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Direct detection of *Prevotella intermedia* and *P. nigrescens* in suppurative oral infection by amplification of 16S rRNA gene

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A specific 16S rDNA PCR and subsequent hybridisation reaction was designed to discriminate between strains of *Prevotella intermedia* (n = 15) and *P. nigrescens* (n = 15). This technique was then used to detect the presence of these two bacterial species in acute suppurative oral infection. A total of 36 pus samples aspirated from 26 peri-apical abscesses, three root canals, three periodontal abscesses, two cases of refractory periodontitis, one cyst and one haematoma was examined. A portion of the pus sample was processed by PCR and the remainder of the specimen was subjected to routine culture. The PCR-based technique gave an identical pattern of detection of *P. intermedia* or *P. nigrescens* to that obtained by culture for 30 of the 36 specimens. Either *P. intermedia* or *P. nigrescens* was present in 14 samples and neither species was detected in 16 samples. In the remaining six samples the PCR method indicated the presence of one (n = 3) or both (n = 3) of the *Prevotella* species but neither or only one species was isolated by culture. It is concluded that the presence of *P. intermedia* and *P. nigrescens* in pus can be detected rapidly and specifically by direct PCR amplification of 16S rDNA. *P. nigrescens* was detected more frequently than *P. intermedia* in suppurative peri-apical infection both by culture and PCR.

Introduction

Pigmented species of *Prevotella* are frequently isolated from various oral and extra-oral infections [1, 2]. Many such infections are suppurative, encompassing infected wounds and abscesses of the head, neck, thorax and abdomen [2]. *P. intermedia* and *P. nigrescens* have been the pigmenting anaerobes encountered most frequently in pleuropulmonary infections, gangrenous or perforative appendicitis and suppurative dental infection [1–5]. Furthermore, studies have reported the isolation of *P. intermedia* from active periodontal lesions and dentoalveolar abscesses, whereas *P. nigrescens* seems to be associated with endodontic infection and gingival sites in both health and disease [2, 6–9].

Conventional biochemical tests do not discriminate between *P. intermedia* and *P. nigrescens* reliably, although these species can be differentiated by cellular protein profiles [7], malate and glutamate dehydrogen-

ase electrophoretic mobility [10, 11], PCR-restriction fragment length polymorphism (PCR-RFLP) [9], oligonucleotide probes [11, 12], 16S rDNA sequencing [13], whole-cell DNA hybridisation [11], surface antigenicity [14] and ribotyping [15]. However, these techniques have several disadvantages, including expense, subjective interpretation of results, requirement for culture, lengthy process time, lack of sensitivity or the need for complex equipment [11, 12, 14, 16–18]. Conventional microbiological processing of pus from oral infection requires prolonged primary anaerobic incubation for up to 7 days followed by subculture. Therefore, microbiological reports for acute dental infections often have little impact on clinical management, because of the late availability of culture results.

Traditionally, members of the penicillin group of antibiotics have been regarded as the agents of choice in the treatment of suppurative oral infection, because the majority of isolates from such infections have been found to be sensitive to these antimicrobial agents. However, the incidence of resistance to penicillin appears to be increasing. A multicentre study reported that one or more penicillin-resistant isolates were isolated from pus samples obtained from 43 (55%) of

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78 patients with acute suppurative oral infections. Resistance was encountered most frequently among *Prevotella* spp., with 41 (61%) of 67 isolates exhibiting resistance to penicillin [19]. To date, the presence of penicillin-resistant strains within the mixed flora of acute suppurative dental infection has rarely produced a clinical problem. However, an increased incidence of resistance in *Prevotella* spp. may have implications for patient management in the future, as these micro-organisms have been shown to be the major pathogens in the mixed flora of acute dentoalveolar abscess [20].

A method for detecting the presence of certain *Prevotella* spp. and, therefore, the possibility of penicillin resistance, may be helpful, particularly in cases where the patient fails to respond within the first 48 h of initial therapy. Two recent studies have reported the detection and differentiation of *P. intermedia* and *P. nigrescens* in dental plaque with the 16S rRNA gene as a target for PCR [8, 17]. However, this approach has not been applied to pus from acute suppurative oral infections. Therefore, the aim of the present study was to develop a novel single-step PCR reaction, targeted to the 16S rRNA gene, to allow rapid detection of *P. intermedia* and *P. nigrescens* in suppurative oral infections.

