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Antibiotic Resistance Testing of the Total Implant-associated Micro-flora and its Pure Isolates

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Abstract
Objective: The aim of the present study was to examine antibiotic resistant strains among the implant-associated microorganisms in vitro, first as mixed cultures and again as pure isolates for resistance to one of five antibiotics.
Methods: Samples were taken with sterile paper points from the deepest pocket of one implant per patient (n = 24) to culture the total oral micro-flora. The samples were streaked on agar (Schaedler or BHI) and incubated for 7 d in an anaerobic atmosphere. All colonies were rinsed off the plates, aliquots were added to top-agar. Susceptibility against antibiotics (ampicillin, ampicillin + sulbactam, azithromycin and penicillin, moxifloxacin) was determined using the Etest®. Resistant strains were picked, purified and characterized, and the Etests® were repeated with a selection of the pure isolates.
Result: The majority of the mixed cultures (67 – 100 %) showed complete antibiotic resistance. No association with clinical parameters like pocket depth, bleeding on probing or insertion of implants into transplanted bone could be found. Smoking and the surface of the implant also had no influence.

23 % of the 597 resistant colonies contained only yeasts, mostly isolated from irradiated tumour patients. Of the 458 resistant bacteria, the majority were Gram-positive cocci or rods. Staphylococci and *M. micros* were detected occasionally. The resistance for the 138 selected pure isolates was in most cases lower than for the total micro-flora, irrespective of the antibiotic.
Conclusions: The higher resistance of the total flora might be explained by synergistic interactions between its members.

Key words: antibiotic resistance, implant-associated micro-flora, mixed cultures, pure isolates

Introduction

About 500-700 species of bacteria inhabit the human oral cavity [1, 46], and the causal relationship between bacterial plaque composition and gingivitis, periodontitis or peri-implantitis is well established [6, 27, 31, 37, 45]. The micro-floras associated with successful or failing implants differ distinctly in their total numbers and their composition [32, 41].

Healthy peri-implant sites are populated by high proportions of Gram-positive coccoid cells, whereas Gram-negative and strictly anaerobic species are present in low numbers [27, 41, 28]. In contrast, infected pockets are densely populated with Gram-negative obligately anaerobic rods, fusiform bacteria, spirochetes and facultatively anaerobic bacteria [5, 19, 29, 40, 42], *Staphylococcus* spp., enterics and *Candida* spp. have also been found in peri-implant infection in partially edentulous patients [3, 39] .

If a patient presents with plaque accumulation at the implant site, bleeding on probing, pus, pocket depths ≥ 5 mm, bone loss, mechanical and antiseptic treatment with 0.1 - 0.2 % chlorhexidine digluconate solution, as proposed in the concept of Cumulative Interceptive Supportive Therapy (CIST), is followed by systemic antibiotic therapy [21, 27]. However, such an approach is not always successful. Its failure might be attributed to a worldwide increase in resistance to antibiotics, including those prescribed in the dental practice. Especially in patients with head and neck cancer, who have undergone radiotherapy, immunosuppression can occur, and therefore it is of great importance to avoid or successfully treat any infection. In order to choose the right antimicrobial drug, it is necessary to obtain a profound knowledge of the infectious agents, their susceptibilities, the pharmacology of the drug and the medical and/or dental history of the patient. More modern macrolide antibiotics, like azithromycin or third or fourth generation fluoroquinolones, e.g. moxifloxacin might be more effective for treatment than penicillins or other more conventional drugs.

Mixed cultures are microbial communities and differ from pure cultures in numerous aspects [17]. Intra-abdominal infections are one example of poly-microbial infections, involving *Escherichia coli*, enterococci and strictly anaerobic bacteria. Considerations for an effective therapy of this mixed infection are described by Brook 2003 [10] and DiPiro 1995 [12]. Abscess formation is induced by anaerobic and facultatively anaerobic organisms, acting synergistically, and more readily than when mixtures of facultatively anaerobic or of anerobic bacteria alone are present. This may protect the facultatively anaerobes from phagocytosis and other body defences [14].

