Occurrence and pattern of antibiotic resistance among dental plaque bacteria from gingivitis patients and their clinical correlation

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ABSTRACT

Background: A diverse group of bacteria live in biofilms in the oral cavity. On dental surfaces biofilms form plaque that is potentially involved in caries and periodontal diseases. Periodic studying of plaque microflora and their antimicrobial sensitivity patterns strongly affects the clinical practice in plaque-induced oral diseases.

Materials and methods: Dental plaque samples were collected from 22 patients having ages ranged between 33 and 49 years with gingivitis that met the study criteria. Plaque, gingival and gingival bleeding indices (PI, GI, GBI) were measured for each patient. Laboratory procedures included microbiological examination of plaque samples followed by antibiotic sensitivity testing using disc diffusion method were also proceeded.

Results: All patients were categorized as moderate gingivitis (GI: 1.1-2.0), the recorded PI were 1.2-2.7. Bleeding was observed in all subjects. Gingivitis was significantly higher in males (P=0.021). A total of 121 bacterial species were isolated from plaque samples, Facultative anaerobes constitute 83%. The most frequently isolated bacteria were α-hemolytic streptococci (36.36%) and *Enterococcus faecalis* (14.87%) among facultative, and *Fusobacterium* sp., *Actinomyces* sp., *Veillonella* sp. among obligate anaerobes (3.31%, 2.48%, 2.48%, respectively). Imipenem (77.2%) and Ciprofloxacin (59.4%) were the most effective agents against both bacterial groups. Multi-drug resistance (MDR) was recorded in most of the isolates (> 90%). A very highly significant relation between MDR with each of the above clinical criteria was recorded (P-value= 0.000).

Conclusions: The high level of MDR isolates is of great clinical concern and requires an urgent reassessment of the policies of antibiotic prescription in dental settings.

Key words: Dental plaque, antibiotic resistance, gingivitis. **(J Bagh Coll Dentistry 2018; 30(2): 51-58)**

INTRODUCTION

The oral cavity is colonized by a complex and unique bacterial flora. Different anatomical surfaces, physical and chemical factors in the oral cavity favor the growth of more than 300 Gram positive and Gram negative bacterial species, that are mostly facultative anaerobic with few obligate anaerobic and aerobic species (1). These bacteria usually grow as diverse biofilms. On dental surfaces, if biofilms left unwashed for days, plaque is formed (2). Bacteria in dental plaque have been implicated in oral diseases such as caries, gingivitis and periodontitis; the most prevalent bacterial infections in humans (3). Surprisingly, oral bacteria have also been involved in serious systemic diseases, such as cardiovascular diseases, pneumonia, preterm low birth weight babies and osteomyelitis in children (4). Antibiotics were first introduced into routine medicine in the 1940s. Since then, they have been cardinal to healthcare in treating bacterial infections and preventing infections in susceptible patients (prophylactic action). The degree of resistance to antibiotics is proportional to the degree of their use; the greater the use of an antibiotic, the greater the chance of emerging of resistant bacterial populations to that antibiotic (5)

Increased morbidity and mortality in the United States due to antibiotic resistance was reported by the U.S. Centers for Disease Control and Prevention (CDC) in 2013, and it was greater than 2 million infections and nearly 23,000 deaths each year (6). Unfortunately, the number of resistant bacterial strains that are able to cause infections is increasing, most of which are resistant to more than one antibiotic, a state known as multi-drug resistance (MDR). These multi-resistant strains comprise an increasingly serious danger to the current antimicrobial therapy (7). Bacteria acquire resistance to the antimicrobial agents through different mechanisms; it may be due to mutation in chromosomal DNA or plasmids. Plasmid (small circular DNA strand in the cytoplasm of bacteria) has the ability to transfer from one strain of bacteria to another of the same or different species. Accordingly, bacterial cells in biofilms are 10-1000 times more resistant to antimicrobial agents, when compared to their planktonic equivalents (8). A bacteriological assessment of dental plaque is essential to identify those agents that are involved in the development of periodontal diseases. Moreover, knowledge about plaque bacteriology and their antibiotic resistant patterns are significant in guiding antibiotic selection and appropriate therapy that will help health care professionals to manage local and/or

