Research Square

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Antimicrobial Susceptibility of Porphyromonas Gingivalis Strains Isolated From Periodontal Patients by Three Testing Protocols

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Abstract

Background: The main goal of antimicrobials is to eliminate microorganisms that persists despite mechanical treatment. This is the case of *Porphyromonas gingivalis (Pg)*, frequently isolated in patients with periodontitis. Global antibiotic studies evaluated in randomized clinical trials and *in vitro* studies have shown mixed results regarding effectiveness and susceptibility, even with different protocols where it is not clear if the laboratory test applied can affect the results. This information is relevant in order to obtain clinical outcomes and prevent antimicrobial resistance for their over-prescription or inadequate choice. The objective of this study was to describe the antimicrobial susceptibility *in vitro* of *Pg* to metronidazole, clindamycin, amoxicillin plus clavulanate, moxifloxacin and azithromycin in periodontal patients by three testing protocols.

Methods: Microbiological samples were obtained in patients with a diagnosis of generalized moderate or severe periodontitis. They were incubated in anaerobic conditions for up to 7 days, and those morphologically compatible with *Pg* were isolated and identified by a mass spectrometer (MALDI-TOF MS). The three most frequently protocols for antimicrobials susceptibility tests (Blood agar- McFarland 0.5- Epsilometer test; Brucella blood agar- McFarland 1.0- Epsilometer test; Brucella blood agar- McFarland 0.5-Agar dilution) were applied to the same strain describing their profile and reporting any difference between the tests. The breakpoints considered the guidelines of CLSI and previous publications.

Results: 50 patients (25 women, 25 men) with periodontitis between 34-69 years were selected. Finally, 25 *Pg* positives strains (50%) were recovered for the susceptibility analysis and all of them were highly sensitive to all antibiotics (range 96%-100%). Only one strain was resistant to azithromycin in one protocol, and no differences were found in the susceptibility results between the three tests.

Conclusion: The *Pg* strains were highly susceptible to the five antibiotics evaluated in this population, showed a high level of susceptibility and significant agreement between the three tests applied, therefore the type of laboratory test used had not impacted on clinical interpretation. These findings are positives in terms of susceptibility and would provide several antibiotics treatment alternatives, and its prescription could be the best choice for the patient's specific context.

1. Introduction

Periodontitis is a microbially-associated and host-mediated inflammatory disease that results in loss of periodontal attachment and finally the loss of teeth [1,2]. This chronic disease has become a worldwide public health problem due to its high prevalence in adults and elevated cost of treatment, if it is not treated timely generates a decrease in quality of life of affected patients [3,4].

Periodontitis treatment is mainly mechanical, with the purpose of disrupting the established subgingival biofilm and allowing the change towards a microbiota compatible with health [5]. Evidence has shown that some patients need antibiotics as an adjunct to conventional mechanical treatment, under specific

conditions like severe cases of periodontitis or in patients with systemic diseases [6,7]. The main goal of antimicrobials is to eliminate microorganisms that persist despite the mechanical treatment. It is the case of *Porphyromonas gingivalis (Pg)* [8], a highly pathogenic bacteria frequently isolated in patients with periodontitis that demonstrate a strong association in the initiation and progression of the disease [9]. This pathogen owns complex virulence factors like fimbriae presence, modulation of the immune response and capacity for tissue invasion [10]. The "keystone bacteria" model showed that *Pg* induces subgingival biofilm dysbiosis and allows the development of some bacteria over other modulating commensal microbiota raising the pathogenicity of the entire community. Thus, individual bacteria triggers a series of collective events associated with tissue destruction by the host response [11,12].

In Chile, the prevalence of severe periodontitis in the adult population is 38.65% between 35 and 44 years and 69.35% between 65 and 74 years, mostly low socioeconomic status [13]. Several studies established a high predominance of strict anaerobic bacteria in those patients where *Pg* appears frequently isolated. Its prevalence differs according to the identification method used [14–17].

The microbiological profile of periodontal patients is well known, and antimicrobial treatment is an evidence-based clinical decision. Nevertheless, the antibiotics evaluated in randomized clinical trials and *in vitro* studies have shown mixed results regarding the effectiveness and susceptibility, even with different protocols where it is not clear if the applied laboratory test can affect the results [16,18–21]. Local susceptibility has not been evaluated, relevant information for obtaining clinical outcomes and preventing antimicrobials resistance due to their over-prescription or inappropriate choice [22,23].

