

Detection of pathogens from periodontal lesions

Detecção de patógenos de lesões periodontais

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Keywords

A. actinomycetemcomitans. *F. nucleatum*. Periodontopathogens. Diagnosis. Periodontal disease. PCR.

Abstract

Objective

To comparatively detect *A. actinomycetemcomitans* and *F. nucleatum* from periodontal and healthy sites.

Methods

Subgingival clinical samples from 50 periodontitis adult patients and 50 healthy subjects were analyzed. Both organisms were isolated using a trypticase soy agar-bacitracin-vancomycin (TSBV) medium and detected by PCR. Conventional biochemical tests were used for bacteria identification.

Results

A. actinomycetemcomitans and *F. nucleatum* were isolated in 18% and 20% of the patients, respectively, and in 2% and 24% of healthy subjects. Among *A. actinomycetemcomitans* isolates, biotype II was the most prevalent. Primer pair AA was 100% sensitive in the detection of *A. actinomycetemcomitans* from both subject groups. Primers ASH and FU were also 100% sensitive to detect this organism in healthy subject samples. Primer pair FN5047 was more sensitive to detect *F. nucleatum* in patients or in healthy samples than primer 5059S. Primers ASH and 5059S were more specific in the detection of *A. actinomycetemcomitans* and *F. nucleatum*, respectively, in patients and in healthy subject samples.

Conclusions

PCR is an effective tool for detecting periodontal pathogens in subgingival samples, providing a faster and safer diagnostic tool of periodontal diseases. The method's sensitivity and specificity is conditioned by the choice of the set of primers used.

Descritores

A. actinomycetemcomitans. *F. nucleatum*. Periodontopatógenos. Diagnóstico. Doença periodontal. PCR.

Resumo

Objetivo

Realizar a detecção comparativa de *A. actinomycetemcomitans* e *F. nucleatum* de sítios periodontais e sadios.

Métodos

Foram analisadas amostras subgingivais de 50 pacientes com periodontite do adulto e de 50 indivíduos sadios. Ambos os organismos foram isolados em meio ágar de soja tripticaseína-bacitracina-vancomicina e detectados por PCR. Testes bioquímicos convencionais foram usados para a identificação bacteriana.

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Resultados

A. actinomycetemcomitans e *F. nucleatum* foram isolados em 18 e 20% dos pacientes, respectivamente, e em 2 e 24% dos indivíduos sadios. Entre os isolados de *A. actinomycetemcomitans*, o biótipo II foi o mais prevalente. O par de iniciadores AA mostrou 100% de sensibilidade na detecção de *A. actinomycetemcomitans*, em ambos os grupos de indivíduos. Iniciadores ASH e FU foram também 100% sensíveis para detectar esse organismo em amostras de indivíduos sadios. O iniciador FN5047 foi mais sensível para detectar *F. nucleatum* em amostras de pacientes ou de sadios que o 5059S. Iniciadores ASH e 5059S foram mais específicos na detecção de *A. actinomycetemcomitans* e *F. nucleatum*, respectivamente, em amostras de pacientes e de sadios.

Conclusões

PCR constitui-se uma ferramenta efetiva na detecção de patógenos periodontais de espécimes clínicos, fornecendo um diagnóstico rápido e seguro da doença periodontal. Entretanto, esse método depende da escolha dos iniciadores específicos utilizados.

INTRODUCTION

It is known that a great number of different microorganisms exist in the oral cavity but only some species, particularly anaerobic bacteria, have been implicated in the etiology of periodontal disease. Both *Actinobacillus actinomycetemcomitans* and *Fusobacterium nucleatum* are Gram-negative bacteria involved in the pathogenesis of human periodontitis but they can also be associated to other endogenous infections.⁹

A. actinomycetemcomitans is an important periodontopathogen that is involved in the etiology of different forms of periodontal diseases, particularly in the localized juvenile and adult periodontitis, and also in several extra-oral infections such as endocarditis, pericarditis, pneumonias, septicemias, and abscesses.^{3,10,17}

