Int. J. Odontostomat.,
7(2):193-198, 2013.

Antimicrobial Susceptibility to b-lactams and Metronidazole of Microorganisms Isolated from Chronic and Aggressive Periodontitis

Susceptibilidad Antimicrobiana a b-lactámicos y Metronidazol de Microorganismos Aislados de Periodontitis Crónica y Agresiva

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GAETTI-JARDIM JÚNIOR, E. & SCHEITZER, C. M. Antimicrobial susceptibility to b-lactams and metronidazole of microorganisms isolated from chronic and aggressive periodontitis. ***Int. J. Odontostomat., 7(2)***:193-198, 2013.

ABSTRACT: The objective of this study was to evaluate the microbial susceptibility to ß-lactams and metronidazole, and evaluate the production of ß-lactamases by microorganisms isolated from patients with chronic or aggressive periodontitis. The samples were obtained from 50 patients with periodontitis and microorganisms were isolated onto selective and nonselective culture media, identified by biochemical methods and tested for susceptibility to antimicrobial agents (amoxicillin, amoxicillin/clavulanate, cefoxitin, imipenem, metronidazole, penicillin G). The isolates were resistant to at least 1 mg/ml of any drug tested were evaluated to verify the production of ß-lactamases by the method of double layer (or biological) and chromogenic cephalosporin using nitrocefin. The results evidenced resistance to amoxicillin and penicillin G, while the susceptibility to association amoxicillin/clavulanate, imipenem and cefoxitin was widely disseminated among the organisms. Resistance to these drugs showed a clear correlation with the production of ß-lactamase in the majority of microbial groups.

KEYWORDS: periodontitis, antimicrobials, b-lactams, bacteria, metronidazole.

INTRODUCTION

Periodontal diseases represent serious health problems and affect all ethnic groups, regardless gender and socio-economic conditions of the population. They result from the loss of equilibrium between the immune response and virulence factors of the resident microbiota. The control of the oral biofilm is a prerequisite to the health maintenance in the periodontium (Eick *et al*., 2011). In general, antimicrobial drugs are not indicated as monotherapy for patients with periodontitis, but some aggressive forms of periodontitis may benefit with adjunctive use of systemic antimicrobials (Ahuja *et al*., 2012).

Several microorganisms have been implicated as pathogens in periodontitis, such as Porphyromonas gingivalis, Prevotella intermedia, P. nigrescens, Tannerella forsythia, spirochetes, Aggregatibacter actinomycetemcomitans and Fusobacterium nucleatum, frequently associated with other Gram-positive and Gram-negative bacteria (López *et al*., 2011).

Antimicrobials and antibiotics used during treatment of patients unable to maintain oral hygiene, mentally or physically handicapped, suffering from dental and/or surgical trauma, and with refractory, aggressive or advanced periodontitis have been prescribed, but most of these compounds have several side effects. Moreover, the widespread use of antimicrobial agents against oral anaerobes has been associated with a significant increase of the antimicrobial resistance, particularly against b-lactams (Senhorinho *et al*., 2012). In addition, most of microorganisms recovered from periodontal sites are strict anaerobes and culture and susceptibility tests of anaerobes are not frequently performed in Brazil, what constitutes a serious problem since clinicians have to choose antimicrobials just using international literature.

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Grants: Fundação de Amparo à Pesquisa do Estado de São Paulo, FAPESP. Process number: 2007/51016-3.

Thus, the aim of this study was to evaluate the susceptibility of microorganisms isolated from chronic and aggressive periodontitis to b-lactams and metronidazole, and evaluate the production of b-lactamases.

MATERIAL AND METHOD

Patients. This study was approved by the Ethics Committee in Research of the School of Dentistry of Araçatuba. The population of this study consisted of 50 patients with clinical and radiographic characteristics of chronic periodontitis, seen at the School of Dentistry of Araçatuba and private clinics for initial trial for treatment. Patient ages ranged from 26 to 59 years (mean 43.3). Additionally, 8 patients with clinical and radiographic features of aggressive periodontitis, aged from 4.5 to 13 years were included. Medical data revealed that all patients were in good general health. The diagnostic and evaluation of the periodontal status of the patients were carried out by specialists in periodontology and pediatrics.