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systemic infections associated with plaque bacteria and to prevent subsequent complications [9]. Locally, little is known regarding the bacterial etiology of gingivitis and periodontitis. In addition to that, antibiotics are usually prescribed blindly for the treatment of oral infections without any updated information about their antimicrobial efficiency. Therefore, The purpose of this study was to investigate bacterial diversity in dental plaque in patients with gingivitis and to determine the antibiotic susceptibility pattern of the isolated bacteria species, then to find out a possible relation between drug resistance and the clinical state of the disease through studying the clinical criteria of gingivitis; gingival index (GI) plaque index (PI) and gingival bleeding index (GBI).

MATERIALS AND METHODS

1. Patients

Dental plaque samples were obtained from 22 patients having ages ranged from 33 to 49 years diagnosed clinically as having gingivitis and representing both gender attending the Department of Periodontics at College of Dentistry/Hawler Medical University during the period 12th Nov. to 31st Dec. 2016. All the patients showed the clinical signs of gingival inflammation; gingival enlargement, false pocket formation (probing pocket depth PPD ≤ 3 mm) and had no signs of attachment loss (clinical attachment loss, CAL, = 0) or radiographic bone loss in the affected area. All patients were systemically healthy according to their medical history. Exclusion criteria comprised the presence of less than 20 natural teeth, pregnancy, smoking, any systemic condition that could affect the host's periodontal status, use of antibiotics and/or anti-inflammatory drugs within the last 3 months; and professional cleaning or periodontal treatment within the last 6 months. The approval of the local Ethics Committee has been obtained prior to the study. Participants who signed an informed consent were accepted into the study.

2. Clinical examination and sample collection

The clinical assessment was performed for each patient before scaling and polishing by a periodontologist. Periodontal assessment included the following indices: plaque index (PI), gingival index (GI) and gingival bleeding index (GBI). Silness and Löe [10] plaque index (PI) was developed in 1964 and estimated the quantity of plaque in terms of tooth area covered. According to this method, each of the four gingival areas of the tooth was assessed and marked with a score from 0 to 3, in which a PI score 0 means no plaque whereas 3 means a considerable amount. Assessing the severity of gingival inflammation, the Gingival Index (GI) and bleeding index were proposed by Löe (1963) [11]. Scoring of GI and GBI is identical to PI scoring (0 no inflammation, 1 mild gingivitis, 2 moderate gingivitis and 3 sever gingivitis). GBI is usually expressed as a percentage value. Probing pocket depth (PPD) and Clinical attachment loss (CAL) were also measured using WHO periodontal probe (Dental care, USA) [12]. Materials for microbiological examinations were collected from supragingival plaque near the gingival margin using curette without touching the adjacent tissue and pooled in eppendorf tubes containing 1 ml physiological saline (NaCl 0.9%). Samples then were immediately transported to the laboratory.

