MOLECULAR DIAGNOSIS

Detection of *Peptostreptococcus micros* DNA in clinical samples by PCR

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Peptostreptococcus micros is a gram-positive anaerobic coccus which, although considered to be a natural commensal of the human oral cavity, is associated with periodontal, endodontal and peritonsillar infections. Identification of the organism has to date relied upon conventional culture methods and biochemical analyses. The purpose of this study was to develop a PCR method for rapid and specific identification of this organism in clinical samples. A pair of primers was selected, each of which was specific at the 3' end for *P. micros* DNA; they were used in the PCR assay, resulting in a 1074-bp product. The primers were shown to be specific for *P. micros* DNA as no PCR products were obtained when genomic DNA extracts from a wide range of other *Peptostreptococcus* species and other oral bacteria were used as templates. The PCR assay was then applied to the identification of *P. micros* DNA in subgingival plaque samples from adult periodontitis patients and pus samples from subjects with acute dento-alveolar abscesses. Confirmation of specific amplification of *P. micros* DNA was obtained by digestion of PCR products with the restriction endonuclease *RsaI*, which gives a unique restriction profile for *P. micros*, and DNA sequencing. Sixty-eight subgingival plaque samples from 18 patients were analysed, of which 19 (28%) were positive for *P. micros* DNA; the proportion of patients carrying *P. micros* DNA in at least one sampled site was 11 (61%) of 18. Twenty (71%) of 28 pus samples analysed by PCR contained *P. micros* DNA. These results confirm that *P. micros* may be involved in the aetiology of acute dento-alveolar abscesses and adult periodontitis. The PCR assay provides a more rapid and reliable alternative to conventional methods for identification of *P. micros* in clinical samples.

Introduction

Peptostreptococcus micros is a gram-positive anaerobic coccus which is primarily found in the human oral cavity [1]. The organism is considered to be a commensal of the oral cavity, as it comprises <3% of the subgingival flora in periodontally healthy adults [2]. However, it has been isolated more frequently and in higher numbers from patients with periodontitis and has been shown to be associated with periodontal destruction, particularly in periodontal sites from disease-active patients [3–5]. *P. micros* is also a known pathogen in other mixed anaerobic infections of the oral cavity, including endodontic abscesses [6, 7], periodontal abscesses [8] and peritonsillar infections

[9]. It is also often involved in severe mixed anaerobic infections throughout the human body. These include infections of the ear, sinuses and gynaecological tract [10], prosthetic joint infections [11] and abdominal abscesses [12]. However, the true extent of its pathogenic nature remains to be determined. *P. micros* exists as three morphotypes (smooth, rough and smooth variant of the rough type) which differ in the nature of their surface structures, with the rough type possessing large fibrillar surface structures that are absent in the other morphotypes [13, 14].

To date, identification of *P. micros* in clinical samples has been achieved principally by the use of bacterial culture coupled to biochemical tests, and serological methods. A selective and differential medium for the primary isolation of *P. micros* has been described [15]. Monoclonal antibodies (MAbs) recognising specific surface antigens of *P. micros* have been generated and successfully applied to its detection in clinical samples in an indirect immunofluorescence assay [16]. How-

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ever, no molecular methods have been described for detection of this organism in clinical samples.

The purpose of this study was to develop a novel PCR-based method for the detection of *P. micros* in clinical samples as an alternative to currently used conventional methods. Although conventional detection methods such as bacterial culture and serology have been used successfully for *P. micros* detection, they are time-consuming and expensive and can give rise to ambiguous results. Furthermore, MAbs directed against *P. micros* are not readily available. An earlier study showed PCR to be more sensitive and specific than culture methods for detection of periodontal pathogens in subgingival plaque [17]. The present study describes the development of PCR primers specific for *P. micros* and their application in a PCR assay for detection of this organism in both subgingival plaque from adult periodontitis patients and in pus aspirates from acute dento-alveolar abscesses.

Materials and methods

Bacterial culture and genomic DNA purification

P. micros ATCC 33270 was inoculated on to Fastidious Anaerobe Agar (Life Technologies, Paisley) supplemented with defibrinated horse blood 7.5% v/v and incubated at 37°C for 4–5 days in an anaerobic chamber (Don Whitley Scientific, Shipley) with an atmosphere of N₂ 85%, CO₂ 10% and H₂ 5%. Bacterial growth was harvested from the plates and genomic DNA was extracted with the Puregene DNA Purification Kit (Flowgen, Ashby de la Zouch) according to the manufacturer's instructions.

