs solution, 2.5 mM of $MgCl_2$, and 1 unit of Taq polymerase (all reagents from Takara, Tokyo, Japan). Amplification was carried out using a T3000 Thermocycler (Biometra, Goettingen, Germany) under the following conditions: 5 min at 95 °C, followed by 35 cycles of 95 °C for 60 s, 57 °C for 60 s, and 72 °C for 60 s, and followed by 10 min at 72 °C. The PCR products were purified using the E.Z.N.A Gel Extraction Kit (Omega Bio-Tek, Norcross, GA, USA) and sent for sequencing to Macrogen Inc. (Amsterdam, Netherlands). The sequences obtained were aligned using Clustal Omega Software and analyzed using NCBI's BLAST (available at http://www.ebi.acuk/ and https://blast.ncbi.nlm.nih.gov/Blast.cgi, respectively). Only sequences with ≥ 99% identity were accepted as proof to identify to the species level.

Detection of β-lactamase genes

Detection of β-lactamase genes was achieved using the primers described in Table 1. To ensure that most of the genetic variants would be detected in the screening, universal primers were used in the detection of bla_{CfxA}, bla_{TEM}, bla_{SHV}, and $bla_{CTX\text{-}M}$. PCR reactions were performed with 30–100 ng of DNA, 1X PCR buffer, 1X dNTPs solution, 2.5 mM $MgCl_2$, 0.5 μM of each primer, and 1 unit Taq polymerase (Takara, Tokyo, Japan). Amplifications were carried out in a T3000 Thermocycler. The thermocycling conditions were as follows: (i) for the detection of bla_{CfxA}, 1 min of denaturation at 95 °C, 1 min of annealing at 58 °C, and 1 min of extension at 72 °C for 26 cycles; (ii) for the detection of $bla_{CepA/CblA}$, 30 s of denaturation at 95 °C, 30 s of annealing at 58 °C, and 30 s of extension at 72 °C for 29 cycles; (iii) for the detection of bla_{ampC}, 1 min of denaturation at 95 °C, 1 min of annealing at 50 °C, and 1 min of extension at 72 °C for 30 cycles; (iv) for the detection of bla_{OXA}, 40 s of denaturation at 95 °C, 40 s of annealing at 55 °C, and 40 s of extension at 72 °C for 30 cycles; (v) for the detection of

Table 1 Primers and polymerase chain reaction conditions for the detection of β-lactamase and 16S rRNA genes

Gene	Primer name	Sequence 5′ – 3′	Tm °C	Product size (bp)	Reference
16S rRNA	27 F 1544 R	GAG TTT GAT CCT GGC TCA G AGA AAG GAG GTG ATC CAG CC	57	1500	[25]
bla_{CfxA}	CFXA F CFXA R	GCA AGT GCA GTT TAA GAT T GCT TTA GTT TGC ATT TTC ATC	58	934	[14]
$bla_{CepA/CblA}$	CepA/CblA F CepA/CblA R	CAA AGY GAC AAY AAT GCC TGC G TSA CGA AGR CGG CWA T	58	426	[26]
bla_{ampC}	AMPC F AMPC R	TAA ACA CCA CAT ATG TTC CG ACT TAC TTC AAC TCG CGA CG	50	769	[27]
bla_{OXA}	OXA F OXA R	TAT CGC GTG TCT TTC GAG TA TTA GCC ACC AAT GAT GCC	55	700	[28]
bla_{TEM}	TEM F TEM R	TCG CCG CAT ACA CTA TTC TCA GAA TGA ACG CTC ACC GGC TCC AGA TTT AT	60	445	[25]
bla_{SHV}	SHV F SHV R	ATG CGT TAT ATT CGC CTG TG TGC TTT GTT ATT CGG GCC AA	60	747	[29]
$bla_{CTX\text{-}M}$	CTX-M F CTX-M R	ATG TGC AGY ACC AGT AAR GTK ATG GC TGG GTR AAR TAR GTS ACC AGA AYC AGC GG	60	593	[30]

bla_{TEM}, bla_{SHV}, and $bla_{CTX\text{-}M}$, a multiplex was performed as previously described [31]. In all reactions an initial, 5-min denaturation at 95 °C and a final 10 min extension at 72 °C were applied. DNA of the isolates carrying the genes screened was used as positive controls. To confirm the presence of the genes in the controls, PCR amplicons of the expected size were sequenced and annealed with reference sequences available at NCBI's GenBank. Negative controls included water in place of DNA in the PCR mix.