During the first visit, written informed consent was obtained from each patient or their responsible before enrolment in the study. Patients who had received antibiotics 6 months before the initial clinical trial were excluded. The clinical samples were collected from the 3 deepest non-contiguous periodontal sites (probing depth ≥ 5 mm) presenting clinical characteristics of inflammation and loss of attachment. Sample collection was performed by mean of sterile paper points, which were introduced into periodontal pockets and remained there for 30s. After this procedure, the paper points were pooled and transferred to transport medium VMGA III (Gaetti-Jardim *et al.*, 2012).

Microbial isolation and identification. In the laboratory, aliquots of VMGA III were submitted to serial 10 fold dilutions in VMG I and were plated on fastidious anaerobic agar (FAA) supplemented with yeast extract (0.5%), hemin (1 mg/mL), menadione (5 mg/mL) and 5% horse blood, tryptic soy serum bacitracin vancomycin agar (TSBVA), and incubated in anaerobiosis (90% N2 + 10% CO_2), at 37ºC, for 14 and 3 days, respectively. Pure cultures were obtained and the isolates were subjected to microbial speciation. Initially, the isolates were subjected to Gram staining, evaluation colony morphology on blood agar plates, identification by biochemical tests and amplification of DNA by PCR (Gaetti-Jardim *et al*.).

A total of 187 isolates of strict and facultative anaerobic bacteria were submitted to the susceptibility tests. Susceptibility to antimicrobial agents was evaluated on 21 isolates of *Aggregatibacter actinomycetemcomitans*, 11 isolates of *Bacteroides sp.,* 13 isolates of *Eubacterium sp*., 3 isolates of *E. lentum*, 4 isolates of *Fusobacterium sp.*, 33 isolates of *F. nucleatum*, 5 *F. periodonticum*, 12 *Parvimonas micra*, 9 *Peptostreptococcus sp.*, 4 isolates of *P. anaerobius*, 10 *Porphyromonas sp*., 23 *Porphyromonas gingivalis,* 5 *Prevotella sp.*, 16 *P. intermedia*, 7 *P. nigrescens*, and 11 isolates of *Veillonella sp.*

Antimicrobial susceptibility tests. The minimal inhibitory concentrations were determined by agar dilution method using Wilkins-Chalgren agar supplemented with horse blood, hemin (1 mg/mL), and menadione (5 mg/mL). The bacterial inoculum was standardized in 105 cells/spot and transferred to the Petri plates containing the antimicrobials and the control plates (without drugs), using a Steer`s replicator (Cefar Ltda, São Paulo, Brazil). The minimum inhibitory concentration (MIC) was defined as the smallest concentration of the drug that completely inhibited the bacterial growth. The following antimicrobial drugs were tested: amoxicillin (Bayer S.A., São Paulo, Brazil), amoxicillin/clavulanate (Smithkline Beecham Brasil Ltda, São Paulo, Brazil), cefoxitin (Merck Sharp & Dohme, São Paulo, Brazil), imipenem (Merck Sharp & Dohme, São Paulo, Brazil), benzylpenicillin (Fontoura-Wyeth S.A., São Paulo, Brazil) and metronidazole (Laboratórios Pfizer Ltda, Guarulhos, Brazil). The breakpoints used for interpretation were those recommended by the Clinical and Laboratory Standards Institute (2007). The test and control dishes were incubated anaerobically (90% N2 + 10% CO2) at 37ºC, for 48 hours.

F. nucleatum ATCC 10953, *F. nucleatum* ATCC 25586, *E. lentum* ATCC 43055, *B. fragilis* ATCC 23745, *E. coli* ATCC 25922, *S. aureus* ATCC 29213, *P. aeruginosa* ATCC 27853, and *E. faecalis* ATCC 29212 were used as reference strains for quality control in the susceptibility tests.

Detection of b-lactamases. All the microorganisms able to resistant to at least 1,0 mg/mL of the tested b-lactms were submitted to assays to evaluate the production of b-lactamases, using biological and chromogenic cephalosporin methods (Gaetti-Jardim *et al.,* 2007). The tested strains included 9 *Prevotella*

spp., 2 *Peptostreptococcus spp*., 12 fusobacteria, and 2 *Bacteroides sp*. A chromogenic cephalosporin b-lactamase assay using Cefinase disks was performed according to the manufacturer's instructions.