This study aimed to describe the antimicrobial susceptibility *in vitro* of *Porphyromonas gingivalis* to metronidazole, clindamycin, amoxicillin plus clavulanate, moxifloxacin and azithromycin in periodontal patients by three testing protocols.

2. Materials And Methods

2.1. Subjects

Microbiological samples were obtained in patients selected in the clinic of Escuela de Odontología, Pontificia Universidad Católica de Chile, carefully selected according to inclusion and exclusion criteria (table 1). We included patients with a diagnosis of generalized moderate or severe periodontitis, according to former AAP classification [24]. The case definition for moderate periodontitis was “two or more interproximal sites with clinical attachment loss ≥ 4 mm, not on the same tooth, or two or more interproximal sites with probing depths ≥ 5 mm, not on the same tooth” and for severe periodontitis was “two or more interproximal sites with clinical attachment loss ≥ 6mm, not on the same tooth, and one or more interproximal sites with probing depth ≥ 5mm”. Periodontitis was considered generalized when it was present in 30% or more of the sites examined. Alveolar bone loss was evaluated with radiographs [25]. Once patients were selected, we proceeded to explain the research protocol and requested voluntary participation in the study, and they signed an informed consent document.

2.2 Clinical periodontal parameters

Clinical records were obtained, and six sites by tooth were measured with North Carolina probes (UNC 15, Hu-Friedy Corp., Chicago IL, USA). Periodontal parameters were evaluated in all teeth presents (with exception third molars), including probing depth, clinical attachment level, plaque index (O'Leary), bleeding on probing (BOP) and suppuration.

2.3 Sampling of subgingival plaque technique

The site selected for sampling was the deepest bleeding pocket with the highest clinical attachment level loss was selected in each quadrant of the mouth. The supragingival plaque was removed with a sterile curette, and then the zone was isolated with sterile cotton rolls and air-dried. Subgingival microbiological samples were obtained, inserting two consecutively sterile paper points N^{o} 30 (Maillefer, Switzerland) for at least 20 seconds within each site of the teeth. Samples of each patient were transported in a tube with 1 ml of RTF (reduced transport fluid) at 4°C to Microbiology Laboratory within 2 hours to begin their processing [26].

2.4 Microbiological laboratory tests

In the laboratory, the samples were vortexed for 30 seconds. Then serial dilutions were then made and plated homogeneously on blood agar (5% sterile lamb blood, 5 µg hemin/mL, 1 µg vitamin K /mL). They were incubated at 37°C in anaerobic jars for up to 7 days, and those morphologically compatible with *Pg* were isolated and passed to a second incubation. Finally, bacterial identification was confirmed through the mass spectrometer (MALDI-TOF MS; Biotyper, Bruker Daltonik, Germany).

2.5 Antimicrobial susceptibility test

We applied the three most widely used protocols for antimicrobial sensitivity tests to the same strain, described their susceptibility profile and reported any difference detected between the tests. Table 3 shows breakpoints for interpretation of *Pg* susceptibility considering the guidelines of the Clinical Laboratory Standards Institute (CLSI) and previous publications.

a)First protocol (Blood agar- McFarland 0.5- Epsilometer test)

The isolated samples of *Pg* were placed inside a tube with sterile reduced thioglycolate broth to obtain a 0.5 McFarland standard (1-2 x 10^{8} UFC/mL). Then the final suspension was plated on blood agar (5% sterile lamb blood, 5 µg hemin/mL, 1 µg vitamin K /mL) [19].

The Epsilometer Test (E-test®, AB Biodisk, Solna, Sweden) for metronidazole, clindamycin, amoxicillin plus clavulanate, moxifloxacin and azithromycin was applied by placing only two antibiotics strips per plate, following the manufacturer's instructions [27].

The plates were incubated at 37°C in anaerobic atmosphere for 2 days, and quality control was *Bacteroides fragilis* strain (ATCC 25285) used as a reference to confirm the correct implementation. The minimum inhibitory concentration (MIC), defined as the lowest concentration of antibiotics that inhibits microorganisms growth, was measured at the intersection of the inhibition area with the strips where was reading and interpreted according to the guidelines of the CLSI and previous publications [19,28].

b)Second protocol (Brucella blood agar- McFarland 1.0- Epsilometer test)

The isolated samples of *Pg* were placed inside a tube with sterile reduced thioglycolate broth in order to obtain a 1.0 McFarland standard (3.0 x 10^8 UFC/mL). After that, the final suspension was plated on Brucella blood agar (Becton Dickinson®) supplemented with 5% sterile lamb blood, 5 µg hemin/mL, 1 µg vitamin K /mL. These are the culture media and turbidimetry suggested by the manufacturer (E-test®, AB Biodisk, Solna, Sweden) [29]. Then E-test® was applied in the same way as in the first protocol [27].