PCR products were observed through electrophoresis using a 2% agarose gel with ethidium bromide. Gels were photographed using a UV light transilluminator GEL DOC™ XR+ system (Bio-Rad Laboratories Inc. Hercules, CA, USA).

In vitro antimicrobial resistance testing

MDR was determined for all isolates using blood agar plates containing 5% of horse blood, hemin (5 mg/l), and menadione (1 mg/l) and supplemented with (i) 1 μg/ml of erythromycin (ERY), (ii) 64 μg/ml of kanamycin (KAN), (iii) 8 μg/ml of chloramphenicol (CHL), (iv) 128 μg/ml of streptomycin (STR), (v) 8 μg/ml of tetracycline (TET), and (vi) 2 μg/ml of CTX or 8 μg/ml of AMX (all antimicrobials were obtained as pure powder from Sigma Aldrich, St. Louis, MO, USA). Incubation was performed at 37 °C in anaerobic and microaerophilic conditions for 48–72 h. The antibiotic concentrations used were set according to the recommendations provided by the EUCAST and CLSI and following the same criteria used for the initial selection for AMX and CTX.

Statistical analysis

The chi-square test was used as a paired statistical test for discrete variables to find statistical differences between the prevalence of the genes screened and phenotypical resistance to the antibiotics tested among the identified bacterial species of this study. p-values of < 0.05 were accepted for statistical significance.

Results

One hundred and thirty subgingival samples were obtained from patients with generalized periodontitis stage III or IV. Patients were between the ages of 24 and 82 years old (mean of 51.3) and showed a mean probing depth of 6.6 ± 1.8 mm, a mean clinical attachment loss of 7.6 ± 2.1 mm, and 92.1% of gingival sites with bleeding on probing. From the 130 samples, 63 (48.5%) and 33 (25.4%) samples had AMX-resistant isolates in anaerobic (ARIA) and in microaerophilic (ARIM) conditions, respectively, while 98 (75.4%) and 70 (53.6%) samples had CTX-resistant isolates in anaerobic (CRIA) and in microaerophilic (CRIM) conditions, respectively. Twenty-eight (21.5%) samples grown in anaerobiosis and 53 (40.8%) samples grown in microaerophilic conditions did not present any β-lactam-resistant isolates. Twenty-two samples (16.9%) did not present any β-lactam-resistant isolate in either microaerophilia or anaerobiosis. Samples grown in blood agar without antibiotics showed a mean bacterial load of 7.3 log10 colony-forming units per milliliter (cfu/ml) (± 1.3) in anaerobic conditions and 7.1 log10 cfu/ml (± 0.9) in microaerophilic conditions. The number of β-lactam-resistant bacteria averaged a 1 logarithmic reduction (Table 2).

The subgingival samples yielded 181 ARIAs, 84 ARIMs, 293 CRIAs, and 179 CRIMs, making a total of 737 isolates. Isolated bacteria were identified by the sequencing of their 16S rRNA gene, and β-lactam resistance genes were screened by PCR. Additionally, phenotypic resistance to CHL, STR, ERY, TET, and KAN was determined. Furthermore, AMX and CTX resistance was also tested depending on which antibiotic the isolates were selected for (Table 3). Data regarding the species detected, their phenotypic resistance, and their screened genes is shown in the Supplementary data (Table S1). Bacteria from the genus *Prevotella* were the most frequently isolated ($n = 213$), mainly the species *Prevotella nigrescens* ($n = 70$) and *Prevotella intermedia* ($n = 65$), followed by the genus *Streptococcus* ($n = 153$) with a wide variety of species within this genus (see Table S1). The genus *Prevotella* was significantly ($p < 0.01$) more prevalent in the ARIAs, while the genus *Veillonella* was more prevalent in the CRIAs ($p < 0.01$). Regarding bacteria grown in microaerophilic conditions, the genus *Neisseria* was significantly more prevalent in ARIMs ($p < 0.05$), while the genus *Micrococcus* was more prevalent in CRIMs ($p < 0.01$). More than 12 % (12.1%) of the isolates were identified at a genus level, and 8% could not be identified at all (Tables 3 and S1).

Regarding the *bla* genes, bla_{CfxA} was the most prevalent gene (24.8%), followed by bla_{TEM} (12.9%), $bla_{CepA/CblA}$ (1.1%), and bla_{SHV} (0.8%). The $bla_{CTX\text{-}M}$, bla_{OXA}, and bla_{ampC} genes were not detected. A significantly higher ($p < 0.01$) percentage of bla_{CfxA} was observed in those isolates grown in anaerobic conditions compared to those grown in microaerophilic conditions.