Like most orofacial infections, peri-implantitis is of

poly-microbial nature, consisting of a variable combination of potential pathogens [19, 44]. Not all of these organisms will be uniformly susceptible to any given antibiotic. Surviving bacteria will have an evolutionary advantage in colonizing the site, which in turn can lead to persistence of the infection. Therefore, a promising concept to learn more about the nature of poly-microbial infections was described by using mixed cultures instead of pure cultures from periodontal pockets for susceptibility testing of antibiotics [49, 50].

To our knowledge, few data exist on the antibiotic resistance of mixed cultures of implant-associated micro-floras.

The purpose of this in vitro investigation was to evaluate the degree of antibiotic susceptibility of mixed implant-associated micro-floras and compare it with that of their pure isolates.

Materials and Methods

For this diagnostic study, 24 patients (13 males, 11 females) of the routine implant recall in the second half of the year 2000 were screened in the Department of Oral and Maxillofacial Surgery, University of Mainz, Germany. Inclusion criteria comprised the following: more than six residual teeth, removable supra-structures in situ for more than half a year, no antibiotic therapy three weeks prior to the examination, no cortisone treatment or chemotherapy, and no use of antibacterial mouthwash 24 h before the examination. Immunocompromised patients (with HIV infection or leukemia) were excluded from this study. According to these criteria twenty-four patients were included who had received a dental implant between 1990 and 1999 at our clinic. 13 (54 %) implants had a rough (four IMZ, two ITI, five Astra, one Ankylos, one Frialit) and 11 (46 %) implants had a smooth surface (Branemark).

An oral status was obtained for each patient, including the determination of the following parameters:

- modified sulcular bleeding-index [33]
- pocket probing depth (PPD) in millimeters
- mobility of the implants
- smoking of the patients
- implants placed in local or transplanted bone
- radiation therapy after resection of a head and neck tumour

After removal of supra-gingival plaque with a light curette for implants (Straumann, Germany) and drying of the surface, a sterile paper point was inserted into the deepest peri-implant pocket, where it was left in place for 10 s. In this manner, one sub-gingival plaque sample was taken per patient (n = 24). The paper points were placed into sterile tubes containing sterile saline and immediately processed. Aliquots of 0.1 ml were streaked on agar plates, prepared from Schaedler broth or BHI (Becton Dickinson, Heidelberg, Germany), and incubated in an atmosphere of H_2 and CO_2 (GasPakPlus™, Becton Dickinson, Heidelberg, Germany) at 37 °C. After 7 d, the plates were taken out and inspected. The colonies were rinsed off with sterile saline, and the resulting suspension was termed the total cultivable implant-associated micro-flora. Aliquots of these suspensions were mixed with top agar and poured onto agar plates (Schaedler or BHI, depending on the previous culturing) to determine possible resistance of the various strains in mixed culture to four or five antibiotics.

The susceptibility of the mixed cultures (n = 48) was tested against azithromycin (0.016 – 256 μg/ml),

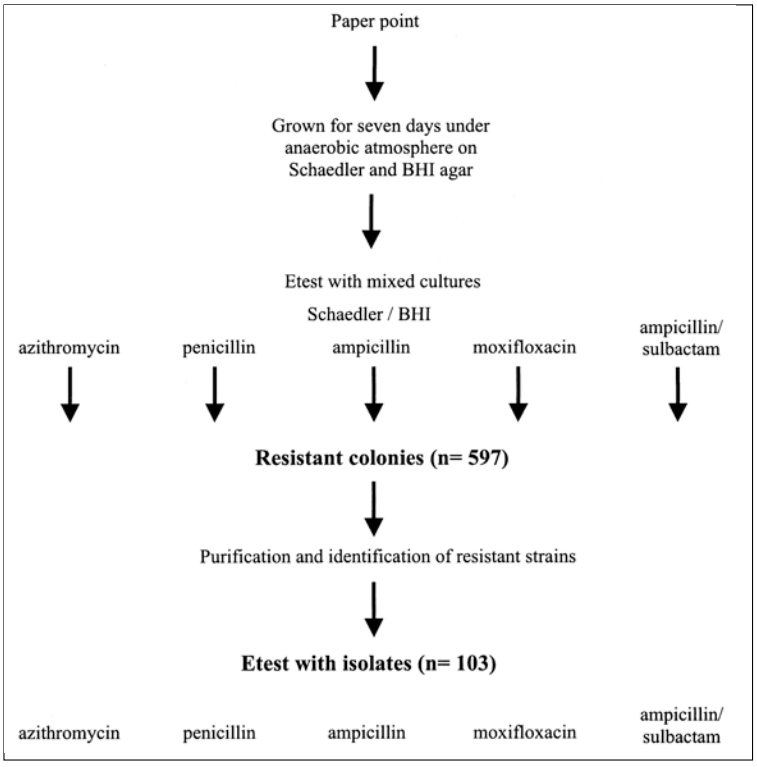


Fig. 1. (Flow chart) course of the microbiological methods starting with the paper point (taken from 24 patients).

