

POLYMERASE CHAIN REACTION IN THE IDENTIFICATION OF PERIODONTOPATHOGENS – A RELIABLE AND SATISFACTORY METHOD?

NATAŠA NIKOLIĆ JAKOBA¹, SANDRA VOJNOVIĆ², A. PAVIĆ², S. JANKOVIĆ¹,
V. LEKOVIĆ¹ and BRANKA VASILJEVIĆ²

¹ Department of Periodontology and Oral Medicine, Faculty of Dental Medicine, University of Belgrade,
11000 Belgrade, Serbia

² Institute of Molecular Genetics and Genetic Engineering, University of Belgrade, 11000 Belgrade, Serbia

Abstract - *Aggregatibacter actinomycetemcomitans* is considered one of the bacterial species of etiological importance in periodontitis. The aim of this study was to evaluate the serotype of *A. actinomycetemcomitans* in the subgingival biofilm in subjects with periodontal health and disease. Pooled samples of subgingival plaque were taken for culture-based identification of microorganisms. Colonies suspected to be *A. actinomycetemcomitans* were selected for molecular identification using either multiplex or conventional PCR in serotype-specific genotyping and 16S rRNA gene sequencing. *In silico* analysis showed that most selected colonies belong to the genus *Campylobacter*, although positive signals for serotypes of *A. actinomycetemcomitans* were obtained with these samples. Identification of *A. actinomycetemcomitans* by conventional PCR for 16S rRNA with one species-specific and one universal primer was inconclusive because an almost identical signal with *Campylobacter gracilis* was obtained. Although PCR-based methods for the identification of *A. actinomycetemcomitans* are more rapid, sequencing should not be omitted.

Key words: *Aggregatibacter actinomycetemcomitans*, periodontitis, PCR

INTRODUCTION

Periodontal diseases are widely distributed in the world and represent a major oral health problem both in developed and in developing countries. These chronic inflammatory diseases are characterized by the destruction of tooth-supporting tissues. It is commonly accepted that dental plaque bacteria are the primary etiologic agents of periodontal disease. More than 700 species have been detected in the oral cavity of different individuals. Approximately 400 of these species have been isolated from different subgingival microenvironments. However, only a few species have been associated with the disease (Paster et al., 2006). Of the bacteria specifically

associated with destructive disease, *Aggregatibacter actinomycetemcomitans* is considered an etiologically important bacterial species in periodontitis and has contributed to the initiation and/or progression of destructive forms of periodontitis (Slots and Ting, 1999).

Aggregatibacter (formerly: *Actinobacillus*) *actinomycetemcomitans* is a Gram-negative, facultatively anaerobic coccobacillus that can be isolated from various intraoral and extraoral infections (Zambon et al., 1983a; Zambon, 1985; Nørskov-Lauritsen and Killian, 2006). *A. actinomycetemcomitans* isolates are classified into six serotypes, from a to f (Asikainen et al., 1991; Kaplan et al., 2001; Saarela et al., 1999).

Data on the prevalence of *A. actinomycetemcomitans* serotypes in populations from geographically distant regions show a predominance of serotypes a, b, c and e, as well as a minor frequency of serotypes d and f (Yoshida et al., 2003; Thiha et al., 2007; Kittichotirat et al., 2010). It seems plausible that *A. actinomycetemcomitans* strains are distinct in their phenotypes, pathogenic mechanisms and functional roles in the subgingival microbial communities, which may result in different patterns of disease association (Kittichotirat et al., 2010).

Several methods to assess bacterial flora have been developed. These include microscopy, bacterial culture, enzymatic analysis, immunoassay, DNA probes, and polymerase chain reaction (PCR), but there is no gold-standard methodology for the identification of a bacterial community in the subgingival environment.

Differences in periodontopathogens have been detected among populations of different geographical origin, in spite of having a similar clinical presentation. The study of subgingival microbiota in a particular country becomes relevant not only for understanding its implications in the pathogenesis of periodontal disease but also in identifying its possible impact on outcomes after treatment. The aim of this study was to determine the serotype of *A. actinomycetemcomitans* in the subgingival biofilm in subjects with periodontal health and disease.

To identify *A. actinomycetemcomitans* in clinical samples we used classical microbiological approaches, such as isolation and characterization of pure cultures as well as molecular characterization of isolates that included PCR and sequencing. It was of utmost importance to obtain a pure culture of *A. actinomycetemcomitans* for use as a positive control in all further PCR analyses.

MATERIALS AND METHODS

Patient selection

A total of 42 subjects who had been referred to the

Department of Periodontology, School of Dental Medicine, University of Belgrade, Serbia, and diagnosed with chronic (CP, n=18) or generalized aggressive periodontitis (GAP, n=19), and periodontal healthy subjects (PH, n=15), were enrolled in the study.

