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16S rRNA REGION BASED PCR PROTOCOL FOR IDENTIFICATION AND SUBTYPING OF *PARVIMONAS MICRA*

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SHORT COMMUNICATION

ABSTRACT

The present study established a PCR protocol in order to identify *Parvimonas micra* and to evaluate the intra-species diversity by PCR-RFLP of *16S rRNA* partial sequence. The data indicated that the protocol was able to identify this species which could be clustered in five genotypes.

Key words: *Parvimonas micra,* PCR-RFLP, *16S rRNA*, anaerobic cocci.

Parvimonas micra (formerly *Peptostreptococcus micros*) are gram positive anaerobic cocci (GPAC) originally classified in the genus *Peptostreptococcus.* These organisms are part of the indigenous microbiota of the oral cavity and gut of humans and animals and are frequently associated with polymicrobial infections (5). The species is present in high proportion in subgingival dental plaque from most periodontitis patients, mainly in those with diseased active sites (3,6) and smokers (14).

Three morphotypes of *P. micra* were described (smooth, rough and a smooth variant of rough type) (2-13), each one differing from the others on colonial morphology, hemolytic activity, hydrophobicity, surface structures and efficiency of adhesion to epithelial cells (3-13).

The identification of *P. micra* is usually performed by phenotypic methods based on colonial morphology in blood agar plates and biochemical tests which are time consuming, laborious and sometimes inconclusive (4). Polymerase chain reaction (PCR) protocols based on *16S rRNA* gene sequences were developed for identification of *P. micra* by using species-specific primers (7-9) and for genotyping by employing RFLP analysis of amplicons obtained with universal primers for the *16S rRNA* gene (8).

In order to facilitate the identification and subtyping of these organisms, the present study describes the identification of *P. micra* by PCR using a specific primer pair directed to *16S rRNA* followed by RFLP analysis of the amplicons.

Seventy seven subgingival clinical isolates were obtained and comprised 22 isolates from chronic periodontitis patients, 10 from agressive periodontitis patients, 30 from gingivitis patients and 15 from healthy subjects. The primary identification was done on the basis of colonial morphology on PMM agar medium (12) and was further confirmed by using Rapid Id 32A kit (bioMérieux ®sa, Marcy l'Etoile, France).

P. micra strains HG 1467 (smooth), HG1259 (rough) (gently given by Dr. T.J. M. van Steenbergen) and ATCC 33270 were used as reference strains. Bacteria from other species as *Porphyromonas gingivalis* (ATCC 33277), *Streptococcus pyogenes* (ATCC10096), *Streptococcus mutans* (GS5) and *Finegoldia magna* (CCUG12461) were used to test the specificity of the PCR.

This project was approved by the Ethical Commitee on Human Research of the Institute of Biomedical Sciences, University of São Paulo, Brazil.

In order to design species-specific primers and to determine the restriction sites to be used in the RFLP analysis of amplicons, *16S rRNA* partial sequences of the reference strains HG1467 and HG1259 were cloned and sequenced.

The *16S rRNA* partial sequences were obtained by PCR performed using Triple Master Kit (Eppendorff, Hamburg,

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Germany) according to manufacturer's instructions, 1pM of each universal primers fD1 (5' AGAGTTTGATCCTGGCTCAG 3') and rP2 (5' ACGGCTACCTTGTTACGACTT 3')(Invitrogen, São Paulo, Brazil) (15) and template DNA from reference strains HG1467 and HG1259. The PCR amplicons were cloned into the pCR2.1-TOPO vector using TOPO TA Cloning kit - version P (Invitrogen, Carlsbad, CA, USA) and sequenced (ESPECIFICAR COMO. POR EX EM QUE APARELHO).

The cloned HG1467 and HG1259 partial sequences of the *16S rRNA* gene were analyzed using DNA Star software (DNASTAR Inc., Madison, WI, USA) which compared them to sequences available in data base, including: *Parvimonas micra* strain (ATCC 33270 GenBank accession number), *Finegoldia magna* (GenBank accession number), the closest species related to *P. micra*, and *Peptostreptococcus anaerobius* (GenBank accession number). Based on multiple alignment analysis, two primers (PM2-upper and PM2-lower), which were conserved among *P. micra* and unique enough to differentiate it from other species, were designed. The amplicon positions are based on corresponding sequence data: ATCC 33270 (position 445 to 1220; GenBank accession number); HG1467 (position 49 to 816, GenBank accession number) and HG 1259 (position 384 to 1166, GenBank accession number).

Reactions with the selected primers PM2 Upper (5' GTA TCA TAG GAG GAA GCC 3') and PM2 Lower (5'TCT AGC TTC TCG TTG TAC C 3') were performed with *P. micra* reference strains (ATCC 33270, HG1467 and HG 1259) and also with template DNA of other bacterial species reported above. Amplification reaction was performed in 100 µl reactions comprising 20 µl template DNA obtained by a boiling method (1), 5U Taq DNA polymerase (Invitrogen), 1X Taq Buffer, 100 µM of each dNTP(Invitrogen), 1.5 mM $MgCl_2$ and 2 pmol of each primer (PM2 Upper and PM2 Lower). Restriction analysis were performed by digesting the resulting amplicon with *Hae* III and *Hinf* I (Invitrogen, Carlsbad, USA), according to the manufacturer´s instructions. The products were resolved by electrophoresis in 1.5% agarose gels in Tris-acetate-EDTA buffer (TAE) and stained with ethidium bromide. Then, the same PCR protocol using template DNA from the 77 clinical *P. micra* isolates were done and the amplicons were digested with the restriction enzymes mentioned above.