F. nucleatum has also been considered an important periodontopathogen for the development of gingivitis and periodontitis and as the most common anaerobic species found in human and animal infections, particularly in the oral cavity.²

The isolation and identification of periodontal pathogens characterize an important tool for increasing knowledge on periodontal microbiota as well as on the etiology and progression of periodontal infections. However, molecular methods have contributed to the detection of putative periodontopathogens in several oral or extra-oral infections.¹⁵

Even so, there have been some limitations of bacterial culture such as high cost and time-consuming procedures, besides the fact that it may fails to

uncultivable organisms. Additionally, cell viability is necessary for isolation but can be partially lost during transport and in the sampling procedure.⁶

Several methods for the rapid detection of periodontal pathogens have been reported such as immunologic and immunoenzymatic assays, protein electrophoresis, and DNA-DNA hybridization. However, these methods show different limitations leading to false-positive results as well as cross-reactivity.^{1,4}

Polymerase chain reaction (PCR) is an excellent tool used to identify putative periodontopathogens directly from subgingival samples. Also, it is a fast and efficient method to detect, identify, and differentiate periodontal organisms due to its sensitivity and specificity but appropriate standardization is necessary.⁵

The aim of this study was detect the presence of *A. actinomycetemcomitans* and *F. nucleatum* from clinical subgingival samples of periodontal patients using two different methods.

METHODS

Fifty patients with adult periodontitis and 50 healthy subjects aged between 20 and 60 years old, of any sex or race, seen at an university of dentistry, in Brazil, were selected. Periodontal patients showed pockets deeper than 5 mm and bone loss, as diagnosed by radiographic examination. None of them was taking antibiotics during a three-month-period prior to sample collection. All subjects involved in this study were informed concerning the study's nature and procedures, and a consent form was obtained.

Table 1 - Specific primers used in the detection of *A. actinomycetemcomitans* and *F. nucleatum*.

Primer	Oligonucleotide sequence (5'→3')	Amplicon	Annealing Temperature	Reference
<i>A. actinomycetemcomitans</i>				
ASH1/ASH2	AAA CCC ATC TCT GAG TTC TTC TTC ATG CCA ACT TGA CGT TAA AT	0.5 kb	55°C	Ashimoto et al ¹ (1996)
AA1/AA2	CGT GCC AGC AGC CGC GGT AAT ACG CTT TGC ACA TCA GCG TCA G GTA CAT CCC CAA GG	0.2 kb	70°C	Garcia et al ⁷ (1998)
FU1/FU2	GTT TAG CCC TGG TGC CCG AGG TGA CGG GCG GTG TGT ACA AGG	0.5 kb	55°C	Macheleidt et al ⁹ (1999)
<i>F. nucleatum</i>				
FN 5047	CAA ATG CTT GTG TCA ATA ATA CT TTT AGA AAT GGT AGA ATA AT	0.5 kb	40°C	Avila-Campos et al ³ (1999)
5059 S	ATTGG GCT AAA AAT TAT AGTT ACC CTC ACT TTG AGG ATT ATA G	1 kb	40°C	Avila-Campos et al ³ (1999)

The Ethics Commission of the Instituto de Ciências Biomédicas, University of São Paulo, approved this study.

Initially, the supragingival plaque was removed and subgingival samples were obtained using three sterilized paper points inserted in the deepest site of periodontal pocket or gingival crevice. After 60 seconds, the paper points were transferred into tubes containing 3.5 ml of the viability maintaining microbiostatic medium (VMGA III) transport medium.⁷

Samples were mixed and diluted 10-fold in VMGA I solution,⁷ and then plated on trypticase soy agar-bacitracin-vancomycin (TSBV).¹³ Plates were incubated in anaerobiosis (90% N₂ + 10% CO₂) at 37°C for four days.

To obtain a pure culture, characteristic colonies of both organisms were subcultured on blood agar. Then, they were presumptively identified by Gram-staining, and catalase, H₂S, and indole production as well as by their susceptibility to sodium fluoride.¹⁴ Definitive identification was achieved using biochemical tests.¹⁴

Reference strains of *A. actinomycetemcomitans* ATCC 29522, ATCC 33384, ATCC 43718, and KC 517 CDC (Centers for Disease Control and Prevention, GA, USA) and of *F. nucleatum* ATCC 10953 were used as controls.