The study protocol was approved by the Institutional Ethics Committee. Informed written consent was given by all participating subjects.

To be included into the study, the patients had to meet the following criteria:

- >18-years-old,
- more than 20 natural teeth present, without prosthetic reconstruction,
- never received periodontal treatment,
- no antibiotics during the last 3 months,
- no pregnancy and lactation,
- no systemic diseases with possible periodontal complications (hematological diseases, immunological defects, and diabetes mellitus). Data with regard to these diseases were collected with an anamnesis; further clinical examinations were not conducted.

Fifteen periodontitis-free university students (control group) were recruited, who had:

- sound first molars and incisors (≤ 1 surface restoration, not endodontically treated or showing signs of pulpal disease),
- no probing pocket depth >3 mm,
- no bleeding upon probing.

Clinical examination and plaque sampling

The periodontal status of all subjects was evaluated

during the initial screening visit. A full-mouth clinical examination was performed on each patient using a manual probe (UNC 15, Hu Friedy, Chicago, IL, USA) and the following parameters were recorded at six sites per tooth: probing depth (PD in mm), clinical attachment loss (CAL in mm), bleeding on probing (BOP in percentage of sites) and plaque index (PI in percentage of sites).

Patients were screened by one periodontist (NNJ). The periodontal status was defined according to the American Academy of Periodontology consensus report on the Classification of Periodontal Diseases (Armitage, 1999).

Selection of sampling sites

Four sites with the highest value of PD were selected in each quadrant. The selection of sites was based on the data recorded during the screening visit. In periodontally healthy subjects, the mesial aspect of the first molar in each quadrant was selected for plaque sampling.

Microbiological sampling

To avoid contamination with saliva, the selected sites were isolated with cotton rolls and gently air-dried. Supragingival plaque was carefully removed using curettes. Two consecutive sterile paper points (Mailfer, Balligues, Switzerland) were inserted as deep as possible into the pocket, and left in place for 10 s. One paper point was transferred to a vial containing 1 ml of saline, while the other one was transferred to a vial containing 1 ml 10% glycerol. Samples from each quadrant were pooled and the vials were sent to the laboratory and kept at -20°C until they were analyzed.

Bacterial culture

The samples stored in 10% glycerol were spread over plates with Tryptic Soy-Serum-Bacitracin-Vancomycin Agar – TSBV (Slots, 1982) with used paper points. The plates were studied after 3-5 days of incubation at 37°C in air with 5% CO₂ in an anaerobic cham-

ber. The plates were carefully examined to identify *A. actinomycetemcomitans*. Colonies suspected to be *A. actinomycetemcomitans*, based on colony morphology, were selected for molecular identification using either multiplex or conventional PCR methods. The cells diluted in 100 µl sterile distilled water were boiled at 100°C for 5 min. The supernatants were subsequently used as a PCR template for multiplex or conventional PCR assays.

Genotyping

Serotype-specific genotyping using multiplex and conventional PCR was performed with primers previously described (Suzuki et al., 2001): SA-F (5'-GCAATGATGTATTGTCTTCTTTTGGGA-3') and SA-R (5'-TTCAGTTGAATGGGGATTGACTAAAAC-3') for serotype a; SB-F (5'-CGGAAATGGAATGCTTGC-3') and SB-R (5'-CTGAGGAAGCCTAGCAAT-3') for serotype b; SC-F (5'-AATGACTGCTGTCGGAGT-3') and SC-R (5'-CGCTGAAGGTAATGTCAG-3') for serotype c; SD-F (5'-TTACCAGGTGTCTAGTCGGA-3') and SD-R (5'-GGCTCCTGACAACATTGGAT-3') for serotype d; SE-F (5'-CGTAAGCAGAAGAATAGTAAACGT-3') and SE-R (5'-AATAACGATGGCACATCAGACTTT-3') for serotype e; and SF-F (5'-ARA AYTTCWTCGGGAATG-3') and SF-R (5'-CCTTTATCAATCCAGACAGC-3') for serotype f (Kaplan et al., 2001). The amplification conditions were as follows: an initial denaturation at 94°C for 5 min, 35 cycles at 95°C for 30 s, 54°C for 30 s, 72°C for 60 s, and a final extension at 72°C for 5 min. In parallel with multiplex PCR, conventional PCR with primers specific for each serotype was performed under the same conditions using two different DNA templates: bacterial colonies and whole-plaque suspension.

In order to identify *A. actinomycetemcomitans*, colony PCR was performed using the universal primer 1492R (5'-AAGGAGGTGATCCAGCCGCA-3') and species-specific primer for *A. actinomycetemcomitans* (CACTTAAAGGTCCGCCTACGTGC), thereby amplifying part of the 16S rRNA gene. PCR amplification was performed as described by Pucar et al. (2007).

