FARMACIA, 2018, Vol. 66, 6

DOI: HTTP://DOI.ORG/10.31925/FARMACIA.2018.6.21 **ORIGINAL ARTICLE**

INVESTIGATION OF ANTIBIOTIC SUSCEPTIBILITY OF THE BACTERIAL ISOLATES AND LOCAL FLORA CHANGES AFTER COMPLEX THERAPY IN CHRONIC PERIODONTITIS AND PERIIMPLANTITIS

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Manuscript received: June 2018

Abstract

The aim of the study was to test the antibiotic susceptibility of the subgingival biofilm (SGBF) isolates from chronic periodontitis (PD) and periimplantitis (PI) sites, and to investigate the changes of local flora after complex therapy including, amoxicillin-clavulanate (XL). SGBF from periodontal pocket (*A* sample) and around the dental implant (*B* sample) was collected before (t_1) and after (t_2) therapy. Gram smears and cultures on blood media were done. E-test was used for the antibiotic susceptibility testing. The smears indicated at t_2 a significant decrease of polymorphonuclears number and microbial load with marked reduction of spirochetes and Gram-negative bacilli number. In *A* sample, *P. intermedia* and *Capnocytophaga* predominated at t_1 and *S. oralis* at t_2, while in *B* sample, *Capnocytophaga* predominated at t_1 and *S. mitis* at t_2. All except 2 isolates (not recovered at t_2) were XL-susceptible. The clinical and microbiological findings showed local status improvement at t_2. The antibiogram is helpful when antibiotics are needed in PD/PI therapy.

Rezumat

Scopul studiului a fost testarea sensibilităţii la antibiotice a izolatelor din biofilmul subgingival (BFSG) al situsurilor cu parodontită marginală cronică (PD) şi periimplantită (PI) și investigarea modificării florei locale după terapia complexă, incluzând amoxicilină-clavulanate (XL). S-a recoltat BFSG de la nivelul pungii parodontale şi din jurul implantului dentar (probele *A* şi *B*), înainte (t_1) și după (t_2) terapie. S-au făcut frotiuri Gram și însămânțări pe medii cu sânge. Sensibilitatea tulpinilor la antibiotice a fost investigată prin E-test. La t_2, frotiurile au arătat o scădere semnificativă a polimorfonuclearelor și a încărcăturii microbiene, cu reducerea marcantă a numărului de spirochete şi bacili Gram-negativi. În proba *A* au predominat *P. intermedia* și *Capnocytophaga* la t_1 şi *S. oralis* la t_2, iar în proba *B*, *Capnocytophaga* la t_1 și *S. mitis* la t_2. Izolatele au fost sensibile la XL, exceptând 2 tulpini neizolate la t_2. Constatările clinico-microbiologice au indicat îmbunătăţirea statusului local la t_2. Antibiograma este necesară când antibioterapia se impune în PD/PI.

Keywords: antibiotic susceptibility, periodontitis, periimplantitis

Introduction

In the human body most bacteria are organized in biofilms, which may be involved in many infections, when local homeostasis breaks down [4, 7, 17, 19]. The subgingival biofilm (SGBF) is the main factor incriminated in the appearance and evolution of chronic periodontitis (PD), but a susceptible host is essential [28]. PD is an inflammatory disease leading to destruction of the teeth supporting tissues [14]. The ecological plaque hypothesis states that periodontal disease occurs as a consequence of an exaggerated host inflammatory response towards a subgingival dysbiosis induced by the local environment changes [21]. Periodontal disease may have a large manifestation area, starting from gingival tissues affection up to destruction of periodontium and in certain cases may lead even to tooth loss [20, 22]. Nowadays, dental implant became a current choice in the treatment of missing teeth [32]. However, serious complications might appear and it is estimated that after 10 years, periimplant mucositis (inflammation of the implant soft tissue) affect 63% of patients and 31% of implants, while periimplantitis (PI), inflammatory process affecting the soft tissue and surrounding bone of the implant, affect 19% of patients and 10% of implants [3]. PI onset is mainly the consequence of an imbalance occurred between microbial load and host defence factors [34]. PI is generally associated with PD history,

smoking, systemic diseases or iatrogenic factors. Since *in vitro* antimicrobial susceptibility testing is not usually done in current practice [30], antibiotics are often given empirically when PD and PI require treatment with such drugs.