Amplification reactions using the primers pair PM2-upper and PM2-lower established in this study resulted in amplicons, as expected, ranging from 776bp (ATCC 33270) to 786 (HG1467). None of the PCR using DNA template of other species including the most related species *Finegoldia magna* yielded amplicons, confirming the reaction specificity.

Amplicon identities were confirmed by restriction analysis with *Hinf* I and *Hae* III, resulting in three patterns as expected (Fig. 1 and Table 1). Alignment analysis revealed that the region of the primer (Pmic 2) also based on 16S rRNA partial sequence and described by Riggio *et al.* (2001) for *P. micra* identification

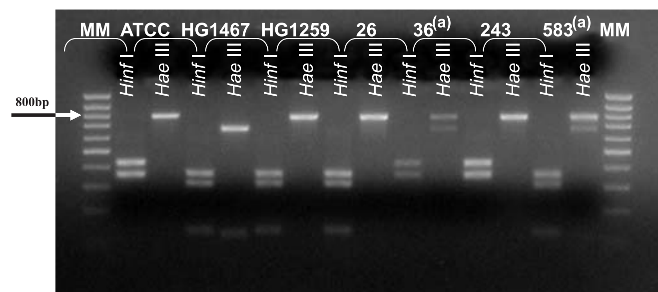

Figure 1. Restriction fragments obtained by digestion with *Hinf* I and *Hae* III of PCR's amplicon using primers PM2 lower and PM2 upper and template DNA of reference strains ATCC 33270, HG 1467 and HG1259 and some clinical isolates (26, 36, 243 and 583). (MM - molecular marker 100bp -Fermentas).
[a] profiles not expected and observed during the tests.

Table 1. Expected fragment sizes of PM2 lower and PM2 upper PCRs-RFLP.

Enzyme	Expected sizes (bp) of fragments of sequence from:		
	ATCC 33270	HG1467	HG1259
Hinf I	423, 353	119, 314, 353	114, 309, 360
*Hae*III	776	681, 105	783

(7) differed in four of twenty bases in the sequences of strains HG1467 and HG1259. These data were corroborated by the finding that primers reported by Riggio *et al.* (2001) (7) was able to identify only a subset of *P. micra* strains, since no amplicon could be obtained by using DNA isolated from 41 out 77 clinical isolates tested in the present study (data not shown) by employing the reaction reported by the authors.

PCR–RFLP analysis using DNA from the 77 clinical isolates confirmed the intra-species polymorphism of the *16S rRNA* region. Two additional unexpected PCR-RFLP profiles (isolates 36 and 583) were observed, indicating that at least 5 distinct genotypes among *P. micra* isolates (Fig. 1) can occur. As shown in Fig. 1, the profile obtained by *Hae* III digestion of amplicons obtained from isolates number 36 and number 583 revealed a band corresponding to an undigested amplicon (~780 bp). The species *P. micra* possess four copies of this *rRNA* operon (10). *Finegoldia magna*, the phylogenetic species most related to *P. micra*, also possess 4 different copies of this operon (11). The presence of digested and undigested amplicons in the same reaction suggests that *P. micra* strains may harbor *16S rRNA* gene copies differing in region of *Hae* III restriction site.

16S rRNA gene polymorphism among *P. micra* isolates has already been reported by Riggio and Lennon (2003) (8), who

had proposed a PCR-RFLP for the identification of *Peptostreptococcus* species. These authors reported that from 22 *P. micra* isolates, 7 exhibited PCR-RFLP divergent from what was expected for this species, suggesting the existence of variants of *P. micra* or even another possibly unidentified bacterial species, very closed related to *P. micra*. The present data confirmed this variability in *16S rRNA* gene among isolates phenotypically identified as *P. micra* and suggest that this variability can be wider than previously reported (8), since 5 genotypes of *P. micra* could be observed.

In the present study, PCR protocol using species-specific primers pairs **PM2 lower** and **PM2 upper** was able to identify all tested *P. micra* isolates. In addition, polymorphism of *16S rRNA* gene could be observed by PCR-RFLP using *Hae*III and *Hinf*I restriction analysis. Further studies should be done in order to confirm the copy number of *rRNA* operons in *P. micra* species and to correlate the different genotypes with phenotypic traits and virulence.

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RESUMO

PCR baseada na região *16S rRNA* para identificação e genotipagem de *Parvimonas micra*

O presente estudo estabeleceu um protocolo de PCR com a finalidade de identificar a espécie *Parvimonas micra* e avaliar a diversidade intra-espécie utilizando a técnica PCR-RFLP do gene que codifica o *rRNA 16S*. Os dados indicaram que o protocolo possibilitou a identificação da espécie e a distinção de 5 grupos genotípicos.

Palavras chave: *Parvimonas micra,* PCR, *16S rRNA*, PCR-RFLP, anaeróbio.

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