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RESEARCH ARTICLE

PORPHYROMONAS GINGIVALIS: BIOFILM FORMATION, ANTIMICROBIAL SUSCEPTIBILITY OF ISOLATES FROM CASES OF LOCALIZED AGGRESSIVE PERIODONTITIS (LAP)

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Abstract

Background: No information is available on the antimicrobial sensitivity pattern and guidelines for oral antibiotic therapy for Yemeni patients for those with localized aggressive periodontitis (LAP) due to *Porphyromonas gingivalis*, a condition that often requires complementary antibiotic treatment.
Aim: The primary aim of the current study was to examine the antibiotic patterns and the potential relationship between *P. gingivalis* biofilm formation and the incidence of antibiotic resistance of clinical isolates on a group of antibiotics commonly used in oral/systemic therapy.
Subjects and Methods: The study included 30 clinically diagnosed patients, and 30 strains of *P. gingivalis* were isolated from them. Microbial sampling, isolation, and identification of bacteria were performed using culture methods appropriate to anaerobic species. Biofilm production was evaluated by the phenotypic method, that is, tissue culture methods (TCPM). Also; each isolate was tested against 12 antibiotics using the disc diffusion method.
Results: After isolated *P. gingivalis* were subjected to biofilm detection by TCP method, 7 (23.3%) showed high, 6 (20%) moderate, while 17 (56.7%) showed non/weak biofilm-forming ability. *P. gingivalis* biofilms showed a higher resistance rate than forming non/weak biofilms e.g. amoxicillin (92.3% vs 64.7%, p=0.08), azithromycin (58.8% vs 11.7, p=0.003), metronidazole (76.9% vs 29.4%, p=0.08), 0.01) and clindamycin (84.6% vs 47.1, p=0.03).
Conclusions: It was found that the drug-resistant factor in *P. gingivalis* isolates is associated with the formation of *P. gingivalis* biofilm. Even though the current results show a high sensitivity result for *P. gingivalis* strains, some resistance has been observed. Antibiotic resistance patterns can modify over the years, make susceptibility testing essential and promoting careful choice of preliminary antibiotic treatment, the same as an adjuvant to endodontic therapy.
Keywords: antibiotic resistance, biofilm formation, disc diffusion method, *Porphyromonas gingivalis*.

INTRODUCTION

Amongst the most important periodontal pathogens, *P. gingivalis* shows to be one of the major etiological factors in the pathogenesis and development of inflammatory proceedings of periodontal disease[1]. These bacteria were obtained in 85.75% of subgingival plaque samples from chronic periodontitis patients. This Gram-negative, non-motile, non-saccharolytic bacterium is a compulsory anaerobic rod that forms black-coloured colonies on blood agar plates and has an obvious prerequisite for iron in its growth[2]. The main habitat of *P. gingivalis* is the subgingival sulcus in the oral cavity of human. It depends on the fermentation of amino acids to produce energy, a property that is necessary for its survival in the deep

pocket of the teeth, where the availability of sugar is low[3]. Being a binding anaerobic, *P. gingivalis* acts as a secondary colonizer for dental plaques, often observing primary colonizers such as *Streptococcus gordonii* and *P. intermedia*[3]. The maintenance and growth of *P. gingivalis* on various surfaces is assisted by a group of adhesives including fimbriae, hemagglutinins and enzyme proteins; and this makes it capable of biofilm formation[4]. A biofilm consists of any synthetic association of microorganisms (including yeast and bacteria) in which cells adhere to each other and often also to the surface[5,6]. These supporter cells develop into fixed in a sticky extracellular matrix composed of extracellular polymeric substances (EPSs). Cells contained by biofilms generate components of EPS, which are typically a polymeric conglomerate of proteins, sugars, lipids, and extracellular DNA[5]. Porphyromonas acquires a considerable number of recognized virulence determinants, suggesting that these bacteria may be one of the most pathogenic types found in the oral cavity. These contain haemagglutinin, fimbriae, outer membrane vesicles, capsule, lipopolysaccharide (LPS), and have strong hydrolytic activities that can disturb host defense mechanisms plus initiate tissue impairment[2,3].

Even though *P. gingivalis clinical* isolates tend to be sensitive to nearly all antimicrobial agents and usually do not create *β-lactamase*, comparatively insignificant information is obtainable on antibiotic susceptibility *in vitro*. Moreover, antibiotic resistance between anaerobes is constantly increasing which may be connected to the selective pressure exerted by antibiotic use. Determination of susceptibility to antimicrobials *in vitro* can be vital in specific circumstances, for example, to monitor patterns of sensitivity and resistance in a population and to assist in the assortment of a suitable antibiotic whilst implied in endodontic therapy[7]. The disc diffusion method was assessed to determine the sensitivity pattern of different antimicrobial agents against oral anaerobic bacteria and it was found to be appropriate[8-10].