The plates were incubated at 37°C in anaerobic atmosphere for 2 days, and quality control was *Bacteroides fragilis* strain (ATCC 25285) used as a reference to confirm the correct implementation. The MIC was reading and interpreted according to the guidelines of the CLSI and preceding studies (CLSI, 2018; Van Winkelhoff et al., 2005).

c) Third protocol (Brucella blood agar- McFarland 0.5-Agar dilution)

The isolated samples of *Pg* were deposited inside a tube with reduced thioglycolate broth until to obtain a 0.5 McFarland standard. Then employing Cathra replicator were successively plated on Brucella blood agar (Becton Dickinson®) supplemented with 5% sterile lamb blood, 5 µg hemin/mL, 1 µg vitamin K /mL plus the individually evaluated antibiotics: metronidazole, clindamycin, amoxicillin plus clavulanate, moxifloxacin and azithromycin, in different concentrations. The plates were incubated at 37°C in anaerobic atmosphere for 2 days, and quality controls were *Bacteroides fragilis* strain (ATCC 25285), *Clostridium difficile* (ATCC 700057) and *Porphyromonas gingivalis* (ATCC 33277), used as a reference to confirm the correct procedure. Finally, the lowest concentration of each antibiotic that inhibits the growth of a microorganism was reported as its MIC [28].

2.6 Data analyses

Descriptive data was recorded for the three testing protocols. The antibiotic was interpreted as susceptible when concentration was below the defined breakpoints the strain, and it was considered resistant if it was equal or higher than the reference value (CLSI; previously publications). The concentrations at which 50% and 90% of strains were susceptible were defined as MIC_{50} and MIC_{90}, respectively.

*This study was approved by Scientific Ethical Committee of the Facultad de Medicina, Pontificia Universidad Católica de Chile (ID 13-137).

3. Results

Microbiological samples were obtained from 50 patients (25 women, 25 men) with periodontitis, between 34-69 years (age mean 51.4 ± 8.19). From the total, 27 (57%) were positives to the presence of *Pg;* 14 male (age mean 54.1 ± 7.38) and 13 female (age mean 47.9 ± 9.93) patients. Table 2 describes the characteristics and clinical data for the total sample and subdivided in a positive and negative culture. Finally, 25 strains positives to *Pg* (50%) were recovered for susceptibility test analysis. The breakpoints for *Pg* susceptibility considered the guidelines of the CLSI and previous publications and table 4 shows the quantitative results in terms of MIC range, MIC_{50} and MIC_{90} percentage of susceptibility, for each antibiotic in the three testing protocols.

All the selected strains were highly sensitive to metronidazole, clindamycin, amoxicillin plus clavulanate and moxifloxacin (range 96%-100%). Only one strain was resistant to azithromycin in the first protocol, and no differences were found in the susceptibility results between the three tests.

4. Discussion

Previous studies identified the microbiological profile of Chilean patients with periodontitis and their comparison with other countries, establishing a high *Pg* prevalence and a geographical difference in their results [14,15,17,30,31]. Also, some randomized clinical trials evaluated the use of some antibiotics and their impact on clinical and microbiological results [32,33]. This study is the first that evaluates *in vitro Pg* susceptibility isolated from Chilean adults with periodontitis to the main antibiotics used in periodontal treatment.

Furthermore, due to the scientific literature has reported different protocols for susceptibility testing, this study evaluated if there are differences in the results obtained through three main laboratory protocols. A descriptive methodology was used in this pilot study; therefore, 50 patients were able to obtain clinical records and microbiological samples for testing.

In a fist laboratory stage, count with the MALDI-TOF MS (Matrix-Assisted Laser Desorption/Ionization-Time-Of-Flight) was an advantage because it could identify the microorganisms in a few minutes by reading their previously ionized peptides and proteins, with a high-confidence (92.9% of the isolates anaerobic bacteria), being simpler than traditional techniques [34,35].