3. Laboratory diagnosis and antimicrobial sensitivity

In the laboratory, the obtained samples were subjected to double dilution and inoculated onto Blood agar (5%), Chocolate agar, and MacConkey agar plates (Lab M Limited, UK). Plates were incubated at 37°C aerobically, under 5% carbon dioxides and anaerobically for 24-72 h. Discrete colonies were identified using microscopy and biochemical examination [13]. Then the identified isolates were subjected to antimicrobial sensitivity testing using Kirby-Bauer disc diffusion method according to the criteria set by the Clinical and Laboratory Standard Institute (CLSI) 2011 [14]. In this procedure, antibiotics were selected based on the availability and prescription frequency in the study area. Facultative anaerobic bacteria were tested for Amoxycillin (10µg), Amoxycillin/Clavulanic acid (30µg), Ampicillin (10µg), Cefotaxime (30µg), Ciprofloxacin (5µg), Erythromycin (15µg) and Imipenem (10µg). Additionally, Tetracycline (30µg) discs were used for Staphylococci. Obligate anaerobic bacteria were examined for their sensitivity to Amoxycillin/Clavulanic acid (30µg), Ampicillin (10µg), Cefotaxime (30µg), Chloramphenicol (30µg), Ciprofloxacin (5µg), Clindamycin (2µg), Imipenem (10µg), Metronidazole (30µg) and Tetracycline (30µg). Bacterial inoculum was prepared by selecting 4-5 pure colonies of a specific isolate using a sterile wire loop and emulsified in sterile 5 ml of physiological saline (NaCl 0.9%), and the concentration was adjusted to 0.5 McFarland standard. Then, the inoculum was taken and uniformly distributed onto Blood agar plates using a sterile swab. Later, antibiotic discs were placed onto the agar medium and incubated at 37°C for 24 h. Diameters of the zone of inhibition around each disc were measured in millimeters (mm) and classified as sensitive,

intermediate, and resistant according to the standardized table provided [14].

4. Statistical analysis

SPSS (Statistical Package for Science Services) version 23.0 under windows 2010 was used for data analysis. Difference between means and the coefficient estimation was tested using t-test and ANOVA. A *P-value* less than 0.05 was considered as statistically significant and less than 0.01 as highly significant.

RESULTS

Demographic and clinical criteria of the study population are shown in Table 1. A total of 22 patients met the inclusion criteria were included in this study. The mean age ± SD of the study group was 40.32±5.31 years (range: 33-49 years). Gingivitis was significantly higher in males (64%) than females (34%) (P=0.021). The Mean ± SD of the studied clinical criteria gingival index (GI), plaque index (PI) and gingival bleeding index (GBI) were 1.45±0.40, 1.94±0.40, 43.73%±24.83, respectively. According to the obtained data, all the patients were categorized as moderate gingivitis or score 2 (GI: 1.1-2.0), whereas the recorded PI of the patients ranged from 1.2-2.7; score 3 and 4. Gingival bleeding was recoded in all of the patients.

One hundred and twenty one (121) bacterial species were isolated in the obtained plaque samples (Table 2). Among the facultative anaerobic bacteria, α-hemolytic streptococci constituted the most frequently isolated group (36.36%), followed by *Enterococcus faecalis* (14.87%). On the other hand *Fusobacterium* sp., *Actinomyces* sp., *Veillonella* sp. were the most common isolates among obligate anaerobic bacteria (3.31%, 2.48%, 2.48%, respectively). Facultative anaerobic bacteria constitute 83% of the isolates, and the remaining (17%) were obligate anaerobes (Figure 1), with the predominance of Gram positive cocci in the former (88/100) and Gram negative rods in the later (11/21) (Figure 2). The overall antibiotic sensitivity pattern of the bacterial isolates is presented in Table 3. The most effective agent was Imipenem (77.2%), followed by Ciprofloxacin (59.4%). Chloramphenicol and Clindamycin were superior to other agents against obligate anaerobes and showed the same antibacterial activity (62.5%), followed by tetracycline (57.7%). Occurrence and prevalence of multi-drug resistance (MDR) among the bacterial isolates is shown in Table 4. Approximately 22.8% and 20.8% of the isolates showed resistance to 4 and 5 antibiotics, respectively. None of the isolates were fully sensitive to all of the tested agents.

Table 1: Demographic and clinical criteria of the study population (GI: gingival index, PI: plaque index, GBI: gingival bleeding index, SD: standard deviation).