Materials and methods

Bacterial culture and identification of test strains

Bacteria were maintained on Fastidious Anaerobe Agar (FAA; Lab M, Amersham) supplemented with sheep blood 5% v/v in an anaerobic atmosphere (CO₂ 10%, H₂ 10%, N₂ 80%) at 37°C. The identity of strains of *P. intermedia* and *P. nigrescens* (Table 1), isolated from various oral and non-oral sites, had been confirmed previously by biochemical analysis, electrophoretic enzyme analysis, 16S rDNA sequencing, SDS-PAGE protein profiling and 16S rDNA PCR-RFLP [9–11, 13, 21]. Further strains were analysed to detect possible cross-reactivity in the PCR assay. These included: *P. corporis* R8579 (facial wound), *P. oris* ATCC 33573^T, *P. denticola* R9102 (oral isolate), *P. loescheii* NCTC 11321^T, *Porphyromonas gingivalis* W50 (periodontal pocket; H. Shah, Eastman Dental School, London), *Por. levii* NCTC 11028^T, *Por. endodontalis* R8754 (oral abscess), *Por. asaccharolytica* NCTC 9337 (haemorrhoid) and R9170 (gangrenous scrotum), *Fusobacterium nucleatum* C81496 and C2796 (dental abscess), *Eubacterium timidum* ATCC 33093^T, *Propionibacterium acnes* NCTC 737^T, *Peptostreptococcus anaerobius* NCTC 11460^T, *Streptococcus constellatus* C27496 and C22897 (dental abscess), *S. anginosus* C50095 and C38397 (dental abscess), *S. intermedius* C4896, *Actinomyces israelii* C886 (dental plaque), C2796 and C38297 (dental abscess), *S. agalactiae* C22297 (dental abscess), *Eikenella corrodens* C22397 (oral isolate), *Pseudomonas aeruginosa* C29197 (dental abscess) and *Candida albicans* C22297

Table 1. Identity and origin of isolates of *P. intermedia* and *P. nigrescens* used to validate PCR and hybridisation reactions

Species	Isolate no.	Origin
<i>P. intermedia</i>	ATCC 25611 ^T	Empyema
	OMZ 311	Periodontal pocket
	OMZ 326	Transtracheal aspirate
	SF 10254	Empyema
	BH 20-30	Supragingival plaque
	DAL 100	Dental plaque
	R 15	Peri-apical abscess
	R 22	Peri-apical abscess
	R 78	Peri-apical abscess
	C 66395	Peri-apical abscess
	C 22996	Peri-apical abscess
	C 56696	Peri-apical abscess
	C 78596	Infected salivary gland
	C 10997	Rapidly progressive periodontitis
	C 16697	Periodontal pocket
<i>P. nigrescens</i>	ATCC 33563 ^T	Vincent's gingivitis
	DAL 10	Dental plaque
	T 588	Dental plaque
	SF 10256	Transtracheal aspirate
	BH 18-23	Periodontal pocket
	MUI 32	Rapidly progressive periodontitis
	R 20	Peri-apical abscess
	R 35	Peri-apical abscess
	R 53	Peri-apical abscess
	K 65	Peri-apical abscess
	K 79	Peri-apical abscess
	R 119	Peri-apical abscess
	C 6996	Peri-apical abscess
	C 7796	Peri-apical abscess
	C 76396	Peri-apical abscess

(oral isolate). Strains designated R were obtained from the Anaerobe Reference Unit, Cardiff and those designated C were from the Oral Microbiology Department, Dental School, Cardiff. All strains were identified according to methods documented in the VPI manual [22] and subsequent updates.

Nucleic acid preparation

Crude template nucleic acid was prepared by boiling 1 volume of bacterial cells (harvested after 40-h culture on FAA) in 10 volumes of a 5% w/v solution of Chelex-100 (BioRad, Hemel Hempstead) for 10 min [23]. The solution was centrifuged (12 000 g, 15 min) and 5 µl of supernate were assayed by PCR. The suitability of the nucleic acid extract for PCR was assessed with the universal eubacterial 16S rDNA primers pA (5' AGAGTTTGATCCTGGCTCAG; 8–27, *Escherichia coli* numbering) and gamma (5' ACT-GCTGCCTCCCGTAGGAG; 358–339), as described previously [24].

