

ANTIBIOTIC RESISTANCE OF PERIODONTAL PATHOGENS OBTAINED FROM FREQUENT ANTIBIOTIC USERS

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ABSTRACT

The occurrence of bacterial strains resistant to different antimicrobials is a growing problem in Latin American countries. The aim was to measure the antimicrobial susceptibility of Fusobacterium nucleatum, Prevotella intermedia and Propyromona gingivalis, isolated from chronic periodontitis patients.

Twenty-five patients diagnosed with generalized- moderate or advanced- chronic periodontitis were consecutively recruited from patients attending the Periodontal Specialist Program, Javeriana University, according to specific criteria, including no recent antibiotic consumption. All patients filled out a questionnaire on antibiotic intake. The deepest periodontal pocket in each quadrant was sampled. Pooled samples were mixed, diluted and plated on enriched brucella agar plates. After anaerobic incubation, identification of the target bacteria was performed, based on colony morphology, gram staining, aero-tolerance and biochemical reactions (RapID Ana II, Remel, U.S.A.). Following identification, the bacteria were subjected to antimicrobial testing, using amoxicillin, tetracycline, doxy-

cycline, azithromycin and metronidazole (E-test, AbBiodisk, Sweden). The minimal inhibitory concentrations obtained were compared with a reference standard to determine antimicrobial resistance. Amoxicillin-resistant species were tested for β -lactamase production.

Forty-four percent of the patients used antibiotics without any medical prescription, 40% used antibiotics at least once a year. The presence of eleven species was confirmed after a series of biochemical tests: four Fusobacterium nucleatum, five Prevotella intermedia and two Propyromona gingivalis. All strains were resistant to metronidazole, five were resistant to tetracycline and azithromycin, and two strains were resistant to doxycycline and amoxicillin. The strains resistant to amoxicillin were positive for β -lactamase production.

Antimicrobial resistance, particularly against metronidazole, was a common phenomenon for the bacterial isolates analyzed in this Colombian sample.

Key words: drug resistance, bacterial, chronic periodontitis.

RESISTENCIA A ANTIBIÓTICOS DE PERIODONTOPATÓGENOS AISLADOS DE UNA MUESTRA DE SUJETOS CON USO FRECUENTE DE ANTIMICROBIANOS

RESUMEN

La presencia de especies bacterianas que demuestran resistencia a múltiples antimicrobianos es un problema creciente en Latinoamérica. El objetivo de este estudio fue el medir la susceptibilidad antibiótica de las bacterias Fusobacterium nucleatum, Prevotella intermedia y Propyromona gingivalis aisladas de pacientes afectados por Periodontitis Crónica.

Un total de venti-cinco pacientes diagnosticados con Periodontitis Crónica Generalizada con severidad de Moderada a Avanzada fueron incluidos en el estudio siguiendo criterios específicos, todos los pacientes respondieron una encuesta acerca de su patrón de uso de antibióticos. En la bolsa más profunda de cada cuadrante dental se tomó una muestra microbiológica, la cual fue procesada siguiendo procedimientos de: Mezcla, dilución y siembra en platos de agar Brucela enriquecidos. Después de un periodo de incubación anaeróbica las bacterias objeto de estudio fueron identificadas de acuerdo a las siguientes técnicas: Morfología de las colonias, coloración de Gram, prueba de aero-tolerancia y la aplicación de un kit de identificación bacteriana que utiliza diversas pruebas bioquímicas (RapID Ana II, Remel, U.S.A.). Después de la identificación, las bacterias fueron sometidas a pruebas de antibiograma usando los siguientes antibióticos: Amoxicilina,

tetraciclina, doxiciclina, azitromicina y metronidazol (E-test, AbBiodisk, Suecia). La concentración inhibitoria mínima se comparó con la referencia estándar para determinar la presencia de la resistencia antibiótica. En las especies bacterianas que mostraron resistencia a la amoxicilina se realizó una prueba de laboratorio para establecer la presencia de β -lactamasa. Un 44% de los pacientes encuestados refirió haber utilizado antibióticos sin prescripción médica, un 40% de ellos usa antibióticos al menos una vez al año. La presencia de once especies bacterianas pudo ser confirmada después de realizar las pruebas de identificación: Cuatro aislamientos de Fusobacterium nucleatum, cinco de Prevotella intermedia y dos de Propyromona gingivalis. Todas las especies bacterianas mostraron resistencia al metronidazol, cinco fueron resistentes a la tetraciclina y a la azitromicina, finalmente dos especies fueron resistentes a la doxiciclina y la amoxicilina. Las especies que mostraron resistencia a la amoxicilina demostraron producción de β -lactamasa.