Biotypes of *A. actinomycetemcomitans* isolates were detected by examining the ability to ferment dextrose, maltose, xylose, and mannitol.¹²

VMGA III media containing clinical samples were warmed at 37°C for 10 minutes and then homogenized. Next 300 µl aliquots were washed three times with sterilized Milli-Q water. Pellets were resuspended in 300 µl water and then boiled for 10 minutes; supernatants were used as templates after centrifugation (14,000 x g, 10 minutes).¹

Also, bacterial DNA was extracted from 10 colonies of *A. actinomycetemcomitans* and five colonies of *F. nucleatum*. They were suspended in 500 µl sterile Milli-Q water, homogenized, and then boiled for 10 minutes. Then, after centrifugation, the supernatant (DNA) was used as template.

DNA amplification was performed in volumes of 25 µl containing 8.25 µl sterile Milli-Q water, 2.5 µl 10x PCR buffer, 1 µl MgCl₂ (50 mM), 1 µl dNTP (0.2 mM), 1 µl of each primer (0.4 M), 0.25 µl *Taq* DNA polymerase (0.5 U), and 10 µl DNA.

A thermocycler Perkin Elmer, Gene Amp PCR System 2,400, was programmed to: 1 cycle at 94°C (5 minutes); 30 cycles at 94°C (30 seconds), annealing temperature for each specific primer pair (Table 1), and 72°C (30 seconds); 1 cycle at 72°C (5 minutes) until the final DNA extension. Specific primers used for identifying *A. actinomycetemcomitans* and *F. nucleatum* are listed in Table 1.

PCR products were detected by electrophoresis in 1% agarose gel in 1x TBE at 70 V for 2.5 hours. Gels were stained with an ethidium bromide solution (0.5 µg/ml), and then photographed on a UV transilluminator using the Electrophoresis Documentation and the Analysis System 120. A 1 kb DNA ladder was used as molecular mass standard.

To assess concordance between both detection methods (culture and PCR) as well as to determine their sensitivity and specificity, data were analyzed using a Kappa Index (K).

RESULTS

To detect *A. actinomycetemcomitans* and *F. nucleatum*, 50 subgingival samples from adult periodontitis patients and 50 from healthy subjects were analyzed. In a trypticase soy agar bacitracin-vancomycin (TSBV), nine (18%) clinical samples from peri-

odontal patients were positive to *A. actinomycetemcomitans*, with 17 isolates recovered, and ten (20%) were positive to *F. nucleatum* with 19 isolates. Two *A. actinomycetemcomitans* were isolated from one (2%) healthy subject and 18 *F. nucleatum* isolates were recovered from 12 (24%) healthy subjects.

A. actinomycetemcomitans isolates were grouped in five biotypes (II, VI, VIII, IX and X). The most prevalent was biotype II.

Table 2 shows the sensitivity and specificity of the primer sets used for detecting *A. actinomycetemcomitans* and *F. nucleatum* from clinical samples. Primers AA were highly sensitive (100%) to detect *A. actinomycetemcomitans* from both subject groups. Furthermore, primers ASH and FU were also 100% sensitive to detect this organism in healthy subject samples. Primer FN5047, used to detect *F. nucleatum*, was more sensitive to detect this organism in patients or healthy samples than primer 5059S. On the other hand, primer ASH and 5059S were more specific to detect *A. actinomycetemcomitans* and *F. nucleatum*, respectively, in patients and healthy subject samples (Table 2).

All amplified products were compared and the primer pairs produced amplicons of predicted size (data not shown). Table 3 shows a comparison of the bacterial detection between culture method and each

primer used in PCR. It can also be observed that the detection values were as significant to detect *F. nucleatum* in periodontal patients using primer FN5047 ($p < 0.002$) as to detect *A. actinomycetemcomitans* in healthy subjects using primer ASH ($p < 0.000$).