Phenotypic resistance to other antibiotics was observed in 599 isolates (81.3%). These isolates were resistant to, at least, one antimicrobial besides the β-lactam for which they were previously selected. Fifty-six point eighty-five percent (56.9%) of the isolates showed resistance to KAN, 54.6% to ERY, 29.4% to TET, 27.7% to STR, and 9.9% to CHL. Isolates selected for AMX resistance showed resistance to CTX more frequently (53.6%) than conversely (32.2%). Most of the MDR isolates were resistant to 1–3 antimicrobials (22.2%, 26.4%, and 25.7%, respectively), and 15 isolates (2.5%) were resistant to the 6 antimicrobials tested (Table 4). Statistical differences were observed between the percentage of ARIAs and ARIMs resistant to CHL and KAN

Table 2 Bacterial counts expressed in colony-forming units per milliliter

	Anaerobic			Microaerophilic		
	AMX-resistant	CTX-resistant	Total bacteria	AMX-resistant	CTX-resistant	Total bacteria
Bacterial counts	1.3×10^6	2.3×10^6	2.0×10^7	1.3×10^6	1.2×10^6	1.4×10^7
Resistance %	6.5	11.6	–	9.6	8.8	–

AMX amoxicillin, *CTX* cefotaxime

and between the percentage of CRIAs and CRIMs resistant to AMX and KAN.

Discussion

This study analyzed the β-lactam-resistant subgingival microbiota isolated from patients with severe forms of periodontitis. Bacteria were isolated based on their resistance to AMX or to CTX. AMX was chosen for being the first-choice β-lactam for many bacterial infections including periodontitis [2, 32, 33]. The use of CTX, as a third-generation cephalosporin, is much more restricted, and therefore, resistance to this antibiotic should be scarcer. However, ESBLs such as CTX-M, which are active against it, are prevalent worldwide [34, 35], making them interesting targets to study when analyzing β-lactam resistance. An average of 7.8% of the culturable microbiota isolated in this study was resistant to AMX, slightly higher that what a previous study found in Spanish samples comparing subgingival microbiota from Spain and the Netherlands [36]. In our study population, CTX resistance turned out to be more prevalent, with 82.3% of the patients having at least one resistant isolate, than AMX resistance (55.4%). We detected a large amount of *Veillonella* isolates resistant to CTX ($n = 76$) and very few resistant to AMX ($n = 5$), which might be an explanation for the higher prevalence of CTX resistance. Previous studies have already shown that the genus *Veillonella* has a low susceptibility for β-lactams, probably due to the presence of penicillin-binding proteins with lower affinity for β-lactams [37–40]. Furthermore, other studies analyzed *Veillonella* isolates and found higher MICs of cefoxitin than AMX and ampicillin [38, 39], suggesting that *Veillonella* spp. might be more resistant to cephalosporins than aminopenicillins. *P. nigrescens* ($n = 70$), *P. intermedia* ($n = 65$), and *Veillonella parvula* ($n = 60$) were the species most often isolated. This agrees with previous reports of *Prevotella* being the main genus in the oral environment expressing β-lactam resistance [41–43]. Most of the studies analyzing β-lactam resistance have based their selection of isolates on the production of β-lactamases, and therefore, despite being β-lactam-resistant, the *Veillonella* genus has been ruled out because it does not produce β-lactamases [37, 39, 44].

In this study, MDR was analyzed in each isolate using breakpoint concentrations suggested by the EUCAST and the CLSI. All the isolates that grew on agar plates with antibiotics were considered resistant. Of the 737 β-lactam-resistant isolates, 81.3% were MDR. From these, 63.3% were selected for CTX resistance and 36.7% for AMX resistance, these values being very similar to the percentage of total CTX- and AMX-resistant isolates obtained (64% and 35%, respectively) with little difference regarding whether they were cultured in anaerobic or in microaerophilic conditions; this suggests that MDR does not depend on the initial AMX or CTX selection. Few differences were observed between the 4 groups of isolates, except for KAN, CHL, and AMX resistance. Kanamycin resistance showed a higher prevalence in anaerobic isolates due mainly to the inherent resistance of anaerobic bacteria to this antibiotic [45] and to the *Prevotella* isolates, which are known to present a higher tolerance to kanamycin than other oral bacteria [46]. Chloramphenicol resistance was observed in higher percentages in microaerophilic isolates due mainly to the *Pseudomonas* isolates, which are able to exhibit high resistance levels thanks to their multidrug efflux pumps [47, 48]. On the other hand, AMX resistance was less often observed in the CTX microaerophilic isolates, probably due to the lack of *Prevotella* isolates, which increased the prevalence of AMX resistance in the CTX-resistant anaerobic group.