La presencia de resistencia a los antibióticos fue un fenómeno común para las bacterias analizadas, en especial la resistencia al metronidazol.

Palabras clave: resistencia a los antibióticos, bacterias, periodontitis crónica.

INTRODUCTION

The development of bacterial strains resistant to antibiotics in Latin America has been considered a threat to the treatment of common infections¹, this has led to the creation of surveillance programs for respiratory, intestinal, genito-urinary and hospital-acquired pathogens. In Colombia, a varying level of antibiotic resistance to different medical pathogens has been reported; for example, 8.5% of *Neisseria meningitidis*, and 12.1% of *Pneumococcus* isolates were resistant to penicillin^{2,3}. Community resistant microorganisms could be the result of the extensive use of antibiotics in hospitals, surgical prophylaxis and treatment of nosocomial infections; but more importantly self-medication and the lack of policies regarding drug selling⁴. Several mechanisms of resistance have been described in the literature; mainly drug target alterations, bacterial membrane resistance, efflux pumps and enzymatic modification and destruction of antibiotics by processes such as β -lactamase production⁵. It is accepted that traditional periodontal therapy consisting of subgingival debridement in conjunction with supragingival plaque control is effective in controlling the clinical signs of periodontal pathology⁶. Nevertheless, the use of antibiotics as an adjunct to scaling and root planing and surgical procedures is reported to offer additional benefits in terms of probing pocket depth reduction and clinical attachment level gains^{7,8}. Disinfection of periodontal pockets is relevant, since the main cause of disease recurrence has been identified as the recolonization by periodontal pathogens⁹. Commonly used antibiotics in periodontal therapy include amoxicillin, metronidazole, tetracycline and doxycycline. A thorough understanding of prevalence and mechanisms for antibiotic resistance would allow more effective treatment planning for the periodontal patient.

The purpose of this study was to determine the prevalence of antibiotic resistance to different antibiotics of three targeted periodontal pathogens (*Porphyromonas gingivalis*, *Prevotella intermedia* and *Fusobacterium nucleatum*) isolated from chronic periodontitis patients. Bacterial strains resistant to amoxicillin were tested for presence of β -lactamase.

MATERIALS AND METHODS

Subject population

This cross-sectional descriptive study included twenty-five patients diagnosed with generalized

chronic periodontitis, according to the periodontal disease classification¹⁰; subjects were consecutively recruited from the new patients seeking treatment at the Periodontal Specialist Program, School of Dentistry, Javeriana University. The study was approved by a university ethics committee. After being informed of the objectives and procedures of the study, all participants signed an informed-consent form. The criteria for inclusion in the study were: age ≥ 25 years, at least 15 natural teeth, loss of clinical attachment level ≥ 3 mm in all jaw quadrants, and presence of at least two periodontal pockets ≥ 6 mm deep in all jaw quadrants. Patients were excluded if they had had any previous periodontal treatment, had taken any antibiotics during the previous three months, were pregnant or breastfeeding. All patients had a complete periodontal examination at baseline, and filled out a survey regarding antibiotic consumption, including frequency of intake, causes for consumption, antibiotic most frequently taken and self-medication.

Microbiological sampling

After cotton roll isolation and removal of supragingival plaque, pooled microbial samples were taken with paper cones from periodontal pockets ≥ 6 mm in each quadrant for 15 seconds. Samples were placed in vials containing anaerobic VMGA III transport medium and transferred for processing.