Sample collection and preparation

Archival subgingival plaque samples were analysed. Previously, the samples had been collected in sterile tubes containing 0.5 ml of freshly prepared Fastidious Anaerobe Broth (Bioconnections, Leeds, W. Yorks) using a single stroke of a separate sterile curette for each sample to prevent cross-contamination. Samples were vortex mixed for 30 s and lysates were prepared for use in PCR by adding 3 µl of achromopeptidase (20 U/µl in 10 mM Tris-HCl, 1 mM EDTA, pH 7.0) to 100 µl of vortex mixed subgingival plaque. Samples were then incubated at 56°C for 30 min, boiled for 5 min and stored at –70°C until required.

Archival pus aspirates previously obtained from patients with acute dento-alveolar abscesses were analysed. Samples of pus (50 µl) were diluted 10–100-fold in PCR diluent (10 mM Tris-HCl, pH 8.0, 10 mM NaCl, 1 mM EDTA). SDS 10% (30 µl) and proteinase K (3 µl at 10 mg/ml) were added to 300 µl of diluted pus and incubated at 55°C for 3 h. Lysates were extracted twice with an equal volume of phenol:chloroform (1:1) and once with an equal volume of chloroform:isoamyl

alcohol (24:1). DNA was precipitated by addition of 0.1 volume of 3 M sodium acetate, pH 5.3, and 2 volumes of ethanol 100%, mixing and holding at –70°C for 30 min. DNA was collected by microcentrifugation and the dried pellet was resuspended in 100 µl of sterile molecular biology grade water.

Sample details

For subgingival plaque analysis, subjects were untreated patients with chronic inflammatory adult periodontitis who were newly referred to Glasgow Dental Hospital. Criteria for use of patient samples in the study were the presence of at least three periodontal pockets with a minimum depth of 5 mm and bleeding on probing, coupled with no history of antibiotic treatment in the preceding 6 months. A total of 68 samples from 18 patients was analysed. The mean pocket depth of samples analysed was 7.1 mm (range 5–11 mm); the age range of the patients was 32–60 years with a mean age of 43.6 years. Also, 28 pus samples were analysed from 28 patients, whose age range was 30–56 years with a mean age of 41.8 years.

PCR primer selection

Primers for use in the *P. micros* PCR assay were selected by alignment of the 16S ribosomal RNA (rRNA) gene sequences of members of the *Peptostreptococcus* genus and of several other oral bacteria. Two primers were selected from the 16S rRNA gene sequence of *P. micros* ATCC 33270 that were sufficiently unique in sequence at their 3' ends, compared with the corresponding regions of all other 16S rRNA genes analysed, to confidently predict their specificity for *P. micros* DNA. The *P. micros* 16S rRNA primers selected were 5'-TCG AAC GTG ATT TTT GTG GA-3' (Pmic-1; base position 55–74) and 5'-TCC AGA GTT CCC ACC TCT-3' (Pmic-2; base position 1128–1109), which yield an expected amplification product of 1074 bp.

PCR

The PCR reaction mixture used was essentially as described previously [17]. Each PCR reaction mixture comprised either 5 µl of plaque sample lysate or 1 µl of bacterial genomic DNA and either 95 or 99 µl, respectively, of reaction mixture that contained MgCl₂ at the optimal concentration of 1.5 mM. PCR cycling conditions comprised an initial denaturation step of 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min and extension at 72°C for 1.5 min, and then a final extension step at 72°C for 10 min.

Stringent anti-contamination procedures were employed when performing PCR, as described previously [17]. For each batch of samples being analysed, negative and positive controls were included. The positive control

used was a standard PCR reaction mixture containing 100 ng of *P. micros* genomic DNA instead of sample, whereas the negative control contained sterile water instead of sample. PCR products were visualised after electrophoresis on agarose 2% gels as described previously [17].

Restriction endonuclease digestion of PCR products

PCR products were purified with the Wizard PCR Purification Kit (Promega) in accordance with the manufacturer's instructions. Briefly, 0.5 µg of purified DNA was separately digested in a total volume of 20 µl with 5.0 units of each of the restriction endonucleases *Rsa*I and *Hinf*I (Promega) at 37°C for 3 h. Restriction fragments generated were visualised by agarose gel electrophoresis.