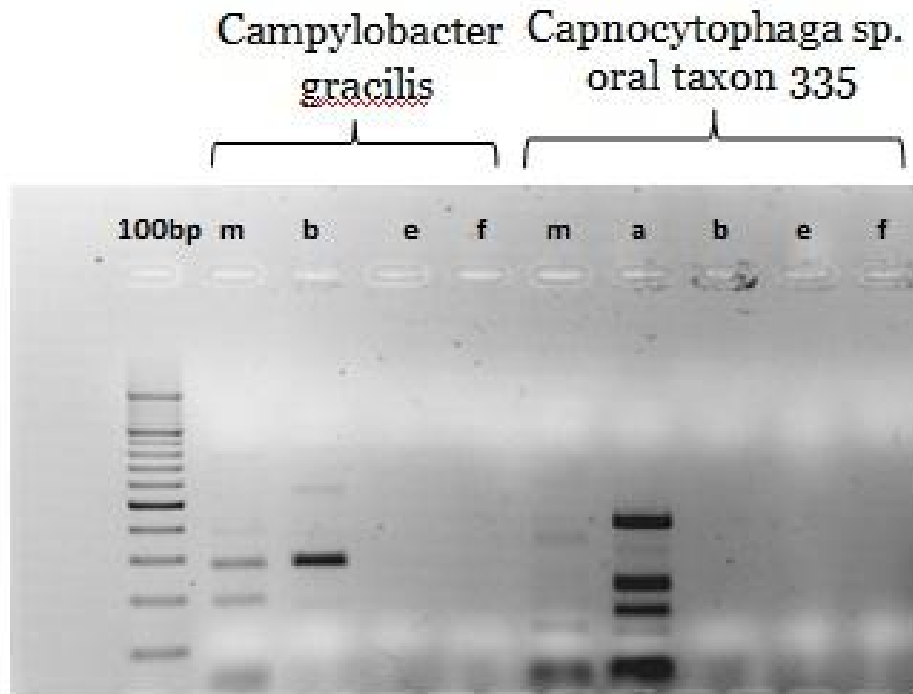


Fig. 1. Electrophoretic analysis of PCR products for serotypes of the suspected *A. actinomycetemcomitans*; m - multiplex PCR ; a, b, e, f - conventional PCR for each serotype; 100 bp - 100 bp Ladder (Fermentas).

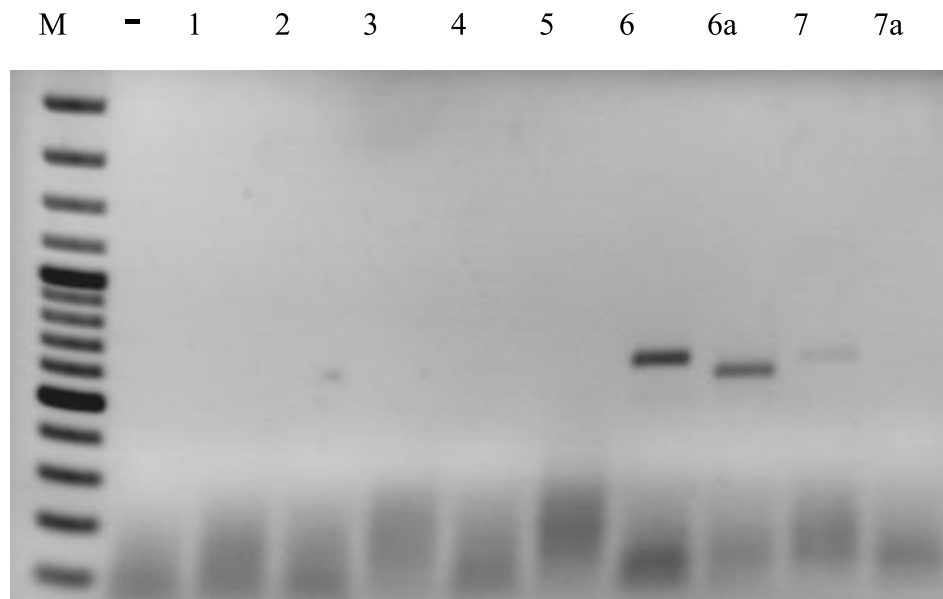


Fig. 2. Polymerase chain reaction (PCR) for 16s rRNA; M - O Gene 100 bp DNA Ladder; - negative control; 1-6, 7 - different subgingival plaque samples as templates; 6a - colony suspected to be *A. actinomycetemcomitans* as a template (later identified as a *Campylobacter gracilis*); 7a - colony suspected to be *Aggregatibacter actinomycetemcomitans* as a template (later identified as a *Eikenella corrodens*).