Statistical analysis showed that primers AA and FU had higher sensitivity, and primers ASH had higher specificity for detecting *A. actinomycetemcomitans*. However, primer FN 5047 showed higher sensitivity and primer 5059S showed higher specificity for detecting *F. nucleatum* (Table 3).

DISCUSSION

A. actinomycetemcomitans and *F. nucleatum* are important organisms of both human and animal indigenous microbiota, and they have been involved in several oral cavity infections. It is well known that improvements in diagnostic methods are useful in the prevention and treatment of periodontal disease and contribute for increasing knowledge on subgingival microbiota. Bacterial cultures are used for allowing to recovering cultivable organisms, although being a time-and-labor-consuming method. Several molecular tools are often used to identify periodontopathogens but PCR is considered to be an easy and fast detection method even in clinical samples.¹¹

In this study, two methods of detecting *A. actinomycetemcomitans* and to *F. nucleatum* were compared. The primary isolation of both organisms studied was performed on selective TSBV medium.¹³ It is important to mention that although TSBV agar is used as the first choice to isolate *A. actinomycetemcomitans*, a high recovery rate of *F. nucleatum* was also observed.

Studies have detected *A. actinomycetemcomitans* using a PCR method but at different rates in populations with (19%) and without (70%) periodontal disease. These studies have also shown PCR sensitivity and the specificity in comparison to traditional bacterial culture.^{8,16}

Table 2 - Sensitivity and specificity of specific primer pairs used to detect *A. actinomycetemcomitans* and *F. nucleatum* from clinical samples in VMGA III.

Primer	Sensitivity* (%)		Specificity** (%)	
	Patient	Healthy	Patient	Healthy
<i>A. actinomycetemcomitans</i>				
ASH	5	100	75	95
AA	100	100	5	15
FU	77	100	59	60
<i>F. nucleatum</i>				
FN 5047	50	34	67	87
5059 S	30	25	97	97

*Sensitivity (positive-true) = (Number of positive samples by PCR and culture/Number of positive samples by culture) x 100

**Specificity (negative-true) = (Number of negative samples by PCR and culture/Number of negative samples by culture) x 100

VMGA: Viability maintaining microbiostatic medium

Table 3 - Detection of *A. actinomycetemcomitans* and *F. nucleatum* strains from periodontal patients and healthy subjects.

Clinical specimens (N)	Culture	ASH [†]		FU* [†]		AA* [†]		FN 5047** [†]		5059S** [†]	
		+	-	+	-	+	-	+	-	+	-
Periodontal patients (50)	+	2	7	7	2	9	0	3	7	5	5
	-	10	31	17	24	39	2	1	39	13	27
K (P)		0.019 (0.555)		0.220 (0.024)		0.018 (0.250)		0.355 (0.002)		0.135 (0.151)	
Healthy subjects (50)	+	1	0	1	0	1	0	3	9	4	8
	-	2	47	20	29	42	7	1	37	5	33
K (P)		0.485 (0.000)		0.055 (0.118)		0.007 (0.342)		0.221 (0.056)		0.290 (0.006)	

[†]Primers to detect *A. actinomycetemcomitans*

^{††}Primers to detect *F. nucleatum*

K = Kappa index; K≠0 = P<0.005

In the present study, primers AA and FU had higher sensitivity for detecting *A. actinomycetemcomitans* but primers ASH had higher specificity (Table 3). On the other hand, when compared to culture for detecting *A. actinomycetemcomitans*, primers AA and FU showed similarly sensitivity (100%), which confirms their specificity.

DNA from *A. actinomycetemcomitans* and *F. nucleatum* strains were amplified by specific methods and produced predicted bands. It is important to note that both the annealing temperature and magnesium concentration are critical factors in PCR detection.¹ *F. nucleatum* detection by PCR (primer 5059S) was similar to culture methods; however, when primer FN 5047 was used, PCR showed higher sensitivity

than culture. The study results suggest that PCR is a sensitive and highly specific technique to detect *A. actinomycetemcomitans* and *F. nucleatum* in the subgingival plaque.