In Romania, only few data have been published regarding the susceptibility of bacteria isolated from oral infections [5, 6]. The aim of this study was the susceptibility testing against amoxicillin-clavulanate (XL) and other 5 commonly used antibiotics of the isolates from SGBF samples in both PD and PI. In addition, the changes in local flora profile were investigated after combined surgical and non-surgical treatment, including administration of systemic antibiotic.

Materials and Methods

The samples for this microbiological investigation were provided from sites with chronic periodontitis and periimplantitis, before and after complex therapy. Patient's informed consent was obtained. The samples received at time 1 *(t1)* were collected before treatment and the samples received at time 2 *(t2)* were collected at the end of one month of combined therapy (scaling and root planning, antibiotic treatment with XL 1000 mg for 10 days, anti-inflammatory treatment with prednisone 5 mg for one month).

SGBF samples were collected by calibrated loop (0.001 mL) from the deepest periodontal pocket (*A* sample), and around the implant (*B* sample), at t_1 and t_2. Glass slide smears and seeding on: Columbia agar with 5% sheep blood (COS) (BioMérieux, France), chocolate agar with PolyViteX (PVX) (BioMérieux, France), Schedler agar with 5% sheep blood, hemin and vitamin K3 (SCS) (BioMérieux, France), and selective Schaedler agar with neomycin and vancomycin (SNVS) (BioMérieux, France), were performed chair side from *A* and *B* sample. The 0.001 mL inoculum was discharged on SCS and spread on the surface by another loop, while the remained material on the collecting loop was spread on COS, PVX and SNVS. SCS and SNVS were introduced into a jar (GENbox) (BioMérieux, France) with an anaerobic atmosphere generator sachet (GEN-box anaer sachet) (BioMérieux, France) and anaerobic indicator strip (*Anaer* indicator) (BioMérieux, France), while COS and PVX were put into a GENbox with a CO_2 sachet (GENbox CO_2 sachet) (BioMérieux, France). The jars and glass slide smears were transported within half an hour to the laboratory of the Microbiology Discipline, Faculty of Dental Medicine, "Carol Davila" University of Medicine and Pharmacy, Bucharest, where the jars were incubated at 35ºC and the smears were stained by Gram's method. COS and PVX were incubated for 48 h, with first examination at 24 h, while SCS and SNVS were incubated for 10 days, with culture examination every 48 h. All the isolates were identified at genus or species level by conventional microbiological methods and by the Rapid ID 32 STREP, Rapid ID 32 A or Api NH system (BioMérieux, France). In addition, the Slidex Strepto Plus kit (BioMérieux, France) was used for serogrouping the streptococcal strains belonging to *anginosus* group and MAST ID XV MIRROR RING (Mast Group Ltd., U.K.) for searching the growth factors requirement of *Haemophilus* isolates.

The semi-quantitative bacterial growth appreciation on SCS (performed at 48h and revised every 48 h during the whole incubation period) was based on the following arbitrary 1 - 4+ score scale: 0 for no colony, 1+ for 1 - 10 colonies, 2+ for 11 - 50 colonies, 3+ for 51 - 100 colonies and 4+ for more than 100 colonies.

The isolates were tested against: penicillin G (PG), ampicillin (AM), XL, clindamycin (CM), tetracycline (TC) and metronidazole (MZ) by Etest (BioMérieux, France). The antibiograms were done on: *Brucella* blood agar (BBA) (BioMérieux, France) for *Capnocytophaga* spp. and anaerobes, including *Actinomyces* isolates, Müller-Hinton agar with 5% sheep blood (MHS) (BioMérieux, France) for streptococcal and *Neisseria* isolates, and *Haemophilus* test medium (HTM) (Becton Dickinson GmbH, Germany) for *Haemophilus* isolates. The BBA plates were seeded by swab with the inoculum prepared in broth and adjusted to the turbidity of 0.5 or 1 McFarland standard (according to the instructions given by the manufacturer for the respective bacterial isolate). After placing the E-test strips onto the seeded media, the plates were anaerobically incubated at 35ºC for 48 - 72 h, while HTM and MHS plates were incubated in 5% CO_2 atmosphere at 35ºC for 24 h. The quality controls were: *Streptococcus pneumoniae* ATCC 49619, *Haemophilus influenzae* ATCC 49247, *Bacteroides fragilis* ATCC 25285 and *Bacteroides thetaiotaomicron* ATCC 29741. The minimum inhibitory concentration (MIC) was interpreted as indicated by Clinical and Laboratory Standards Institute (CLSI) [11]. The Cefinase-test (BioMérieux, France) was applied on the putative beta-lactamase producers, while the streptococcal isolates were checked for inducible CM resistance by the double disk test (the "D" test), using 15 µg erythromycin (EM) and 2 µg CM disks (Oxoid, UK).