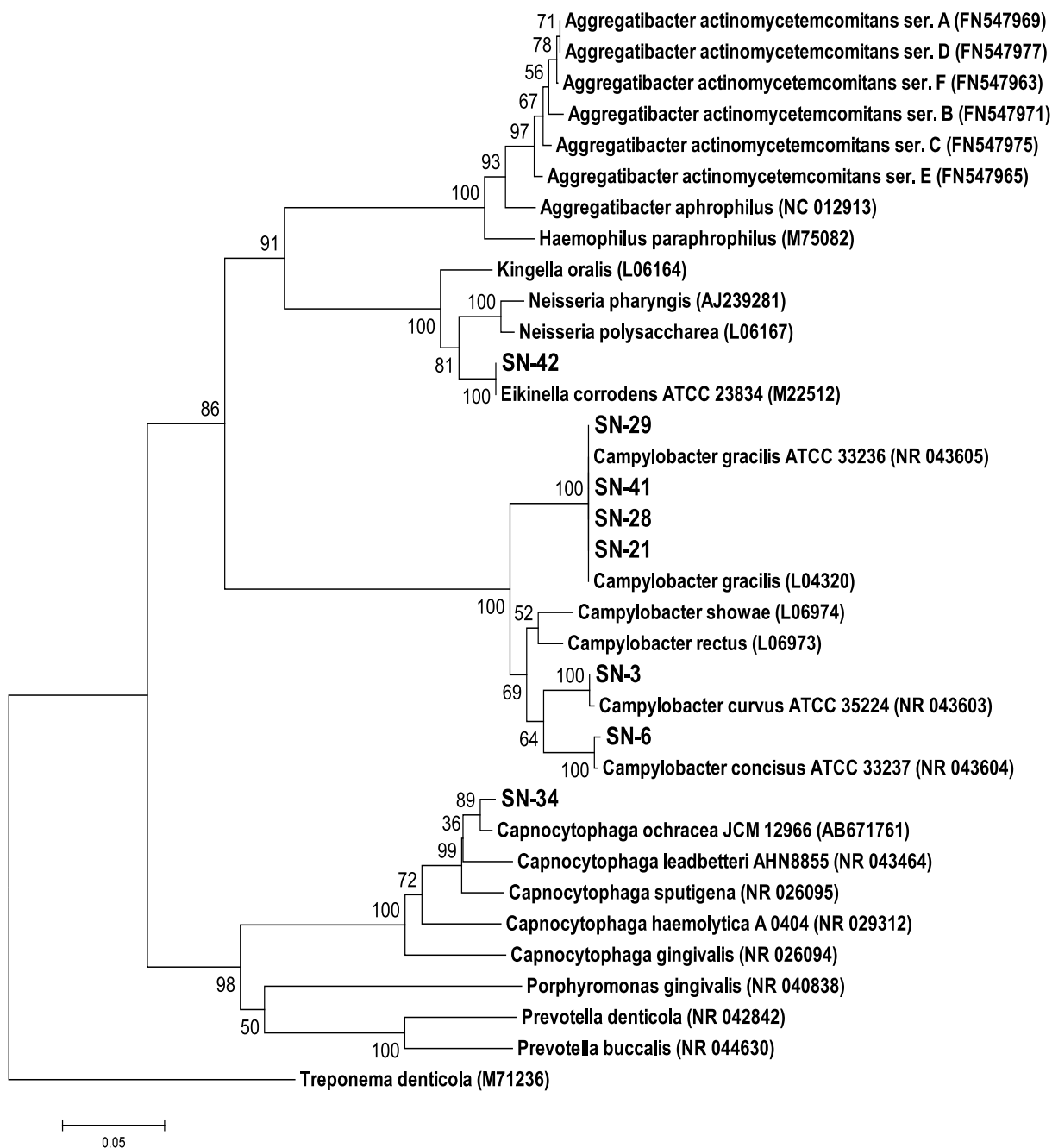


Fig. 3. The neighbor-joining phylogenetic tree of cultivable bacteria belonging to different phyla isolated from infected root of the tooth canal with periodontitis and refractory periodontitis. Bootstrap values (1000 replicate runs) of at least 51% are given. *Treponema denticola* (M71236) was used as the outgroup. Bacterial strains from this research were designated as SN (bold type).

Table 1. Clinical characteristics of the samples.

