Development of a novel PCR assay for detection of *Prevotella oris* in clinical specimens

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**Abstract**

*Prevotella oris* is a nonpigmented, Gram-negative, anaerobic bacterium that has been associated with several serious oral and systemic infections. *Prevotella oris* has been identified in clinical specimens by bacterial culture and biochemical tests, which are generally unreliable. The aim of this study was to develop a PCR assay for the direct detection of *P. oris* in clinical specimens. PCR primers specific for *P. oris* were identified by alignment of bacterial 16S rRNA genes from closely related species and selection of PCR primers specific for *P. oris* at their 3' ends. Amplification of a 1110-bp product indicated PCR positivity for *P. oris*. The primers were shown to be specific for *P. oris* DNA, because no PCR products were obtained when DNA from other oral bacteria, including closely related *Prevotella* species, were used as test species, and this was confirmed by digestion of PCR products with RsI and MnI. *Prevotella oris* DNA was detected in 17 (36.2%) of 47 pus samples from subjects with dentoalveolar abscesses and in all three pus samples from subjects with spreading odontogenic infections. This PCR assay provides a sensitive, specific and reliable method for identifying *P. oris* in clinical specimens.

**Introduction**

Many members of the *Prevotella* genus are black-pigmented anaerobic species that comprise a major proportion of the human oral microbial communities and have been implicated in oral disease. *Prevotella intermedia* is strongly associated with acute necrotizing ulcerative gingivitis (Loesche et al., 1982), pregnancy gingivitis (Kornman & Loesche, 1980) and adult periodontitis (Slots et al., 1986; Slots & Listgarten, 1988). However, *P. intermedia* and the closely related species *Prevotella nigrescens* are also present in the oral cavity of periodontally healthy subjects (Dahlin et al., 1992; König, 1993). *Prevotella intermedia*, *P. nigrescens* and *Prevotella tannerae* have also been identified in endodontic infections (Xia et al., 2000; Gomes et al., 2005).

Nonpigmented species of the *Prevotella* genus include *Prevotella oris*, *Prevotella oralis*, *Prevotella veroralis*, *Prevotella buccalis*, *Prevotella buccae*, *Prevotella zoogloeformans*, *Prevotella bivia*, *Prevotella disiens* and *Prevotella oulora* (Yousefimashouf et al., 1993). *Prevotella oris* is known to be associated with several important oral and systemic infections. These include dentoalveolar abscesses (Dymock et al., 1996), epidural spinal abscesses (Frat et al., 2004), chronic otitis media and brain abscesses (Marina et al., 1997), infected tonsils (Rajasuo et al., 1996) and anaerobic empyema (Civen et al., 1995). *Prevotella oris* has also been associated with bacteraemia in a patient with extensive tooth decay (Bein et al., 2003).

PCR-based methods are increasingly being used in tandem with, or even to replace, conventional microbiological culture for the identification of bacteria within clinical specimens (Song, 2005). However, no such methods have been described to date for the direct identification of *P. oris* within clinical specimens. The purpose of this study, therefore, was to develop a novel PCR assay that could specifically detect *P. oris* DNA within clinical specimens. The PCR method was then applied to the identification of *P. oris* DNA within pus specimens from subjects with dentoalveolar abscesses and spreading odontogenic infections.
Materials and methods

Culture of bacterial strains and extraction of genomic DNA

The *Prevotella* reference strains used in this study were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ; Braunschweig, Germany) and the American Type Culture Collection (ATCC; LGC Promocochem, Teddington, UK). Bacteria were cultured on fastidious anaerobe agar plates (Life Technologies, Paisley) supplemented with 7.5% v/v defibrinated horse blood and incubated at 37 °C for 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 extracted with the Puregene DNA Isolation Kit (Novara Flowgen, Ashby de la Zouch).

Collection of pus samples

Before sampling, the oral mucosa was disinfected with a solution of 0.2% w/v chlorhexidine gluconate. Sampling was accomplished intraorally through intact oral mucosa, using a sterile, disposable 5 mL syringe through a 21G needle.