Anaerobic susceptibility is not frequently implemented in laboratories, but test results are relevant to direct appropriate therapy and identify anaerobic resistance, which is increasing globally [36,37]. Another important concern is that the variety of susceptibility test methods for anaerobes have significant advantages and disadvantages [38] and it is unclear whether the type of process could bring different results and, therefore, different clinical interpretations. For the present study, we selected the E-test® and agar dilution.

The E-test® is a simple laboratory technique that read the MICs in a strip containing a gradient of antibiotic. However, there are published different process to determine susceptibility, with several media culture as well as the concentration of colony-forming units determined by the McFarland turbidimetry. The two E-test® protocols performed in this study are the most frequently published, where brucella blood agar and blood agar were used, as well as McFarland 1 and 0.5 turbidimetry, respectively [19,29]. However, agar dilution remains the gold standard for determining susceptibility since it has the standardization by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [39] and CLSI [28], so we also select this protocol, although it is a more complex technique and requires considerable time in the process [38].

In general, E-test® results have a strong correlation with MICs assessed by broth or agar dilution methods; nevertheless, there is some inherent bias toward higher or lower MICs determined by some test-microorganism-antimicrobial agent association [38]. For example, metronidazole resistance in E-test® can be overestimated if anaerobiosis is inadequate [40] or discrepancies were reported for this antibiotic when compared E-test® and agar dilution for anaerobes (13 to 14%; FDA category of very major error) [41].

In the dilution in agar, not changing the culture media or the inoculum concentration is essential, because minor methodological adaptations to the protocols cannot guarantee reliable results and allow them to be compared with other publications [42]. Therefore, to achieve accurate and reproducible results, the test medium must accomplish a serial of requirements for certain antibiotic/species combinations. Also, the concentration of colony-forming units must be as indicated. A higher inoculum can increase MIC, specifically if the tested bacteria produce an enzyme that destroys the antibiotic. Conversely, a lighter inoculum may artificially lower MICs [43]. Thus, studies should be careful when comparing results because emerging mechanisms of resistance require constant vigilance about the sensitivity of each test to detect resistance accurately [44].

In the field of periodontal microbiology, it has been demonstrated that several antibiotics need to be much higher concentrated on reaching the MIC in a biofilm compared with microorganisms grown in a planktonic culture [45,46], therefore *in vitro* test can predict the clinical efficacy *(in vivo*) [47].

Despite the limitations that this study, we could interpret positives results in terms of a high level of susceptibility in all antibiotics studied against *Pg*, and significant agreement in the results between the three tests so would not have an impact on changing the clinical decision when choosing among antibiotics. These results are in accordance with previous reports where E-test® determined the susceptibility of *Pg* strains isolates from periodontitis. In the Netherlands and Spain periodontal population were susceptible to all antibiotics tested including metronidazole, clindamycin, azithromycin, amoxicillin plus clavulanate, amoxicillin, penicillin, tetracycline and ciprofloxacin [19]. Another study, also in the Netherlands *Pg* was highly susceptible to metronidazole, clindamycin, amoxicillin plus clavulanate, azithromycin, amoxicillin and tetracycline [48]. In Switzerland periodontal population, they were susceptible to metronidazole, clindamycin, amoxicillin plus clavulanate, phenoxymethylpenicillin and tetracycline [20].

Different results were found in Colombia with E-test® for *Pg*, showing high susceptibility to amoxicillin plus clavulanate and moxifloxacin, but resistance to clindamycin (23.52%), metronidazole (21.56%) and amoxicillin (25.49%)[29]. In Iran, E-test® results revealed 100% susceptibility of *Pg* to azithromycin, doxycycline and amoxicillin/clavulanic acid but lower susceptibilities for the rest of antibiotic agents evaluated: clindamycin (96%), metronidazole (94%), penicillin (92%), amoxicillin (88%) and ciprofloxacin (60%)[49].

For other tests, in the Swedish periodontal population, no resistance was found using disc diffusion, and agar dilution among strains of *Pg* isolates against the antibiotics commonly used in the treatment of periodontal disease: clindamycin, metronidazole, penicillin, amoxicillin and tetracycline [21].