In the biological method, 20 μL of cultures of the resistant isolate were plated on the surface of Wilkins-Chalgren agar, containing 1,0 μg/mL of the tested b-lactam (benzylpenicillin or cefoxitin). These plates were then incubated anaerobically at 37°C for 48-72 h. After incubation, the cultures were exposed to chloroform fumes for 20 min and then covered with 3 mL of semi-solid brain heart infusion (BHI) agar previously inoculated with 106 cells of *Streptococcus pyogenes* FOA-94F14, sensitive to all tested b-lactams in a concentration of ≤ 0.06 mg/mL. The Petri dishes were then incubated under aerobiosis for 24 h at 37°C. After incubation, the presence of this halo of growth was indicative of the degradation of the b-lactam. Bacteroides fragilis ATCC 43858 was used as the positive control of b-lactamase production.

RESULTS

It was not verified any difference in the susceptibility patterns of the isolates recovered from chronic or aggressive periodontitis and the results are presented regardless the source of the clinical samples, in Tables I and II. All strains tested were susceptible to amoxicillin/clavulanate and imipenem, but variable levels of resistance to amoxicillin, benzylpenicillin, and cefoxitin were detected. Resistance to b-lactams was frequently detected in Gram-negative strict anaerobes, particularly in the genera *Fusobacterium, Prevotella* and *Bacteroides*, although some anaerobic cocci also evidenced resistance to these drugs. Resistance to metronidazole was observed in *A. actinomycetemcomitans* (9.5%); in addition, 19% of A. actinomycetemcomitans and 8% of *Peptostreptococcus spp.* presented intermediate susceptibility to this antimicrobial agent.

Out of 25 bacterial isolates resistant or presenting intermediate susceptibility to to b-lactams, 20 (80%) were b-lactamase producers (10.7% of the isolated bacteria). The production of these hydrolytic enzymes seems to be the major mechanism of resistance to b-lactams, excluding Bacteroides sp. and peptostreptococci, where b-lactamases were not detected (Table II).

DISCUSSION

The effectiveness of the antimicrobials in the treatment of mixed anaerobic infections of the head and neck is undermined by empirical selection of drugs and the problem of self-medication. In addition, microorganisms producing b-lactamases are frequently detected in dental biofilm from childhood to adulthood particularly in infectious processes (Fosse *et al*., 2002; Kuriyama *et al*., 2002).

The strict anaerobes play a major role in the pathogenesis of chronic or aggressive periodontitis and the systemic use of antimicrobial agents may improve the treatment of such infections when associated to local procedures and modification of oral hygiene standards. Moreover, periodontitis is a reservoir of opportunistic pathogens and their eradication depends on use of antimicrobials, particularly b-lactams (Ahuja *et al*.), while metronidazole is the frequently used in the therapy of anaerobic mixed infections, specially due to its action on Gram-negative anaerobes associated with oral infections, and its association with amoxicillin has been evaluated in the treatment of aggressive periodontitis (Casarin *et al*., 2012).

Most studies that deal with the emergence of antimicrobial resistance of oral bacteria have been done in the developed countries and little information is available from south hemisphere (Gaetti-Jardim Júnior *et al.*, 2007). In addition, antimicrobial resistance has expanded considerably, which has rendered traditional prescription habits critical in the absence of laboratorial tests (Baumgartner & Xia, 2003). This fact is further aggravated when one considers the relevance of the phenomenon of self-medication, which limits the efficacy of treatment and can make prescribing a difficult task, as it modifies the local patterns of susceptibility to drugs.

The resistance to amoxicillin and benzylpenicillin was similar to previously reported in the literature from developing countries (Brescó-Salinas *et al.*, 2006; Gaetti-Jardim Júnior *et al*., 2007) and evidenced that most of resistant isolates belonged to a peculiar group of Gram Clinical and Laboratory Standards Institute Clinical -negative strict anaerobes, as also reported to bacteria isolated from endodontic infections (Gaetti-Jardim Júnior *et al.*, 2007). The bacteria that accounted for the significantly increased proportion of resistance to amoxicillin and penicillin G in clinical samples were Gram-negative rods, susceptible to metronidazole. The results of Table II suggest that b-lactamases from

Table I. Susceptibility to antimicrobials of 187 clinical isolates recovered from chronic and aggressive periodontitis.