In conclusion, the present study demonstrated the usefulness of specific primers based on the PCR detection of periodontal organisms in subgingival samples. The use of molecular tools in the bacterial detection will provide faster and safer diagnostics of periodontal diseases and PCR reaction may be helpful in detecting putative periodontopathogens from subgingival samples. A single method could not be ideal, and using both traditional and molecular methods is recommended in the bacterial detection.

REFERENCES

1. Ashimoto A, Chen C, Bakker I, Slots J. Polymerase chain reaction detection of 8 putative periodontal pathogens in subgingival plaque of gingivitis and advanced periodontitis lesions. *Oral Microbiol Immunol* 1996;11:266-73.
2. Avila-Campos MJ, Sacchi CT, Whitney AM, Steigerwalt AG, Mayer LW. Arbitrarily primed-polymerase chain reaction for identification and epidemiologic subtyping of oral isolates of *F. nucleatum*. *J Periodontol* 1999;70:1202-8.
3. Chen YC, Chang SC, Luh KT, Hsieh WC. *Actinobacillus actinomycetemcomitans* endocarditis: a report of four cases and review of the literature. *Q J Med* 1991;81:871-8.
4. Chen C, Slots J. Microbiological tests for *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis*. *Periodontology* 2000 1999;20:53-64.
5. Doungudomdacha S, Rawlison A, Douglas WI. Enumeration of *Porphyromonas gingivalis*, *Prevotella intermedia* and *Actinobacillus actinomycetemcomitans* in subgingival plaque samples by a quantitative-competitive PCR method. *J Med Microbiol* 2000;49:861-74.
6. Lopez NJ. Occurrence of *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia* in progressive adult periodontitis. *J Periodontol* 2000;71:948-54.
7. Möller AJR. Microbiological examination of root canals and periapical tissues of human teeth. *Odontol Tidskr* 1966;74:1-380.
8. Mullally BH, Dace B, Shelburne CE, Wolff LF, Coulter WA. Prevalence of periodontal pathogens in localized and generalized forms of early-onset periodontitis. *J Periodont Res* 2000;35:232-41.
9. Paster BJ, Boche SK, Galvin JL, Ericson RE, Lau CN, Levanos VA, Sahasrabudhe A, Dewhirst FE. Bacterial diversity in human subgingival plaque. *J Bacteriol* 2001;183:3770-83.
10. Preus HR, Haraaszthy VI, Zambon JJ, Genco RJ. Differentiation of strains of *Actinobacillus actinomycetemcomitans* by arbitrarily primed polymerase chain reaction. *J Clin Microbiol* 1993;31:2773-6.
11. Riggio MP, Lennon A. Rapid identification of *Actinobacillus actinomycetemcomitans*, *haemophilus aphrophilus*, and *haemophilus paraphrophilus* by restriction enzyme analysis of PCR-amplified. *J Clin Microbiol* 1997;168:1-8.
12. Slots J, Reynolds HS, Genco RJ. *Actinobacillus actinomycetemcomitans* in human periodontal disease: a cross-sectional microbiological. *Infect Immun* 1980;29:1013-20.
13. Slots J. Selective medium for isolation of *Actinobacillus actinomycetemcomitans*. *J Clin Microbiol* 1982;15:606-9.

14. Slots J. Salient biochemical characters of *Actinobacillus actinomycetemcomitans*. *Arch Microbiol* 1982;131:60-7.
15. Slots J, Ashimoto A, Flynn MJ, Li G, Chen C. Detection of putative periodontal pathogens in subgingival specimens by 16S ribosomal DNA amplification with the polymerase chain reaction. *Clin Infect Dis* 1995;20:304-7.
16. Tran SD, Rudney JD. Improved multiplex PCR using conserved and species-specific 16S rRNA gene primer for simultaneous detection. *J Clin Microbiol* 1999;37:3504-8.
17. van Winkelhoff AJ, Overbeek BP, Pavicic MJAM, van den Bergh JPA, Ernest JPMG, Graaff J. Long standing bacteremia caused by oral *Actinobacillus actinomycetemcomitans* in a patient with a pacemaker. *Clin Infect Dis* 1993;16:216-8.