MICROBIOLOGICAL DIAGNOSIS OF THE SEVERE CHRONIC PERIODONTITIS

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ABSTRACT

In total, 14 adult patients with severe chronic periodontitis were evaluated for the presence of associated anaerobic and aerobic bacteria. Subgingival plaque specimens from three pocket depths per patient were obtained. Microaerophilic and facultative anaerobic bacteria, probably involved in the periodontitis, were isolated in six (42.9%) patients. These were Gram negative species involving *Aggregatibacter* (*Haemophilus*) *aphrophilus* (14.3%), *Haemophilus parainfluenzae* (7.1%), *Kingella denitrificans* (7.1%) and *Moraxella osloensis* (7.1%) as well as Gram-positive species, including *Arcanobacterium* (*Actinomyces*) *pyogenes* (7.1%) and *Rhodococcus equi* (7.1%). Anaerobic microbiology was completed for 12 patients. Of them, suspected periodontopathogens were isolated in seven (58.3%) patients and comprised *Prevotella intermedia* (in 41.7% of the patients) and *Porphyromonas gingivalis* (25%) as well as *Porphyromonas endodontalis* (8.3%). *Tannerella forsythia* was detected by PCR in half of the 12 cases. In conclusion, the presence of periodontopathogens as well as other bacterial species of possible importance should be considered in the patients with severe chronic periodontitis.

Key words: periodontitis, microbiological diagnosis, periodontal pathogens, subgingival plaque, Susceptibility testing

Periodontal diseases are serious diseases in humans and, if untreated, can cause the tooth to loosen and then to be lost. Up to 30% of the adults have been reported to have periodontitis with presence of ≥3 teeth with pockets of ≥4 mm (Kumar et al., 2005). Chronic periodontitis have been considered to be chromic infections, involving anaerobic, microaerophilic and aerobic bacteria. Many putative periodontal pathogens have been extensively evaluated and involved *Aggregatibacter actinomycetem-comitans, Prevotella intermedia, Tannerella forsythia, Porphyromonas gingivalis, Porphyromonas endodontalis, Treponema denticola* etc. (Kumar et al., 2003; Kumar et al., 2005).

AIM

The aim of this study was to assess the incidence and susceptibility patterns of the anaerobic and aerobic bacteria as periodontopathogens as well as other bacterial species of possible importance in the patients with severe chronic periodontitis.

MATERIAL AND METHODS

In total, 27 adult patients with severe chronic periodontitis were evaluated for the presence of associated anaerobic and aerobic bacteria. Of them, 22 patients were untreated and 5 were treated for the periodontitis. Anaerobic microbiology was performed to detect the growth of the suspected peiodondontopathogens such as *P. intermedia, P. gingivalis* and *P. endodontalis,* as well as the presence of *T. forsythia* by PCR. Additionally, the presence of non-*intermedia* pigmented *Prevotella* spp. (NIPPS), and *Actinomyces odontolyticus* (AO) spp. was evaluated.

Subgingival plaque specimens from three pocket depths per patient were obtained. The pocket depths were 3-5, 5-7 and >7 mm. For the first four patients, one subgingival pocket specimen per patient was taken for both aerobic and anaerobic microbiology. For the other patients, two subgingival pocket specimens per patient (one for aerobic and one for anaerobic microbiology) were taken with sterile paper-points (35Roeko). The specimens for anaerobic microbiology were placed in Stuart transport medium (BBL) or Portagerm (bio Merieux) and were sent to the laboratory within 1-2 h.

Anaerobic microbiology

The anaerobic media used were:

1. Schaedler agar with kanamycin 100 mg/l, vancomycin 7.5 mg/l and 5% sheep blood (BBL);

2. Columbia CNA agar with 5% sheep blood (BBL), colistin 10 mg/L and nalidixic acid 10 mg/l (BBL), and

3. Non-selective medium for anaerobes, also suitable for growth of T. forsythia - Brucella blood agar with hemin and vitamin K (BBL), supplemented with yeast extract (Oxoid) 4 g/l, sodium pyruvate 1g/l (Applichem Germany) and N acetylmuramic acid (Sigma-Aldrich). NAM was

dissolved to 10 mg/ml in distilled water, stored at -20°C, and added to the medium to a final concentration of 10 mg/l after autoclaving (Jousimies-Somer et al., 2002; Takemoto et al., 1997). Anaerobic media were incubated anaerobically using GasPak EZ Anaerobe Container System Sachets (BBL) at 37° C for up to 10 days.