	Aggressive periodontitis	Chronic periodontitis	Healthy subjects	P -value
BOP	6.00±0 .00	5,42 ± 1.52	0.00±0.00	0.825 AP/CP 0.004* AP>H 0.011* CP>H 0.000* AP>CP
PD	6.56 ±1.41	4.78 ± 0.81	1.33±0.57	0.002* AP>H 0.002* CP>H
CAL	7.87± 2.63	5.39 ±1.29	0.00±0.00	0.003* AP>CP 0.002* AP>H 0.002* CP>H
PI	2.08±0 .511	1.94 ±0 .39	0.80±0.60	0.825 AP/CP 0.002* AP>H 0.002* CP>H

All values are expressed as mean ± standard deviation; AP- aggressive periodontitis group; CP- chronic periodontitis group; H- healthy subjects group; *p-value<0.05 Wilcoxon rank sum test

Identification of bacteria

Selected colonies were analyzed by PCR amplification with the 16S rDNA bacteria-specific primers: 27F (5' AGAGTTTGATCMTGGCTCAG-3') and 1492R (5' ACGGGCGGTGTGTGTRC-3') (Marchesi et al., 1998). PCR amplification was performed as described by Marchesi et al. (1998).

Amplified fragments were purified (QIAquick PCR Purification Kit, Qiagen) and sequenced using an Applied Biosystems 3130 Genetic Analyzer (Foster City, CA, USA).

To identify the bacterial strains, the 16S rRNA gene sequences were compared with published sequences in the NCBI GenBank using the BLAST program (Altschul et al. 1997) and checked manually for nucleotide positions. To secure taxonomic relevance, the most related sequences of type strains or those previously confirmed to be associated with the root of the tooth canal being infected with periodontitis and refractory periodontitis, were used for phylogenetic analyses. All sequences were aligned using CLUSTAL W (Thomson et al. 1997) implemented in the BioEdit 7.1.3 programme (Hall, 1999). Phylogenetic trees were constructed in MEGA 5 (Tamura *et al.* 2011) using the neighbor-joining method based on a pair-wise distance matrix with the Kimura two-

parameter nucleotide substitution model. The topology of the trees was evaluated by the bootstrap resampling method with 1000 replicates.

Data analysis

Statistical analysis was performed using commercial software (SAS Enterprise Guide 4.1, SAS Institute Inc., 2008) with the significance level established at 5% (p <0.05).

For clinical parameters (BOP, PI, PPD and CAL), indicators of descriptive statistics were used. The mean values of the six sites were calculated per tooth and then by patient and by group. All data were expressed by mean and standard deviations (SD), together with a confidence level interval (for 95% of confidence) for all variables, patient/subject being the unit of analysis.

After descriptive statistical analysis of all data, the Kolmogorov-Smirnov test was performed for each variable in order to test the normality of distribution, and in accordance with this, the appropriate statistical test (parametric or non-parametric) was chosen. Inter-group comparisons of the clinical parameters were tested with the Kruskal-Wallis test, while differences in the mean values of the clinical parameters were tested by the Wilcoxon rank-sum test.

Table 2. *In silico* analysis of 16S rRNA gene sequences.

Strain	The most related strains	Acc. No	Maximal identity (%)
SN-3	<i>Campylobacter curvus</i> LMG 11127	AF550651	99.87
	<i>Campylobacter curvus</i> ATCC 35224	NR_043603	99.73
SN-6	<i>Campylobacter concisus</i> CHR3152	HM536952	99.71
SN-21	<i>Campylobacter gracilis</i> LMG 7616	AF550656	99.72
	<i>Campylobacter gracilis</i> ATCC 33236	NR_043605	99.72
SN-22	<i>Campylobacter gracilis</i> CCUG 27721	AF550658	98.83
	<i>Campylobacter gracilis</i> CCUG 13143	AF550657	98.83
	<i>Campylobacter gracilis</i> LMG 7616	AF550656	98.83
	<i>Campylobacter gracilis</i> ATCC 33236	NR_043605	98.83
SN-28	<i>Campylobacter gracilis</i> CCUG 27721	AF550658	99.73
	<i>Campylobacter gracilis</i> CCUG 13143	AF550657	99.73
	<i>Campylobacter gracilis</i> LMG 7616	AF550656	99.73
	<i>Campylobacter gracilis</i> ATCC 33236	NR_043605	99.73
SN-29	<i>Campylobacter gracilis</i> LMG 7616	AF550656	98.96
	<i>Campylobacter gracilis</i> ATCC 33236	NR_043605	98.96
SN-34	<i>Capnocytophaga</i> sp. WWP_SS2_G57	GU412132	97.93
	<i>Capnocytophaga ochracea</i> DSM 7271	CP001632	97.77
SN-41	<i>Campylobacter gracilis</i> LMG 7616	AF550656	99.57
	<i>Campylobacter gracilis</i> ATCC 33236	NR_043605	99.57
SN-42	<i>Eikenella corrodens</i> JCM12952	AB525415	99.74

