**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.

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Germany) according to manufacturer’s instructions, 1pM of each universal primers fD1 (5’AGAGTTTGATCCTGAGCAG 3’) and rP2 (5’ACGGCTACCTTGTTACGACCTT 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 EXEMPLE 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: *Peptostreptococcus anaerobius* (GenBank accession number), *Peptostreptococcus anaerobius* (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). 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’TCTAGTCC TTC TCG TTG TAC C 3’) were performed with *P. micra* reference strains (ATCC 33270, HG1467 and HG1259) 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 MgCl2 and 2 pmol of each primer (PM2-upper and PM2-lower), Restriction analysis was 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 (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.

**REFERENCES**

1. Alam, S.; Brailsford, S.R.; Whiley, R.A.; Beighton, D. (1999). PCR-based methods for genotyping viridans group streptococci. *J. Clin. Microbiol.*, 37, 2772-2776.
2. Kremer, B.H.A.; Magee, J.T.; van Dalen, P.J.; van Steenbergen, M.T.J. (1997). Characterization of smooth and rough morphotypes of *Peptostreptococcus micros*. *Int. J. Syst. Bacteriol.*, 47, 363-368.
3. Moore, W.E.C.; Moore, L.H.; Ranney, R.R.; Smibert, R.M.; Burmeister, J.A.; Schenkein, H.A. (1991). The microflora of periodontal sites showing active destructive progression. *J. Clin. Periodontol.*, 18, 729-739.
4. Murdoch, D.A.; Mitchelmore, I.J. (1991). The laboratory identification of gram-positive anaerobic cocci. *J. Med. Microbiol.*, 34, 295-308.
5. Riggio, M.P.; Lennon, A. (2003). Identification of *Peptostreptococcus micros* isolates by PCR-restriction fragment length polymorphism analysis of 16S rRNA genes. *J. Clin. Microbiol.*, 41, 4475-4479.
6. Rams, T.E.; Feik, D.; Listgarten, M.A.; Slots, J. (1992). *Peptostreptococcus micros* in human periodontitis. *Oral Microbiol. Immunol.*, 7, 1-6.
7. Riggio, M.P.; Lennon, A.; Smith, A. (2001). Detection of *Peptostreptococcus micros* DNA in clinical samples by PCR. *J. Med. Microbiol.*, 50, 249-254.
8. Riggio, M.P.; Lennon, A. (2003). Identification of oral *Peptostreptococcus micros* isolates by PCR-restriction segment length polymorphism analysis of 16S rRNA genes. *J. Clin. Microbiol.*, 41, 4475-4479.
9. Song, Y.; Liu, C.; McTeague, M.; Vu, A.; Liu, J.Y.; Finegold, S.M. (2003). Rapid identification of gram-positive anaerobic coccal species originally classified in the genus *Peptostreptococcus* by multiplex PCR assays using genus- and species-specific primers. *Microbiology*, 19, 1719-1727.
10. Todo, K.; Goto, T.; Miyamoto, K.; Akimoto, S. (2002). Physical and genetic map of *Finegoldia magna* (formerly *Peptostreptococcus magnus*) ATCC 29328 genome. *FEMS Microbiol. Lett.*, 210, 33-37.
11. Todo, K.; Goto, T.; Honda, A.; Tamura, M.; Miyamoto, K.; Fujita, S.; Akimoto, S. (2004). Comparative analysis of the four rRNA operons in *Finegoldia magna* ATCC 29328. *System. Appl. Microbiol.*, 27, 18-26.
12. Turng, B.F.; Guthmiller, J.M.; Minah, G.E.; Falkner, W.A. Jr. (1996). Development and evaluation of selective medium for primary isolation of *Peptostreptococcus micros*. *Oral Microbiol. Immunol.*, 5, 356-361.
13. van Dalen, P.J.; van Steenbergen, T.J.M.; Cowan, M.M.; Busscher, H.J.; Graaf, J. (1993). Description of two morphotypes of *Peptostreptococcus micros*. *Int. J. Syst. Bacteriol.*, 43, 789-793.
14. van Winkelhoff, A.J.; Bosch-Tihojof, C.J.; Winkel, E.G.; van der Reijden, W.A. (2001). Smoking affects the subgingival microflora in periodontitis. *J. Periodontol.*, 72, 666-671.
15. Weisburg, W.G.; Barns, S.M.; Pelletier, D.A.; Lane, D.J. (1991). 16S ribosomal DNA amplification for phylogenetic study. *J. Bacteriol.*, 173, 697-703.