DNA extraction from pus samples

Fifty microliters of each pus sample was diluted 10–100-fold in PCR diluent (10 mM Tris-HCl, pH 8.0, 10 mM NaCl, 1 mM EDTA). Thirty microliters of 10% sodium dodecyl-sulfate and 3 μL proteinase K (10 mg mL⁻¹ stock) were added to 300 μL of diluted pus and incubation was carried out at 55 °C for 3 h. Samples were extracted twice with an equal volume of phenol:chloroform (1:1) and once with an equal volume of chloroform. DNA was precipitated by adding 0.1 volume of 3 M sodium acetate, pH 5.3, and 2 volumes of 100% ethanol, mixing and incubation at −70 °C for 30 min. Precipitated DNA was recovered by centrifugation and the pellet suspended in 100 μL of sterile molecular biology grade water.

Selection of PCR primers

Sequence alignment of the 16S rRNA genes of members of the *Prevotella* genus and several other oral bacteria permitted the identification of primers specific for *P. oris*. The primers selected that demonstrated absolute specificity for *P. oris* at their 3’ ends were 5’-CGG CCT AAT ACC TCA TGG CA-3’ (PO1; 169–188 nt) and 5’-AGA TCG TGT TTG CAC AAA AT-3’ (PO2; 1278–1259 nt), which yield an amplification product of 1110 bp.

PCR

All PCR reactions were carried out in a total volume of 50 μL, which comprised bacterial DNA template and 45 μL reaction mixture containing 1 × PCR buffer (10 mM Tris-HCl pH 9.0, 50 mM KCl, 1.5 mM MgCl₂, 0.1% Triton X-100), 1.0 U Taq DNA polymerase (Promega, Southampton), 0.2 mM dNTPs and each primer at a concentration of 0.2 μM. Reactions were carried out in a Hybaid OmniGene thermal cycler. After an initial denaturation step at 94 °C for 5 min, 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1.5 min were carried out, followed by a final extension step at 72 °C for 10 min.

PCR anticontamination procedures

In order to avoid contamination, PCR was carried out under stringent conditions as described previously (Riggio et al., 2000). Each batch of samples being analyzed contained both negative and positive controls. A standard PCR reaction, which contained water instead of sample, was included as a negative control, while the positive control contained 10 ng of *P. oris* genomic DNA instead of sample.

Visualization of PCR products

PCR products were electrophoresed on 2% agarose gels, stained with ethidium bromide (0.5 μg mL⁻¹) and visualized with an ImageMaster® Video Documentation System.

Bacterial strains used as PCR controls

Bacterial cells (100 and 1000) from the following *Prevotella* strains were used as controls when testing the specificity of the *P. oris* primers: *P. oris* ATCC 33573 T, *P. intermedia* ATCC 25611 T, *P. nigrescens* ATCC 35363 T, *Prevotella dentalis* ATCC 49559 T, *Prevotella melaninogenica* ATCC 25845 T, *Prevotella shahii* DSM 15611 T, *Prevotella multiformis* DSM 16608 T, *Prevotella baronae* DSM 16972 T, *Prevotella denticola* ATCC 35308 T, *P. buccalis* ATCC 20615, *P. buccalis* ATCC 35310 T, *P. oralis* ATCC 33269 T, *Prevotella salivae* DSM 15606 T, *Prevotella marshii* DSM 16973 T. In addition, cells (100 and 1000) of the following oral bacteria were also used as controls: *Helicobacter pylori* ATCC 43504 T, *Escherichia coli* NCTC 10418; *Peptostreptococcus anaerobius* ATCC 11460 T; *Tannarella forsythensis* ATCC 43037 T, *Streptococcus mitis* NCTC 12261; *Streptococcus intermedius* NCTC 11324 T; *Actinobacillus actinomyctematum* ATCC 33843 T, *Fusobacterium nucleatum* ATCC 25586 T, *Actinomyces naeslundii* ATCC 12104 T, *Staphylococcus aureus* ATCC 12600 T.
Restriction enzyme digestion of PCR products

PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Crawley) in accordance with the manufacturer's instructions. Approximately 0.5 μg of each purified PCR product was digested in a total volume of 20 μL with 2.0 U RsaI (Promega) and MnlI (Helena Biosciences, Sunderland) at 37 °C for 3 h. The restriction fragments generated were visualized by agarose gel electrophoresis.