penicillin (0.016 – 32 µg/ml), ampicillin (0.016 – 256 µg/ml), ampicillin + sulbactam (2: 1; 0.016 – 256 µg/ml) and moxifloxacin (0.016 – 32 µg/ml; n = 10) using the Etest (AB BIODISK, Solna, Sweden) on Schaedler- and BHI- agar plates in an anaerobic atmosphere. After 24 – 48 h, the Etest was read according to the manufacturers' instructions. Three or more of the most resistant colonies were taken from each plate, purified and further characterized (n = 597). From these, 138 isolates were chosen and were grown on Schaedler, Brain Heart Infusion (BHI) or MRS in an anaerobic atmosphere for 24 – 48 h. The Etest was repeated with these isolates accordingly. The flow chart (Fig. 1) illustrates the course of the microbial method starting with the sample taking. To define which of the microbial samples from the peri-implant pocket have to be considered as resistant or sensitive, concentrations of 3.3 – 6.47 µg/ml azithromycin in gingival crevicular fluid were chosen as cut-off. Since the concentrations of the remaining antibiotics in crevicular fluid are not known, the peak concentration of the respective antibiotic in the serum (3 µg/ml penicillin, 12 µg/ml ampicillin, 177 – 200 µg/ml ampicillin + 82 – 102 µg/ml sulbactam, and 0.55 µg/ml moxifloxacin) was used.

Microbial Analysis

Microbial isolates were divided according to their cellular morphology into rods, cocci and yeasts. Yeasts were not further identified. All isolates were Gram stained (Difco Laboratories, Detroit, MI, USA). To obtain the hemolysis type, the bacterial isolates were spread on blood agar (Columbia-agar with 5 % sheepblood), and to judge if they were aerobic, facultatively anaerobic or strictly anaerobic, they were immediately incubated in an aerobic and anaerobic atmosphere. Gram-positive rods with the typical morphology of lactobacilli were streaked on MRS agar and incubated at 32 °C to confirm their identity. Streptococci were tested for the presence of oxidase (Macherey-Nagel, Düren, Germany) and catalase, and the type of Lancefield antigen was determined (Prolex™, Hain Diagnostica, Nehren, Germany). Enterococci were identified as blue-green colonies growing on CPS agar (bioMérieux, Nürtingen, Germany). Other Gram-positive cocci (staphylococci) were tested for the presence of coagulase (Dryspot Staphytect Plus, Oxoid Ltd, Basingstoke, UK) oxidase (Macherey-Nagel, Düren, Germany) and catalase.

Statistical Analysis

The statistical analysis of the experimental data was performed in a descriptive way only. The MIC's of the mixed cultures and their pure isolates were compared using a pair-wise descriptive method without confirmatory testing. Since the concentrations on the Etests are not linear but exponential, a logarithmic transformation was performed.

Results

Description of the Patient Collective

12 patients participating in this study were smokers, 13 patients were provided with implants with a rough implant surface, 11 patients received implants with a smooth surface. In 13 patients the local bone supply was sufficient to insert the implants into local bone, whereas in 11 patients it was necessary to insert the implants into transplanted bone. Out of the 24 patients 13 had head and neck cancer, 8 of these were treated with radiation after the operation. None of the implants was mobile (Table 1).