Though there are various studies of oral and dental problems in Yemen[9-17], the antimicrobial susceptibility pattern of *P. gingivalis* isolated from localized aggressive periodontitis (LAP), no study discusses the antimicrobial susceptibility pattern of *P. gingivalis*. The current study was designed in an adult population in Sana'a city, in Yemen (1). to determine the levels of *P. gingivalis* biofilm formation (2). also to detect antibacterial sensitivity of *P. gingivalis* isolate (3). and study the association between biofilm formation and antibiotic resistance.

SUBJECTS AND METHODS

Patients: This study included 30 patients clinically and radiologically confirmed with LAP, who were admitted to the dental clinic at the Republican University Hospital and private dental clinics (Al-Mortadda dental clinics, Al-Abany dental clinics and Al-Kahara dental clinics) in Sana'a, during a period of more than a year, which started in December 2019 and ended in February 2021, which is the time that provided by the faculty of dentistry to conduct this study. Informed consent was taken from all subjects. The culturing and antibiotic sensitivity were conducted in the Microbiology Department of the National Center of Public Health Laboratories (NCPHL) Sana'a, Yemen. Cultures were obtained from the collected pocket by probes in order to isolate the various bacterial causative agents. First, the supragingival plaque was removed (without disturbing the subgingival plaque) and a bacterial sample was collected from the deepest periodontal pockets with a sterile probe. The samples were then placed in a vial containing 2 ml of liquid thioglycolate enriched medium, sealed immediately and transported to the laboratory within 30 minutes. Bacteriological procedures were performed within one hour of sample collection. For germ cultures, the following media and conditions were used: Tryptic Soy Agar (TSA) with blood (5%) and MacConkey agar plates - incubated at 35°C under 5% CO_2 and examined at 24 and 48 hours; Brucella agar enriched with Vitamin K1 and CDC + amikacin blood agar - incubated at 35°C anaerobically in a Gaspak jar (Oxoid Ltd). Cultures were examined for the presence of bacteria at 48 and 96 h. Plates showing bacterial growth were retained until final processing and organism identification by classical standard techniques including culture colonies morphology, microscopy staining methods, and biochemical tests[18].

Biofilm production detection

The biofilm was detected by the tissue culture method / microtiter plate method (TCA)[19]. *P. gingivalis* isolates were inoculated from fresh agar plates in 2 mL of Brain Heart Infusion (BHI) broth and incubated for 24 h at 37°C anaerobically. Then the cultures were diluted 1:40 with fresh medium (BHI broth with 1% glucose added); 200 µl of the sample was dispensed in the individual microtitration plate and incubated further 24 h at 37°C anaerobically. The content was removed again with subsequent washing with phosphate saline (pH 7.2) three times to remove free-floating sessile bacteria with gentle pecking then the adherent bacteria, a biofilm product, was fixed with sodium acetate (2%) and stained with crystal violet (0.1% w/w) for 10-15 minutes. The liberated violet crystal solution was removed with triplicate wash with PBS, and then the plate was kept for drying. Finally, with 200µl ethanol (95%) all wells were filled to release the dye from the well and the optical density (OD) was measured at the wavelength of 630 nm. The OD value was calculated for each negative test and control strain, and the OD cutoff values (ODc) were evaluated as previously described[19].

Antimicrobial susceptibility testing was performed using the disk diffusion method according to CLSI[20]. Antimicrobial agents used in the study included amoxicillin (AM) 10µg, amoxicillin/clavulanic acid (2:1) (amoxy-clav) (AM-C) 10µg, tetracycline (TE 30µg), doxycycline (DO 30µg), clindamycin (DA 2 µg), azithromycin (AZM 15 µg), moxifloxacin (MFX 5µg), cefazolin (KZ 30µg), ceftriaxone (CRO 30µg), cefuroxime (CXM 30µg), cefotaxime (CTX 30µg) and metronidazole (MET 5µg) (Oxoid Ltd). Inocula of test strains were prepared in thioglycollate broth to a

concentration of 0.5 MacFarland standards and inoculated onto brucella blood agar plates supplemented with hemin and menadione. The antibiotic discs were placed in the plate and the plates were then incubated anaerobically in a gas-pak jar at 37°C overnight. The inhibition zones were measured and interpreted according to Clinical and Laboratory Standards Institute (CLSI) methodology[20].

