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Antimicrobial susceptibility tests on anaerobic oral mixed cultures in periodontal diseases

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Abstract. The ecosystem of the dental plaque in periodontal diseases is very complex: the study of such micro-organisms, which are mostly strict anaerobes, requires the use of specific techniques under conditions of strict anaerobiosis. The aim of the present study was to design a rapid method to evaluate the activity of antimicrobials on mixed bacterial plaque of subjects with periodontal diseases. The study was carried out using a computerised instrument generally used for simultaneous diagnostic tests with aerobic bacteria. Operative and methodological modifications were made to obtain conditions of strict anaerobiosis and the balanced growth of all the microbial forms present in the mixed cultures of the plaque. Penicillins and cephalosporins were active on all the samples, whereas colistin, gentamicin, kanamycin and nalidixic acid showed no activity. Clindamycin, tetracycline, erythromycin and penicillin G were effective only against some samples. The activity of the antimicrobials towards isolated strains was analogous to that towards the corresponding mixed culture.

Key words: mixed culture; anaerobic bacteria; antimicrobial test; periodontal diseases

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Bacteria are the primary etiologic agents in periodontal disease (Socransky 1970, Listgarten 1976). It was thought that the amount of plaque rather than its composition was responsible for inflammation of periodontal tissues (Loe et al. 1965), but successive studies with more refined microbiological methods revealed that not only the amount but also the quality of the plaque was important (Tanner et al. 1979, Slots et al. 1986, Tanner & Goodson 1986, Marsh 1995). The composition of bacterial plaque is different in a state of periodontal health, in cases of gingivitis, and in various forms of periodontitis (Bragd et al. 1987, Slots & Listgarten 1988, Petsios et al. 1995). The predominant bacteria in periodontal pocket are obligately anaerobic Gram-negative rods, filaments or spiral-shaped bacteria, many of which are nutritionally fastidious and difficult or, till now impossible, to grow in pure culture.

It has been shown in animal models that, in the presence of gingivitis, the bacterial plaque increases in diversity, but no study has demonstrated the specificity of any bacteria for these pathologic forms (Page & Schroeder 1982, Page 1991). Different micro-organisms have been found in adult chronic periodontitis, but no correlation has been demonstrated between the presence of such bacteria and the pathologic phenomena typical of this form (Slots 1979, Slots et al. 1988). In many cases of localised juvenile periodontitis, specific micro-organisms, whose virulence factors can worsen the pathology, have been observed, but this does not occur in all patients or at all sites; moreover, it is not certain that other micro-organisms do not provoke the same disease at other sites and in other patients (Cianciola et al. 1977, Van Dyke et al. 1980, Vandesteen et al. 1984, Boughman et al. 1986, Beaty et al. 1987).

Recently has been hypothesized that the etiology of various periodontal diseases could be due to the individual reaction of the host and to the synergetic action of many microbial forms, rather than to the presence of individual specific pathogenic micro-organisms: periodontal diseases are probably an example of a polymicrobial infection, perhaps involving pathogenic synergism (Maiden et al. 1990, Marsh & Martin 1992, Gilbert & Brown 1995, Marsh & Bradshaw 1995).

In cases of refractory periodontitis, which does not respond to common surgical or mechanical therapy, treatment with antibiotics requires preliminary microbiological tests (Bonta et al. 1985, Tanner & Goodson 1986). Isolation of the pathogens and study of their susceptibility to various antibiotics is a decisive factor in the treatment outcome; caution is warranted in prescribing antibiotics without a preliminary microbiological screening (Listgarten et al. 1978, Slots et al. 1988). Moreover the spectrum of antimicrobials known to be effective in treating anaerobic infections has

changed significantly in recent years: there is occurrence of resistance to various antimicrobials in certain groups of anaerobes (Finegold 1988), and some agents previously regarded as therapeutic mainstays, such as tetracycline, are no longer considered useful (Rosenblatt 1986).

Although the study of anaerobic micro-organisms has been simplified by the availability of anaerobic techniques that have been refined in recent years, the isolation of the various microbial forms and study of their sensitivity to antimicrobials agents are complex and time-consuming; moreover these tests often merely involve simple determinations of the minimum inhibitory concentration of antimicrobials, using bacteria grown in pure culture, usually under conditions that do not reflect their natural environment. Consequently, there is a need for improved methods of evaluating antimicrobials (McDermid et al. 1987, Herles et al. 1994).

The primary object of our study was to define a rapid and automatic method to determine, in anaerobic conditions, the activity of antimicrobials on mixed bacterial plaque of subjects affected by various periodontal diseases. We used a new method for antibiograms in anaerobic conditions and verified the reliability of the results by following the growth curves of mixed cultures in the presence of the tested antimicrobial agents. The results obtained on a sample of plaque were then compared with those obtained on strains isolated from the same plaque sample.

