Specific PCR detection of *Peptostreptococcus magnus*

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Peptostreptococcus magnus is the most pathogenic and one of the most common Gram-positive anaerobic cocci found in human clinical specimens. The organism has been isolated in pure culture from a range of serious infections, including meningitis and endocarditis. However, isolation of *Peptostreptococcus magnus* from the oral cavity has rarely been attempted. Identification of *Peptostreptococcus magnus* in clinical specimens is reliant upon microbiological culture and biochemical methods, which often give ambiguous results. The aim of this study was to develop a PCR assay for the specific detection of *Peptostreptococcus magnus* in oral clinical specimens. PCR primers specific for *Peptostreptococcus magnus* DNA were derived by comparison of 16S rRNA gene sequences and selection of primers that demonstrated specificity at their 3' ends for *Peptostreptococcus magnus*. PCR positivity for *Peptostreptococcus magnus* DNA was indicated by the amplification of a 553 bp product. The PCR assay was then used to attempt detection of *Peptostreptococcus magnus* DNA in subgingival plaque samples from adult periodontitis patients and pus aspirates from subjects with acute dento-alveolar abscesses. The PCR assay was demonstrated to be highly specific for *Peptostreptococcus magnus* DNA, since no PCR products were obtained when genomic DNA from a wide range of other oral bacteria, including closely related *Peptostreptococcus* species, was used in the PCR assay. Confirmation of specific amplification of *Peptostreptococcus magnus* DNA was obtained by digestion of PCR products with the restriction endonuclease *RsaI*, which gives a unique restriction profile for *Peptostreptococcus magnus*. Of the 33 subgingival plaque samples analysed, 2 (6 %) were positive for *Peptostreptococcus magnus* DNA. None of the 60 pus aspirates analysed was positive for *Peptostreptococcus magnus* DNA. It is concluded that *Peptostreptococcus magnus* is not a major pathogen in adult periodontitis or dento-alveolar abscesses. The PCR assay provides a more rapid, specific and sensitive alternative to conventional methods for identification of *Peptostreptococcus magnus* in clinical specimens.

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INTRODUCTION

Peptostreptococcus magnus is a Gram-positive anaerobic coccus (GPAC) and is the most pathogenic and one of the most frequently isolated GPAC species found in human clinical specimens. *Peptostreptococcus magnus* is regarded as part of the normal flora of the skin, urogenital tract and prepubertal vaginal flora (Murdoch, 1998) and has been isolated from a wide variety of body sites in pure culture. These include cases of endocarditis (van der Vorm *et al.*, 2000), meningitis (Brown *et al.*, 1994) and pneumonia (Panagou *et al.*, 1991), some of which have been fatal. *Peptostreptococcus magnus* is most commonly associated with infection of skin and soft tissue and bone and joint infections (Bourgault *et al.*, 1980), but has also been isolated from cases of septic arthritis following prosthetic joint replacement (Fitzgerald *et al.*, 1982), prosthetic implant

infections (Davies *et al.*, 1988), breast abscesses (Edmiston *et al.*, 1990), diabetic foot infections (Johnson *et al.*, 1995) and upper respiratory tract infections such as sinusitis and otitis media (Brook, 1994; Murdoch *et al.*, 1994). *Peptostreptococcus magnus* was recently isolated from the subgingival plaque of children with primary dentition (Kamma *et al.*, 2000), and this represents the first report to date of *Peptostreptococcus magnus* isolation from the oral cavity. However, it has been observed that other GPAC species are more commonly found in infections of the central nervous system, abdomen, female genitourinary tract and the mouth (Brook, 1988, 1994; Murdoch *et al.*, 1994).

The identification of *Peptostreptococcus magnus* in clinical samples has been particularly problematic, most notably because of the difficulty in differentiating it from *Peptostreptococcus micros*. This has been attempted primarily by using conventional bacterial culture in combination with biochemical tests. Initial tests used, such as colony morph-

Abbreviation: GPAC, Gram-positive anaerobic coccus.

ology, alkaline phosphatase production and carbohydrate fermentation profiles, have proved inadequate for identification of *Peptostreptococcus magnus* and, indeed, other GPAC species, often giving rise to ambiguous results (Murdoch, 1998). Identification of GPAC species by assessment of their proteolytic activities using pre-formed enzyme kits has been more favourable, differentiating extremely well between *Peptostreptococcus magnus* and *Peptostreptococcus micros* (Murdoch, 1998). Gas-liquid chromatography (GLC) has been used to detect volatile fatty acid production by GPAC species and thus classify them into three groups based on the volatile fatty acids produced (Murdoch, 1998). However, this is a time-consuming and costly procedure that is beyond the capabilities of most diagnostic laboratories.