Microbial Resistance in Mixed Culture

Resistant strains were detected in each sample taken from the deepest site of the peri-implant pocket from all patients. When testing the inhibitory concentrations of the mixed cultures from these 24 samples for ampi-

Table 1. Characteristics of the patients with and without radiation therapy.

	number of patients	cancer	mean pocket depth	BOP positive	transplanted bone	smokers	implant surface rough	smooth
Total	24	12	4.8	16	11	11	13	11
with radiation therapy	8	8	4.0	6	2	3	4	4
without radiation therapy	16	4	4.6	10	9	8	9	7

Table 2. Numbers of patients with completely resistant colonies (265 µg/ml for azithromycin, ampicillin and ampicillin/sulbactam or 32 µg/ml for penicillin G and moxifloxacin) grown in mixed cultures on BHI- and Schaedler-Agar.

antibiotic	azithromycin (n = 24)	ampicillin (n = 24)	amp./sulb (n = 24)	penicillin G (n = 24)	moxifloxacin (n = 5)
BHI	22 (92 %)	16 (67 %)	16 (67 %)	17 (71 %)	5 (100 %)
Schaedler	20 (83 %)	22 (92 %)	19 (79 %)	23 (96 %)	5 (100 %)

Table 3. Highest antibiotic resistance of colonies grown in mixed cultures tested on BHI and Schaedler agar.

patient	azithromycin		ampicillin		ampicillin/ sulbactam		penicillin G		moxifloxacin	
	BHI	S	BHI	S	BHI	S	BHI	S	BHI	S
1	256	256	32	256	**0.25**	**0.25**	4	**3**	-	-
2	256	256	256	256	**2**	**128**	32	32	-	-
3	256	6	256	192	256	**128**	32	32	-	-
4	256	256	256	256	256	256	32	32	-	-
5	256	256	96	256	**1**	256	24	32	-	-
6	256	256	256	256	256	256	32	32	-	-
7	256	64	256	256	256	256	24	32	-	-
8	**4**	256	128	192	**48**	256	8	32	-	-
9	256	256	**1**	256	256	256	**0.75**	32	-	-
10	256	256	256	256	**16**	256	32	32	-	-
11	256	256	256	256	256	256	32	32	-	-
12	256	256	256	256	**192**	256	32	32	-	-
13	256	6	**5**	256	256	**192**	8	32	-	-
14	8	256	256	256	**128**	256	32	32	-	-
15	256	256	256	256	256	256	32	32	-	-
16	256	256	32	256	**32**	256	32	32	-	-
17	256	256	256	256	256	256	32	32	-	-
18	256	256	256	256	256	256	32	32	-	-
19	256	256	256	256	256	256	32	32	-	-
20	256	256	256	256	256	256	32	32	32	32
21	256	256	**8**	256	256	256	10	32	32	32
22	256	256	48	256	256	256	32	32	32	32
23	256	256	256	256	256	256	32	32	32	32
24	256	**1**	256	256	256	**0.19**	32	32	32	32

Bold = samples which are susceptible to the antibiotic (peak concentrations in serum or crevicular fluid)

cillin, ampicillin/sulbactam, azithromycin, penicillin G and moxifloxacin (in five cases) by the Etest, strains with different degrees of resistance were detected.

The numbers of completely resistant mixed cultures (ampicillin, ampicillin/sulbactam and azithromycin 256 µg/ml, penicillin G and moxifloxacin 32 µg/ml) are listed in Table 2.

67 – 100 % of the samples on BHI-Agar and 79 – 100 % of the samples on Schaedler-Agar showed resistance up to the highest concentrations. In nine patients, all mixed cultures were completely resistant to all tested antibiotics (Table 3).