Currently, the indication of antibiotics in chronic periodontitis is performed empirically in most cases based on clinical criteria, but for this critical decision, having information about laboratory identification or at least recognize predominant flora would be desirable. Other clinical concern is antibiotic monotherapy prescription, if it is possible, considering the risk of resistance, adverse drug reactions and patient compliance [50]. Besides, consider choosing broad-spectrum antibiotics instead of broad-spectrum [51] and having pharmacological alternatives in case of allergies or risk of drug interactions, for example, in patients with polypharmacy.

Periodontal pathogens in the Chilean population-as in other countries-showed a marked predominance of strict anaerobic bacteria; therefore, the real need to use broad-spectrum antibiotics or their combinations should be evaluated, contemplating possible adverse reactions or risk of resistance. Therefore, a single antibiotic or a synergic combination of them must achieve an optimal result in periodontal health, but the cost/benefit decision for the patient should be based ideally on microbiological profile and individual clinical context.

Similar to other countries, Chile implemented a national plan for antimicrobial resistance that involves a series of regulations, including its acquisition only under prescription since 1999 [52]. Nevertheless, it is estimated an increase of 55% in antibiotic consumption between 2000 and 2016, and the antimicrobial resistance increased in 21% for priority bacterial-antibiotic pairs and in general 4.6 percentage points (average) in 2005–2015 period [22,53].

Taking all this evidence into account, it is essential to consider the impact of antimicrobial protocols on patient care health policies and, therefore, update the clinical guidelines [54].

Conclusions

This study suggests that *Pg* strains were highly susceptible to all antibiotics evaluated in this population. Through descriptive data, it was determined that the three methods to assess susceptibility in anaerobes most commonly used in periodontics found no difference in their results and provide the basis for future randomized clinical trials.

Declarations

This study was approved by Scientific Ethical Committee of the Facultad de Medicina, Pontificia Universidad Católica de Chile (ID 13-137).

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

The authors declare that they have no competing interests.

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Authors' contributions:

LI: Corresponding author, was a major contributor in writing the manuscript.

NO: Performed the clinical examination, completed clinical records and took the microbiological samples from patients.

MJC: Performed the clinical examination, completed clinical records and took the microbiological samples from patients.

DO: Performed study design and data analysis.

ML: Performed laboratory test for all the samples and delivered the results and their interpretation.

PG: Senior advisor in microbiological protocols and critical reviewer of the manuscript.

All authors read and approved the final manuscript.

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References

1. Caton J, Armitage G, Berglundh T, et al. A new classification scheme for periodontal and peri-implant diseases and conditions – Introduction and key changes from the 1999 classification. *J Clin Periodontol* 2018; 45: S1–S8.
2. Tonetti MS, Greenwell H, Kornman KS. Staging and grading of periodontitis: Framework and proposal of a new classification and case definition. *J Periodontol* 2018; 89: S159–S172.
3. Kassebaum NJ, Bernabé E, Dahiya M, et al. Global burden of severe periodontitis in 1990-2010: A systematic review and meta-regression. *J Dent Res* 2014; 93: 1045–1053.