The aim of the current study was to develop a more reliable, specific and rapid alternative to those methods used previously for identification of *Peptostreptococcus magnus* in clinical specimens. A PCR assay was developed using a primer pair targeting the 16S rRNA gene of *Peptostreptococcus magnus* and used to attempt detection of *Peptostreptococcus magnus* DNA in subgingival plaque samples from adult periodontitis patients and pus aspirates from subjects with acute dento-alveolar abscesses. Although *Peptostreptococcus magnus* has not been found in the oral cavity using traditional, less-sensitive identification methods, PCR could be used to determine more accurately its true prevalence in the oral cavity.

METHODS

Bacterial culture and genomic DNA extraction. *Peptostreptococcus magnus* NCTC 11804^T was cultured on fastidious anaerobe agar (Life Technologies) supplemented with defibrinated horse blood (7.5%, v/v) and incubated at 37 °C for 4–5 days in an anaerobic chamber under an atmosphere of 85% N₂, 10% CO₂ and 5% H₂. Bacteria were harvested from the plates and genomic DNA was extracted using the Puregene DNA isolation kit (Novara Flowgen) in accordance with the manufacturer's instructions.

Sample details. Subgingival plaque was obtained from patients with untreated chronic inflammatory adult periodontitis who had been newly referred to Glasgow Dental Hospital. Criteria for inclusion in the study were the presence of at least three periodontal pockets with a depth of at least 5 mm and bleeding on probing, together with no history of antibiotic treatment during the preceding 6 months. In total, 39 subgingival plaque samples from 10 patients were analysed. The mean pocket depth of analysed samples was 7.0 mm (range 5–9 mm) and the age range of the patients was 28–58 years (mean 42.0 years). One pus sample from each of 60 subjects (age range 32–57 years; mean 43.9 years) was analysed.

Sample collection and preparation. Subgingival plaque samples were collected using a sterile curette into sterile tubes containing 0.5 ml freshly prepared fastidious anaerobe broth (Bioconnections). Samples were mixed for 30 s and lysates were prepared by adding 3 µl achromopeptidase (20 U µl⁻¹ in 10 mM Tris/HCl, 1 mM EDTA, pH 7.0) to 100 µl of plaque sample. Samples were incubated at 56 °C for 30 min, boiled for 5 min and stored at –70 °C until required.

Aliquots (50 µl) of each pus sample were diluted 10- to 100-fold in PCR diluent (10 mM Tris/HCl, pH 8.0, 10 mM NaCl, 1 mM EDTA). Next, 30 µl 10% SDS and 3 µl proteinase K (10 mg ml⁻¹) were added to 300 µl

diluted pus and incubation was carried out at 55 °C for 3 h. Lysed samples were extracted twice with equal volumes of phenol/chloroform (1:1) and once with an equal volume of chloroform. DNA was precipitated by adding 0.1 vol. 3 M sodium acetate, pH 5.3, and 2 vols 100% ethanol, mixing and incubation at –70 °C for 30 min. Precipitated DNA was recovered by centrifugation and the pellet was resuspended in 100 µl sterile molecular-biology-grade water.

Selection of PCR primers. Primers targeting the 16S rRNA gene of *Peptostreptococcus magnus* were developed for use in the PCR assay. This was achieved by alignment of the 16S rRNA gene sequences of *Peptostreptococcus* species and several other oral bacteria, using version 8 of the GCG sequence analysis package (Devereux *et al.*, 1984). Primers with demonstrable 3' sequence specificity for *Peptostreptococcus magnus* were selected from appropriate regions of the *Peptostreptococcus magnus* 16S rRNA gene sequence. The primer sequences selected were Pmag-1 (5'-CGGGNTTTAGTAGACAGAAG-3'; base positions 60–79; N = A, C, G or T) and Pmag-2 (5'-CAGTTTCCAATGCTTTACGG-3'; base positions 612–593). The expected size of the amplification product is 553 bp.