Materials and Methods

Samples

We collected samples of bacterial plaque from 9 adults (aged 30 to 60 years), of which 5 were affected by adult chronic periodontitis, 1 by rapidly progressive periodontitis, 2 by gingivitis and 1 by pseudomembranous stomatitis (Table 1). None of the patients had been given antibiotic therapy within the last 3 months.

Table 1. Characteristics of tested subjects

No.	Sex	Age (year)	Subject periodontal disease	pocket depths (mm)
1	F	40	adult chronic periodontitis	6, 5, 5, 7
2	M	50	adult chronic periodontitis	6, 5, 5, 5
3	M	45	adult chronic periodontitis	6, 5, 6, 6
4	F	40	adult chronic periodontitis	6, 5, 6, 5
5	M	55	adult chronic periodontitis	7, 7, 5, 7
6	M	30	rapidly progressive periodontitis	8, 7, 6, 8
7	M	30	gingivitis	5, 4, 4, 5
8	F	30	gingivitis	5, 5, 4, 4
9	F	60	pseudomembranous stomatitis	5, 4, 4, 4

Operative conditions

Each subject underwent four samplings of dental plaque from pockets 4 to 8 mm deep, one in each quadrant; the four samples, obtained by sterile curettes (Socransky et al. 1963), were pooled and immediately placed in a vial containing 1 ml of pre reduced Aranki medium (Aranki et al. 1969) in Anapak jars (Scott Laboratories, Fiskeville, Rhode Island, USA). The resultant initial suspension (McFarland turbidity no. 1) was transferred to the laboratory and introduced into an anaerobic cabinet (Forma Scientific, model 2024, Marietta, Ohio, USA) in an atmosphere of N_2, H_2 and CO_2 (85/10/5, v/v/v) at an oxygen concentration <20 ppm (Brusa & Ferrari 1985) and processed in less than 2 h after sampling. All successive manipulations of samples for microbiological analysis were carried out in the same cabinet, and the media used were previously reduced in the cabinet for 48 h.

Starter broth

MT medium (Marcus & Talalay 1956), modified by the addition of 5 g/ml of hemin and 1 g/ml of vitamin K, was inoculated at a concentration of 6% of the initial suspension of plaque samples and used to perform the antibiograms and the growth curves. For the same purpose, the isolated strain cultures, grown overnight at 37°C in modified MT medium, were diluted with modified MT medium to rich a turbidity comparable to McFarland no. 0.5 and inoculated in the medium at a concentration of 1.2%.

Phase-contrast microscopy

Small aliquots of initial plaque suspensions, immediately after sampling, and of inoculated medium, before and after 6-8 h of incubation, were aspirated in a sterile syringe, placed onto a microscope slide and then analysed with a differential phase-contrast microscope (Axioskop, Zeiss, Germany) at a magnification of 1200×, equipped with a high-definition telecamera (model CCD 2/3″, Panasonic) connected to an electronic 12 inc. video (Grounding). One hundred microorganism, from fields selected at random, were classified into seven morphological categories (Mousquès et al. 1980): motile rods, spirochetes, filaments, fusiforms, coccoid cells, curved and regular rods.

Isolation and identification of the micro-organisms

The cultivable flora present in the sample of plaque no. 8 (taken as an example) was isolated using different media. Specifically: numerically predominant microorganisms were isolated on agarized modified MT medium; *Prevotella* and *Fusobacterium* on tryptic soy agar to which 5 μg/ml hemin, 0.5 μg/ml menadione, 100 μg/ml kanamycin, 7.5 μg/ml vancomycin and 5% defibrinated horse blood (Unipath SpA, Milano, Italy) had been added (Zambon et al. 1981); spirochetes on blood agar (Holdeman & Moore 1973) to which 50 μg/ml cocarboxylase, 2 μg/ml rifampicin, 800 U/ml polymyxin B, and 75 μg/ml nalidixic acid (Fiehn & Frandsen 1994) had been added; *Campylobacter* on brain heart infusion agar (Holdeman & Moore 1973) to which 5 μg/ml trimethoprim lactate, 10 μg/ml vancomycin, 2.5 U/ml polymyxin and 10% defibrinated hourse blood (Unipath SpA, Milano, Italy) had been added (Skirrow 1977); *Leptotrichia* on CVE agar medium (Walker et al. 1979); *Capnocytophaga* on TS-blood agar to which 50 μg/ml bacitracin and 100 μg/ml polymyxin B (Mashimo et al. 1983) had been added.

Appropriate dilutions of the sample suspension were spread (0.1 ml) with a bent glass rod on duplicate pre reduced agar plates with the different medium. After incubation for 24–72 h at 37°C in the anaerobic cabinet, a suitable dilution plate with isolated colonies was selected, and the colonies were picked up, grown in cooked-meat medium

(BBL, Cockeysville, MD, USA) and stored at −80° C. All the procedures were performed in the anaerobic chamber. The isolated strains were subjected to morphological (description of the colonies, observation at the phase-contrast microscope and Gram staining) and biochemical (API System 20 A, bio-Mérieux, France) evaluations as well as gas chromatography of volatile acid products (Variel et al. 1982). Identification was made according to the API 20 A analytical profile index and Bergey's Manual of Determinative Bacteriology (Holt et al. 1994).