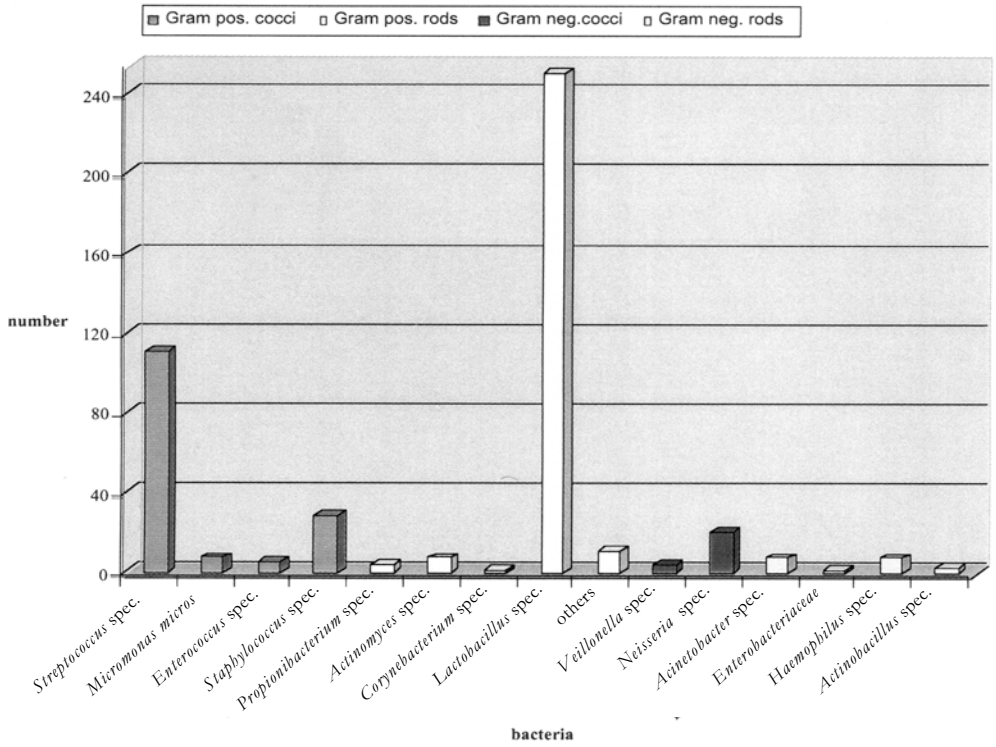
In two patients, one of the mixed cultures was susceptible to azithromycin at its peak concentration in serum (2 µg/ml), and both were susceptible to azithromycin at its peak concentration in gingival crevicular fluid (3.3 – 6.47 µg/ml). Three patients had mixed cultures, which were susceptible to ampicillin at its peak concentration in serum (12 µg/ml). Two patients had mixed cultures, which were susceptible to penicillin G at its peak concentration in serum (3 – 3.8 µg/ml). In 13 patients, mixed cultures were found, which were susceptible to concentrations of ampicillin + sulbactam in the range of 0.19 – 192 µg/ml (peak serum concentration). None of the five tested patients had mixed cultures susceptible to moxifloxacin.

From each plate several of the most highly resistant samples were taken and purified, which makes up a total number of 597 isolates.

Characterization of the Purified Resistant Isolates

139 (23 %) samples of the 597 isolates were yeasts, which were not further examined. They originated from 10 patients, six of which had received radiation therapy.

168 (37 %) of the 458 remaining isolates were cocci and 290 (63 %) were rods. 432 (94 %) of the isolates were Gram-positive determined by means of Gram-stain testing. The 155 Gram-positive cocci were isolates of *Streptococcus* spec. (112), *Staphylococcus* spec. (29), *Micromonas micros* (8), and *Enterococcus* spec. (6). The 277 (61 %) Gram-positive rods are isolates of *Lactobacillus* spec. (251), *Actinomyces* spec. (8), *Propionibacterium* spec. (4), *Corynebacterium* spec. (2), and 12 other Gram-positive rods. 26 (6 %) Gram-negative isolates could be cultivated and identified. 5 (1 %) of them are cocci, *Veillonella* spec. (4) and one *Neisseria* spec. and 21 (5 %) are rods, *Haemophilus* spec. (8), *Acinetobacter* spec. (8), *Actinobacillus* spec. (3), and *Enterobacteriaceae* (2) (Fig. 2).



Gram positive cocci	*Enterococcus* spec.	*Staphylococcus* spec.	*Micromonas micros*	*Streptococcus* spec.	
number	6	29	8	112	
Gram positive rods	*Propionibacterium* spec.	*Actinomyces* spec.	*Corynebacterium* spec.	*Lactobacillus* spec.	others
number	4	8	2	251	12
Gram negative cocci	*Veillonella* spec.	*Neisseria* spec.			
number	4	21			
Gram negative rods	*Acinetobacter* spec.	*Enterobacteriaceae* spec.	*Haemophilus* spec.	*Actinobacillus* spec.	
number	8	2	8	3	

Fig. 2. Distribution of the identified Gram-positive cocci (155), Gram-positive rods (277), Gram-negative cocci (21) and Gram-negative rods (5).

concentrations higher than 2 μg/ml (peak concentration in serum); and 15 isolates even showed resistance higher than 3.3 – 6.47 μg/ml (peak concentration in gingival crevicular fluid).