Fifteen isolates were resistant to the 7 antibiotics tested in this study and were all obtained from different patients. The isolates identified were *V. parvula* ($n = 3$), *Pseudomonas aeruginosa* ($n = 2$), *Acinetobacter guillouiae* ($n = 3$), and *Stenotrophomonas maltophilia* ($n = 1$). Of the 15 isolates, 6 could not be identified through 16S rRNA gene sequencing. It has been reported that *Pseudomonas*, *Acinetobacter*, and *Stenotrophomonas* genera are rich in efflux pumps, which confer on them resistance to multiple antimicrobials [49]. Furthermore, tetracycline, kanamycin, and erythromycin resistance can be linked to the presence of transposons of the Tn*916/1545* family, which are ubiquitous in the oral microbiota and carry genes that confer resistance to these antibiotics [50, 51]. These transposons have been described in *Veillonella* [52, 53], which might be responsible for the observed MDR.

The bla_{CfxA} gene was detected in 24.8% of the isolates, of which 71.0% ($n = 136$) were bacteria from the *Prevotella*

Table 3 Number (*N*) of β-lactam-resistant bacteria isolated from subgingival microbiota in patients with periodontitis, classified by the culture conditions in which they grew and the genus to which they pertained

Genera	Antimicrobial resistance													
	N	*bla*$_{TEM}$	*bla*$_{SHV}$	*bla*$_{CTX-M}$	*bla*$_{CfxA}$	*bla*$_{CepA/CblA}$	*bla*$_{OXA}$	*bla*$_{ampC}$	TET	CTX/AMX	CHL	STR	ERY	KAN
Amoxicillin anaerobiosis[†]														
Actinomyces sp.	4	1	0	0	0	0	0	0	1	0	0	1	0	4
Alloprevotella sp.	4	0	0	0	1	0	0	0	0	1	0	1	1	1
Anaerococcus sp.	1	0	0	0	0	0	0	0	0	1	0	0	1	0
Eikenella sp.	1	0	0	0	0	0	0	0	0	1	0	0	1	1
Escherichia sp.	4	2	2	0	0	0	0	0	2	2	1	1	3	3
Fusobacterium sp.	3	0	0	0	0	0	0	0	0	2	0	2	2	1
Klebsiella sp.	2	0	0	0	0	0	0	0	0	0	0	1	2	2
Leptotrichia sp.	1	0	0	0	0	0	0	0	0	0	0	1	1	1
Prevotella sp.	106	14	0	0	66	1	0	0	42	71	1	25	47	80
Pseudomonas sp.	1	0	0	0	0	0	0	0	0	1	1	1	1	1
Staphylococcus sp.	5	0	0	0	1	0	0	0	1	3	1	5	5	2
Streptococcus sp.	29	2	0	0	1	0	0	0	16	7	0	2	21	24
Veillonella sp.	5	1	0	0	1	0	0	0	0	2	0	4	5	0
Not determined	15	3	0	0	5	0	0	0	2	5	0	3	5	5
Total	181	23	2	0	75	1	0	0	64	96	4	47	95	125
Amoxicillin microaerophile[‡]														
Acinetobacter sp.	4	0	0	0	0	0	0	0	0	4	0	2	4	0
Actinomyces sp.	2	1	0	0	0	0	0	0	0	0	0	0	0	1
Bacillus sp.	1	0	0	0	0	0	0	0	0	1	0	0	0	0
Capnocytophaga sp.	5	1	0	0	4	0	0	0	0	5	1	1	0	5
Dermacoccus sp.	1	0	0	0	0	0	0	0	0	1	0	1	0	0
Haemophilus sp.	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Micrococcus sp.	1	0	0	0	0	0	0	0	0	1	1	1	0	0
Neisseria sp.	13	3	0	0	0	2	0	0	2	1	0	0	12	1
Paenibacillus sp.	2	0	0	0	0	0	0	0	0	2	0	2	0	0
Pantoea sp.	2	0	0	0	0	0	0	0	0	2	0	0	2	0
Pseudomonas sp.	10	0	1	0	0	0	0	0	7	10	10	1	10	0
Roseomonas sp.	1	1	0	0	0	0	0	0	0	1	0	0	1	0
Sphingomonas sp.	3	0	0	0	0	0	0	0	0	3	0	3	1	0
Stenotrophomonas sp.	3	0	0	0	0	0	0	0	3	3	1	3	3	3
Streptococcus sp.	22	1	0	0	0	0	0	0	5	3	1	0	8	16
Not determined	13	1	0	0	0	0	0	0	5	9	5	6	6	5
Total	84	8	1	0	4	2	0	0	22	46	19	20	47	31
Cefotaxime anaerobiosis[§]														
Acinetobacter sp.	2	0	0	0	0	0	0	0	0	0	0	0	0	0
Bifidobacterium sp.	3	1	0	0	0	0	0	0	0	1	0	0	0	3
Campylobacter sp.	9	0	0	0	0	0	0	0	1	1	0	1	7	1
Capnocytophaga sp.	3	0	0	0	3	0	0	0	0	3	0	0	0	3
Dialister sp.	1	0	0	0	0	0	0	0	0	0	0	1	1	1
Klebsiella sp.	2	0	2	0	0	0	0	0	2	2	0	1	2	0