Autoanalyser

We used an instrument (Abbott Avantage MS2) designed for studies involving kinetic changes in optical density measurements. The instrument (Thornsberry 1986) consists of analyser module, cuvets and control module. The analyser module contains an electro-optical scanning system which every 5 min automatically monitors the growth of organisms in cuvets, by reading changes in trasmittance at 670 nm; it also provides a temperature-controlled environment and the agitation of the inoculated broth medium. During the short time it takes for reading the trasmittance the shaker stops rotating. The system utilises appropriate plastic disposable cuvette assemblies, named cartridge (cm 12×2): the analyser module, which measures cm 45×50, may hold seven cartridge, composed by eleven individual cuvets.

The analysis module is linked to a control module; data concerning the culture is entered into the computer of the control module, which prints out the receptacle in the analyser module, where the cuvets are to be placed. The computer records transmission data and calculates the optical density ($OD = -\log T_n/T_0$); by means of the continuous monitoring of optical density in each cuvets, growth curves are constructed. The AVANTAGE antimicrobial system programme utilises sequential turbidimetric analysis of microbial growth in presence and absence of antimicrobials, to determine both qualitative antimicrobial susceptibilities and clinically relevant minimum inhibitory concentrations.

Antimicrobials

The activity of 20 antimicrobials was assayed: amoxicillin,ampicillin, penicillin, ticarcillin, cephalothin, cefamandole, cefoperazone, cefotetan, cefoxitin, cefuroxime, imipenem, moxalactam, gentamicin, kanamycin, nalidixic acid, clindamycin, chloramphenicol, colistin, erythromycin, tetracycline. We used antimicrobial saturated disks manufactured by Abbott (Diagnostic Division, Irving, Texas, USA).

Antibiogram

The antibiogram was obtained in a type of cuvette assemblies named "transfer cartridge" (T), composed of one big upper growth chamber, into which the inoculated medium is initially introduced through a small opening, and, in the lower portion, of 11 cuvets into which the bacterial culture is automatically transferred once it reaches the log-phase growth (predetermined changes in trasmittance value) and where the individual tests are run. The cartridges were prepared by placing antimicrobial disks into the bottom of each of the ten lower cuvets (one cuvet without antimicrobial was the reference blank) by the aid of the automatic dispenser and then forcibly sealing there with plastic sealers. To completely eliminate oxygen from this type of cartridges, it is therefore necessary to insufflate into its single hole, for 10 min, a mixture of $N_2/H_2/CO_2$ (85/10/5, v/v/v) before the introduction in the anaerobic cabinet. The inoculated media (13.5 ml) was then introduced in the upper chamber and covered with 1.5 ml of paraffin oil. Each cartridge, sealed with the appropriate stopper, was removed from the anaerobic cabinet and placed into the analyser module.

A program for antibiograms (MS-2 Susceptibility System, Abbott Laboratories, Diagnostic Division, Irving, Texas, USA) compared growth obtained in the presence and in the absence (reference blank) of the antimicrobials. Using appropriate algorithms, the results were printed out qualitatively (resistant or susceptible to the antimicrobials).

To assay the validity of the anaerobiosis conditions, a strain of *Eubacterium formicigerans*, extremely sensitive to oxygen (Brusa et al. 1989), was used as a test micro-organism.

Growth curves

We used a cuvette assemblies named "research cartridge" (R), composed of 11 individual cuvets with a lid; in this type of cuvette the antibiotic-saturated disks had been manually placed (except the reference blank). The inoculated medium was distributed, at the rate of 1 ml, in each cuvets of the cartridge, and covered with 0.3 ml of paraffin oil. The cartridges were than closed by their lids, taken from the anaerobic cabinet, and introduced into the analyser module.

Results

Phase-contrast microscopy

Phase-contrast microscopy was performed to evaluate the qualitative-quantitative aspect of the microflora present and thus to allow a comparison between microflora initially present in the plaque and its capacity to grow in culture medium. The phase-contrast

Table 2. Relative proportion of bacterial morphotypes (differential phase-contrast microscopy) in plaque specimen suspensions

Subject no.	Motile rods (%)	Spirochetes (%)	Filaments (%)	Fusiforms (%)	Coccoid cells (%)	Curved rods (%)	Regular rods (%)
1	10	10	10	8	20	2	40
2	10	10	15	5	20	5	35
3	5	15	20	8	20	2	30
4	5	8	15	15	15	2	40
5	20	5	15	10	15	5	30
6	3	8	10	12	25	3	40
7	2.5	5	25	10	30	2.5	25
8	5	5	15	18	15	2	40
9	2	5	15	15	25	2	40

microscopic analysis (Table 2) did not show significant changes of the relative proportion of bacterial morphotypes in initial plaque suspensions and in the inoculated MT medium before and after 6–8 h of incubation.