Processing of microbial samples

Samples were vortex-mixed for 30 seconds and serially ten-fold diluted five times. 100 μ L of 10^{-3} and 10^{-4} dilutions were plated on Brucella agar (Brucella agar, bacto agar, yeast extract, 5% defibrinated sheep's blood, 5 μ g/mL haemin and 0.3 μ g/mL menadione). Brucella plates were incubated for 7 days at 37°C in 80% N₂, 10% CO₂ and 10% H₂. Presumptive identification of bacteria was performed based on colony morphology, Gram staining, aerotolerance test and a commercial identification micromethod system (RapID ANA II, Remel, Norcross, USA) for *Porphyromonas gingivalis*, *Prevotella intermedia* and *Fusobacterium nucleatum*.

Antimicrobial susceptibility

Selected colonies of *Porphyromonas gingivalis*, *Prevotella intermedia* and *Fusobacterium nucleatum* taken from pure cultures were used for antimicrobial

Table 1: Antibiotic Resistance of Isolated Bacteria.

Level of resistance	Tetracycline		Doxycycline		Azithromycin		Amoxicillin		Metronidazole	
	N	%	N	%	N	%	N	%	N	%
Resistant	5	45.5	2	18.2	5	45.5	2	18.2	11	100
Intermediate			2	18.2			1	9.1		
Sensitive	6	54.5	7	63.6	6	54.5	8	72.7		
Total	11	100	11	100	11	100	11	100	11	100

icrobial susceptibility testing, using five different commercially available antibiotic strips: amoxicillin, metronidazole, azithromycin, tetracycline and doxycycline (E-test, AB Biodisk, Solna, Sweden). Viable colonies were homogenized in 0.85% saline solution and adjusted to the MacFarland turbidity standard of 1.0. Using a sterile glass rod, 0.1 mL of the inoculum was spread over Brucella agar plates and allowed to dry for 15 minutes. E-test strips were gently placed over the agar surface and incubated under anaerobic conditions for 3 days. The intersection between the zone of bacterial inhibition and the E-test strip represented the minimal inhibitory concentration (MIC).

β-lactamase production in amoxicillin-resistant bacteria

All bacterial isolates showing resistance to amoxicillin E-test testing were subjected to a commercially available test for production of β-lactamase (β-lactamase identification cones, Oxoid, United Kingdom). The identification kit is based on a color change reaction for the substrate nitrocefin, a chromogenic cephalosporin, to red in the presence of β-lactamase.

Data analysis

Descriptive statistics including mean and standard deviation for quantitative variables, and frequency distribution for qualitative variables were calculated. The percentage of resistant bacteria from the total number of isolates was reported. All calculations were made using the Windows SPSS software statistical package, version 11.0.

RESULTS

Patient description and antibiotic consumption survey

The mean age of the twenty-five subjects fulfilling the inclusion criteria was 47.2 ± 8 years; there

were 16 males and 9 females. The antibiotic survey revealed that 16 subjects did not use antibiotics every year; whereas the other 9 subjects used antibiotics at least once a year on average. The most common reported reason for antibiotic consumption was a severe cold in 10 subjects, followed by tonsillitis in 6 subjects. The majority of the subjects, 16 patients, used amoxicillin. Finally, eleven subjects reported use of antibiotics without medical prescription.

Identification of targeted bacteria

In four of the twenty-five patients, none of the three targeted bacteria could be identified. A total of thirty-six bacterial isolates fulfilled the presumptive identification criteria and were subjected to analysis using the RapID Ana II system; the results of the confirmatory system were: fourteen samples of *Prevotella intermedia*, four of *Fusobacterium nucleatum*, two of *Porphyromona gingivalis*, six of *Porphyromona endodontalis*, and ten samples in which the biochemical tests indicated a 60/40% probability for *Fusobacterium necrophorum* and *Fusobacterium nucleatum*.

Antibiotic susceptibility results and β-lactamase production

E-test antimicrobial testing was performed on eleven bacterial isolates: five *Prevotella intermedia*, four *Fusobacterium nucleatum* and two *Porphyromona gingivalis*.