Table 3 (continued)

Genera	Antimicrobial resistance													
	N	*bla*$_{TEM}$	*bla*$_{SHV}$	*bla*$_{CTX-M}$	*bla*$_{CfxA}$	*bla*$_{CepA/CblA}$	*bla*$_{OXA}$	*bla*$_{ampC}$	TET	CTX/AMX	CHL	STR	ERY	KAN
Morganella sp.	1	1	0	0	0	0	0	0	1	1	0	1	1	1
Olsenella sp.	2	0	0	0	0	0	0	0	0	1	0	2	0	0
Peptostreptococcus sp.	6	2	0	0	0	0	0	0	2	1	0	2	3	4
Prevotella sp.	107	11	0	0	70	3	0	0	33	75	5	28	42	81
Pseudomonas sp.	6	0	0	0	0	0	0	0	2	3	2	0	3	4
Rothia sp.	2	2	0	0	0	0	0	0	0	0	1	1	0	1
Serratia sp.	4	0	0	0	0	0	0	0	3	3	1	2	3	1
Staphylococcus sp.	11	3	0	0	4	0	0	0	4	5	4	3	9	5
Stenotrophomonas sp.	2	0	0	0	0	0	0	0	0	0	0	0	0	0
Streptococcus sp.	47	2	1	0	5	0	0	0	22	8	5	11	27	43
Veillonella sp.	74	19	0	0	7	1	0	0	12	16	4	39	69	43
Not determined	11	2	0	0	0	0	0	0	3	1	0	4	3	3
Total	293	43	3	0	89	4	0	0	85	121	22	96	170	194
Cefotaxime microaerophile[¶]														
Acinetobacter sp.	7	0	0	0	0	0	0	0	4	4	3	5	5	3
Capnocytophaga sp.	10	0	0	0	8	0	0	0	0	2	0	1	0	10
Corynebacterium sp.	2	1	0	0	0	0	0	0	0	0	2	0	2	2
Dermacoccus sp.	6	0	0	0	0	0	0	0	3	0	2	2	3	2
Haemophilus sp.	1	0	0	0	0	0	0	0	1	0	1	0	1	1
Janibacter sp.	2	2	0	0	0	0	0	0	0	0	0	0	0	0
Micrococcus sp.	27	3	0	0	0	0	0	0	0	0	5	9	7	4
Neisseria sp.	13	3	0	0	0	0	0	0	4	2	0	1	11	1
Paenibacillus sp.	2	0	0	0	0	0	0	0	0	0	0	0	2	0
Pseudomonas sp.	11	0	0	0	0	1	0	0	5	8	7	3	10	2
Roseomonas sp.	2	0	0	0	0	0	0	0	0	0	0	0	2	0
Rothia sp.	8	2	0	0	1	0	0	0	0	0	0	0	0	0
Sphingomonas sp.	2	1	0	0	0	0	0	0	0	2	0	2	2	0
Staphylococcus sp.	8	1	0	0	1	0	0	0	2	4	0	0	2	1
Stenotrophomonas sp.	2	0	0	0	0	0	0	0	2	2	0	2	2	2
Streptococcus sp.	55	7	0	0	4	0	0	0	18	1	1	9	28	32
Veillonella sp.	2	1	0	0	0	0	0	0	2	2	2	2	2	2
Not determined	19	0	0	0	1	0	0	0	5	4	5	5	11	7
Total	179	21	0	0	15	1	0	0	46	31	28	41	90	69