The pigmented anaerobes were subcultured and tested with DMACA indol dropper (BBL).

The indol-positive strains were identified by Gram stain, colonial morphology, aerobic control, susceptibility to special potency discs, catalase, spot indol, and Crystal Anaerobe ID kit (BBL), (Jousimies-Somer et al., 2002). The special potency disks, (Rosco and Becton Dickinson), contained oxgall, kanamycin (1000 μg), vancomycin (5 μg), colistin (10 μg) and metronidazole (5 μg).

The isolated *P. intermedia* and *P. gingivalis* strains were tested for susceptibility to amoxicillin, metronidazole, clindamycin, azithromycin, tetracycline and ciprofloxacin with M.I.C.Evaluator (Oxoid, England) as well as with breakpoint susceptibility testing method (BST) as described previously (Boyanova et al., 2006). *Actinomyces odontolyticus*, *Porphyromonas endodontalis* and non-*intermedia* pigmented *Prevotella* strains (NIPPS) were additionally evaluated. The bacterial inoculum corresponded to 0.5 McFarland standard and the final inoculum was about 105 c.f.u. per spot (CLSI-NCCLS, 2004). When no growth was observed on the plate after 48 h of anaerobic incubation, the isolate was considered to be susceptible to the agent. Breakpoints for resistance were amoxicillin ≥2 mg/L, metronidazole ≥32 mg/L, clindamycin ≥8 mg/L (CLSI-NCCLS, 2004), tetracycline >4, ciprofloxacin >1 mg/L, respectively, and azithromycin >2 mg/L.

Beta-lactamase activity was evaluated by using nitrocefin disc test (Cefinase BBL, Becton Dickinson).

All colonies from the non-selective anaerobic medium after 10-day-anaerobic-inculation were collected in PBS 1 mL to perform PCR for *T. forsythia. T. forsythia*-specific primers, which amplify a 641-bp amplicon from the 16 rRNA gene, were used (Sharma et al., 2005), (Table 1).

Table 1. Primers for *T. forsythia*.

Primers for *T. forsythia*	**Size**
Tf –F 5'-GCGTATGTAACCTGCCCGCA-3',	
Tf -R 5'-TGCTTCAGTGTCAGTTATACCT-3'	641 bp

PCR products were analyzed by agarose (1%) gel electrophoresis and ethidium bromide staining.

Aerobic microbiology

The aerobic media used were:

For the isolation of the pathogenic species *Aggregatibacter actinomycetemcomitans* and *Aggregatibacter (Haemophilus) aphrophilus* (commonly associated with periodontal disease), we used selective medium with vancomycin, nonselective chocolate agar (GC agar with 1% haemoglobin and 1% isovitalex) and blood agar, and the agar of McConkey served as a negative control. The selective medium contains brain heart infusion arap (BHIA), to 1 liter of which are added: 5 g yeast extract, 1.5 g sodium fumarate, 1g sodium formiate and 9 mg vancomycin. The medium favors the oral species of the genus Aggregatibacter. The specimens were then incubated for 48h in microaerophilic conditions. For identification of the isolates, we used BBL Crystal N/H and apiNH of bioMerieux, as well as ONP tests of PLIVA-Lachema.

The antimicrobial susceptibility testing was made by DDM (disk diffusion method) on Haemophilus test medium with antibiotic discs for the antimicrobials amoxicillin, trimetoprim/sulfamethoxazole, ceftriaxone, ciprofloxacin (Oxoid). The MIC determining was done for azithromycin and tetracycline by using E-test strips (AB Biodisk, Solna, Sweden).

RESULTS

Anaerobic microbiology was completed for the 27 patients. Of them, Gram-negative pigmented anaerobic bacteria (*P. intermedia, P. gingivalis,* non-*intermedia* pigmented *Prevotella* spp. (NIPPS) and *P. endodontalis*, alone or in combinations) were isolated in 92.6% (25 of 27 patients), involving 90.9% (20 of 22 cases) of the untreated patients and all the five treated patients (Fig. 1).

Table 2. Isolation of *P. intermedia,* non-*intermedia* pigmented *Prevotella* sp. (NIPPS), *P. gingivalis, P. endodontalis* and *Actinomyces odontolyticus* (AO) from patients with severe periodontitis before and after treatment.