Data analysis

The interpretative criteria for anaerobic sensitivity were applied to determine the breakpoints for amoxicillin, amoxicillin/clavulanic acid (2:1) (amoxy-clav), tetracycline, doxycycline, clindamycin, azithromycin, moxifloxacin, cefazolin, ceftriaxone, cefuroxime, cefotaxime and metronidazole[20]. Since guidelines for the antibiotics used were not available for *P. gangivilis*' interpretive criteria for facultative anaerobic organisms, they were applied to these antibiotics. For the level of statistical significance of antibiotic resistance rate between *P. gingivalis* biofilm producers and non-producers, significance was assumed at $p<0.05$. The p value was made by z-test for hypotheses concerning the mean of a normal distribution with known variance.

Table 1: Biofilm detection by TCP method for *P. gingivalis* isolates from localized aggressive periodontitis (LAP) cases.

Biofilm formation	Number	Percentage
High*	7	23.3
Moderate *	6	20
Total biofilm	13	43.3
Non/weak	17	56.7
Total *P. gingivalis* isolates	30	100

*TCP-High OD ≥ 0.24; Moderate OD =0.127-0.24, Non/weak OD<0.12

RESULTS

When isolated *P. gangivilis* were exposed to biofilm detection by TCP method, 7 (23.3%) showed high biofilm formation capacity, 6 (20%) showed moderate biofilm formation capacity, while 17 (56.7%) showed non/weak formation capacity of biofilm. The overall rate biofilm formation was 43.3% (Table 1). *P. gingivalis* biofilms producing isolates showed a higher resistance rate than non/weak producing biofilms e.g amoxicillin (92.3% vs 64.7%, p=0.08), azithromycin (58.8% vs 11.7, p=0.003), metronidazole (76.9% vs 29.4%, p=0.08), 0.01) and clindamycin (84.6% vs 47.1, p=0.03) (Table 2). Regarding all antibiotic sensitivity findings: cefotaxime, ceftriaxone and moxifloxacin showed excellent activity at 100% sensitivity, followed by amoxiclav (90%), tetracycline (83.3%), cefuroxime (80%), cefazolin (73.3%) and azithromycin (63.3%). In addition, bacterial strains showed poor sensitivity to clindamycin, doxycycline, metronidazole and amoxicillin (Table 3).

Table 2: Antimicrobial susceptibility of *P. gingivalis* isolates from localized aggressive periodontitis (LAP) cases.

Tested Antibiotics (Disc concentration µg) Inhibition Zone by mm (R I S)	Resistance No	Resistance %	Intermediate No	Intermediate %	Sensitive No	Sensitive (%)
Amoxicillin (AM 10µg) ≤13 14-17 ≥18	23	76.7	4	13.3	3	10
Amoxicillin-clavulanic acid (10 µg) ≤13 14-17 ≥18	2	6.7	1	3.3	27	90
Azithromycin (AZM 15 µg) ≤13 14-17 ≥18	9	30	2	6.7	19	63.3
Clindamycin (DA 2 µg) ≤14 15-16 ≥17	19	63.3	4	13.3	7	23.3
Cefazolin (KZ 30 µg) ≤14 15-17 ≥18	4	13.3	4	13.3	22	73.3
Cefotaxime (CTX 30 µg) ≤15 16-18 ≥19	00	00	00	00	30	100
Ceftriaxone (CRO 30 µg) ≤19 20-22 ≥23	00	00	00	00	30	100
Cefuroxime (CXM 30 µg) ≤14 15-22 ≥23	1	3.3	5	16.7	24	80
Doxycycline (DO 30 µg) ≤15 16-18 ≥19	00	00	16	53.3	14	46.7
Metronidazole (MET 5 µg) ≤20 20-25 ≥26	15	50	4	13.3	11	36.7
Moxifloxacin (MFX 5 µg) ≤15 16-18 ≥19	00	00	00	00	30	100
Tetracycline (TE 30 µg) ≤15 16-18 ≥19	1	3.3	4	13.3	25	83.3

R=Resistance, I= intermediate, S=Sensitive, mm=millimetre

Table 3: Antibacterial resistance pattern of *P. gingivalis* associated with biofilm formation.