PCR. All PCRs were carried out in a total volume of 50 µl and the PCR mixture was essentially as described previously (Riggio *et al.*, 2000). Each PCR mixture comprised either 5 µl lysed plaque sample/pus extract or 1 µl bacterial genomic DNA and either 45 or 49 µl reaction mixture containing MgCl₂ at the optimum concentration of 1.5 mM. The PCR cycling conditions were 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 60 °C for 1 min and extension at 72 °C for 1 min, with a final extension step at 72 °C for 10 min.

Stringent anti-contamination procedures were employed when carrying out PCR, as described previously (Riggio *et al.*, 2000). Negative and positive controls were included with each batch of samples analysed. The positive control was a standard PCR mixture containing 10 ng *Peptostreptococcus magnus* genomic DNA instead of sample, whereas the negative control contained sterile water instead of sample. PCR products were visualized by electrophoresis on 2% agarose gels as described previously (Riggio *et al.*, 2000).

Restriction endonuclease digestion of PCR products. PCR products were purified using the Wizard PCR purification kit (Promega) in accordance with the manufacturer's instructions. Aliquots containing 0.5 µg purified DNA were digested in a total volume of 20 µl with 5.0 U *RsaI* (Promega) at 37 °C for 3 h. Restriction fragments generated were visualized by agarose gel electrophoresis.

Bacterial species used as PCR controls. Bacterial strains used as controls for PCR were from the *Peptostreptococcus* species *Peptostreptococcus magnus* (NCTC 11804^T, ATCC 29328 and three clinical isolates), *Peptostreptococcus anaerobius* ATCC 27337^T, *Peptostreptococcus micros* ATCC 33270^T, *Peptostreptococcus asaccharolyticus* NCTC 11461^T, *Peptostreptococcus tetradius* ATCC 35098^T, *Peptostreptococcus productus* NCTC 11829^T, *Peptostreptococcus indolicus* NCTC 11088^T and *Peptostreptococcus prevotii* NCTC 11806^T. In addition, the following oral bacteria were also used as PCR controls: *Prevotella intermedia* ATCC 25611^T, *Porphyromonas gingivalis* ATCC 33277^T, *Bacteroides forsythus* ATCC 43037^T, *Helicobacter pylori* ATCC 43504^T, *Actinobacillus actinomycetemcomitans* ATCC 33384^T, *Fusobacterium nucleatum* ATCC 25586^T, *Eikenella corrodens* ATCC 23834^T, *Streptococcus mutans* ATCC 25175^T, *Staphylococcus aureus* ATCC 12600^T and *Actinomyces naeslundii* ATCC 12104^T. With the exception of *Peptostreptococcus magnus* ATCC 29328 and the three clinical isolates of *Peptostreptococcus magnus*, all strains tested were type strains.

RESULTS

Sensitivity and specificity of the *Peptostreptococcus magnus* PCR assay

Following 35 cycles of amplification, 50 *Peptostreptococcus magnus* cells were detectable by PCR (data not shown). The specificity of the PCR assay was confirmed by carrying out PCR under standard conditions using 10 ng genomic DNA from each of the bacterial species selected for use as controls. A PCR product of the expected size (553 bp) was only obtained when DNA from each of the five *Peptostreptococcus magnus* strains tested was used as a template (data not shown), thereby confirming the specificity of the PCR assay for *Peptostreptococcus magnus* DNA.

PCR detection of *Peptostreptococcus magnus* DNA in oral specimens

The *Peptostreptococcus magnus* PCR assay was applied in the attempted detection of *Peptostreptococcus magnus* in oral specimens. Of 33 subgingival plaque samples from patients with adult periodontal disease that were analysed by PCR, 2 (6%) contained *Peptostreptococcus magnus* DNA (Fig. 1). None of 60 pus aspirates from subjects with acute dento-alveolar abscesses was positive for the presence of *Peptostreptococcus magnus* DNA by PCR. Sample inhibition of PCR was discounted, since the spiking of subgingival plaque and pus samples with 1000 whole cells of *Peptostreptococcus magnus* yielded a PCR product of the expected size (data not shown).