15 out of 31 isolates tested against ampicillin showed resistance to a concentration higher than 12 μg/ml (peak concentration in serum). 10 out of 31 isolates tested against ampicillin/sulbactam showed resistance to a concentration higher than 177 – 200 μg/ml ampicillin + 82 – 102 μg/ml sulbactam μg/ml (peak concentration in serum). 10 out of 22 isolates tested against penicillin G showed resistance higher than 3 μg/ml (peak concentration in serum). Four out of 10 isolates tested against moxifloxacin showed resistance higher than 0.55 μg/ml (peak concentration in serum).

Comparison of the Degree of Antibiotic Resistance of the Mixed Cultures and the

found to be resistant to only 0.032 μg/ml when tested again as purified isolates. Accordingly, mixed cultures, completely resistant to penicillin or moxifloxacin (> 32 μg/ml) were only resistant to 0.19 μg/ml.

The statistical analysis of the differences in resistance to four of the antibiotics was performed, using the number of patients harbouring resistant strains (Fig. 4). Due to the small number of patients (n = 5) with moxifloxacin resistant strains, the respective data were omitted from further evaluation.

In accordance with Table 3, the data were analyzed separately for samples grown on BHI or Schaedler agar. Fig. 4 shows the differences in resistance (logarithmic MIC values of the mixed cultures minus the MIC values) for the isolates per patient and antibiotic.

If a difference in resistance between the mixed culture and the pure isolates can be detected, a positive logarithmic value means the isolates are more susceptible than the mixed culture, a negative value means they

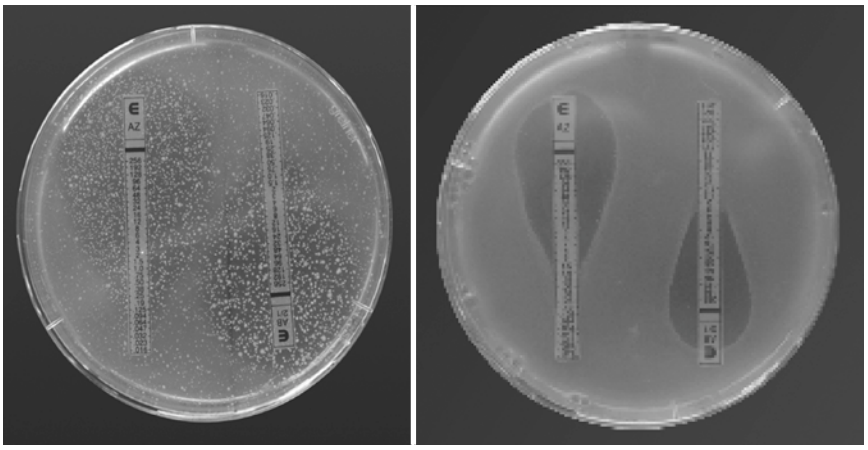


Fig. 3. Example of an Etest testing azithromycin and ampicillin/sulbactam with a mixed culture on the left, showing resistant colonies, and with purified isolates on the right, showing the inhibition zones.