RESULTS AND DISCUSSION

The role of subgingival microbial species in the etiology of periodontal disease has been extensively documented (Curtis et al., 2005, Socransky and Haffajee, 1994; van Winkelhoff and Boutaga, 2005). *A. actinomycetemcomitans* has long been implicated in periodontal disease and existing data in the literature strongly support its role as an etiological factor in localized aggressive periodontitis, while the relationship with other forms of periodontal disease or periodontal health remains unclear (Socransky and Haffajee, 2008). However, periodontally healthy individuals are also colonized by *A. actinomycetemcomitans*, usually in the order of <20% (Rylev and Kilian, 2008). The goal of the present study was to determine the occurrence and serotype of *A. actinomycetemcomitans* in the subgingival biofilm in subjects with periodontal health and disease.

The hallmark of periodontitis is alveolar bone loss and different clinical parameters, such as probing depth, clinical attachment level and bleeding on probing, are generally used to facilitate diagnosis and detect inflammatory lesions.

Clinical data for participants are presented in Table 1. The three groups demonstrated statistically significant differences regarding the clinical parameters examined in the whole dentition. Subjects in the AP group showed higher mean PD and CAL values in comparison with the CP group, indicating a more severe level of periodontal disease, while the BOP values did not show significant difference. Diseased subjects had a significantly higher full-mouth bleeding score compared with healthy controls.

In addition to these conventional examinations, pathogenic factors are also important in monitoring the potential activity of periodontal disease and there are various methods for the detection of periodontal pathogens. Traditional culture growth of bacteria has some inherent advantages, such as the ability to detect multiple bacterial species coincidentally, allowing for antibiotic susceptibility testing and detecting unexpected bacteria, which is an important advan-

tage of culture not shared by any other available techniques. Such capabilities make this diagnostic tool the reference method for identification of periodontal pathogens. However, culture techniques have serious shortcomings, such as the need to preserve bacterial viability, the inability to detect low numbers of microorganisms with a detection limit averaging 10^3 to 10^4 bacterial cells, labor intensiveness, the need for experienced personnel, strict sampling and transport conditions, and a prolonged period of time before results can be obtained (Lamster et al., 1993; Armitage, 1996).

In this study, a selective medium, TSBV agar, was used for the isolation of *A. actinomycetemcomitans*. The TSBV medium suppresses most oral species and permits a significantly higher recovery of *A. actinomycetemcomitans* than a nonselective blood agar medium. We would like to highlight that without an microscopic examination of plates, colonies of *A. actinomycetemcomitans* could not be recognized. Although we selected colonies that were small, circular, convex, translucent, glistening and 0.5 to 1.0 mm in diameter after incubation for 3 days, *in silico* analysis showed that most of these colonies belong to the genus *Campylobacter*, as well as to *Capnocytophaga* and *Eikenella* (Table 2). It is only theoretically possible that among the 42 subjects enrolled into the study, none of them was colonized with *A. actinomycetemcomitans*. More likely, colonies of *A. actinomycetemcomitans* were readily overlooked because other organisms outnumbered it significantly.

PCR technology offers a quicker detection time and increased accuracy compared to traditional culture and represents the most sensitive and rapid test available for determining the prevalence of periodontal bacteria (Ashimoto, 1996; Riggio et al., 1996; Eick et al., 2002). It can amplify exceedingly small amounts of bacterial nucleic acid and can detect as few as 10 organisms in a plaque sample (Tran and Rudney, 1999).

Molecular biological methods using 16S rRNA gene sequences are now commonly used for the identification and classification of bacteria. Ribos-

omal RNA sequences are widely used to rank biological phylogenetic taxonomy including that of microorganisms (Fox et al., 1980). Since the 16S rRNA contains some conserved regions among biological species, the comparison of 16S rRNA sequences is possible in studies of molecular evolution. Such 16S rRNA sequences also enable the identification of microorganisms because 16S rRNA contains other more variable sequences that change according to species or family (Rossello-Mora and Amann, 2001). 16S rRNA is therefore used for phylogenetic analysis, including the species-specific identification of bacteria.