Antibiotics	Total		Biofilm producing *P. gingivalis*		Non-Biofilm producing *P. gingivalis*		*p*
	No	%	NO	%	No	%	
Amoxicillin	23	76.7	12	92.3	11	64.7	0.08
Amoxicillin-clavulanic acid	2	6.7	1	7.7	1	5.9	0.84
Azithromycin	9	30	7	53.8	2	11.7	0.003*
Clindamycin	19	63.3	11	84.6	8	47.1	0.03*
Cefazolin	4	13.3	3	23.1	1	5.9	0.17
Cefotaxime	00	00	00	00	00	00	1.0
Ceftriaxone	00	00	00	00	00	00	1.0
Cefuroxime	1	3.3	1	7.7	00	00	0.25
Doxycycline	00	00	00	00	00	00	1.0
Metronidazole	15	50	10	76.9	5	29.4	0.01*
Moxifloxacin	00	00	00	00	00	00	1.0
Tetracycline	1	3.3	1	7.7	00	00	0.25
Total	30	100	13	43.3	17	56.7	-

DISCUSSION

Even though clinical isolates of *P. gingivalis* are likely to be sensitive to the majority antimicrobial agents and usually do not generate *β-lactamase*, fairly modest information is obtainable on susceptibility of antibiotic on *in-vitro*. Moreover, antibiotic resistance among anaerobes is constantly rising which may be associated to the selective pressure applied by the use of antibiotics[10,21]. Determination of susceptibility to antimicrobials *in vitro* can be essential in certain conditions, for example, to screen patterns of sensitivity and resistance in a population and to assist in the choice of a suitable antibiotic when implied in endodontic therapy. The disc diffusion assay was evaluated for determination of the sensitivity of anaerobic bacteria against different antimicrobial agents and was found to be suitable[10,21]. Recent indication shows that endodontic infection is straight related to bacterial invasion of dentine, the root canal system, and peri-root tissue. Treatment is mainly reliant on mechano-chemical, remove infected pulp and dentine remnants to avoid re-infection. Failure rates due to contamination are alterable from 11-20%[22,23]. The therapeutic method for endodontic infection is comparable to that of an anaerobic infection in common. Surgery is known to play a major role; Debridement of necrotic tissue and drainage of pus collections is essential. On the other hand, in some cases such as puffiness, weakness, superficial erythema, lymphadenopathy, fever, or if surgical treatment cannot be carried out due to the general condition of the patient, antimicrobial therapy should be determined with careful selection of the antibiotic to be used[21,24]. Establishing drug sensitivity patterns in pathogenic bacteria is a vital step for rising a rational antimicrobial guide. In dentistry, once the use of antimicrobial drugs is required, the selection of antibiotic is established on sensitivity pattern research described from the international literature. It has been revealed that anaerobic bacteria can cause a number of critical human infections, and that they are becoming gradually more resistant to numerous of the conventional anti-anaerobic antibiotics currently in use. In reality, over latest years, resistance to various antimicrobial agents has been commonly described intercontinental and sensitivity patterns have become less predictable[10,25]. Therefore in patients with aggressive periodontitis, the majority clinicians commend matching antibiotic therapy[10,26]. Nevertheless, regrettably, neither adequate guidelines nor pattern of antimicrobial susceptibility to *P. gingivalis* are obtainable from Yemen for suitable antimicrobial treatment. The most familiar drugs used as part of periodontal therapy include, amoxicillin-clavulanic acid, tetracycline, amoxicillin, azithromycin, moxifloxacin, clindamycin and metronidazole[10,26,27]. Conversely, taking into account the most common antibiotics prescribed for systemic diseases, there were additives to this panel that included cefazolin, cefuroxime, doxycycline, cefotaxime and ceftriaxone. Even though *P. gingivilis* are facultative anaerobic organisms, antimicrobial susceptibility testing is performed for anaerobic bacteria. There are three different methods for this purpose including agar dilution, broth microdilution and MIC gradient method by E-test strips[28,29]. In this study, a disc diffusion assay was used to test the antimicrobials for *P. gingivilis* as the results are similar to those of anaerobic bacteria disc diffusion methods with their standard scales for inhibition zone diameter being considered the "gold standard". Several studies have examined the effect of different periodontal treatments on clinical and microbiological parameters in LAP[10,30,31]. To my knowledge, there are no publications on the pattern of antimicrobial susceptibility to *P. gingivilis* from Yemen.