Results

Sensitivity and specificity of the P. oris PCR assay

Following 35 cycles of amplification, c. 50 P. oris cells were detectable by PCR (data not shown). To confirm the specificity of the PCR assay, PCR was carried out under standard conditions using 100 and 1000 cells from each of the bacterial strains selected for use as controls. A PCR product of the expected size (1110 bp) was only obtained when P. oris ATCC 33573T was used as a template (data not shown). This confirmed the absolute specificity of the PCR assay for P. oris DNA.

Detection of P. oris DNA in oral specimens by PCR

Prevotella oris DNA was detected in 17 (36.2%) of 47 pus samples from subjects with dentoalveolar abscesses. All three pus samples from subjects with spreading odontogenic infections, which were analyzed for the presence of P. oris DNA, were positive for this species. Some of the positive samples obtained by PCR are shown in Fig. 1.

Given the low prevalence of P. oris within the pus samples analyzed, the 30 pus samples shown to be negative for P. oris by PCR were spiked with 100 and 1000 P. oris cells. Prevotella oris DNA was detected in all spiked samples by PCR. Typical results obtained are shown in Fig. 2. This confirmed that there was no inhibition of the PCR assay and that the PCR-negative samples truly did not contain P. oris.

Confirmation of P. oris PCR product specificity

In order to confirm that the successfully amplified products obtained were indeed from P. oris, each PCR product was digested with the restriction enzymes RsaI and MnlI. These enzymes produce a restriction profile unique for P. oris, which permits its discrimination from other bacterial species, including closely related Prevotella species. Digestion of PCR products obtained from P. oris yielded DNA fragments (only sizes of at least 100 bp are indicated) of 403, 387 and 320 bp for RsaI, and fragments of 478, 127, 105 and 100 bp for MnlI (Fig. 3). All 20 PCR products obtained from the clinical specimens examined gave restriction patterns with both RsaI and MnlI that matched those expected for P. oris.

Discussion

The purpose of this study was to develop a rapid PCR-based method that could specifically detect P. oris DNA in clinical
specimens. The PCR assay was shown to be both specific and highly sensitive, and its applicability in detecting P. oris DNA directly in pus aspirates from subjects with dentoalveolar abscesses and spreading odontogenic infections was also demonstrated. Confirmation of amplification of the correct product was obtained by digestion of PCR products with the restriction enzymes Rsal and MnlI, which yielded restriction patterns unique for P. oris and could therefore distinguish P. oris from closely related Prevotella species. The P. oris-specific PCR primers used in this study would be suitable for use in a quantitative real-time PCR assay for P. oris, which would be highly desirable so that relative levels of this organism in clinical specimens could be more accurately assessed.

Previously, P. oris has been identified in clinical specimens using conventional microbiological culture methods. This requires isolation of the organism by culture, followed by identification on the basis of colony morphology, Gram staining characteristics and biochemical analyses, usually using the Rapid ID 32 A system, which analyzes bacterial sugar fermentation characteristics and enzymatic activities. Although such methods for identification of bacteria have undoubtedly been of considerable use, ambiguous results are often obtained due to the emergence of phenotypically variable strains with altered biochemical characteristics. Other considerations are the lengthy processes involved and the high overall costs of carrying out such tests. Because PCR can unequivocally detect phenotypically variable strains, it can be considered as a more specific and reliable method for bacterial identification. Furthermore, PCR is a rapid and relatively inexpensive identification method. The pus samples analyzed by PCR in this study were archival, having previously been analyzed in a routine diagnostic microbiology laboratory using microbiological culture methods. This approach identified Prevotella species in only two of the 50 samples analyzed, none of which were P. oris.