DNA sequencing

PCR products were cloned into the pCR2.1-TOPO vector with the TOPO TA Cloning Kit (Invitrogen BV, Groningen, The Netherlands) in accordance with the manufacturer's instructions. Plasmid DNA from recombinant clones was purified with the Wizard Plus Minipreps DNA Purification System (Promega). Sequencing reactions were performed with Thermo Sequenase Fluorescent Labelled Primer Cycle Sequencing Kit with 7-deaza-dGTP (Amersham Pharmacia Biotech, Little Chalfont, Bucks) and IRD800-labelled M13 universal (-21) primer. Sequence data was collected and analysed in an automated DNA sequencing system.

Results

Sensitivity and specificity of the *P. micros* PCR assay

After a single round of 35 cycles of amplification, the lower limit of detection of *P. micros* DNA was 50 fg or c. 50 bacterial cells (data not shown). To confirm the specificity of the *P. micros* PCR assay, 100 ng of genomic DNA from each of the following *Peptostreptococcus* species was used as a template in a standard PCR: *P. micros* (ATCC 33270, ATCC 23195), *P. magnus* NCTC 11804, *P. anaerobius* NCTC 11460, *P. asaccharolyticus* NCTC 11461, *P. prevotii* NCTC 11806, *P. tetradius* ATCC 35098, *P. productus* NCTC 11829 and *P. indolicus* NCTC 11088. DNA extracted from each of the following oral species was also used as a template in the PCR assay: *Prevotella intermedia* ATCC 25611, *Porphyromonas gingivalis* ATCC 33277, *Bacteroides forsythus* ATCC 43037, *Helicobacter pylori* ATCC 43504, *Actinobacillus actinomycetemcomitans* ATCC 33384, *Fusobacterium nucleatum* ATCC 25586, *Eikenella corrodens* ATCC 23834, *Streptococcus mutans* ATCC 25175 and *Staphylococcus aureus* ATCC 12600. PCR products were obtained with the

two strains of *P. micros* but not with any other species tested (data not shown), thus confirming the specificity of the primers used in the PCR assay.

PCR detection of *P. micros* in clinical samples

The *P. micros* PCR assay was applied to the detection of this bacterial species in subgingival plaque samples from adult periodontitis patients and pus samples from subjects with acute dento-alveolar abscesses. *P. micros* DNA was detected by PCR in 19 (28%) of the 68 samples analysed. Eleven (61%) of 18 patients harboured *P. micros* DNA in at least one analysed site. Twenty (71%) of 28 pus samples analysed (one per subject) contained *P. micros* DNA. Figs. 1a and b show representative results obtained with PCR to detect *P. micros* DNA in subgingival plaque samples and pus samples, respectively.

Confirmation of PCR product specificity

To confirm that successfully amplified products were derived from *P. micros*, PCR products were digested with the restriction endonuclease *Rsa*I, which gives a restriction profile unique to *P. micros* and, therefore, allows its distinction from other closely related *Peptostreptococcus* species. Digestion of PCR products obtained from *P. micros* gave DNA fragments with predicted sizes of 504, 261, 183 and 126 bp (Fig. 2). A further confirmatory digestion with *Hinf*I yielded fragment sizes of 388, 312, 234 and 140 bp (Fig. 2). All PCR products gave restriction patterns with both *Rsa*I and *Hinf*I that were expected for *P. micros*.

Four PCR products were cloned into the pCR2.1-TOPO plasmid and four recombinant clones obtained from each cloned product were sequenced. The DNA sequence data confirmed that all 16 recombinant clones sequenced were derived from *P. micros*, thereby further demonstrating the specificity of the PCR assay.

Discussion

The purpose of this study was to design and implement a novel, direct PCR assay for the specific detection of *P. micros* DNA in clinical samples. Current methods for identification of this organism are heavily reliant on traditional microbiological culture and biochemical analyses. *P. micros* is commonly identified on the basis of colony morphology, oxygen tolerance and Gram's staining characteristics, followed by analysis of its enzymic activities (API ZYM, Rapid ID 32A) and its capacity to hydrolyse amino acid and phosphate substrates (RapID ANA II) [5, 18]. A selective and differential medium has been described that facilitates the primary isolation of *P. micros* from clinical samples which is based upon a Columbia CNA agar, supplemented with glutathione and lead acetate [15], that is selective for gram-positive cocci. When grown on this

Fig. 1. Electrophoresis on agarose 2% gels of PCR products obtained from analysis of clinical samples for *P. micros* DNA. **(a)** Detection of *P. micros* DNA in subgingival plaque samples: lanes 1–17, subgingival plaque samples; 18, positive control; 19, negative control; 20, 100-bp DNA ladder. **(b)** Detection of *P. micros* DNA in pus samples: lanes 1–12, pus samples; 13, 100-bp DNA ladder; 14, positive control; 15, negative control. A positive PCR result is indicated by the presence of a 1074-bp product.