In order to discount the possibility of cross-reactivity of the PCR primers with the human genome, PCR was also carried out using genomic DNA purified from primary oral epithelial cells as a template. No PCR product was obtained (data not shown), indicating the lack of cross-reactivity of these primers with human genomic DNA.

Confirmation of PCR product specificity

In order to confirm that successfully amplified products were derived from *Peptostreptococcus magnus*, PCR products were digested with the restriction endonuclease *RsaI*, which gives a restriction profile unique to *Peptostreptococcus magnus* and

therefore allows its distinction from other closely related *Peptostreptococcus* species. Digestion of PCR products obtained from *Peptostreptococcus magnus* gives DNA fragments with predicted sizes of 407, 80 and 66 bp (Fig. 2). All PCR products obtained from clinical specimens gave restriction patterns with *RsaI* that matched that expected for *Peptostreptococcus magnus*.

DISCUSSION

Peptostreptococcus magnus is one of the most common GPAC found in human clinical specimens and is regarded as being the most pathogenic. The purpose of this study was to develop a novel PCR assay for the specific detection of this organism and to apply this assay to its attempted detection in oral specimens. The PCR assay was demonstrated to be highly sensitive and specific for *Peptostreptococcus magnus* DNA. Conventional methods for identification of *Peptostreptococcus magnus* and indeed other members of the genus *Peptostreptococcus* are reliant upon bacterial culture coupled to biochemical identification of clinical isolates. *Peptostrep-*

Fig. 2. Electrophoresis on a 2% agarose gel of 553 bp *Peptostreptococcus magnus* PCR products obtained from subgingival plaque samples following digestion with the restriction enzyme *RsaI*. Lanes: 1 and 3, 100 bp DNA ladder; 2 and 4, PCR products digested with *RsaI*. Confirmation of amplification of *Peptostreptococcus magnus* DNA was observed by the appearance of restriction fragments of 407, 80 and 66 bp.

Fig. 1. Electrophoresis on a 2% agarose gel of PCR products obtained from analysis of subgingival plaque samples for *Peptostreptococcus magnus* DNA. Lanes: 1, 100 bp DNA ladder (fragment sizes 100–1000 bp in 100 bp increments); 2–18, subgingival plaque samples; 19, negative control; 20, positive control. A positive PCR result is indicated by the presence of a 553 bp product.

Peptostreptococcus magnus is commonly identified on the basis of colony morphology and Gram-staining characteristics, followed by biochemical analyses, such as carbohydrate fermentation tests, alkaline phosphatase production and detection of bacterially encoded proteolytic enzyme activities (Murdoch, 1998). The most useful of these tests for identification of *Peptostreptococcus magnus* are those that analyse bacterial proteolytic enzyme profiles using pre-formed kits such as RapID ANA II (Marler *et al.*, 1991), Rapid ID32A (Ng *et al.*, 1994) and ATB 32A (Murdoch & Mitchelmore, 1991).

More recently, the use of GLC to detect the production of volatile fatty acid by-products of metabolism has been attempted as a means of identification of GPAC species (Murdoch & Mitchelmore, 1991; Murdoch, 1998; Wilson *et al.*, 2000). However, this method alone is not sufficient to identify *Peptostreptococcus magnus*, since *Peptostreptococcus magnus*, *Peptostreptococcus micros* and *Peptostreptococcus heliotrinreducens* all produce acetic acid alone as their terminal volatile fatty acid. It has been concluded that a combination of colony morphology, proteolytic enzyme profiles and Gram-stained cell morphology to assess cell size can be used to differentiate adequately between *Peptostreptococcus magnus* and *Peptostreptococcus micros*, thereby eradicating the need for GLC (Murdoch, 1998).

Whilst these methods have been of significant value, their usefulness is limited by the fact that the results obtained are often ambiguous, primarily because of the emergence of phenotypically atypical strains that may possess different biochemical characteristics. In addition, 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 inexpensive method of bacterial identification.