It was found that antimicrobial resistance was a common phenomenon among the samples analyzed; all eleven samples were resistant to metronidazole; five out of eleven samples were resistant to tetracycline and azithromycin; two samples were resistant to doxycycline and amoxicillin (Table 1, Fig. 1, Fig. 2). Both bacterial isolates resistant to amoxicillin, one of *Prevotella intermedia* and one of *Fusobacterium nucleatum*, were positive for β-lactamase production.

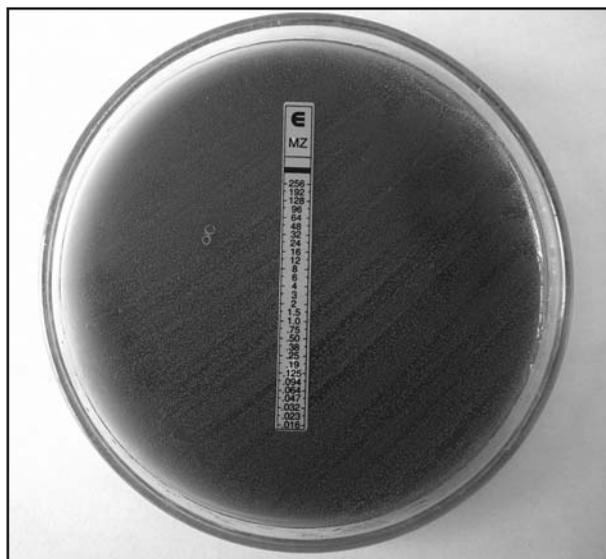


Fig. 1: Microbial isolate showing resistance against metronidazole.

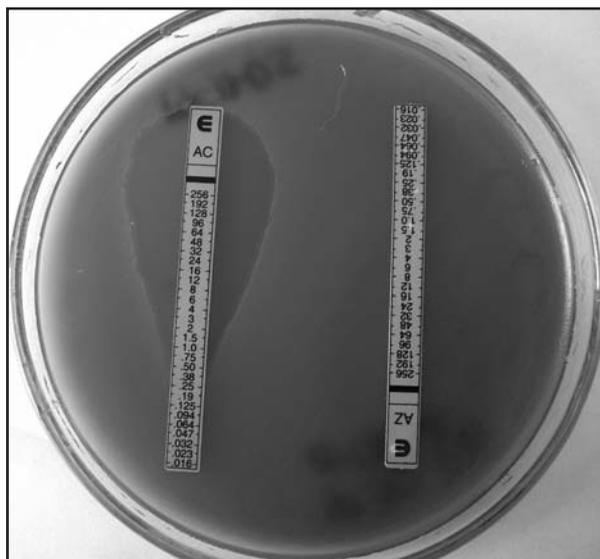


Fig. 2: Microbial isolates showing resistance against azithromycin, and susceptibility to amoxicillin.

DISCUSSION

The studied population was characterized by frequent self-medication with antibiotics, as eleven out of twenty-five patients reported using antibiotics without any medical prescription. This same pattern of medication intake has been referred in the literature¹¹. The Drug Utilization Research Group¹¹ surveyed 8597 individuals in six Latin American countries when seeking medication intended for treatment of several pathologies; the authors found that 66% of the intended-to-buy medicines would be catalogued as prescription-only medicines. An equally large proportion of our patient sample, 44%, mentioned buying antibiotics without any prescription. According to the Research Group¹¹, the second largest group of consumed drugs was antibiotics; 7.4% were antibiotics of the penicillin-group, and the most frequently consumed. Similarly the results of the present study showed that the most frequently used antimicrobial was a penicillin-group antibiotic, amoxicillin. As mentioned by Wolff¹², lack of patient and professional education and stringent policies regarding drug sales can result in antibiotic drug misuse in Latin America.