Description	Study Group
Patients	22
Age (year)	
Range	33-49
Mean ± SD	40.32±5.31
Gender	
Female no. (%)*	8 (36)
Male no. (%)	14(64)
GI (mm)	
Range	1.1-2.0
Mean± SD	1.45±0.40
PI (mm)	
Range	1.2-2.7
Mean ± SD	1.94±0.40
GBI (%)	
Range	12.5-83.3
Mean ± SD	43.73±24.83

*** Significant (t-test P-value=0.021)**

Table 2: Isolated bacteria from supragingival plaque of moderate gingivitis patients

Bacteria	Number	Frequency (%)
Facultative anaerobic bacteria		
Staphylococcus aureus	9	7.44
Staphylococcus epidermidis	2	1.65
Staphylococcus saprophyticus	1	0.83
Enterococcus faecalis	18	14.87
α-hemolytic streptococci	44	36.36
Streptococcus pyogens	2	1.65
***Lactobacillus* sp.**	5	4.13
***Corynebacterium* sp.**	5	4.13
***Neisseria* sp.**	10	8.26
***Haemophilus* sp.**	2	1.65
***Micrococcus* sp.**	2	1.65
Obligate anaerobic or micro-aerophilic bacteria		
***Actinomyces* sp.**	3	2.48
***Fusobacterium* sp.**	4	3.31
***Clostridium* sp.**	3	2.48
***Peptostreptococcus* sp.**	4	3.31
***Bacteroids* sp.**	1	0.83
***Prevotella* sp.**	1	0.83
***Porphyromonas* sp.**	2	1.66
***Veillonella* sp.**	3	2.48
Total	121	100

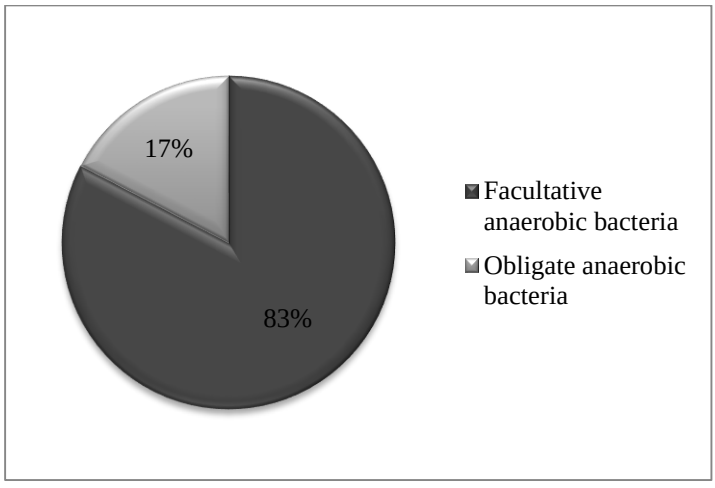

Figure 1: Facultative anaerobic versus obligate anaerobic bacterial isolates

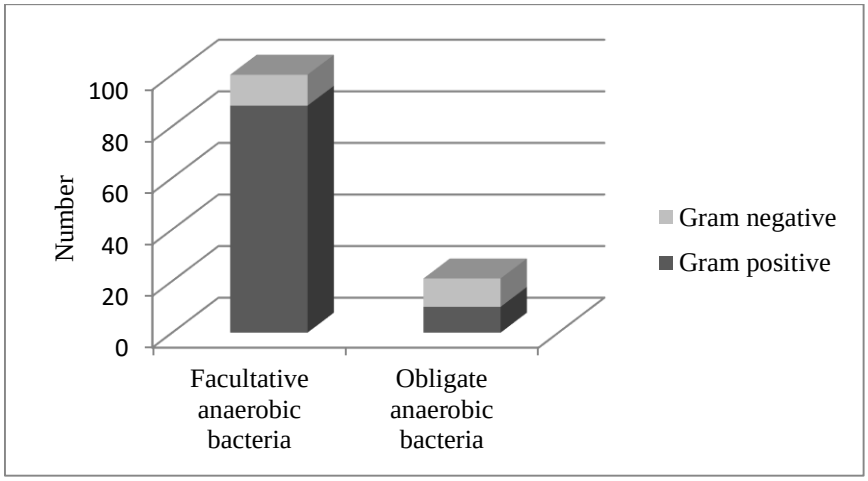

Figure 2: Gram positive versus gram negative bacterial isolates

Table 3: Activity of the tested antimicrobial agents against the bacterial isolates