Sensitivity to antimicrobials of plaque samples

The procedures for use of the instrument, Abbott Advantage MS 2, have been modified to perform analyses in anaerobic conditions. The preliminary test on *Eubacterium formicigerans* (of intestinal origin and extremely sensitive to oxygen) confirmed the validity of the conditions of anaerobiosis we obtained.

The medium used was previously tested for its ability to support balanced growth of mixed cultures of anaerobes.

The results of the automatic antibiograms (cartridges T) on the plaque cultures are reported in Table 3. We generally observed an analogy of sensitivity or resistance to antimicrobials of the different samples of bacterial plaque examined. In fact, amoxicillin, ampicillin, cefamandole, cefoperazone, cefotetan, cefoxitin, cefuroxime, cephalothin, chloramphenicol, imipenem, moxalactam and ticarcillin were active on all the samples, whereas colistin, gentamicin, kanamycin and nalidixic acid were not active in any of the samples. Clindamycin and tetracycline were active against 4 samples, erythromycin against 6 and penicillin against 8.

Similar results were obtained by evaluating the growth curves (cartridges R) of plaque cultures in the presence of the same antimicrobials. Optical densities after 6 h of growth, the time necessary to perform automatic antibiograms (cartridges T), are reported in Table 4. Fig. 1a and 1b are examples of growth curves, showing the development of the control culture and of the cultures in presence of non-active antimicrobials (sample no. 8).

Table 3. Susceptibility test on plaque specimens (transfer cartridge)

Antimicrobial	Quantity (μg/ml)	Susceptibility subject no. 1	2	3	4	5	6	7	8	9	Susceptible specimens (%)
amoxicillin	8	S	S	S	S	S	S	S	S	S	100
ampicillin	2.5	S	S	S	S	S	S	S	S	S	100
penicillin	1	S	S	S	S	S	S	R	S	S	89
ticarcillin	25	S	S	S	S	S	S	S	S	S	100
cephalothin	6	S	S	S	S	S	S	S	S	S	100
cefamandole	9	S	S	S	S	S	S	S	S	S	100
cefoperazone	14	S	S	S	S	S	S	S	S	S	100
cefotetan	10	S	S	S	S	S	S	S	S	S	100
cefoxitin	6	S	S	S	S	S	S	S	S	S	100
cefuroxime	12	S	S	S	S	S	S	S	S	S	100
imipenem	4	S	S	S	S	S	S	S	S	S	100
moxalactam	16	S	S	S	S	S	S	S	S	S	100
gentamicin	2	R	R	R	R	R	R	R	R	R	0
kanamycin	8	R	R	R	R	R	R	R	R	R	0
nalidixic acid	30	R	R	R	R	R	R	R	R	R	0
clindamycin	0.5	R	R	S	S	R	R	S	R	S	44
chloramphenicol	9	S	S	S	S	S	S	S	S	S	100
colistin	6	R	R	R	R	R	R	R	R	R	0
erythromycin	3	S	R	S	R	S	S	S	S	R	67
tetracycline	5	S	R	R	R	S	R	S	S	R	44

S: susceptible; R: resistant.

Table 4. Optical density of cultures of plaque specimens in the presence of antimicrobials (research cartridge) after 6 h of incubation

Antimicrobial	Quantity (μg/ml)	Optical density subject no. 1	2	3	4	5	6	7	8	9
amoxicillin	8	0	0	0	0	0	0	0	0	0
ampicillin	2.5	0	0	0	0	0	0	0	0	0
penicillin	1	0	0	0	0	0	0	0.108	0	0
ticarcillin	25	0	0	0	0	0	0	0	0	0
cephalothin	6	0	0	0	0	0	0	0	0	0
cefamandole	9	0	0	0	0	0	0	0	0	0
cefoperazone	14	0	0	0	0	0	0	0	0	0
cefotetan	10	0	0	0	0	0	0	0	0	0
cefoxitin	6	0	0	0	0	0	0	0	0	0
cefuroxime	12	0	0	0	0	0	0	0	0	0
imipenem	4	0	0	0	0	0	0	0	0	0
moxalactam	16	0	0	0	0	0	0	0	0	0
gentamicin	2	0.50	0.210	0.300	0.183	0.172	0.387	0.121	0.026	0.310
kanamycin	8	0.061	0.218	0.385	0.181	0.074	0.367	0.101	0.020	0.310
nalidixic acid	30	0.072	0.129	0.232	0.220	0.082	0.330	0.090	0.013	0.236
clindamycin	0.5	0.049	0.201	0	0	0.102	0.563	0	0.010	0
chloramphenicol	9	0	0	0	0	0	0	0	0	0
colistin	6	0.083	0.274	0.374	0.147	0.088	0.406	0.143	0.026	0.302
erythromycin	3	0	0.022	0	0.173	0	0	0	0	0.296
tetracycline	5	0	0.218	0.046	0.163	0	0.360	0	0	0.136

0: susceptible; >0: resistant.