Results and Discussion

A and *B* smears at t_1 showed a huge number of: polymorphonuclear leukocytes, spiral-shaped bacteria, Gram-negative coccobacilli, fusiform and rounded ends bacilli, and Gram-positive cocci *in diplo* and chains. The *A* smear presented Gram-negative reniforme diplococci in small number and moderate Gram-positive filamentous bacteria, while the *B* smear showed also Gram-negative reniforme diplococci in high

number, but only few Gram-positive filamentous bacteria. At t_2, the *A* smear showed very few leukocytes, lack of spirochetes, high number of Gram-positive cocci (mostly in chains) and rods, and moderate Gram-negative bacilli and coccobacilli, while *B* smear showed moderate leukocytes and spirochetes, but high number of: Gram-negative coccobacilli/bacilli, Gram-positive cocci (single, *in diplo* and chains) and Gram-negative reniforme diplococci. The Gram smears indicated at t_2 a significant decrease of the number of leukocytes, spirochetes, fusiforms and Gram-negative cocobacilli/ bacilii with rounded ends.

Table I

Culture findings of the subgingival biofilm samples

Sample	Culture results before therapy		Culture results after therapy	
	Isolates	Growth score[a] on SCS[b]	Isolates	Growth score on SCS
SGBF[c] from the deepest periodontal pocket	*P. intermedia*	3+		
	Capnocytophaga spp.	3+	*P. intermedia*	1+
	S. constellatus	2+	*P. buccae*	2+
	S. mitis	2+	*S. anginosus* (F group)	1+
	S. sanguinis	2+	*S. oralis*	3+
	A. viscosus	1+		
SGBF around dental implant	*Capnocytophaga* spp.	4+		
	P. oralis	2+	*P. buccae*	2+
	S. intermedius	2+	*S. constellatus* (C group)	1+
	S. gordonii	2+	*S. mitis*	3+
	H. parainfluenzae[e]	0	*N. mucosa*[d]	0
	Veillonella spp.	1+		

[a]Score of: 0 for no colony, 1+ for 1 - 10 colonies, 2+ for 11 - 50 colonies, 3+ for 51 - 100 colonies and 4+ for more than 100 colonies; [b]non-selective Schaedler agar; [c]subgingival biofilm; [d]species isolated only from the primary Columbia blood agar and chocolate agar; [e]species isolated only from the primary chocolate agar

Table II

The minimum inhibitory concentrations of the tested antibiotics and beta-lactamase production of the strains isolated from the subgingival biofilm samples