Antimicrobial agent	No. of tested isolates	Sensitive No. (%)	Intermediate No. (%)	Resistant No. (%)
Amoxycillin	85	15 (17.6)	-	70 (82.4)
Amoxycillin/Clavulanic acid	101	34 (33.7)	-	67 (66.3)
Ampicillin	101	19 (18.8)	-	82 (81.2)
Cefotaxime	101	12 (11.9)	1 (1)	88 (87.1)
Chloramphenicol	16	10 (62.5)	-	6 (37.5)
Ciprofloxacin	101	60 (59.4)	-	41 (40.6)
Clindamycin	16	10 (62.5)	-	6 (37.5)
Erythromycin	85	20 (23.5)	10 (11.8)	55 (64.7)
Imipenem	101	78 (77.2)	-	23 (32.8)
Metronidazole	16	4 (25.0)	-	12 (75.0)
Tetracycline	26	15 (57.7)	1 (3.9)	10 (38.4)

Table 4: Prevalence of MDR among the bacterial isolates

Category	Number	Frequency (%)
Fully sensitive	0	0
Resistant to 1 antibiotic	6	5.9
Resistant to 2 antibiotic	3	3.0
Resistant to 3 antibiotic	18	17.8
Resistant to 4 antibiotic	23	22.8
Resistant to 5 antibiotic	21	20.8
Resistant to 6 antibiotic	12	11.8
Resistant to 7 antibiotic	13	12.9
Resistant to 8 antibiotic*	3	3.0
Resistant to 9 antibiotic**	2	2.0
Total	101	100

***In case of *Staphylococcus* spp. eight antibiotics were tested**
****In case of anaerobes nine antibiotics were tested**

In an attempt to find out the relation between MDR status of the isolated bacteria and gingivitis through studying the clinical criteria of gingivitis; GI, PI and GBI of the included subjects (Table 5 and 6). A very highly significant relation between MDR with each of the above clinical criteria was recorded P-value= 0.000), in which MDR showed in 74%, 73% and 54% of the obtained GI, PI and GBI, respectively, and the highest frequency of MDR isolates (100%) were recovered from patients with high bleeding index (bleeding rate > 60%) (Table 6).

Table 5: Relation between MDR state of the isolates and the clinical criteria of gingivitis

Clinical	MDR (%)	P-value
GI	74%	0.000**
PI	73%	0.000**
GBI	54%	0.000**

**** Very highly significant (t-test P-value < 0.001)**

Table 6: Relation between the frequency of MDR isolates and GBI

GBI (%)	MDR/Total isolates	
	Number	Frequency (%)
0-20	15/16	94/100
21-40	32/37	87/100
41-60	22/25	88/100
61-80	11/11	100/100
81-100	11/11	100/100