Sensitivity to antimicrobials of pure microbial cultures

To verify whether the antimicrobials active on the mixed cultures of the plaque showed the same activity on isolated microorganisms, 50 strains were isolated in pure culture from a plaque sample (no. 8). The isolated strains belonged to the following species: *Actinomyces israelii*, *A. viscosus*, *Prevotella intermedia*, *Pr. melaninogenica*, *Pr. oralis*, *Fusobacterium* spp, *Leptotrichia buccalis*, *Selenomonas sputigena*, *Streptococcus intermedius* and *Streptococcus* spp. 10 strains, 1 for each isolated species, were tested with the antimicrobial agents.

The data obtained with the automatic antibiogram (cartridge T) in 6 h are reported in Table 5. There was a clear analogy between the sensitivity to antimicrobials of the pure strains from sample 8 and that of the mixed culture obtained from the same plaque sample.

Similar results were obtained by evaluating the growth curves (cartridge R) in the presence of antimicrobials, as shown by the values of optical density reported in Table 6. Fig. 2a and b show,

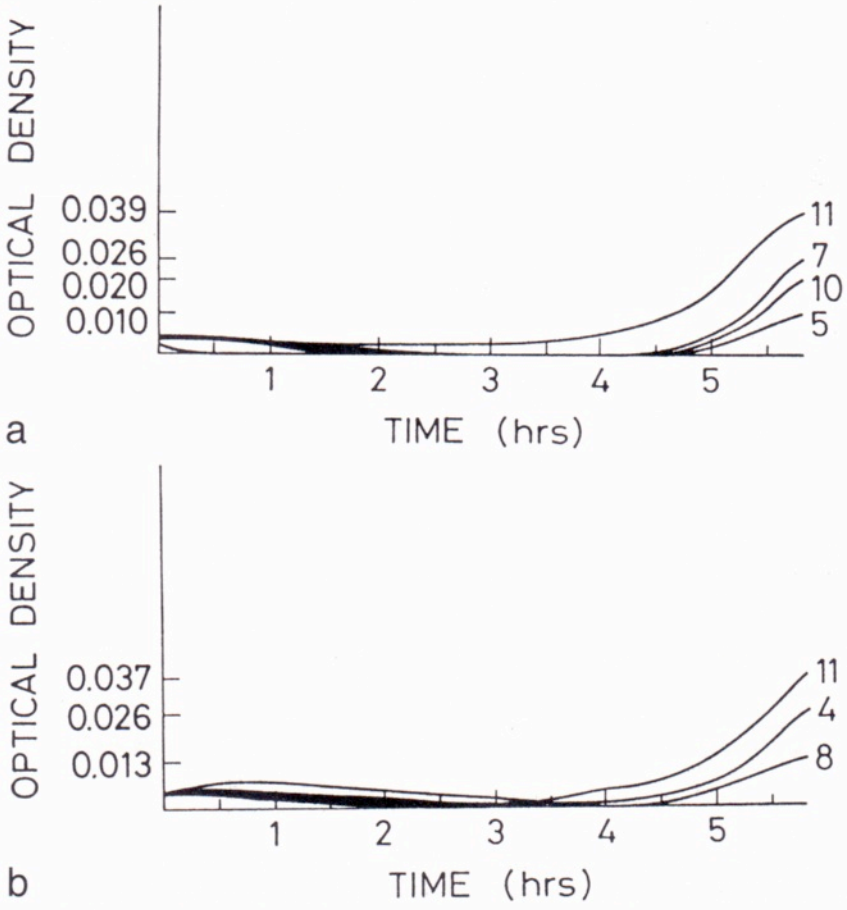

Fig. 1. (a, b) Growth curves of mixed culture of plaque specimen no. 8 in presence of clindamycin (5), gentamycin (7), kanamycin (10), colistin (4), nalidixic acid (8). 11)=reference blank. The bacteria were susceptible to the other tested antimicrobials.

as an example, the growth curves of *Actynomices viscosus* in presence of non-active antimicrobials.

Discussion

Recent years have witnessed continued development of and improvement in methods for susceptibility testing of anaerobes: the method should be simple and quick, efficient for daily testing of small numbers of clinical isolates for individual patient management.

We developed a method to evaluate in anaerobiosis antibiograms on mixed cultures of dental plaque micro-organisms, using an Abbott apparatus designed for serial testing of aerobic bacteria, making the necessary operative modifications to obtain conditions of strict anaerobiosis. The specimen was collected in reduced Aranki salt solution and processed to obtain a chain of strict anaerobiosis throughout all phases of sample handling, from sampling to microbiological analysis. The validity of the conditions of anaerobiosis during sample manipulations was confirmed by growth of a strain of *Eubacterium formicigerans* of intestinal origin and extremely sensitive to oxygen (Brusa et al. 1989).