Fig. 2. Electrophoresis on agarose 2% gel of 1074-bp *P. micros* PCR products after digestion with restriction endonucleases *Rsa*I and *Hinf*I. Lanes 1 and 2, clinical samples, *Rsa*I; 3, *P. micros* ATCC 33270, *Rsa*I; 4, 100-bp DNA ladder; 5 and 6, clinical samples, *Hinf*I; 7, *P. micros* ATCC 33270, *Hinf*I.

medium, *P. micros* utilises the reduced form of glutathione to form H_2S , which reacts with lead acetate to produce a black precipitate that is clearly visible below *P. micros* colonies.

Whilst these methods have been of significant value, their usefulness is limited by the fact that results obtained are often ambiguous, primarily due to the emergence of phenotypically atypical strains which may differ in their biochemical characteristics. Furthermore, traditional identification methods are time-consuming, labour-intensive and expensive. PCR offers an alternative means of bacterial identification that overcomes these limitations. PCR is able to detect phenotypically divergent strains and is a rapid, highly specific and relatively inexpensive identification method.

In the present study, PCR primers were developed that specifically targeted the 16S rRNA gene of *P. micros*. The PCR assay successfully identified *P. micros* DNA in subgingival plaque from patients with adult periodontitis and in pus aspirates from patients with acute dento-alveolar abscesses. An elevated presence of *P. micros* in subgingival samples from patients with

periodontal disease has been demonstrated previously by culture methods [4, 5]. In the study of Rams *et al.* [5], subgingival plaque samples from 907 patients with advanced adult periodontitis, 127 with early-onset periodontitis and 12 with localised juvenile periodontitis were analysed for the presence of *P. micros*. It was found in 58–63% of samples analysed in these patient groups. The present study detected *P. micros* DNA by PCR in the subgingival plaque of 11 (61%) of 18 adult periodontitis patients, a similar figure to the 63% patient positivity rate reported when culture methods were used for detection of *P. micros* [5]. An even higher prevalence of *P. micros* in the subgingival plaque of adult periodontitis patients has been reported, with 112 (91%) of 123 patients shown to harbour the organism as demonstrated by culture methods [19]. The gingival immune response to periodontal pathogens in juvenile periodontitis patients has been examined by a gingival explant culture system, with local IgG levels to *P. micros* shown to be elevated in these patients compared with healthy controls [20].

In a study investigating the associations between microbial species in dental root canal infections *P. micros* was the most prevalent organism, being detected by culture in 22 (34%) of 65 infected human root canals [21]. In the same study, a positive association was found between *P. micros* and *Pr. intermedia*, an association that has also been demonstrated in subgingival plaque [22]. The present study detected *P. micros* DNA by PCR in 20 (71%) of 28 pus aspirates from acute dento-alveolar abscesses, which are a sequel to root canal infection. IgG, IgM and IgA antibodies have been found against several micro-organisms that are present in human peri-apical lesions, with IgG levels for *P. micros* being significantly high [23].

In conclusion, a novel PCR assay for the specific and direct detection of *P. micros* DNA in clinical samples has been developed and applied. PCR detection of *P. micros* DNA in subgingival plaque of adult periodontitis patients and in pus aspirates from acute dento-alveolar abscesses confirms *P. micros* as a putative pathogen in these lesions. The use of this PCR assay as a rapid, more reliable alternative to conventional culture and biochemical methods for identification of *P. micros* in clinical samples is proposed.