Laboratory PatientNo.	**Consecutive patientNo.**	**Treatment**	Specimen 1 pocket depth 3-5 mm	Specimen 2 pocket depth 5-7 mm	Specimen 3 pocket depth >7 mm
1	1	Untreated	NIPPS, AO	Negative*	AO
21		**Treated**	*P. intermedia*	Negative	*P endodontalis, P. intermedia*
2	2	Untreated	Negative	NIPPS	NIPPS
15		**Treated**	AO	Negative	NIPPS
3	3	Untreated	NIPPS	NIPPS, AO	NIPPS , AO
18		**Treated**	AO	Negative	NIPPS
4	4	Untreated	NIPPS	NIPPS	NIPPS
5	5	Untreated	NIPPS	*P. endodontalis*	*P. endodontalis*
19		**Treated**	Negative	Negative	*P. endodontalis*
6	6	Untreated	*P. gingivalis*	*P. gingivalis*	*P. gingivalis, P. intermedia*
22		**Treated**	*P. intermedia*	Negative	NIPPS
7	7	Untreated	NIPPS	Negative	Negative
8	8	Untreated	Negative	Negative	*P. intermedia*
9	9	Untreated	*P. gingivalis*, NIPPS	*P. gingivalis*	*P. gingivalis*
10	10	Untreated	*P. intermedia*	*P. gingivalis*	*P. intermedia, P. gingivalis*
11	11	Untreated	*P. intermedia*	*P. intermedia*	*P. intermedia*
12	12	Untreated	Negative	Negative	*P. intermedia*, AO
13	13	Untreated	NIPPS, AO	AO	AO
14	14	Untreated	NIPPS, AO	*P. intermedia*, NIPPS	NIPPS, AO
16	15	Untreated	Negative	Negative	Negative
17	16	Untreated	Negative	Negative	Negative
20	17	Untreated	NIPPS	Negative	Negative
23	18	Untreated	AO	*P. intermedia*, AO	*P. intermedia, P. gingivalis*
24	19	Untreated	*P. gingivalis*	Negative	*P. gingivalis*
25	20	Untreated	Negative	Negative	NIPPS
26	21	Untreated	*P. gingivalis*	*P. gingivalis*	*P. gingivalis*
27	22	Untreated	*P. intermedia, P. gingivalis,* AO	*P. intermedia, P. gingivalis,* AO	*P. intermedia, P. gingivalis,* AO

NIPPS- Non-*intermedia* pigmented *Prevotella* sp., AO- *Actinomyces odontolyticus*
**negative for *P. intermedia, P. gingivalis* and *P. endodontalis.*

P. intermedia was found in 37.0% (10 of 27 cases) of all patients, including 36.4% (8 of 22) untreated patients and two of five treated patients. *P. gingivalis* was detected in 25.9% (7 of 27) of all patients, including 31.8% (7 of 22) of the untreated subjects and in no specimen from treated patients. *P. endodontalis* was isolated from 11.1% (3 of 27 cases) of all patients, including 4.5% (1 of 22) of the specimens from untreated patients and two of the five specimens from treated patients.

Both *P. intermedia* and *P. gingivalis/P. endodontalis* were present in the specimens from five (four untreated and one treated) patients.

In the 22 untreated patients, *P. intermedia* and *P. gingivalis/P. endodontalis,* alone or in combinations, were found in 31.8% (7 of 22 specimens) of the specimens from pocket depth 3-5 mm, 40.9% (9 of 22) of those from pocket depth 5-7 mm and in 50% (11 of 22) of those from pocket depth >7 mm (Table 4).

In untreated patients, *A. odontolyticus* was detected in five specimens from pocket depth 3-5 mm, four specimens from pocket depth 5-7 mm and six specimens from pocket depth >7 mm. In treated patients, however, *A. odontolyticus* was found only in two specimens from pocket depth 3-5 mm.

In untreated patients, NIPPS were found in nine specimens from pocket depth 3-5 mm, four specimens from

pocket depth 5-7 mm and five specimens from pocket depth >7 mm.

T. forsythia was detected by PCR in 44.4% (8 of 18 cases) of the patients evaluated, involving 50% (8 of 16) of the untreated patients and none of the two treated patients. Within the untreated patients, *T. forsythia* positive were 37.5% (6 of 16 cases) of the specimens from pocket depth 3-5 mm, 50% (8 of 16) of those from pocket depth 5-7 mm and in 37.5% (6 of 16 cases) of those from pocket depth >7 mm.