[†] Amoxicillin-resistant isolates grown in anaerobic conditions. [‡] Amoxicillin-resistant isolates grown in microaerophilic conditions. [§] Cefotaxime-resistant isolates grown in anaerobic conditions. [¶] Cefotaxime-resistant isolates grown in microaerophilic conditions

TET tetracycline, *CTX* cefotaxime, *AMX* amoxicillin, *CHL* chloramphenicol, *STR* streptomycin, *ERY* erythromycin, *KAN* kanamycin

Table 4 Number of isolates resistant to other antimicrobials

	MDR isolates				
N° of antibiotics	CRIA	CRIM	ARIA	ARIM	Total
0	47	46	37	8	138
1	37	56	16	24	133
2	70	29	33	26	158
3	73	23	49	9	154
4	39	11	28	9	87
5	26	6	18	2	52
6	1	8	0	6	15
Total	293	179	181	84	737

CRIA cefotaxime-resistant isolates in anaerobic conditions, *CRIM* cefotaxime-resistant isolates in microaerophilic conditions, *ARIA* amoxicillin-resistant isolates in anaerobic conditions, *ARIM* amoxicillin-resistant isolates in microaerophilic conditions, *MDR* multidrug-resistant

genus. This genus has been previously associated with the bla_{CfxA} gene, acquiring resistance to a variety of penicillins and cephalosporins [54]. As previously reported by other studies [12, 13, 55], we observed a high prevalence of bla_{CfxA} in isolates of the *Capnocytophaga* genus (83.3%). To our knowledge, this is the first report of bla_{CfxA} in the *Staphylococcus*, *Alloprevotella*, *Streptococcus*, and *Veillonella* genera. Since oral biofilm is a favorable environment for horizontal gene transfer [56, 57], it might be possible for bla_{CfxA} to have been transferred from the *Prevotella* or *Capnocytophaga* genera, which are usual carriers of the gene [13]. The bla_{TEM} gene was found in 23.1% of the samples and in 12.9% of the isolates, mainly in *Veillonella* spp. and *Prevotella* spp. The prevalence of this gene in our samples was low when compared to the study conducted by Ioannidis et al. which observed a prevalence of between 46.2 and 72.7% of bla_{TEM} in subgingival and tongue samples from Greek subjects [58]. These differences might be related to the higher consumption of β-lactams by the Greek population according to the European Centre for Disease Prevention and Control [59], or methodologically related, since the detection of bla_{TEM} was done from a pool of subgingival bacteria in each sample, regardless of their resistance patterns or ability to grow in isolation. The detection of the genes bla_{TEM}, bla_{SHV}, and bla_{CfxA} in streptococci is noteworthy, given that there is some controversy about the presence of *bla* genes in this genus. Although it has been suggested that streptococci are unable to acquire foreign *bla* genes [60], at least two studies have reported the presence of these genes in *Streptococcus pneumoniae* [61, 62]. The detection of bla_{TEM} by Ding et al. was questioned due to possible contamination of the Taq polymerase used in the reaction [63]; however, in our study, the negative controls did not suggest the presence of any contamination. Therefore, it would be interesting to conduct further studies to analyze the presence of *bla* genes among oral streptococci. Both $bla_{CepA/CblA}$ and bla_{SHV} were found in low numbers as observed by the previous studies [13, 43, 64], suggesting that they may not play a critical role in β-lactam resistance in the oral environment.

With the data obtained in this study, we conclude that β-lactam resistance is widespread among the subgingival bacteria of Spanish patients with periodontitis. *Prevotella*, *Veillonella*, and *Streptococcus* were the genera with the highest number of β-lactam-resistant isolates, suggesting that in this population, oral commensal microbiota might be a reservoir of β-lactam resistance. Of special importance is the presence of β-lactamases that are coded in transferrable genes such as bla_{CfxA} and bla_{TEM}, which could transfer to other oral or transient bacteria. Moreover, a high prevalence of MDR was observed, constraining the number of antibiotics available against bacterial infections, where these to be needed. Despite the reports warning about increasing antimicrobial resistance, antimicrobials are usually prescribed in the clinical practice without studying microbial profiles and without performing antibiograms, a practice that favors the spread of antimicrobial resistance. The above, together with the high percentages of β-lactam resistance observed in this study, underlines the risk of a currently successful antibiotic treatment becoming ineffective.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the Universitat Internacional de Catalunya research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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