Few studies have analyzed antibiotic susceptibility in bacterial samples taken from chronic periodontitis patients in Colombia. In the present study, a high level of antibiotic resistance was found against metronidazole, tetracycline and azithromycin. In particular, all bacterial isolates showed resistance

against metronidazole. However, few samples were subjected to antimicrobial testing after a series of confirmatory tests. In contrast, Jaramillo et al.¹³ found a low proportion of antibiotic resistance in periodontal pathogens isolated from periodontal abscesses in Colombia. In that study 9 out of the 29 bacterial isolates showed resistance against tetracycline, metronidazole or amoxicillin, the highest incidence corresponding to resistance against metronidazole. No information about the pattern of antibiotic consumption of the analyzed sample was reported. A similar low percentage of antibiotic resistance to amoxicillin and doxycycline was found by Almaguer-Flores et al.¹⁴ in Mexico, with a maximum resistance of 13.4% and 20.4% at the lowest antibiotic concentration tested. However, antibiotics for which bacterial resistance were found in the present study; metronidazole, azithromycin and tetracycline, were not tested by Almaguer-Flores et al.¹⁴. A report on metronidazole resistance for the elimination of *Helicobacter pylori* by Gutierrez and Otero¹⁵ in Colombia shows a great level of resistance; a total of 84 bacterial samples were analyzed using the E-test, showing that 82% were resistant to metronidazole, even when considering a MIC of 32 µg/ml. The authors hypothesized that the high level of resistance could be due to inadequate use of metronidazole for parasite infections.

In industrialized countries, like the U.S.A., Germany, the Netherlands or Spain, a varying level of antibiotic

resistance has been found. A microbiological comparison between the Netherlands and Spain by van Winkelhoff et al.^{16, 17} showed a higher proportion of antibiotic resistance in Spain; the largest proportion of antibiotic resistance in Spanish patients was to metronidazole with nearly 25%, followed by tetracycline, erythromycin and azithromycin; the least resistance was to amoxicillin and amoxicillin/clavulanic acid. Although showing a higher level of antibiotic resistance, the results of the present study showed a similar trend with a high resistance to metronidazole and tetracycline, and a low resistance to amoxicillin. In the study by van Winkelhoff et al.¹⁶, more than half of the Spanish patients (54.8%) reported using antibiotics during the previous year, a large proportion of patients in our sample reported using antibiotics at least once a year. Studies from U.S.A. and Germany have found a low percentage of antibiotic resistance, with a maximum resistance level of 29-30%^{18, 19, 20}. In the U.S.A., Walker et al.¹⁸ and Walker et al.¹⁹ showed greater antimicrobial susceptibility for penicillins, including amoxicillin, and less susceptibility for tetracycline and metronidazole, a similar pattern to the results of the present study. In Germany a different trend of antimicrobial susceptibility was found by Kleinfelder et al.²⁰, as tetracycline and metronidazole were more effective than amoxicillin and amoxicillin plus clavulanic acid.

Two studies by Feres et al.²¹ and Feres et al.²² performed in U.S.A., analyzed the effect of systemic

antibiotic administration on the development of antibiotic resistance. Two out of three antibiotics tested amoxicillin and doxycycline, showed low levels of resistance before antibiotic intake (6% and 0.5% respectively); in contrast resistance against metronidazole was 50% at baseline. Resistance to all antibiotics increased with their consumption, ranging from 30 to 65% of bacterial isolates; 90 days later resistance decreased to initial levels. The high baseline resistance against metronidazole observed in the present study is in keeping with the findings of Feres et al.²². A large proportion of subjects in the present study use antibiotics regularly, but the date of last consumption was not recorded. As a consequence, the impact of recent antibiotic intake on the resistance levels could not be measured, in contrast to the studies of Feres et al.^{21, 22}.

In the present study, both bacterial isolates showing resistance to amoxicillin demonstrated β -lactamase production. Occurrence of β -lactamase activity in subgingival plaque was a common finding in the report of van Winkelhoff et al.²³, i.e. enzyme activity was detected in 26% of *P. intermedia* isolates, 11% of *T. forsythia* and 13% of *F. nucleatum*.

In conclusion, antibiotic resistance was a common phenomenon in this subject population exhibiting frequent antibiotic consumption. All bacterial isolates were resistant to metronidazole, and a large proportion were resistant to tetracycline and azithromycin.

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