Phase-contrast microscopic analysis confirmed the importance of some microbial forms in periodontal diseases; in

Table 5. Susceptibility test on strains isolated from the plaque sample of subject no.8 (transfer cartridge)

		Susceptibility strain										
Antimicrobial	Quantity (μg/ml)	*A. israelii*	*A. viscosus*	*Pr. intermedia*	*Pr. melaninogenica*	*Pr. oralis*	*F. sp.*	*L. buccalis*	*S. sputigena*	*Str. intermedius*	*Str. sp.*	Susceptible strains (%)
amoxicillin	8	S	S	S	S	S	S	S	S	S	S	100
ampicillin	2.5	S	S	S	S	S	S	S	S	S	S	100
penicillin	1	S	S	S	S	S	S	S	S	S	S	100
ticarcillin	25	S	S	S	S	R	S	S	S	S	S	90
cephalothin	6	S	S	S	S	S	S	S	R	S	S	90
cefamandole	9	S	S	S	S	S	S	S	S	S	S	100
cefoperazone	14	S	S	S	S	R	S	S	S	S	S	90
cefotetan	10	S	S	S	S	S	S	S	S	S	S	100
cefoxitin	6	S	S	S	S	S	S	S	S	S	S	100
cefuroxime	12	S	S	S	S	S	S	S	R	S	S	90
imipenem	4	S	S	S	S	S	S	S	S	S	S	100
moxalactam	16	S	S	S	S	S	S	S	S	S	S	100
gentamicin	2	S	R	R	R	R	R	R	R	R	R	10
kanamycin	8	S	R	R	R	R	R	R	R	R	R	10
nalidixic acid	30	S	R	R	R	R	R	R	R	R	R	10
clindamycin	0.5	S	S	R	S	R	S	R	R	S	R	50
chloramphenicol	9	S	S	S	S	S	S	S	S	S	S	100
colistin	6	R	R	R	R	R	R	R	R	R	R	0
erythromycin	3	S	S	S	S	S	S	S	S	S	S	100
tetracycline	5	S	S	S	S	S	S	S	S	S	S	100

A.: Actinomyces; Pr.: Prevotella; F.: Fusobacterium; L.: Leptotrichia; S.: Selenomonas; Str.: Streptococcus; S: susceptible; *R*: resistant.

Table 6. Optical density of cultures of pure strains, isolated from the plaque sample of subject no. 8, in the presence of antimicrobials (research cartridge) after 6 h of incubation

		Optical density strain									
Antimicrobial	Quantity (μg/ml)	*A. israelii*	*A. viscosus*	*Pr. intermedia*	*Pr. melanin-ogenica*	*Pr. oralis*	*F. sp.*	*L. buccalis*	*S. sputigena*	*Str. intermedius*	*Str. sp.*
amoxicillin	8	0	0	0	0	0	0	0	0	0	0
ampicillin	2.5	0	0	0	0	0	0	0	0	0	0
penicillin	1	0	0	0	0	0	0	0	0	0	0
ticarcillin	25	0	0	0	0	0.054	0	0	0	0	0
cephalothin	6	0	0	0	0	0	0	0	0.014	0	0
cefamandole	9	0	0	0	0	0	0	0	0	0	0
cefoperazone	14	0	0	0	0	0.108	0	0	0	0	0
cefotetan	10	0	0	0	0	0	0	0	0	0	0
cefoxitin	6	0	0	0	0	0	0	0	0	0	0
cefuroxime	12	0	0	0	0	0	0	0	0.016	0	0
imipenem	4	0	0	0	0	0	0	0	0	0	0
moxalactam	16	0	0	0	0	0	0	0	0	0	0
gentamicin	2	0	0.142	0.150	0.015	0.080	0.141	0.131	0.015	0.062	0.073
kanamycin	8	0	0.168	0.148	0.028	0.073	0.116	0.110	0.016	0.048	0.090
nalidixic acid	30	0	0.170	0.160	0.043	0.011	0.062	0.090	0.013	0.065	0.084
clindamycin	0.5	0	0	0	0	0.084	0	0.081	0.017	0	0.083
chloramphenicol	9	0	0	0	0	0	0	0	0	0	0
colistin	6	0.023	0.215	0.201	0.056	0.091	0.223	0.211	0.018	0.106	0.088
erythromycin	3	0	0	0	0	0	0	0	0	0	0
tetracycline	5	0	0	0	0	0	0	0	0	0	0

A.: Actinomyces; Pr.: Prevotella; F.: Fusobacterium; L.: Leptotrichia; S.: Selenomonas; Str.: Streptococcus; 0: susceptible; >0: resistant.

fact particular importance has been given to the percentage of filaments, mobile rods and spirochetes present as an index of the diseases status: in normal conditions, mobile microbial forms and spirochetes should not exceeded 5% (Listgarten & Hellden 1978, Armitage et al. 1982, Greenstein & Polson 1985).