In the present study, the studied isolates illustrated a high level of resistance to amoxicillin (76.7%) but excellent efficacy to amoxiclav (90% sensitive). In spite of this several researchers have revealed diverse results with moderate to high sensitivity to amoxicillin and usually excellent efficacy to amoxiclav[10,31-34]. The current study found a high level of resistance to metronidazole among the isolates (50%), and these results are higher than those reported by Kulik *et al.*,[26] where *P. gingivalis* was 9.5% resistant to metronidazole. Metronidazole resistance has not been reported much in the literature, while in the current study 15 strains of *P. gingivalis* were resistant to

metronidazole. The sensitive finding of those studies showing 100% sensitivity to metronidazole may be that such data do not appear in the results because the experiment cannot be repeated because these strains are no longer viable and false positives may have occurred. The discrepancy is usually caused by too much oxygen during incubation, which is the most common mechanism of false-resistance results with metronidazole because the occurrence of a growth inhibiting zone (death of anaerobic bacteria) is due to exposure to too much oxygen during administration of antibiotic discs[10]. Azithromycin is a new generation semi-synthetic macrolide derivative of erythromycin that has been modified to create a broader spectrum of antibacterial activity and improve tissue penetration. However, in this study, 30% of the strains were resistant to azithromycin. Also in the current study, a high level of clindamycin resistance was found (63.3%) and this is different from the results of several other studies where clindamycin resistance was only 38% or less[27,28]. In the current study, doxycycline 46.7% had a sensitive and moderate inhibitory effect (53.3%) on *P. gingivalis* compared with tetracycline (83% sensitive) and 13.3% moderate. These results differ from those reported by Kulik *et al.*, in Swaziland where they reported a sensitivity of 95.2% to doxycycline[26].

Fluoroquinolones are known to have a very good effect against oral bacteria including *P. gingivalis*. Amongst the range of drugs in this group, moxifloxacin is used to treat a number of infections, include: cellulitis, anthrax, intra-abdominal infections, respiratory infections, endocarditis, meningitis, and tuberculosis. Moxifloxacin was original in 1988 and approved for use in the United States in 1999 and is on the World Health Organization's List of Essential Medicines[35]. Almost all researchers have shown that moxifloxacin has excellent activity against oral microbes,[32,33-34,36] results similar to the results of the current study where the drug showed a high sensitivity of 100% (Table 2).

Only a few studies discuss the activity of different cephalosporins on *P. gingivalis* strains[31,32]. The current study found the results to be highly variable. While cefotaxime and ceftriaxone showed very good efficacy (100%), the sensitivity rate for cefazolin was 73.3% and cefuroxime 80%. Although cefoxitin is the drug of choice from the LAB group for the treatment of anaerobic disease[37]. The selection of the current study includes the most common cephalosporins prescribed in Yemen for systemic/non-oral bacterial infections. The results clearly showed that cefotaxime and ceftriaxone, which belong to the third generation of cephalosporins and have a wider range of activity, have a better effect compared to cefazolin (1st generation) and cefuroxime (2nd generation) in inhibiting bacterial growth.

Moreover, recent findings in Yemen highlight the problem of MDR in Gram-positive and Gram-negative bacteria from clinical bacterial isolates in general and oral infections[9,17,38-42]. This warns us of the need for judicious use of different groups of antimicrobials, especially in our resource-poor country. Furthermore, this requires more focus on identifying relevant resistance drivers and implementing effective strategies to combat resistance and MDR problems.

CONCLUSIONS

Even though the present results show a high sensitivity pattern for *P. gingivalis* strains, some resistance has been observed. Antibiotic resistance patterns can change over the years, making susceptibility testing essential and to promote careful selection of initial antibiotic therapy, as an adjuvant to endodontic therapy. Because resistance cannot be prevented, an attempt should be made to modify its progression by limiting the use of antibiotics and restricting prophylaxis to where it is of proven value. In single antibiotics, the development of resistance to azithromycin and cefazolin also appears to be important. The change of resistance between different members of the cephalosporin group is a factor that should be further investigated due to the lack of a sensitivity profile for these antibiotics and the interpretive criteria for oral bacteria. The disc diffusion method is easy to implement, interpret and can be applied up to one isolation at a time. Similar studies with a large sample size and from other parts of the world should be conducted to obtain information on the effect of geographic distribution on the resistance pattern of *P. gingivalis*.

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AUTHOR'S CONTRIBUTIONS

Shoga Al-Deen HM: study design, writing original draft. **Al-Ankoshy AAM:** literature survey, critical review. **Al-Najhi MMA:** critical review. **Al-Kibsi TAM:** methodology, clinical work. **AL-Haddad KA:** visualization, editing. **Al-Akwa AAY:** critical review. **Al-Shamahy HA:** microbiological study, supervision. **Al-labani MA:** data interpretation. The final manuscript was read and approved by all authors.

DATA AVAILABILITY

The datasets generated during this study are available from the corresponding author upon reasonable request.

CONFLICT OF INTEREST

None to declare.

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