DISCUSSION

Twenty two gingivitis patients met the inclusion and exclusion criteria of the study were included. The mean age±SD was 40.32±5.31 years, and males were significantly higher than females (P-value=0.021). This result is highly in accordance with those reported by Marsh (2004) [15] and Nazir et al (2010) [16] in which the occurrence and severity of periodontal diseases is usually increased with age, significantly over 40 years old. It is still uncertain whether aging is a risk factor for the development of severe periodontal diseases, or it is due to the prolonged exposure to real etiological factors in older patients. On the other hand, Ragghianti et al (2004) [17] reported that males are more prone to develop periodontal diseases because they usually display poorer oral hygiene than females. The results of this study showed that isolates were mostly facultative anaerobic Gram positive. The same result was observed by Sharma et al (2011) [18]. Marsh (2003) [19] reported that increased levels of obligate anaerobic bacteria are usually recovered from deep periodontal pockets. According to this fact it is accepted for the balance to be tilted toward facultative anaerobic because all the subjects included in this study had moderate gingivitis. A diverse group of facultative and obligate anaerobic species were isolated from plaque samples. Unfortunately, a number of isolates were missed due to the difficulty in maintaining during sequential identification procedures. Among the facultatives , α-Hemolytic *streptococci* were isolated in the highest frequency (36.36%). It is a broad group of species belonging to the genus *Streptococcus* showing α-hemolysis on blood agar. These bacteria are the most common members of the resident oral flora and behave as opportunistic pathogens. *Streptococcus mutans*, the prominent member of dental plaque bacteria, belongs to this group [20]. The polymicrobial nature of dental plaque and the predominance of Streptococci, more specifically *Streptococcus mutans*, were also mentioned by Saini and co-workers (2003) [21]. *Fusobacterium* sp. was isolated in the highest frequency among obligate anaerobic bacteria (3.31%). In a detailed review by Huang and his colleagues (2011) [22], the important role of *Fusobacterium nucleatum* for the survival of obligate anaerobic species as a bridge bacterium which can aggregate with both aerobic and obligate anaerobes, was discussed. Among the tested antibiotics, Imipenem was the potent one against the whole bacterial isolates (77.2%). It is a carbapenem antibiotic that has a broad spectrum activity against aerobic and anaerobic bacteria and very stable to β-lactamases making it particularly useful in the treatment of serious polymicrobial infections, as well as for initial empirical treatment [23]. A number of worldwide survey and comparative studies have found the increased resistance to the routinely used antimicrobial agents with sustained susceptibility to Imipenem in spite of their old discovery [24]. Among the agents used against obligate anaerobic isolates, Chloramphenicol and Clindamycin were effective (62.5%) with an unexpected high resistance to Metronidazole

(75%). A lower resistance rate to Clindamycin than metronidazole among Gram-negative anaerobes was also found by Boyanova et al (2006) [25]. However, metronidazole was described by Saini et al (2003) [21] as the drug of choice against anaerobes. A relatively high resistance to the tested β-lactam drugs was recorded in this study. Aldridge et al (2001) found that the resistant rates to β-lactams may reach 83%. In another study from Norway by Handal and his colleagues (2003) [26], it was found that nearly 68% of the patients harbored β-lactamase producing bacteria in their subgingival plaque. Resistance to the penicillinase-stable penicillins such as Amoxicillin/Clavulonic acid is usually mediated by other mechanisms, such as changes in the affinity of penicillin-binding proteins [14]. It is obvious that most of the isolates were resistant to multiple drugs. Saini *et al* (2015) [27] reported that microorganisms grow within biofilms show unique phenotypic and genotypic properties, including antibiotic resistance potentials, compared to the same cells grow planktonic in liquid media. The most acceptable explanation for such high frequency of resistance is the genetic exchange that occurs among bacteria growing in high density in the biofilm. Conjugation and transformation are the frequent mechanisms of gene transfer in biofilms. Conjugation, direct cell-cell contact, is the most frequent way of gene transfer among the same species of bacteria and the major contributor in the evolution of new strains with great resistant potential [28]. Transformation is widely happened among oral bacteria possessing systems specialized for the uptake of DNA that could be species-specific or non-specified to a certain genus or species [27].

This study is the first study that has attempted to find out a relation between the frequency of MDR among bacteria isolated from plaque samples and the clinical state of disease that may reflect the isolates' virulence in patients with gingivitis. A very highly significant relation between the MDR state and each of the studied clinical criteria of gingivitis (PI, GI, GBI) was recorded. This is definitely means that resistance to multiple drugs in bacteria plays an important role in disease progression. The results in table 6 supports what statistical analysis have found, as the highest frequency of MDR bacteria were isolated from patients with high bleeding index (GBI > 60%). As it was mentioned above, bacteria growing in biofilms represent distinct phenotypic changes, leading to changes in gene expression, enhancing virulence and the acquisition of antibiotic resistance. In a review article by Schroeder et al (2017) [29], it was recorded that increased virulence and antibiotic resistance arise nearly simultaneously; however, their genetic connection has been relatively disregarded.

CONCLUSIONS:

The high level of multi-drug resistance shown by bacterial isolates cultivated from plaque samples is of great clinical concern and requires an urgent reassessment of the policies of antibiotic prescription in dental settings.

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