Susceptibility testing was performed with 19 strains (15 strains from untreated patients and four strains from treated patients) of the pigmented anaerobic periodontopathogens, isolated from 11 untreated and 3 treated patients: *P. intermedia* (9 strains), *P. gingivalis* (7 strains) and *P. endodontalis* (3 strains), (Fig. 2).

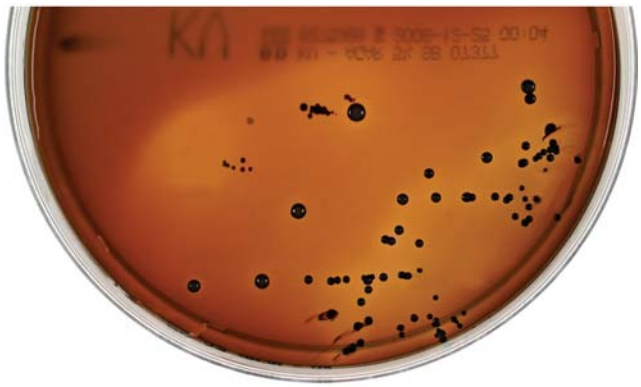

Fig. 1. Growth of *Prevotella intermedia* on Schaedler agar with kanamycin, vancomycin and 5% sheep blood (BBL).

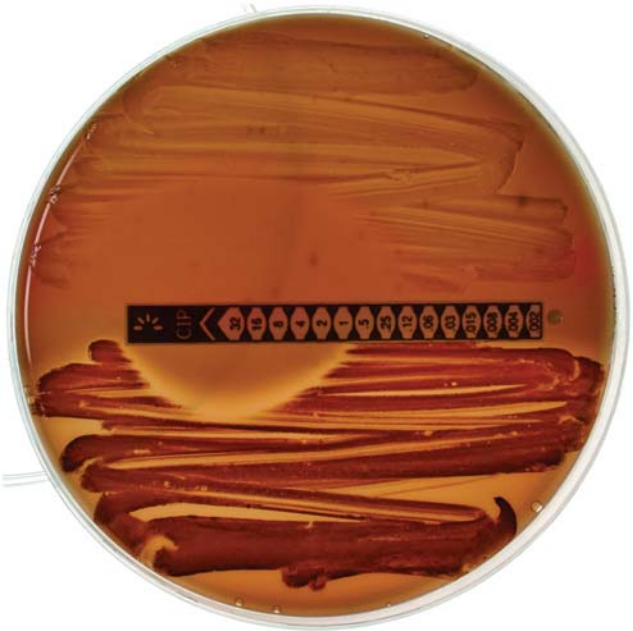

Fig. 2. Susceptibility testing of *P. intermedia* and *P. gingivalis* by M.I.C.Evaluator (Oxoid, England)

Thirteen (86.7%) of the 15 strains from untreated patients were susceptible to amoxicillin (MICs, ≤0.5 mg/L), while amoxicillin resistance was found in one *P. intermedia* strain (amoxicillin MIC, 16 mg/L) and one *P. gingivalis* strain (MIC, 2 mg/L).

All the 15 strains from untreated patients were susceptible to metronidazole (MICs, 0.06-2 mg/L), clindamycin (MICs, 0.016-0.38 mg/L) and azithromycin (MICs, 0.023-1 mg/L). Six (40.0%) strains from the untreated 15 patients were resistant to tetracycline (MICs, >4 mg/L), (Table 1). Three (21.4%) of the 14 strains tested for susceptibility to ciprofloxacin, was resistant to the agent (MIC, 32 mg/L).

It is of note that two of the four strains of the pigmented anaerobic periodontopathogens (*P. intermedia, P. gingivalis* and *P. endodontalis*) from the three treated patients, were resistant to amoxicillin and were beta-lactamase positive. Both resistant strains belonged to the species *P. intermedia*. One *P. intermedia* strain from a treated patient acquired a secondary resistance to amoxicillin (the strain being susceptible to the agent before treatment and resistant after treatment).

The strains from the treated patients were susceptible to metronidazole (MICs, ≤8 mg/L), clindamycin (MICs, ≤2 mg/L), azithromycin (MICs, ≤2 mg/L) and ciprofloxacin (MICs, ≤1 mg/L). One patient' strain, which was tetracycline resistant (MIC, 8 mg/L), remained resistant after treatment with the same MIC concentration.