4. Frencken JE, Sharma P, Stenhouse L, et al. Global epidemiology of dental caries and severe periodontitis – a comprehensive review. *J Clin Periodontol* 2017; 44: S94–S105.
5. Krishna R, De Stefano JA. Ultrasonic vs. hand instrumentation in periodontal therapy: Clinical outcomes. *Periodontol 2000* 2016; 71: 113–127.
6. Herrera D, Sanz M, Jepsen S, et al. A systematic review on the effect of systemic antimicrobials as an adjunct to scaling and root planing in periodontitis patients. *J Clin Periodontol* 2002; 29: 136–159.
7. Feres M, Figueiredo LC, Soares GMS, et al. Systemic antibiotics in the treatment of periodontitis. *Periodontol 2000* 2015; 67: 131–186.
8. Mombelli A, Schmid B, Rutar A, et al. Persistence Patterns of Porphyromonas gingivalis, Prevotella intermedia/ nigrescens, and Actinobacillus actinomycetemcomitans After Mechanical Therapy of Periodontal Disease. *J Periodontol* 2000; 71: 14–21.
9. Ximénez-Fyvie LA, Haffajee AD, Socransky SS. Comparison of the microbiota of supra- and subgingival plaque in health and periodontitis. *J Clin Periodontol* 2000; 27: 648–657.
10. Pathirana RD, O'Brien-Simpson NM, Reynolds EC. Host immune responses to Porphyromonas gingivalis antigens. *Periodontol 2000* 2010; 52: 218–237.
11. Hajishengallis G. Periodontitis: From microbial immune subversion to systemic inflammation. *Nat Rev Immunol* 2015; 15: 30–44.
12. Hajishengallis G, Korostoff JM. Revisiting the Page & Schroeder model: the good, the bad and the unknowns in the periodontal host response 40 years later. *Periodontol 2000* 2017; 75: 116–151.
13. Gamonal J, Mendoza C, Espinoza I, et al. Clinical Attachment Loss in Chilean Adult Population: First Chilean National Dental Examination Survey. *J Periodontol* 2010; 81: 1403–1410.
14. Gajardo M, Silva N, Gómez L, et al. Prevalence of Periodontopathic Bacteria in Aggressive Periodontitis Patients in a Chilean Population. *J Periodontol* 2005; 289–294.
15. Herrera D, Contreras A, Gamonal J, et al. Subgingival microbial profiles in chronic periodontitis patients from Chile, Colombia and Spain. *J Clin Periodontol* 2008; 35: 106–113.
16. Ardila CM, López MA, Guzmán IC. High resistance against clindamycin, metronidazole and amoxicillin in Porphyromonas gingivalis and Aggregatibacter actinomycetemcomitans isolates of periodontal disease. *Med oral Patol Oral Cir Bucal* 2010; 15: 947–951.
17. López NJ, Socransky SS, Silva I Da, et al. Subgingival Microbiota of Chilean Patients With Chronic Periodontitis. *J Periodontol* 2004; 717–725.
18. Van Winkelhoff AJ, Gonzales DH, Winkel EG, et al. Antimicrobial resistance in the subgingival microflora in patients with adult periodontitis. *J Clin Periodontol* 2000; 27: 79–86.
19. Van Winkelhoff AJ, Herrera D, Oteo A, et al. Antimicrobial profiles of periodontal pathogens isolated from periodontitis patients in the Netherlands and Spain. *J Clin Periodontol* 2005; 32: 893–898.
20. Kulik EM, Lenkeit K, Chenaux S, et al. Antimicrobial susceptibility of periodontopathogenic bacteria. *J Antimicrob Chemother* 2008; 61: 1087–1091.

21. Dahlén G, Gmür R, Yoshino T. Phenotypes, serotypes and antibiotic susceptibility of Swedish Porphyromonas gingivalis isolates from periodontitis and periodontal abscesses. *Oral Microbiol Immunol* 2007; 22: 80–86.
22. Klein EY, Boeckel TP Van, Martinez EM, et al. Global increase and geographic convergence in antibiotic consumption between 2000 and 2015. *Proc Natl Acad Sci* 2018; 115: E3463–E3470.
23. Lockhart PB, Tampi MP, Abt E, et al. Evidence-based clinical practice guideline on antibiotic use for the urgent management of pulpal- and periapical-related dental pain and intraoral swelling: A report from the American Dental Association. *J Am Dent Assoc* 2019; 150: 906-921.e12.
24. Armitage G. Development of a classification system for Periodontal Diseases and Conditions. *Ann Periodontol* 1999; 4: 1–6.
25. Eke, P. I., Page, R. C., Wei, L., Thornton-Evans, G., & Genco RJ. Update of the case definitions for population-based surveillance of periodontitis. *J Periodontol* 2012; 83: 1449–1454.
26. Syed SA, Loesche WJ. Survival of human dental plaque flora in various transport media. *Appl Microbiol* 1972; 24: 638–644.
27. Biomeriux. Etest: Antimicrobial Susceptbility Testing. 2012; 1–6.
28. CLSI. Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria. 9th ed. CLSI standard M11. Wayne, PA: Clinical and Laboratory Srandards Institute, www.clsi.org. (2018).
29. Ardila CM, Granada MI, Guzmán IC. Antibiotic resistance of subgingival species in chronic periodontitis patients. *J Periodontal Res* 2010; 45: 557–563.
30. Haffajee AD, Bogren A, Hasturk H, et al. Subgingival microbiota of chronic periodontitis subjects from different geographic locations. *J Clin Periodontol* 2004; 31: 996–1002.
31. López NJ. Occurrence of Actinobacillus actinomycetemcomitans, Porphyromonas gingivalis, and Prevotella intermedia in Progressive Adult Periodontitis. *J Periodontol* 2000; 71: 848–954.
32. López NJ, Gamonal JA, Martinez B. Repeated Metronidazole and Amoxicillin Treatment of Periodontitis. A Follow-Up Study. *J Periodontol* 2000; 71: 79–89.
33. López NJ, Socransky SS, Da Silva I, et al. Effects of metronidazole plus amoxicillin as the only therapy on the microbiological and clinical parameters of untreated chronic periodontitis. *J Clin Periodontol* 2006; 33: 648–660.
34. Kliem M, Sauer S. The essence on mass spectrometry based microbial diagnostics. *Curr Opin Microbiol* 2012; 15: 397–402.
35. Rodríguez-Sánchez B, Alcalá L, Marín M, et al. Evaluation of MALDI-TOF MS (Matrix-Assisted Laser Desorption-Ionization Time-of-Flight Mass Spectrometry) for routine identification of anaerobic bacteria. *Anaerobe* 2016; 42: 101–107.
36. Boyanova L, Kolarov R, Mitov I. Recent evolution of antibiotic resistance in the anaerobes as compared to previous decades. *Anaerobe* 2015; 31: 4–10.
37. Cooley L, Teng J. Anaerobic resistance: Should we be worried? *Curr Opin Infect Dis* 2019; 523–530.