In this study we used oligonucleotide primers specific for the gene clusters involved in the biosynthesis of serotype-specific polysaccharide antigens which were designed to identify *Actinobacillus actinomycetemcomitans* serotypes a to e using multiplex PCR. According to Suzuki et al. (2001), this method may be useful for serotype-specific genotyping rapidly and directly from clinical samples containing various organisms. We obtained nonspecific results when a whole plaque suspension was used as the DNA template (data not shown). Since we did not have 16S rRNA gene sequencing results, further molecular examinations were performed that led us to surprising results. Colonies obtained from the same patients and identical sampling sites were subjected to serotype-specific genotyping, and positive signals were recorded (Fig. 1.). Surprisingly, *in silico* analysis showed that most selected colonies belong to the genus *Campylobacter*, as well as to *Capnocytophaga* and *Eikenella* (Table 2), in spite of the fact that we obtained positive signals for serotypes of *A. actinomycetemcomitans* with these samples. Phylogenetic analysis based on partial 16S rRNA gene sequences of these PCR-serotype-positive strains showed the closest relatedness of strains SN-21, SN-28, SN-29 and SN-41 to *Campylobacter gracilis* ATCC 33236 (NR_043605), strains SN-3 and SN-6 to *Campylobacter curvus* ATCC 35224 (NR_043603) and *Campylobacter concisus* ATCC 33237 (NR_043604), respectively, as well as strain SN-34 to *Capnocytophaga ochracea* JCM 12966 (AB71761) and strain SN-42 to *Eikenella corrodens* ATCC 23834 (M22512) (Fig. 3).

The results of this study were even more confusing when we demonstrated that in conventional PCR for 16S rRNA, with one universal and one species-specific primer, an almost identical signal with *Campylobacter gracilis* was obtained (Fig. 2.).

Although PCR-based methods have the advantage of providing rapid, sensitive and quantitative detection of small numbers of oral pathogens that are under the detection limit of the culture technique, the presented results show that the PCR method is not conclusive *per se* and confirmed that sequencing should not be omitted. A combination of molecular techniques and culture-based identification as a traditional gold standard should be used for precise microbiological diagnosis and in monitoring patients with periodontal disease.

Acknowledgments - This work was supported by the Ministry of Education and Science of the Republic of Serbia (Grants. 41008 and 173048).

REFERENCES

- Altschul, S.F. Madden, T.L. Schaffer, A.A. Zhang, J. Zhang, Z. Miller, W., and D.J. Lipman (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **25**, 3389-3402.
- Armitage, G.C. (1996). Periodontal diseases: Diagnosis. *Ann. Periodontol.* **1**, 37-215.
- Armitage, G. C. (1999). Development of a classification system for periodontal diseases and conditions. *Ann. Periodontol.* **4**, 1-6.
- Ashimoto, A. Chen, C. Bakker, I., and J. Slots (1996). Polymerase chain reaction detection of 8 putative periodontal pathogens in subgingival plaque of gingivitis and advanced periodontitis lesions. *Oral Microbiol. Immunol.* **11**, 266-273.
- Asikainen, S. Lai, C-H. Alaluusua, S., and S. Slots (1991). Distribution of *Actinobacillus actinomycetemcomitans* serotypes in periodontal health and disease. *Oral Microbiol Immunol.* **6**, 115-118.
- Curtis, M. A., Slaney, J. M., and J. Aduse-Opoku (2005). Critical pathways in microbial virulence. *J Clin Periodontol.* **32**, 28-38.
- Eick, S., and W. Pfister (2002). Comparison of microbial cultivation and a commercial PCR based method for detection