DISCUSSION

Boutaga et al. (2007) have reported the real-time polymerase chain reaction (PCR) to be a very sensitive technique to detect bacterial periodontopathogens. Probably, the relatively low detection rate of *Porphyromonas* spp. (36.4%, 8 of 22 cases) in the untreated patients in the present study could be explained by the use of culture instead of PCR-based method as well as by the use of one specimen per patient for both aerobic and anaerobic microbiology laboratories for the first four patients (Table 3).

Table 3. Prevalence of *P. intermedia, P. gingivalis, P. endodontalis* and *T. forsyrthia* in untreated patients with periodontitis acc. to several studies.

Prevalence (%) Acc. to	No. of patients	Method	*P. intermedia*	*P. gingivalis*	*P. endodontalis*	*T. forsyrthia*
Colombo et al., 2006	49	Checkerboard DNA–DNA hybridization method	37.0	42.0	NA	26.0
Ready et al., 2008	107	PCR	NA	78.0	NA	81.0
Rocas & Siqueira, 2008	NA[#]	Reverse-capture checkerboard hybridization assay	NA	NA	56.0[¶]	19.0
Romano et al., 2007	21	PCR	55.5	61.1	NA	72.2
Salari & Kadkhoda, 2004		Culture	10.5	21.9	NA	NA
Yoshida et al., 2005	10	Loop-mediated isothermal amplification method	NA	60.0	NA	50.0
Present study (untreated patients)	22*	Culture for *P. intermedia*, *P. gingivalis,* and *P. endodontalis,* and PCR for *T. forsyrthia*	36.4	31.8	4.5	50.0

NA - non-available, *- 16 patients were tested by PCR for *T. forsythia.* [#]- 43 teeth specimens, [¶]- for bacteria present at levels $>10^5$

The incidence of *P. intermedia* in the present study was similar to those (10.5-55.5%) reported in the literature but was higher than that in the culture-based study of Salari & Kadkhoda (2004). In the present study, however, the detection of both *P. gingivalis* and *P. endodontalis* was lower than that by PCR-based methods but was higher than that performed with culture by Salari & Kadkhoda (2004).

Both *P. intermedia* and *P. gingivalis/P. endodontalis* per specimen were found only in one specimen from pocket depth 3-5 mm, one specimen from pocket depth 5-7 mm and in more (five) specimens from pocket depth >7 mm. The Gram-negative pigmented anaerobic periodontopathogens from untreated patients exhibited similar antibacterial susceptibility patterns to those in the study of Kulik et al. (2008), except for the higher resistance (40.0%) to tetracycline in the present study compared to 0-12.6% in the study of Kulik et al. (2008).

The acquisition of amoxicillin resistance in one *P. intermedia* strain, which was amoxicillin susceptible before treatment, is of clinical significance.

It is important that in untreated patients, more *P. intermedia, P. gingivalis* and *P. endodontalis* isolates were cultured from specimens of pocket depth >7 mm than in those with other depths (Table 4).

Table 4. Distribution of anaerobic periodontopathogens according to the depth of the pocket.

Anaerobic species	Untreated patients (no.=22)*			Treated patients (no.=5)*		
Pocket depth	3-5 mm	5-7 mm	>7 mm	3-5 mm	5-7 mm	>7 mm
P. intermedia	3	4	7	2	0	1
P. gingivalis	5	5	7	0	0	0
P. endodontalis	0	1	2	0	0	1
T. forsythia*	6	8	6	0	0	0
NIPPS	9	4	5	0	0	3
A. odontolyticus	5	4	6	2	0	0

* Only 16 untreated and two treated patients were evaluated for *T. forsythia* by PCR.

CONCLUSION

In conclusion, the presence of periodontopathogens as well as other bacterial species of possible importance should be considered in the patients with severe chronic periodontitis. The specimens with pocket depth >7 mm were associated with higher rate of positive pigmented anaerobic periodontopathogenic bacteria and, in some cases, with more frequent presence of two pigmented anaerobic isolates per specimen. Half of the untreated patients harbored *T. forsythia*. The pigmented anaerobic periodontopathogens from untreated patients had a high susceptibility rate to amoxicillin, metronidazole, clindamycin, and azithromycin but relatively low susceptibility rate to tetracycline. Out of all the isolated aerobic species, *A. aphrophilus* was the most significant one. It can be considered as a part of the causative agents of periodontal disease. The isolates had a high susceptibility rate to amoxicillin, azithromycin, ceftriaxone, tetracycline and ciprofloxacin.

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