16S rDNA specific PCR

Two pairs of diagnostic PCR primers were designed to the respective 16S rRNA genes of *P. intermedia* (Pit1, 5' CAAAGATTCATCGGTGGA; 245–262; Pit2, 5' GCCGGTCCTTATTTCGAAG; 551–534) and *P. nigrescens* (Png1, 5' CAAAGGTTTTCCGGTAAG; 245–

262: Png2, 5' GCCGGTCCTTATTCATGA; 551–534), with significant 3' sequence mismatch to non-homologous templates. Cross-reactivity of primers and probes with 16S rRNA genes from related species was assessed by multiple alignment, and reduced further by a BlastN homology search (Internet address: [Hp://www.dispatch1.nlm.nih.gov](http://www.dispatch1.nlm.nih.gov)) of the EMBL database. Nucleic acid extract was added to a 50- μ l PCR reaction mixture (10 mM Tris-HCl, pH 8.3; 1.5 mM $MgCl_2$; 50 mM KCl; gelatin 0.01%; 125 μ M each dNTP; 1 U Taq DNA polymerase, Promega, Southampton) containing either the *P. intermedia* or *P. nigrescens* specific primer pair (0.18 μ M). Reaction mixtures were denatured for 3 min at 94°C, and subjected to 30 cycles of denaturation at 94°C for 45 s, annealing at 50°C for 1 min and polymerisation at 72°C for 1 min. Amplification products were resolved by agarose (2% w/v) gel electrophoresis in TAE buffer (40 mM Tris-acetate buffer, 1 mM EDTA, pH 8.0) and visualised by washing for 20 min in a solution of ethidium bromide 0.5 μ g/ml. The sensitivity of the reaction was determined by amplification of serially diluted genomic DNA (range 5 fg–500 ng) that had been prepared from the respective type strain of each species by the method of Pitcher *et al.* [25] and quantified by absorbance measurement at 260 nm (GeneQuant; Pharmacia, Amersham). In addition, PCR products generated from the type strains of *P. intermedia* and *P. nigrescens* were cleaned with QIAquick-spin PCR clean-up columns (Qiagen, Crawley, West Sussex) and product identity and size were determined by sequencing with the ABI-PRISM Dye Terminator Cycle Sequencing kit (Perkin-Elmer, Warrington).

Southern blotting and hybridisation

The identity of all PCR products was confirmed by oligonucleotide hybridisation with internal 5' biotinylated probes Pit3 (5' CTTTGTGGGGAGTA; 479–494) and Png3 (5' CTTTATGTGGAATAAA; 479–496) specific for the 16S rRNA genes of *P. intermedia* and *P. nigrescens*, respectively. DNA transfer, hybridisation, washing and visualisation were performed with the Sure Blot[®] Blue Southern kit according to the manufacturer's instructions (Oncor, Gaithersburg, MD, USA). A minor modification to the standard protocol involved removing non-specifically bound probe by washing the membrane three times in 2 $\times$ SSC at room temperature for 15 min, followed by two stringency washes in 2 $\times$ SSC at 42°C for 15 min.

Processing of clinical samples

Pus specimens aspirated from peri-apical abscesses (n = 26), infected root canals (3), infected haematoma (1), infected cyst (1) and periodontal abscesses (3), and plaque samples from two patients with rapidly progressive periodontitis were examined. Duplicate 10- μ l volumes of sample were added directly to 90 μ l of Chelex-100 5% and nucleic acid was extracted and

analysed as above. The remainder of the specimen was processed routinely with traditional culture techniques. Briefly, samples were inoculated on to FAA-blood media, incubated in an anaerobic atmosphere and examined for growth every 24 h over a 7-day period [3]. Six black-pigmented colonies were selected from each primary plate and subcultured for purity. Isolates were identified as either *P. intermedia* or *P. nigrescens* by enzyme electrophoresis [10, 21] and by direct PCR as described above.