Origin of the isolates		Isolates	MIC[a] (mg/L) [interpretation of susceptibility]						Beta-lacta-mase
			PG[b]	AM[c]	XL[d]	CM[e]	TC[f]	MZ[g]	
SGBF[h] from the deepest peri-odontal pocket	Before therapy	*P. intermedia*	1.5 [I[i]]	1 [I]	0.064[S[j]]	0.016 [S]	2 [S]	0.016 [S]	+[k]
		Capnocytophaga	0.064 [S]	0.019 [S]	0.019 [S]	0.016 [S]	0.094 [S]	NT[l]	-[m]
		S. constellatus	0.023 [S]	0.125 [S]	NT	0.064 [S]	0.75 [S]	NT	NT
		S. mitis	2 [I]	1,5 [I]	NT	0.023 [S]	0.75 [S]	NT	NT
		S. sanguinis	1 [I]	1 [I]	NT	0.023 [S]	3 [I]	NT	NT
		A. viscosus	0.094 [S]	0.064 [S]	0.064 [S]	0.016 [S]	0.38 [S]	>256[R[n]]	-
	After therapy	*P. intermedia*	2 [R]	1,5 [I]	0.064 [S]	0.016 [S]	2 [S]	0.016 [S]	+
		P. buccae	0.38 [S]	0.25 [S]	0.25 [S]	0.016 [S]	0.125 [S]	0.016 [S]	-
		S. anginosus	0.047 [S]	0.094 [S]	NT	0.094 [S]	4 [I]	NT	NT
		S. oralis	0.75 [S]	0.094 [S]	NT	0.064 [S]	0.125 [S]	NT	NT
SGBF around dental implant	Before therapy	*Capnocytophaga*	0.047 [S]	0.064 [S]	0.064 [S]	0.016 [S]	0.064 [S]	NT	-
		P. oralis	0.023 [S]	0.023 [S]	0.023 [S]	0.016 [S]	0.125 [S]	0.016 [S]	-
		S. intermedius	0.016 [S]	0.016 [S]	NT	0.016 [S]	0.19 [S]	NT	NT
		S. gordonii	0.064 [S]	0.094 [S]	NT	0.064 [S]	0.50 [S]	NT	NT
		H. parainfluenzae	NT	24 [R]	0.75 [S]	NT	1 [S]	NT	+
		Veillonella	0.023 [S]	0.023 [S]	0.023 [S]	0.016 [S]	0.19 [S]	0.064 [S]	-
	After therapy	*P. buccae*	0.023 [S]	0.064 [S]	0.064 [S]	0.016 [S]	0.125 [S]	0.094 [S]	-
		S. constellatus	0.064 [S]	0.125 [S]	NT	0.125 [S]	0.094 [S]	NT	NT
		S. mitis	0.047 [S]	0.016 [S]	NT	0.094 [S]	0.125 [S]	NT	NT
		N. mucosa	0.094 [S]	0.064 [S]	0.064 [S]	96 [R]	3 [I]	NT	-

[a]MIC = minimum inhibitory concentration, [b]PG = penicillin G, [c]AM = ampicillin, [d]XL = amoxicillin-clavulanate , [e]CM = clindamycin, [f]TC = tetracycline, [g]MZ = metronidazole, [h]SGBF = subgingival biofilm; [i]I = intermediate susceptible, [j]S = susceptible, [k]+ = beta-lactamase positive, [l]NT = not tested (not recommended to be tested); [m]- = beta-lactamase negative, [n]R = resistant.

Mixed cultures were obtained from all samples and the results indicated a significant shift in the local flora profile at t_2 compared to t_1 (Table I). As expected, the colonies of *Actinomyces viscosus* became visible after one week of anaerobic incubation. *Neisseria mucosa* was isolated only from COS (9 colonies) and PVX (11 colonies), while *Haemophilus parainfluenzae* only from PVX, in scanty growth (4 colonies).

The *Capnocytophaga* spp. strains were isolated from SCS in very heavy growth, but not from the primary PVX, incubated in 5% CO_2 atmosphere for 48 h. However, these isolates were further sub-cultivated on PVX in 5% CO_2 atmosphere and grew properly.

Capnocytophaga spp. and *Prevotella intermedia* predominated (score 3+) in *A* sample at t_1, while at t_2 the first were not isolated and the latter grew extremely scanty (2 colonies). *Capnocytophaga* was by far the predominant bacteria (score 4+) isolated from *B* sample at t_1, without being recovered at t_2.

The antibiograms data are mentioned in Table II, together with the results of beta-lactamase detection (Table II). Besides the beta-lactam antibiotics, the isolates were tested against other 3 drugs of different families, chosen as alternative to XL therapy.

XL was active against all the isolates except for *Streptococcus mitis* and *Streptococcus sanguinis* strains from *A* sample, which expressed PG- and AM-reduced susceptibility, and being implicitly XL-intermediate susceptible. *S. mitis* was the only streptococcal strain found with EM-reduced susceptibility and displayed the M phenotype. *S. mitis* and *S. sanguinis* showed a growth score of 2+ at t_1 but were not isolated anymore at t_2.

Of the isolates tested for beta-lactamase production only *P. intermedia* and *H. parainfluenzae* strains showed positive results and were reported as AM- and PG- resistant. *P. intermedia* was isolated in extremely scanty growth (2 colonies on SCS) at t_2 and it was noticed a slightly increase of PG- and AM-MIC values.

N. mucosa is a commensal oral microorganism but may produce infections in immunocompromised patients. Since there are not accepted criteria for antimicrobial susceptibility evaluation of *N. mucosa*, the MIC values of this bacterial isolates were orientatively interpreted based on the CLSI breakpoints for *Moraxella catarrhalis* (previously known as *Neisseria*/ *Branhamella catarrhalis*) [12]. The *N. mucosa* strain was susceptible to the beta-lactam antibiotics, but highly CM-resistant. CM was active against the other isolates but not the *Haemophilus* isolate, due to its intrinsic resistance.