Because P. oris was detected in a total of 20 pus samples in the current study, this confirms the superiority of PCR over conventional culture methods for detecting this species in clinical specimens. The improved detection rate of PCR compared with culture is undoubtedly due primarily to the differing sensitivities of the methods; PCR can detect as few as 50 P. oris cells, whereas culture methods detected no fewer than 10^5 cells.

Prevotella oris, formerly known as Bacteroides oris, was first identified as a potential human pathogen in 1982, when it was associated with cases of human periodontitis (Hodeman et al., 1982). Following subsequent taxonomic reclassification, P. oris was later assigned as a member of the Prevotella genus (Shah & Collins, 1990). Phylogenetic analysis of 16S rRNA genes has demonstrated that P. oris is most closely related (94% similarity) to P. salivae EPSA1^T (Sakamoto et al., 2004), a relatively new member of the Prevotella genus that was first isolated from the saliva of a patient with chronic periodontitis (Sakamoto et al., 2002). Prevotella salivae was one of the species against which the P. oris PCR primers were tested, resulting in a negative outcome. This further confirms the specificity of the P. oris PCR assay. Indeed, alignment of the region of the P. oris 16S rRNA gene to which the PCR primer sequences bind with the corresponding region of the P. salivae 16S rRNA gene confirmed that the P. oris primers are both unique at their 3′ ends and would therefore not be expected to detect P. salivae.

In the current study, P. oris DNA was detected by PCR in 17 (36.2%) of 47 pus specimens obtained from subjects with dentoalveolar abscesses and in all three pus specimens from subjects with spreading odontogenic infections. Prevotella oris DNA has previously been detected by both culture and 16S rRNA gene sequencing methods in dentoalveolar abscesses (Dymock et al., 1996). However, this study is the first to use a direct PCR approach for the specific detection of P. oris in clinical specimens.

The finding that P. oris is associated with dentoalveolar abscesses supports the findings of the authors’ previous study investigating the bacteria associated with spreading odontogenic infections (Riggio et al., 2007). In that study, four pus specimens from subjects with spreading odontogenic infections were analyzed for the presence of bacteria using a 16S rRNA gene gene cloning and sequencing approach. Species from the Prevotella genus accounted for half of the clones identified. More specifically, P. oris was the most abundant species identified, accounting for 22.2% of the clones analyzed, and was present in all four specimens analyzed. Three of these four specimens were used in the P. oris-specific PCR assay in the current study, and all were positive for the presence of P. oris. Thus it is apparent that P. oris may be an etiological agent in both localized and spreading odontogenic infections. However, the precise role...
of *P. oris* in the exacerbation of acute odontogenic infection is unknown. Further research is necessary to establish the virulence factors responsible for the pathogenicity of this species and its relationship with host immune responses.

While *P. oris* has been associated with several oral and systemic infections, its relative pathogenic importance in dental infections is still not clearly understood. This is due to the fact that *P. oris* remains a relatively understudied member of the *Prevotella* genus. Similarly, while many oral *Prevotella* species are known to be associated with microbial resistance through the production of β-lactamases (Bernal et al., 1998; Eick et al., 1999; van Winkelhoff et al., 2005), relatively little information is available in this respect with regard to *P. oris*. However, a recent study demonstrated that five of 12 *P. oris* isolates from dentolaveolar infections produced β-lactamase (Iwahara et al., 2006). The finding that *P. oris* exhibits antimicrobial resistance lends further support to the use of this PCR assay for the detection of *P. oris*, particularly in terms of improving patient management.

In conclusion, a novel PCR assay has been developed and applied for the specific and direct detection of *P. oris* DNA in clinical samples. PCR detection of *P. oris* DNA in pus specimens from subjects with acute dentolaveolar abscesses and spreading odontogenic infections suggests that *P. oris* may be a putative pathogen in these infections. The use of this PCR assay as a rapid, more reliable alternative to conventional culture and biochemical methods for the identification of *P. oris* in clinical specimens can be proposed.

### References