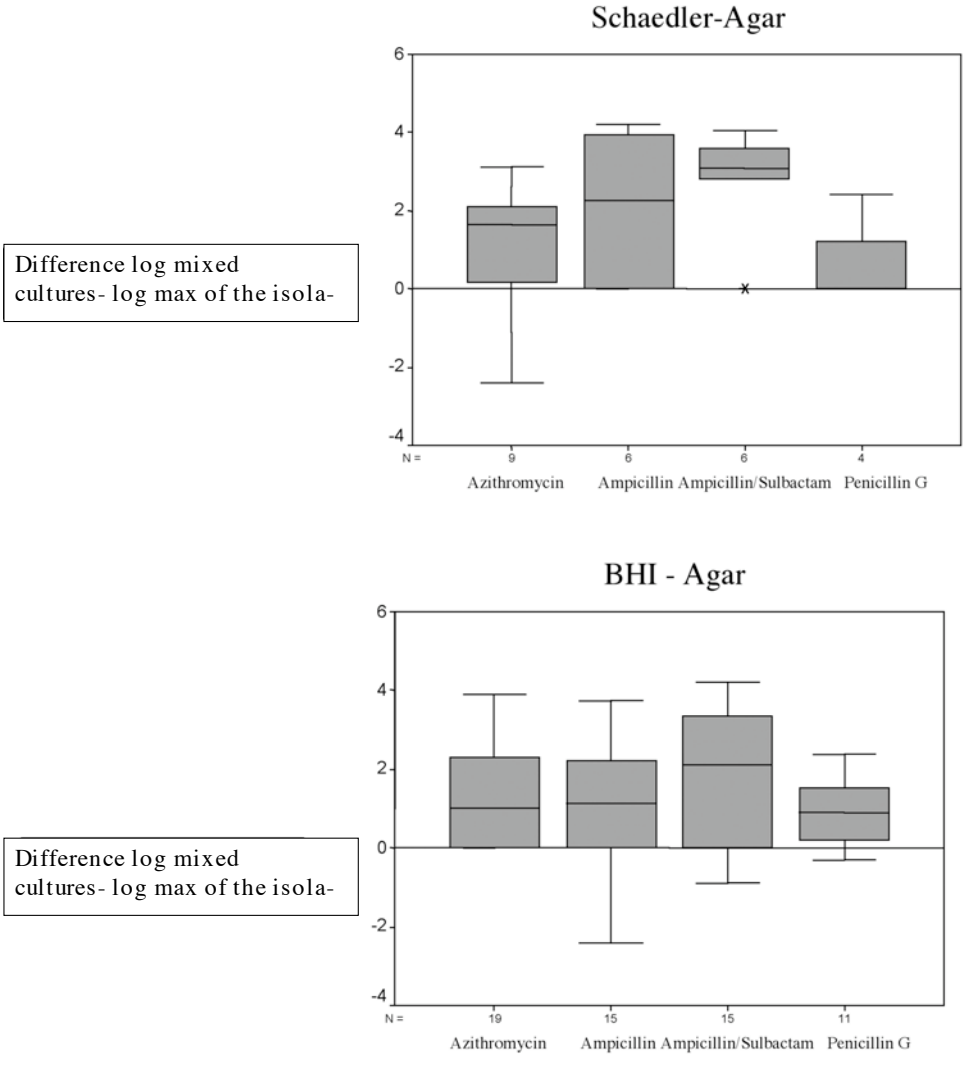


Fig. 4. Difference of the logarithmic MIC values of the mixed cultures – the MIC values of the isolates on Schaedler- and BHI-Agar.

are more resistant. In the majority of the cases, the values were positive. Only in very few cases negative values were found: for the isolates grown on Schaedler agar, this was the case with azithromycin, for those grown on BHI agar, it mainly concerned ampicillin.

Discussion

Important pharmacological determinants, including the degree of absorption, rate of metabolism and duration of effective antimicrobial sulcular levels [44], need to be known in order to select the optimal antimicrobial therapy. Pharmacokinetics of antibiotics in plasma have been well described, but few data exist

about penetration of the drugs into the gingival crevicular fluid [36, 47].

Azithromycin exhibits excellent ability to penetrate into both normal and pathological periodontal tissues. After an oral dosage from 500 mg once daily, the peak serum level was 0.33 ± 0.04 μg/ml, the highest concentration in saliva was 2.14 ± 0.30 μg/ml and in gingival tissue 6.47 ± 0.5 μg/ml and had a half-life of 12 hours [8, 22].

The concentrations of the remaining antibiotics in crevicular fluid are not known Fluoroquinolones also penetrate readily into periodontal tissue and gingival crevicular fluid and may reach there even higher concentrations than in serum. 12 hours after two oral dosages of 400 mg, the peak serum level was 0.55 μg/ml [16] and this is the concentration we used for this study. The peak concentrations of the remaining antibiotics in the serum, 3 μg/ml penicillin [43], 12 μg/ml ampicillin [34] and 177 – 200 μg/ml ampicillin + 82 – 102 μg/ml sulbactam [35], were used.

In the present study, we first investigated the mixed implant-associated micro-flora and again as pure isolates for resistance to one of five antibiotics: azithromycin, ampicillin, ampicillin/sulbactam, penicillin G, moxifloxacin. It was shown that none of the antibiotics was active against all members of the mixed implant-associated micro-flora. In general, resistant organisms isolated from these mixed cultures were more susceptible against the five tested antibiotics, than when they were present in mixed culture.