- of periodontopathogenic species in subgingival plaque samples. *J Clin Periodontol.* **29**, 638-644.
- Fox, G.E. Stackebrandt, E., and R.B. Hespell (1980). The phylogeny of prokaryotes. *Science*, **209**, 457-463.
- Hall, T.A. (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp Series.* **41**, 95-98.
- Kaplan, J.B. Perry, M.B. Maclean, L.L. Furgang, D. Wilson, M.E., and D.H. Fine (2001). Structural and genetic analyses of O-polysaccharide from *Actinobacillus actinomycetemcomitans* serotype f. *Infect. Immun.* **69**, 5375-5384.
- Kittichotirat, W., Bumgarner, R. and C. Che (2010). Markedly different genome arrangements between serotype a strains and serotypes b or c strains of *Aggregatibacter actinomycetemcomitans*. *BMC Genomics.* **11**, 489.
- Lamster, I.B. Celenti, R.S. Jans, H.H. Fine, J.B., and J.T. Grbic (1993). Current status of tests for periodontal disease. *Adv. Dent. Res.* **7**, 182-190.
- Marchesi, J. R. Sato T. Weightman, A. J. Martin, T. A. Fry, J. C. Hiom, S. J., and W. G. Wade (1998). Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA. *Appl. Environ. Microbiol.* **64**, 795-799.
- Nørskov-Lauritsen, N., and M. Kilian (2006). Reclassification of *Actinobacillus actinomycetemcomitans*, *Haemophilus aphrophilus*, *Haemophilus paraphrophilus* and *Haemophilus segnis* as *Aggregatibacter actinomycetemcomitans* gen. nov., comb. nov., *Aggregatibacter aphrophilus* comb. nov. and *Aggregatibacter segnis* comb. nov., and emended description of *Aggregatibacter aphrophilus* to include V factor-dependent and V factor-independent isolates. *Int. J. Syst. Evol. Microbiol.* **56**, 2135-2146.
- Paster, B.J. Olsen, I. Aas, J.A., and F.E. Dewhirst (2006). The breadth of bacterial diversity in human periodontal pocket and other oral sites. *Periodontology 2000.* **42**, 80-87.
- Pucar, A. Milasin, J. Lekovic, V. Vukadinovic, M., Ristic, M. Putnik, S., and E. B. Kenny (2007). Correlation between atherosclerosis and periodontal putative pathogenic bacterial infections in coronary and internal mammary arteries. *J. Periodontol.* **78**(4), 677-82.
- Riggio, M.P. Macfarlane, T.W. Mackenzie, D. Lennon, A. Smith, A.J., and D. Kinane (1996). Comparison of polymerase chain reaction and culture methods for detection of *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* in subgingival plaque samples. *J. Periodontol. Res.* **31**, 496-501.
- Rossello-Mora, R. and R. Amann (2001). The species concept for prokaryotes. *FEMS Microbiol. Rev.* **25**, 39-67.
- Rylev, M., and M. Kilian (2008). Prevalence and distribution of principal periodontal pathogens worldwide. *J. Clin. Periodontol.* **35**, 346-361.
- Saarela, M. Asikainen, S. Alaluusua, S. Pyhälä, L. Lai, C.H., and H. Jousimies-Somer (1992). Frequency and stability of mono- or poly-infection by *Actinobacillus actinomycetemcomitans* serotypes a, b, c, d or e. *Oral Microbiol Immunol.* **7**, 277-279.
- Slots, J. (1982). Selective medium for isolation of *Actinobacillus actinomycetemcomitans*. *J. Clin. Microbiol.* **15**, 606-609.
- Slots, J., and M. Ting (1999). *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* in human periodontal disease: Occurrence and treatment. *Periodontology 2000.* **20**, 82-121.
- Socransky S. S. and A. D. Haffajee (1994). Evidence of bacterial etiology: a historical perspective. *Periodontology 2000.* **5**, 7-25.
- Socransky, S. S., and A. D. Haffajee (2008). Periodontal infections. In: (Eds. Lindhe, J., Lang, N. P. & Karring, T.). *Clinical Periodontology and Implant Dentistry*, pp. 207-267. Oxford, Blackwell Munksgaard.
- Suzuki, N. Nakano, Y. Yoshida, Y. Ikeda, D. and T. Koga (2001). Identification of *Actinobacillus actinomycetemcomitans* serotypes by multiplex PCR. *J. Clin. Microbiol.* **39**(5), 2002-2005.
- Tamura, K. Peterson, D. Peterson, N. Stecher, G. Nei, M., and S. Kumar (2011). MEGA 5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. *Molecular Biology and Evolution* **28**, 2731-2739.
- Thiha, K., Takeuchi, Y., Umeda, M., Huang, Y., Ohnishi, M. and Ishikawa, I. (2007). Identification of periodontopathic bacteria in gingival tissue of Japanese periodontitis patients. *Oral Microbiology and Immunology* **22**, 201-207.
- Thompson, J.D. Higgins, D.G, and T.J. Gibson (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nuc. Acids Res.* **22**, 4673-4680.
- Tran, S.D. and J.D. Rudney (1999). Improved multiplex PCR using conserved and species-specific 16S rRNA gene primers for simultaneous detection of *Actinobacillus actinomycetemcomitans*, *Bacteriodes forsythus*, and *Porphyromonas gingivalis*. *J. Clin. Microbiol.* **37**, 3504-3508.
- van Winkelhoff, A. J. and K. Boutaga (2005). Transmission of periodontal bacteria and models of infection. *J. Clin. Periodontol.* **32**, 16-27.
- Yoshida, Y., Laine, M. L., van Winkelhoff, A. J. and G. Dahlen (2007). Genotype variation and capsular serotypes of Por-

phyromonas gingivalis from chronic periodontitis and periodontal abscesses. *FEMS Microbiology Letters*. **270**, 75-81.

Zambon, J.J. Christersson, L.A., and J. Slots (1983). *Actinobacillus actinomycetemcomitans* in human periodontal disease.

Prevalence in patient groups and distribution of biotypes and serotypes within families. *J. Periodontol.* **12**, 707-711.

Zambon, J.J. (1985). *Actinobacillus actinomycetemcomitans* in human periodontal disease. *J. Clin. Periodontol.* **12**, 1-20.