An oligonucleotide 16S rRNA probe has been described for *Peptostreptococcus magnus*, but was also shown to cross-react with *Peptostreptococcus barnesae* and *Peptostreptococcus prevotii* DNA (Conrads *et al.*, 1997). Hence, this probe could not be used for reliable detection of *Peptostreptococcus magnus* in clinical samples. In the present study, we designed a pair of PCR primers based upon the 16S rRNA gene sequence of *Peptostreptococcus magnus* that were specific for the DNA of this organism. The PCR assay was used to attempt detection of *Peptostreptococcus magnus* DNA in subgingival plaque samples from adult periodontitis patients and in pus aspirates from subjects with acute dento-alveolar abscesses. *Peptostreptococcus magnus* DNA was detected in only 2 (6%) of 33 subgingival plaque samples and in none of 60 pus aspirates analysed. This low incidence of *Peptostreptococcus magnus* in the oral cavity is perhaps not surprising, since previous studies using conventional culture-based methods have not identified *Peptostreptococcus magnus* in the oral cavity on any occasion. However, *Peptostreptococcus anaerobius* and, in particular, *Peptostreptococcus micros* have been identified in the oral cavity. Culture methods have found

Peptostreptococcus micros at elevated levels in a high proportion (58–91%) of subgingival plaque samples from patients with periodontitis (Rams *et al.*, 1992; van Dalen *et al.*, 1998). The association of *Peptostreptococcus anaerobius* with the oral cavity is less striking, although it has been associated with gingivitis and periodontitis (Moore *et al.*, 1987; Wade *et al.*, 1992) and is one of the most frequently found species in the root canals of teeth with periapical periodontitis (Sundqvist, 1992). We have previously used PCR to detect *Peptostreptococcus micros* DNA in the subgingival plaque of 11 (61%) of 18 adult periodontitis patients and in pus aspirates from acute dento-alveolar abscesses of 20 (71%) of 28 subjects (Riggio *et al.*, 2001). The evidence to date clearly suggests that *Peptostreptococcus magnus* is rarely found in the oral cavity and, to our knowledge, the present study is the first to use PCR to detect this organism, albeit at low prevalence, in the oral cavity.

The classification of members of the genus *Peptostreptococcus* is currently the subject of intense review. Several studies have suggested that members of the genus *Peptostreptococcus* should be removed from this genus and reclassified. On the basis of biochemical analysis and 16S rRNA sequence data, it has been proposed that *Peptostreptococcus magnus* be reclassified in the new genus *Finegoldia* as *Finegoldia magna* and that *Peptostreptococcus micros* be reclassified in the new genus 'Micromonas' as 'Micromonas micros' (Murdoch & Shah, 1999) (though the name 'Micromonas micros' is illegitimate because of the pre-existence of the fungal genus *Micromonas*). It has also been proposed that, on the basis of 16S rRNA sequence data, *Peptostreptococcus productus* should be placed in the genus *Ruminococcus* (Ezaki *et al.*, 1994). Three other new genera have been proposed for members of the genus *Peptostreptococcus*, namely *Peptoniphilus* (which includes *Peptostreptococcus asaccharolyticus*, *Peptostreptococcus indolicus* and three other species), *Anaerococcus* (which includes *Peptostreptococcus prevotii* and five other species) and *Gallipicola* (which includes only *Peptostreptococcus barnesae*) (Ezaki *et al.*, 2001). *Peptostreptococcus anaerobius* should be the only remaining member of the genus *Peptostreptococcus* (Murdoch *et al.*, 2000).

In conclusion, we have developed and applied a novel PCR assay for the specific and direct detection of *Peptostreptococcus magnus* DNA in clinical specimens. This PCR assay was used to assess the prevalence of *Peptostreptococcus magnus* in subgingival plaque from adult periodontitis patients and in pus aspirates from subjects with acute dento-alveolar abscesses. *Peptostreptococcus magnus* was only detected at very low prevalence in subgingival plaque and was absent in the pus aspirates analysed, which suggests that this organism is not a putative pathogen in these lesions. The use of this PCR assay is advocated as a rapid, more-reliable alternative to conventional culture and biochemical methods for identification of *Peptostreptococcus magnus* in clinical specimens.

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