The isolates were TC-susceptible except for *S. sanguinis*, *Streptococcus anginosus* and most likely for *N. mucosa* too, which presented reduced susceptibility. MZ was fully active against the anaerobic isolates apart from *A. viscosus*, which is actually a facultative anaerobe. However, several studies reported MZ-susceptible *Actinomyces* isolates [15, 35].

Capnocytophaga isolates were considered to be susceptible to all 6 antibiotics tested, by comparing their MIC values to CLSI breakpoints indicated for the group of the following bacteria: *Haemophilus*, *Aggregatibacter*, *Cardiobacterium*, *Eikenella* and *Kingella* (HACEK group), known at present as the group of: *Aggregatibacter aphrophilus* (formerly *Haemophilus aphrophilus*), *Aggregatibacter*, *Actinomycetemcomitans*, *Cardiobacterium hominis*, *Eikenella corrodens* and *Kingella kingae* (AACEK group) [2].

Many studies have been focused on the efficacy of various periodontal therapy methods in improving the clinical periodontal parameters. However, several studies have investigated the subgingival microbiota changes due to periodontal therapy and their results have indicated that the decrease in frequency, levels and proportions of the periodontal pathogenic bacteria was accompanied by the clinical improvement [1, 26, 29, 31]. The present study indicated also the reduction of the periodontal pathogenic bacteria, although the microbiological investigation was limited at one month after therapy. Like in the findings of Socransky *et al* [31], *S. oralis* was the only bacteria found in a high level after periodontal treatment.

Capnocytophaga spp. are members of the normal oral flora, but may produce oral or systemic infections too. These bacteria were isolated from SGBF in healthy periodontium, but from PD too [8, 23, 24], and sometimes in heavy growth [33].

In the present study *Capnocytophaga* was found at a high level at t_1 and this is in agreement with other authors who reported an increased prevalence of this microorganism in periodontal disease [18, 27]. Several scientific papers have also shown a high prevalence of *Capnocytophaga* spp. in diabetic patients with gingivitis or periodontitis [9, 10, 25]. Ehrmann *et al* reported that the rate of this microorganism isolation among the patients investigated (including 11 periodontitis patients) was 100% [13]. The same authors found that 44% of the subgingival isolates of *Capnocytophaga* were beta-lactamase positive, while *Capnocytophaga* strains isolated during the present study were beta-lactamase negative.

In contrast to this study data, Handal *et al* reported *Capnocytophaga* isolates of oral origin with high resistance (MIC ≥ 256 mg/L) against: amoxicillin, ceftazidime, cefotaxime, cefuroxime, EM, CM and TC [16]. It was found that *cfxA2* and *cfxA3* genes were responsible for the extended spectrum beta-lactamase production in 80% of those isolates, while XL and ceftazidime-clavulanic acid were fully active [16].

The clinical examination at *t2* indicated a significant improvement of the local status: gingival reattachment, absence of bleeding on probing and lack of suppuration.

In this study, all the isolates, either putative periodontal pathogens, like *P. intermedia* and *S. constellatus* (members of the orange complex associated with PD), or non-pathogenic bacteria, were tested against all 6 commonly used antibiotics. This allowed a larger view upon the antibiotic resistance among the isolates from PD and PI in dynamics. It is obviously that the combined therapy was focused on SGBF control and not SGBF elimination, in order to reestablish the normal local flora which is compatible with the periodontal and periimplant health status.

Conclusions

The antibiogram indicated that XL was active against all the isolates, including the beta-lactamase producers, except for a couple of oral streptococcal isolates (not recoved at t_2). Sometimes *Capnocytophaga* spp. seems to be one of the main bacteria involved in PD and PI. The present findings suggest that anaerobic incubation should be performed too, when looking for primary isolation of this capnophilic microorganism. The microbiological and clinical data indicated marked local status improvement following the complex therapy, including XL administration. The antibiogram should be considered a helpful tool when antibiotics are required for PD and PI treatment.

Acknowledgement

This work was supported by the Sectorial Operational Programme Human Resources Development (SOP HRD), financed from the European Social Fund and by the Romanian Government under the contract number POSDRU/187/1.5/S/155463.

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