Results

Validation of PCR method

Results of PCR reactions with primer pairs specific for *P. intermedia* and *P. nigrescens* are shown in Fig. 1a and c, respectively. PCR with the *P. intermedia* primer pair produced an amplification product (size 296 bp) with all 15 strains of *P. intermedia* isolated from various oral and non-oral sites but not with strains of *P. nigrescens* or other similar organisms. *P. intermedia* SF 10254 (a strain producing an atypical cellular protein profile) and a highly proteolytic strain, *P. intermedia* R78 [21], also yielded the specific amplification product. All 15 strains of *P. nigrescens* produced a detectable product (size 291 bp) with the primer pair for this species and no cross-reactivity was observed with strains of *P. intermedia* or similar taxa. The identity of all PCR products was confirmed by specific oligonucleotide hybridisation (Fig. 1b and d, respectively). The sensitivity of the *P. intermedia* and the *P. nigrescens* specific PCR was 5 pg of genomic DNA and sensitivity was increased 10-fold (to 500 fg) with oligonucleotide hybridisation.

Analysis of clinical samples by culture and PCR

Routine culture yielded three isolates of *P. intermedia* and 12 isolates of *P. nigrescens* from 15 samples (Table 2). Chelex-extracted nucleic acid from clinical pus samples provided template suitable for amplification allowing direct detection and discrimination of *P. intermedia* and *P. nigrescens* (Table 2); six samples were PCR-positive for *P. intermedia* and 16 for *P. nigrescens*.

PCR (with subsequent hybridisation) and traditional culture were in agreement for 30 of the 36 pus specimens examined. Discrepancies between the methods occurred in six samples; three yielded PCR product indicating the presence of *P. intermedia* or *P. nigrescens*, but neither species was isolated by culture, and three yielded products indicating the presence of both species, whereas culture yielded only one or neither species. All samples that yielded *P. intermedia* or *P. nigrescens* in culture also gave positive results in the PCR.

P. nigrescens was detected more frequently than

Fig. 1. Amplification products obtained with (a) *P. intermedia* specific primers, Pit1 and Pit2, and (c) *P. nigrescens* specific primers, Png1 and Png2. Confirmation of PCR product identity by hybridisation with (b) *P. intermedia* specific biotinylated probe Pit3, and (d) *P. nigrescens* specific biotinylated probe Png3. Lanes: L, 1-kb marker (Gibco BRL, Paisley); 1–8, strains of *P. intermedia*; 9–20, strains of *P. nigrescens*; 21, *P. corporis*; 22, *P. oris*; 23, *P. denticola*; 24, *P. loescheii*; 25, *Por. gingivalis*; 26, *Por. levii*; 27, *Por. endodontalis*; 28 and 29, *Por. asaccharolytica*; 30, water.

Table 2. Identity of *P. intermedia* and *P. nigrescens* detected by culture and PCR in 36 suppurative oral infections

Sample no.	Infection	Presence of <i>Prevotella</i>	
		Culture	PCR
1074/96	Peri-apical abscess	Pi	Pi
182/97	Periodontal abscess	Pn	Pn
183/97	Peri-apical abscess	Pi	Pi
228/97	Root canal	—	—
236/97	Peri-apical abscess	—	Pn
285/97	Peri-apical abscess	—	—
286/97	Root canal	Pn	Pn
289/97	Peri-apical abscess	Pn	Pn
303/97	Peri-apical abscess	—	—
307/97	Peri-apical abscess	Pn	Pn
330/97	Periodontal abscess	—	Pn
355/97	Peri-apical abscess	Pn	Pn
356/97	Dental cyst	—	—
361/97	Root canal	—	—
366/97	Haematoma	—	—
376/97	Peri-apical abscess	—	Pi
382/97	Peri-apical abscess	—	—
383/97	Peri-apical abscess	Pn	Pn
399/97	Peri-apical abscess	Pn	Pn
408/97	Peri-apical abscess	Pn	Pn
423/97	Peri-apical abscess	—	—
437/97	Periodontal abscess	—	—
480/97	Peri-apical abscess	Pn	Pn
481/97	Peri-apical abscess	Pn	Pn
499/97	Peri-apical abscess	—	—
504/97	Refractory periodontitis	Pi	Pi + Pn
513/97	Peri-apical abscess	—	—
515/97	Peri-apical abscess	—	Pi + Pn
534/97	Refractory periodontitis	Pn	Pn
545/97	Peri-apical abscess	—	—
547/97	Peri-apical abscess	—	—
548/97	Peri-apical abscess	—	—
549/97	Peri-apical abscess	—	—
554/97	Peri-apical abscess	Pn	Pi + Pn
558/97	Peri-apical abscess	—	—
566/97	Peri-apical abscess	—	—

Pi, *P. intermedia*; Pn, *P. nigrescens*; —, neither detected.