38. Schuetz AN. Antimicrobial resistance and susceptibility testing of anaerobic bacteria. *Clin Infect Dis* 2014; 59: 698–705.
39. European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters.Version 9.0., http://www.eucast.org. (2019).
40. Cormican MG, Erwin ME, Jones RN. False Resistance to Metronidazole by E-Test Among Anaerobic Bacteria Investigations of Contributing Test Conditions and Medium Quality. *Diagnostic Microbiol Infect Dis* 1996; 24: 117–119.
41. Rennie RP, Turnbull L, Brosnikoff C, et al. First comprehensive evaluation of the M.I.C. evaluator device compared to etest and CLSI reference dilution methods for antimicrobial susceptibility testing of clinical strains of anaerobes and other fastidious bacterial species. *J Clin Microbiol* 2012; 50: 1153–1157.
42. Balouiri M, Sadiki M, Ibnsouda SK. Methods for in vitro evaluating antimicrobial activity: A review. *J Pharm Anal* 2016; 6: 71–79.
43. Wiegand I, Hilpert K, Hancock REW. Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. *Nat Protoc* 2008; 3: 163–175.
44. Jorgensen JH, Ferraro MJ. Antimicrobial Susceptibility Testing: A Review of General Principles and Contemporary Practices. *Clin Infect Dis* 2009; 49: 1749–1755.
45. Sedlacek MJ WC. Antibiotic resistance in an in vitro subgingival biofilm model. *Oral Microbiol Immunol* 2007; 22: 333–339.
46. Eick S, Seltmann T, Pfister W. Efficacy of antibiotics to strains of periodontopathogenic bacteria within a single species biofilm - An in vitro study. *J Clin Periodontol* 2004; 31: 376–383.
47. Turnidge J, Paterson DL. Setting and revising antibacterial susceptibility breakpoints. *Clin Microbiol Rev* 2007; 20: 391–408.
48. Veloo ACM, Seme K, Raangs E, et al. Antibiotic susceptibility profiles of oral pathogens. *Int J Antimicrob Agents* 2012; 40: 450–454.
49. Japoni A, Vazin A, Noushadi S, et al. Antibacterial susceptibility patterns of porphyromonas gingivalis isolated from chronic periodontitis patients. *Med oral Patol Oral Cir Bucal* 2011; 16: 1031–1035.
50. Michael CA, Dominey-Howes D, Labbate M. The antimicrobial resistance crisis: Causes, consequences, and management. *Front Public Heal* 2014; 2: 1–8.
51. Melander RJ, Zurawski D V., Melander C. Narrow-spectrum antibacterial agents. *Med Chem Comm* 2018; 9: 12–21.
52. García P. Resistencia bacteriana en Chile. *Rev Chil Infectol* 2003; 20: 11–23.
53. OECD. *Stemming the Superbug Tide*. OECD. Epub ahead of print 7 November 2018. DOI: https://doi.org/10.1787/9789264307599-en.
54. Mendelson M, Røttingen JA, Gopinathan U, et al. Maximising access to achieve appropriate human antimicrobial use in low-income and middle-income countries. *Lancet* 2016; 387: 188–198.