References

1. Neut C, Lesieur V, Romond C, Beerens H. Analysis of gram-positive anaerobic cocci in oral, fecal and vaginal flora. *Eur J Clin Microbiol* 1985; 4: 435–437.
2. Moore WEC, Holdeman LV, Smibert RM *et al.* Bacteriology of experimental gingivitis in young adult humans. *Infect Immun* 1982; 38: 651–667.
3. Dzink JL, Socransky SS, Haffajee AD. The predominant cultivable microbiota of active and inactive lesions of destructive periodontal diseases. *J Clin Periodontol* 1988; 15: 316–323.
4. Moore WEC, Moore LH, Ranney RR, Smibert RM, Burmeister JA, Schenkein HA. The microflora of periodontal sites showing

- active destructive progression. *J Clin Periodontol* 1991; **18**: 729–739.
5. Rams TE, Feik D, Listgarten MA, Slots J. *Peptostreptococcus micros* in human periodontitis. *Oral Microbiol Immunol* 1992; **7**: 1–6.
 6. Brook I, Grimm S, Kielich RB. Bacteriology of acute periapical abscess in children. *J Endod* 1981; **7**: 378–380.
 7. Williams BL, McCann GF, Schoenknecht FD. Bacteriology of dental abscesses of endodontic origin. *J Clin Microbiol* 1983; **18**: 770–774.
 8. Newman MG, Sims TN. The predominant cultivable microbiota of the periodontal abscess. *J Periodontol* 1979; **50**: 350–354.
 9. Mitchelmore IJ, Prior AJ, Montgomery PQ, Tabaqchali S. Microbiological features and pathogenesis of peritonsillar abscesses. *Eur J Clin Microbiol Infect Dis* 1995; **14**: 870–877.
 10. Brook I. Recovery of anaerobic bacteria from clinical specimens in 12 years at two military hospitals. *J Clin Microbiol* 1988; **26**: 1181–1188.
 11. Sullivan PM, Johnston RC, Kelley SS. Late infection after total hip replacement, caused by an oral organism after dental manipulation. A case report. *J Bone Joint Surg* 1990; **72A**: 121–123.
 12. Brook I. A 12 year study of aerobic and anaerobic bacteria in intra-abdominal and postsurgical abdominal wound infections. *Surg Gynecol Obstet* 1989; **169**: 387–392.
 13. Kremer BHA, Magee JT, van Dalen PJ, van Steenberg TJM. Characterization of smooth and rough morphotypes of *Peptostreptococcus micros*. *Int J Syst Bacteriol* 1997; **47**: 363–368.
 14. van Dalen PJ, van Deutekom-Mulder EC, de Graaff J, van Steenberg TJM. Pathogenicity of *Peptostreptococcus micros* morphotypes and *Prevotella* species in pure and mixed culture. *J Med Microbiol* 1998; **47**: 135–140.
 15. Turng B-F, Guthmiller JM, minah GE, Falkler WA. Development and evaluation of a selective and differential medium for the primary isolation of *Peptostreptococcus micros*. *Oral Microbiol Immunol* 1996; **11**: 356–361.
 16. Gmur R, Thurnheer T. Monoclonal antibodies for the rapid identification in clinical samples of *Peptostreptococcus micros* and *Actinobacillus actinomycetemcomitans* serotypes a, d, and e. *Med Microbiol Lett* 1996; **5**: 335–349.
 17. Riggio MP, Macfarlane TW, Mackenzie D, Lennon A, Smith AJ, Kinane D. Comparison of polymerase chain reaction and culture methods for detection of *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* in subgingival plaque samples. *J Periodont Res* 1996; **31**: 496–501.
 18. Ng J, Ng L-K, Chow AW, Dillon J-AR. Identification of five *Peptostreptococcus* species isolated predominantly from the female genital tract by using the Rapid ID 32A system. *J Clin Microbiol* 1994; **32**: 1302–1307.
 19. van Dalen PJ, van Winkelhoff AJ, van Steenberg TJM. Prevalence of *Peptostreptococcus micros* morphotypes in patients with adult periodontitis. *Oral Microbiol Immunol* 1998; **13**: 62–64.
 20. Hall ER, Martin SA, Suzuki JB, Falkler WA. The gingival immune response to periodontal pathogens in juvenile periodontitis. *Oral Microbiol Immunol* 1994; **9**: 327–334.
 21. Sundqvist G. Associations between microbial species in dental root canal infections. *Oral Microbiol Immunol* 1992; **7**: 257–262.
 22. Socransky SS, Haffajee AD, Dzink JL, Hillman JD. Associations between microbial species in subgingival plaque samples. *Oral Microbiol Immunol* 1988; **3**: 1–7.
 23. Kettering JD, Torabinejad M, Jones SL. Specificity of antibodies present in human periapical lesions. *J Endod* 1991; **17**: 213–216.