The question as to witch specific anaerobes to test when several are present is difficult . In situations in which multiple organisms are present, the relative importance of each organism and the role, if any, of microbial synergy in a particular infection my be uncertain (Finegold 1988). The mixed nature of anaerobic infections requires that antimicrobial therapy be direct at all components of the infecting flora (Rosenblatt 1986). For these reasons, we thought it was more important to determine the sensitivity to antimicrobials of mixed rather than pure cultures.

We performed the antibiograms using modified MT medium that allows an equilibrated growth of microflora present in the various plaque samples, during the hours necessary to perform the antibiogram (6 h); in fact also in previous studies regarding different microbial ecosystems (Del Puppo et al 1994, Zanchi et al 1994), we observed a constant qualitative-quantitative ratio among the different microbial forms present in the samples during 24 h.

Among the tested antimicrobials, chosen from the antimicrobial list of the Abbott Susceptibility Test Program, amoxicillin, ampicillin, tetracycline and erythromycin are often used in dentistry, but the others generally are not. Only one concentration of each antibiotic was tested, that have been chosen by the Abbott Avantage Center being the critical point separating susceptibility from resistance based on achievable blood levels of each agent.

As regards the sensitivity of the samples to the various antibiotics, 100% of the samples, apart from the pathology, was sensitive to amoxicillin, ampicillin, cefamandole, cefoperazone, cefotetan, cefoxitin, cefuroxime, cephalothin, chloramphenicol, imipenem, moxalactam and ticarcillin, whereas no sample was sensitive to colistin, gentamicin, kanamycin or nalidixic acid. Penicillin was inactive only in one case of gingivitis. Moreover, 67% of the samples were sensitive to erythromycin and 44% to clindamycin and tetracycline: it is noteworthy that the sensitive samples had been obtained from individuals affected by different pathologies. Tetracycline resistance among anaerobes has been recognised for many years, whereas in vitro work by dental researchers showed that isolates from subgingival plaque remain very susceptible to penicillins and only 10% produce penicillase (Laatsch et al. 1982); moreover multicenter study, made by different geographic institutions, has demonstrated variability in susceptibilities to clindamycin and cefoxitin (Bawdon et al. 1979, Tally et al. 1983).

In our experimental conditions the sensitivity to antimicrobials observed for the mixed culture of the sample n. 8, taken as an example, almost reflected the sensitivity of the individual microbial strains isolated from the same plaque sample: in fact, the antimicrobial activity on the pure strains was analogous to that exerted on the bacterial fraction of the total plaque. However the tested strain of *Prevotella oralis* was resistant to ticarcillin and cefoperazone and the strain of *Selelomonas sputigena* was resistant to cephalothin and cefuroxime, in contrast with the mixed culture. In these cases it seems that the growth in mixed culture influence the test: the limited availability of key nutrients in the mixed culture could impose a slowing of the specific growth rate and the expression of phenotypes which are not typical of the