P. intermedia by both methods. In endodontic and peri-apical infections (n = 29), the prevalence of *P. nigrescens* (n = 10 in culture or 12 by PCR) was greater than *P. intermedia* (n = 2 in culture or 5 by PCR).

Discussion

Although *P. intermedia* and *P. nigrescens* exhibit exceptional phenotypic similarity, there are distinct genotypic differences. Recognised techniques for the reliable identification of these organisms are not readily applicable to large-scale clinical studies or in smaller laboratories [9]. However, previous PCR-based studies have indicated that simple, rapid methods for discriminating *P. intermedia* and *P. nigrescens* are helpful in providing information on the relative roles of these two strict anaerobes in oral disease [8, 17].

The PCR primers described in the present study discriminated *P. intermedia* and *P. nigrescens* both from one another and from other similar organisms. The identity of PCR products from strains of *P.*

intermedia and *P. nigrescens* was confirmed subsequently by hybridisation with specific internal oligonucleotide probes. The overall sensitivity of the *P. intermedia* and *P. nigrescens* reactions was 500 fg of target DNA, equating to a detection limit of c. 100 bacterial cells per reaction. In contrast to earlier reports [8, 17, 18], the PCR technique used here did not require stringent annealing conditions to differentiate strains of *P. intermedia* and *P. nigrescens*. Primers were designed with significant mismatching (7 bp with non-homologous template) at the 3' end of oligonucleotides, allowing PCR discrimination at a relatively low annealing temperature and standard MgCl₂ concentration. Also, specific oligonucleotide probes were designed to contain 5-bp mismatches with the non-homologous template, thereby reducing the possibility of cross-reaction and mis-diagnosis to a minimum.

Rapid nucleic acid preparation by boiling in Chelex-100 resin has been reported to remove protein contamination, increase the stability of template [23] and reduce PCR inhibition [26] when compared with boiling in water alone. In the present study, Chelex-100-extracted nucleic acid from cultured bacteria and clinical pus and plaque samples provided template suitable for 16S rDNA PCR with 'universal' primers targeted to 16S rRNA gene sequences. Therefore, it would appear that inhibition of PCR by constituents of oral pus may not be a major problem. A combination of the simple template preparation technique with the specific PCR reaction permitted the direct, rapid detection and discrimination of *P. intermedia* and *P. nigrescens* in oral pus samples. This strategy gave a preliminary indication of the presence of *P. intermedia* or *P. nigrescens*, or both, in oral pus after only 4 h (confirmation by hybridisation after 24 h).

Detection by PCR produced results analogous to traditional culture for the majority of samples (Table 2). However, a small number of discrepancies did occur and comparison with studies employing these techniques individually should be drawn with caution. Previous studies involving the detection of *Por. gingivalis* in saliva and plaque have reported clear differences between the findings of culture and those obtained by PCR [17, 26]. Such discrepancies have been attributed to the presence of DNA from non-viable organisms remaining available for amplification, the higher sensitivity of PCR and the possible loss of slower-growing organisms during culture due to the overgrowth of other bacteria. In the present study, six pigmented colonies were taken from the primary plate for subculture according to routine practice. However, colonies of *P. intermedia* and *P. nigrescens* have a similar appearance and colony type is not helpful in distinguishing between the two species. It is not practical to subculture all pigmented colonies on the primary plate and, therefore, it is possible that one of the species may have been undetected by culture. The PCR method detected the presence of *P. intermedia*

and *P. nigrescens* on each occasion it was cultured from the pus sample. The PCR detection of additional strains in six samples highlights the advantage of the method over routine diagnostic culture methods.

Despite the small number of samples included in the present study, the relative occurrence of *P. intermedia* and *P. nigrescens* indicates that *P. nigrescens* may be more likely to be encountered in suppurative oral infections. These results would support the association of *P. nigrescens* with endodontic and peri-apical abscess infections which has been reported previously [7, 8, 27].

Penicillins remain the antibiotic group of choice in the treatment of acute suppurative oral infection despite the increased incidence of penicillin resistance in strains of *Prevotella* spp. [19]. The PCR-based method described here has the potential to provide useful information to the clinician, as rapid detection of the presence of *P. intermedia* and *P. nigrescens* would give an early indication of the likelihood of penicillin-resistant strains in the infection if a patient was failing to respond to initial antimicrobial therapy.

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