Isolates (N)	%			MIC(µg/mL)		
	S	I	R	range	MIC_{50}	MIC_{90}
A. actinomycetemcomitans (21)						
amoxicillin	100	0.0	0.0	≤0.006-4	0.25	4
amoxicillin/clavulanate	100	0.0	0.0	≤0.006-1	0.25	1
benzylpenicillin	100	0.0	0.0	≤0.006-4	0.25	4
cefoxitin	100	0.0	0.0	≤0.006-2	0.125	1
imipenem	100	0.0	0.0	≤0.006-4	0.125	0.5
metronidazole	71.4	19.0	9.5	≤0.006-64	0.125	4
Bacteroides sp. (11)						
amoxicillin	81.8	0.0	18.2	≤0.006-128	0.25	128
amoxicillin/clavulanate	100	0.0	0.0	≤0.006-1	0.25	1
benzylpenicillin	81.8	0.0	18.2	≤0.006-256	0.25	256
cefoxitin	81.8	0.0	18.2	≤0.006-32	0.125	32
imipenem	100	0.0	0.0	≤0.006-4	0.125	4
metronidazole	100	0.0	0.0	≤0.006-2	0.125	2
Eubacterium spp. (16)						
amoxicillin	100	0.0	0.0	≤0.006-0.25	≤0.006	0.25
amoxicillin/clavulanate	100	0.0	0.0	≤0.006-0.25	≤0.006	0.25
benzylpenicillin	100	0.0	0.0	≤0.006-0.5	≤0.006	0.5
cefoxitin	100	0.0	0.0	≤0.006-0.5	≤0.006	0.5
imipenem	100	0.0	0.0	≤0.006-0.5	≤0.006	0.125
metronidazole	100	0.0	0.0	≤0.006-1	≤0.006	1
Fusobacterium spp. (42)						
amoxicillin	73.8	9.5	16.7	≤0.006-256	0.125	32
amoxicillin/clavulanate	100	0.0	0.0	≤0.006-0.5	≤0.006	0.5
benzylpenicillin	71.4	11.9	16.7	≤0.006-256	0.125	32
cefoxitin	97.6	2.4	0.0	≤0.006-8	≤0.006	8
imipenem	100	0.0	0.0	≤0.006-0.5	≤0.006	0.5
metronidazole	100	0.0	0.0	≤0.006-0.5	≤0.006	0.25
[1] *Peptostreptococcus* spp. (25)						
amoxicillin	92	8	0.0	≤0.006-16	≤0.006	0.5
amoxicillin/clavulanate	100	0.0	0.0	≤0.006-0.5	≤0.006	0.5
benzylpenicillin	88	8	4	≤0.006-16	≤0.006	0.5
cefoxitin	100	0.0	0.0	≤0.006-2	≤0.006	1
imipenem	100	0.0	0.0	≤0.006-0.5	≤0.006	0.5
metronidazole	92	8	0.0	≤0.006-16	0.125	1
Porphyromonas spp. (33)						
amoxicillin	100	0.0	0.0	≤0.006-0.25	≤0.006	0.25
amoxicillin/clavulanate	100	0.0	0.0	≤0.006-0.25	≤0.006	0.25
benzylpenicillin	100	0.0	0.0	≤0.006-0.5	≤0.006	0.5
cefoxitin	100	0.0	0.0	≤0.006-0.5	≤0.006	0.5
imipenem	100	0.0	0.0	≤0.006-0.25	≤0.006	0.25
metronidazole	100	0.0	0.0	≤0.006-0.25	≤0.006	0.25
Prevotella spp. (28)						
amoxicillin	71.4	14.3	14.3	≤0.006-256	0.25	128
amoxicillin/clavulanate	96.4	3.6	0.0	≤0.006-8	0.125	2
benzylpenicillin	67.9	17.9	14.3	≤0.006-512	0.25	128
cefoxitin	78.6	7.1	14.3	≤0.006-128	0.5	64
imipenem	100	0.0	0.0	≤0.006-1	≤0.006	1
metronidazole	100	0.0	0.0	≤0.006-0.5	≤0.006	0.5
Veillonella sp. (11)						
amoxicillin	100	0.0	0.0	≤0.006-2	0.25	1
amoxicillin/clavulanate	100	0.0	0.0	≤0.006-0.25	≤0.006	0.25
benzylpenicillin	100	0.0	0.0	≤0.006-2	0.25	1
cefoxitin	100	0.0	0.0	≤0.006-0.5	≤0.006	0.5
imipenem	100	0.0	0.0	≤0.006-0.25	≤0.006	0.25
metronidazole	100	0.0	0.0	≤0.006-0.25	≤0.006	0.25