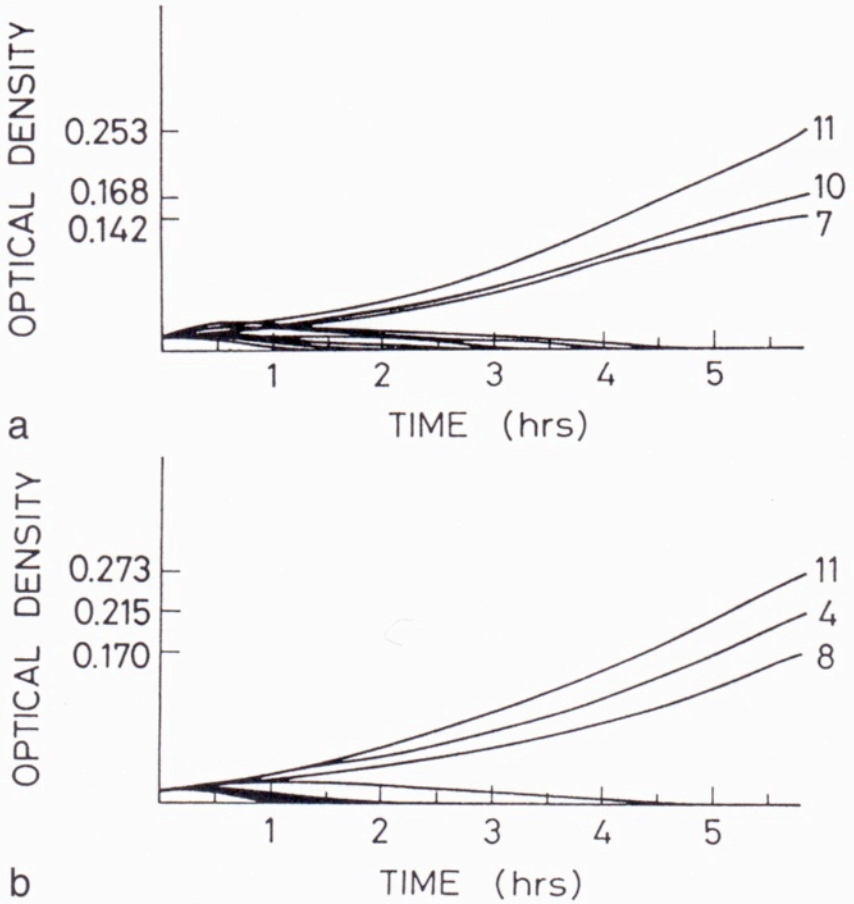

Fig. 2. (a, b) Growth curves of strain *Actynomices viscosus* isolated from plaque specimen no. 8 in presence of gentamycin (7), kanamycin (10), colistin (4), nalidixic acid (8). 11)=reference blank. The strain was susceptible to the other tested antimicrobials.

pure culture in the same medium. Probably the use of an environmentally-related laboratory model can affect the results of antimicrobials susceptibility test: for example, among other things, recent studies show that mixed culture biofilms are less sensitive to antimicrobials than the planktonic cultures (Marsh 1995).

There are many problems involved in correlating in vitro susceptibility test and clinical response and there is some disagreement in the relevance of in vitro susceptibility test in predicting in vivo responses: a test should have a good predictive value on the basis of in vivo correlations (Finegold 1988). We notify a preliminary remark: the patient no. 9, at first treated by tetracicline and eritromicyne without improving, was than successful treated by amoxicillina, antimicrobial chosen after our susceptibility test.

Therefore, our experimental results indicate that the antimicrobials particularly active on the examined samples are those belonging to the group of penicillins, cephalosporins and chloramphenicol. Our results also demonstrated the validity of the use of antibiograms on mixed cultures of dental plaque and of the method, which allowed identification in 3-6 h of the antimicrobials active against anaerobes for the treatment of periodontal disease. The availability of speedy susceptibility test results for anaerobe infections, allow specific therapy and avoid empirically treated patients who received inappropriate antimicrobials.

Zusammenfassung

Antibiogramm anaerober oraler Mischkulturen von Patienten mit Parodontalerkrankungen

Das Ökosystem der Zahnplaque bei Parodontalerkrankungen ist sehr komplex: Das Studium dieser Mikroorganismen, die meistens strikte Anaerobier sind, benötigt besondere Techniken mit strikter Anaerobiose. Das Ziel dieser Studie war es, eine schnelle Methode der Beurteilung der Aktivität antimikrobieller Substanzen auf bakterielle Mischkulturen von Patienten mit Parodontalerkrankungen zu entwickeln. Die Studie wurde unter Verwendung eines computer-unterstützten Instrumentes durchgeführt, das im allgemeinen für simultane Diagnostiktests mit aeroben Bakterien verwendet wird. Technische und methodologische Modifikation wurden durchgeführt, um strikt anaerobe Bedingungen einzuhalten und das gleichmäßige Wachstum aller mikrobieller Formen der Plaque-Mischkulturen zu gewährleisten. Penicilline und Cephalosporine wirkten auf alle Proben, während Colistin, Gentamycin, Kanamycin und Nalidixinsäure keine Wirkung zeigten. Clindamycin, Tetracyclin, Erythromycin und Penicillin G waren nur gegen einige Proben wirksam. Die Wirkung der antimikrobiellen Substanzen gegen isolierte Stämme war analog zur Wirkung auf die entsprechende Mischkultur.

Résumé

Tests antimicrobiens de susceptibilité sur des cultures anaérobies buccales mixtes dans les maladies parodontales

L'écosystème de la plaque dentaire dans les maladies parodontales est très complexe: l'étude de ces micro-organismes, qui pour la plupart sont strictement anaérobies, exige l'utilisation de techniques spécifiques dans des conditions d'anaérobiose stricte. Le but du présent travail était d'élaborer une méthode rapide pour évaluer l'activité de produits antimicrobiens sur la plaque bactérienne mixte de sujets atteints de maladies parodontales. Cette étude a été pratiquée à l'aide d'un dispositif assisté par ordinateur, généralement utilisé pour les tests diagnostiques simultanés avec les bactéries aérobies. Des modifications opératoires et méthodologiques ont été apportées pour obtenir des conditions d'anaérobiose stricte et la croissance équilibrée de toutes les formes microbiennes présentes dans les cultures mixtes de la plaque. Les pénicillines et les céphalosporines agissaient sur tous les échantillons, tandis que la colistine, la gentamycine, la kanamycine et l'acide nalidixique restaient sans activité. La clindamycine, la tétracycline, l'érythromycine et la pénicilline G n'étaient efficaces qu'envers certains échantillons. L'activité des produits antimicrobiens envers les souches isolées étaient analogue à l'activité observée envers les cultures mixtes correspondantes.

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