Tables

Table 1
Patients inclusion and exclusion criteria

Inclusion criteria	Exclusion criteria
1. Patients age ≥ 30 years	1. Patients who need premedication with antibiotics prior to clinical examination
2. At least three natural teeth per quadrant at the time of examination	2. Periodontal treatment in the last 12 months
3. Clinical diagnosis of moderate or generalized advanced periodontitis untreated	3. Consumption of antibiotics in the last 3 months
4. Radiographic evidence of bone loss in all quadrant of the dentition	4. Pregnancy
	5. Diabetes type 1

Table 2
Characteristics and clinical data for total sample and culture positive and negative to *Pg*

	Total sample	Culture (+) *Pg*		Culture (-) *Pg*	
Parameter		Male	Female	Male	Female
Age range	39–69	41–63	34–63	41–63	34–63
Mean age	51.4 ± 8.19	54.1 ± 7.38	47.9 ± 9.93	54.1 ± 7.38	47.9 ± 9.93
% Smokers	44.0	50.0	23.0	50.0	23.0
Cigarettes smoking/day	4.06 ± 6.92	6.8 ± 10.91	1 ± 2.24	6.8 ± 10.91	1 ± 2.24
*CAL (mm)	6.6 ± 1.36	6.6 ± 1.62	6.4 ± 1.30	6.6 ± 1.62	6.4 ± 1.30
Probing pocket depth (mm)	5.9 ± 0.74	5.7 ± 0.54	6.0 ± 0.79	5.7 ± 0.54	6.0 ± 0.79
Bleeding on probing (% sites)	56.4 ± 0.24	59.3 ± 0.24	56.8 ± 0.26	59.3 ± 0.24	56.8 ± 0.26
Suppuration (% sites)	1.9 ± 0.03	1.1 ± 0.02	2.1 ± 0.03	1.1 ± 0.02	2.1 ± 0.03
**Plaque index (% sites)	81.8 ± 17.86	80.3 ± 21.32	78.8 ± 15.33	80.3 ± 21.32	78.8 ± 15.33
*CAL (clinical attachment level); **O´Leary Plaque index					

Table 3
Breakpoints for interpretation of *Pg* susceptibility

	S - I - R (µg/mL)(*)
Metronidazole(**)	≤ 8 - 16 - ≥ 32
Clindamycin(**)	≤ 2 - 4 - ≥ 8
Amoxicillin + Clavulanate(**)	≤ 4/2 - 8/4 - ≥16/8
Moxifloxacin(**)	≤ 2 - 4 - ≥ 8
Azithromycin(***)	≤ 2
* S: Susceptible I: Intermediate R: Resistant ** Clinical Laboratory Standards Institute (CLSI) ***Van Winkelhoff et al., 2005[18]	

Table 4
Results of three protocols for antimicrobial susceptibility to *Pg*

	First protocol	Second protocol	Third protocol
Metronidazole	0.016-1	0.016	0.125
MIC range (μg/mL)	0.016	0.016	0.125
MIC_{50} (μg/mL)	0.016	0.016	0.125
MIC_{90} (μg/mL)			
Clindamycin	0.016–0.047	0.016	0.125
MIC range (μg/mL)	0.016	0.016	0.125
MIC_{50} (μg/mL)	0.016	0.016	0.125
MIC_{90} (μg/mL)			
Amoxicillin/Clavulanate	0.016-1	0.016–0.125	0.25/0.125
MIC range (μg/mL)	0.016	0.016	0.25/0.125
MIC_{50} (μg/mL)	0.016	0.016	0.25/0.125
MIC_{90} (μg/mL)			
Moxifloxacin	0.002–0.094	0.002-1	0.125
MIC range (μg/mL)	0.002	0.008	0.125
MIC_{50} (μg/mL)	0.016	0.19	0.125
MIC_{90} (μg/mL)			
Azithromycin	0.016-8	0.016-1.5	0.03–0.125
MIC range (μg/mL)	0.094	0.047	0.03
MIC_{50} (μg/mL)	0.19	1	0.03
MIC_{90} (μg/mL)			

*First protocol: Blood agar- McFarland 0.5- E-test; second protocol: Brucella blood agar- McFarland 1.0- E-test; third protocol: Brucella blood agar- McFarland 0.5-Agar dilution.

**All antibiotics showed 100% susceptibility in the tree protocols, except azithromycin in the first protocol (96%)