In each patient, in mixed culture highly resistant microorganisms were found. Their number and degree of resistance was not associated with any clinical parameters like pocket depth, bleeding on probing or insertion of implants into transplanted bone. Smoking and the surface of the implant (rough/smooth) also had no influence.

After microscopic inspection, some of the resistant colonies were identified as yeasts. The majority of these were isolated from tumour patients who had received radiotherapy. An increase of yeasts in irradiated patients has been described by Weischer *et al.* 1996 [51]. A change in the oral micro-flora was observed by Amstahl *et al.* 2003 [4] in patients who had received radiotherapy for head and neck cancer six months earlier with a subsequent reduction in the function of the salivary glands, Especially the proportion of lactobacilli, the numbers of *Candida albicans*, *Staphylococcus aureus* and enterics were increased in the control group.

Little information is available concerning the composition and the level of antibiotic resistance of the normal implant-associated micro-flora. Reports on an increase in antibiotic resistance involve true pathogens as well as microorganisms comprising the normal human flora. For example, Van Winkelhoff *et al.* 2000 [48] could show that the widespread use of eight antibiotics in Spain (including penicillin and azithromycin) was reflected in the increased level of resistance in subgingival bacteria. Prabhu *et al.* 2005 [38] reported that the percentage of fluoroquinolone resistant strains from the oropharynx after quinolone antimicrobial prophylaxis was greatly increased; many of the isolates were viridans group streptococci. Betriu *et al.* 2005 [7] observed in organisms of the *Bacteroides fragilis* group an increase of the rate of resistance to fluoroquinolones of 6 to 16.5 % over a period of five years.

Van Winkelhoff *et al.* 2000 [48] found in patients with adult periodontitis, who had previously been treated with antibiotics, a high percentage of azithromycin resistant bacteria This confirms the occurrence of azithromycin resistant mixed cultures or purified isolates from this study. Known mechanisms of resistance to macrolides include the presence of one of two efflux pumps or methylation of the ribosomal target in bacteria [15].

In mixed culture, all samples from this study were completely resistant to moxifloxacin. Several mechanisms leading to quinolone resistance have been described, e.g. alterations of the quinolone targets and multi-drug efflux pumps, some of which are located on the chromosome or plasmid-borne [11]. When the purified isolates were tested, only one proved to be completely resistant to moxifloxacin and in addition to several other antibiotics (data not shown). It was characterized as belonging to the viridans streptococci, for which multi-drug efflux pumps have been reported [38].

Penicillin resistance in oral pathogens is often associated with the production of β-lactamase [13, 20]. The presence of β-lactamase producing bacteria may lead to treatment failure or disease recurrence and can protect susceptible bacteria from β-lactam antibiotics [9]. This phenomenon was reflected in the present study in the mixed cultures, when comparing the efficacy of penicillin G or ampicillin alone with that of ampicillin in combination with the β-lactamase inhibitor sulbactam.

Currently, the therapy of peri-implant infections is tailored towards the presence of only a few periodontopathogens. The antibiotic susceptibilities of these species have been described for isolated strains *in vitro* [18, 34], and these values are used for the therapy with specific systemic antibiotics. This concept often only leads to a short-term success, but in many cases symptoms like inflammation can return and persist, which could lead later to loss of the implant. However, in the crevicular sulcus almost always a mixed flora is present and might even be organized as a biofilm [18, 48]. The analysis of the resistant implant-associated micro-flora from this study confirmed the existence of a microbial community, which was dominated by various resistant lactobacilli and oral streptococci. Resistant staphylococci and *M. micros* were detected only occasionally. Bacterial resistance to one ore more classes of antibiotics can be due to the presence of efflux pumps or the production of modifying enzymes. Innately sensitive bacteria become resistant because of mutations affecting the drug target or acquisition of resistance genes from other organisms. Of special importance is in this context horizontal gene transfer, for example in multi-species biofilms [26, 52], where antibiotic resistant bacteria from the normal oral flora can serve as reservoir for such genes.

The matrix of a biofilm can impair diffusion of and scavenge or inactivate a significant proportion of the applied active agent and thus protect the bacteria from

the action of the antimicrobial substance [24, 25].

An explanation for the higher resistance, observed in this study for the total mixed flora, might be that similar phenomena occur, which have been already shown for biofilms [23, 30], but further investigations will be necessary to confirm this.

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