[1]Including *Parvimonas micra.*

Table II. Production of b-lactamases by oral microorganisms isolated from chronic or aggressive periodontitis.

*Taxon (N)	Production of β–lactamases N (%)
Bacteroides sp. (2)	0 (0.0)
Fusobacterium spp. (12)	12 (100)
Peptostreptococcus spp. (2)	0 (0.0)
Prevotella spp. (9)	8 (88.9)
Total (25)	20 (80)

*Isolates presenting resistance or intermediate susceptibility to the tested *b-lactams.*

anaerobic Gram-negative bacteria are active on penicillins and cephalosporins, as also described by Wybo *et al*. (2007) and Gaetti-Jardim Júnior *et al*. (2007).

The association of amoxicillin/clavulanate and imipenem were the most effective b-lactams against all tested isolates, including amoxicillin, penicillin G and metronidazole resistant microorganisms. This effectiveness is related to the action of clavulanate in inhibiting most b-lactamases of oral microorganisms (Gaetti-Jardim Júnior *et al*., 2007) and the stability of carbapenems against most frequent b-lactamases. Although imipenem-hydrolyzing class D b-lactamase has been identified, the present investigation evidenced that all b-lactamase producers were highly susceptible to this carbapenem. However, due to its effectiveness on pseudomonads and other resistant microorganisms, the use of carbapenems should be restrict to severe infections, particularly life-threatening diseases, to avoid dissemination of resistance (Gaetti-Jardim Júnior *et al*., 2007).

In the present investigation, some isolates that are resistant to b-lactams were not to be producers of b-lactamase by the methodology employed in this study (Table II), suggesting suggests that these microorganisms are producers of non-exportable b-lactamases, as previously reported in Gram-negative bacteria (Handal *et al*., 2004; Gaetti-Jardim Júnior *et al*., 2007).

CONCLUSION

The results of this study evidenced that resistance to b-lactams among microorganisms isolated from aggressive and chronic periodontitis is limited to a few genera of strict obligate anaerobes, which were highly susceptible to metronidazole. Imipenem and the association of amoxicillin and clavulanate may overcome the problem of resistance to b-lactams. The resistance to metronidazole was restricted to some isolates of A. actinomycetemcomitans, a facultative rod.

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RESUMEN: El objetivo fue evaluar la susceptibilidad a ß-lactámicos y metronidazol, y evaluar la producción de ß-lactamasas por microorganismos aislados de pacientes con periodontitis crónica y agresivo. Las muestras fueron obtenido de 50 pacientes con periodontitis y microorganismos aislados en medios de cultivo selectivos y no selectivos, identificados por métodos bioquímicos y probados a la susceptibilidad a los antimicrobianos (amoxicilina, amoxicilina/clavulanato, cefoxitina, imipenem, metronidazol, penicilina G). Los aislados fueron resistentes a por lo menos 1 mg/ml de cualquier drogas analizadas fueron evaluados para verificar la producción de ß-lactamasas por el método de doble capa (o biológico) y nitrocefina. Los resultados mostraron resistencia a amoxicilina y penicilina G, mientras la susceptibilidad a la asociación amoxicilina/clavulanate, cefoxitina y imipenem fue ampliamente difundido entre los microrganismos. Resistencia a estas drogas mostraron una clara correlación con la producción de ß-lactamasas en la mayoría de grupos microbianos.

PALABRAS CLAVE: periodontitis, antimicrobianos, b-lactámicos, bacteria, metronidazol.

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Received: 07-11-2012
Accepted: 22-03-2013