Development of a genus-specific PCR assay for the molecular detection, confirmation and identification of *Fusobacterium* spp

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Accepted: 26 March 2007

ABSTRACT

A genus-specific polymerase chain reaction (PCR)-based assay is developed for the detection and identification of clinically relevant *Fusobacterium* species, including *F. nucleatum* and *F. necrophorum*. Two 16S ribosomal DNA (rDNA) primers, FUSO1 (forward primer: 5′-GAG AGA GCT TTG CGT CC-3′ [17-mer]) and FUSO 2 (reverse primer: 5′-TGG GCG CTG AGG TTC GAC -3′ [18-mer]) are designed to target conserved regions of the 16S rDNA gene for *Fusobacterium* spp. Subsequent proof-of-principle studies employing this assay detected *Fusobacterium* spp. in the faeces of eight (10%) out of 80 patients with suspected gastrointestinal infection. This assay may be used for the genus-specific detection of *Fusobacterium* spp. from clinical specimens and for subsequent species identification.

KEY WORDS: Colitis, ulcerative.
Crohn disease.
Fusobacteria.
Irritable bowel syndrome.
Oligonucleotide primers.
Polymerase chain reaction.

Introduction

Fusobacteria are Gram-negative anaerobic bacilli that are responsible for several clinically important infections, ranging from localised abscesses and throat infections to systemic life-threatening disease. Presently, 15 species are formally recognised within the genus *Fusobacterium* and these are *F. canifelinum, F. equinum, F. gonidiaformans, F. mortiferum, F. naviforme, F. necrogenes, F. necrophorum, F. nucleatum, F. perfoetens, F. periodonticum, F. pseudonecrophorum, F. russii, F. simiae, F. ulcerans* and *F. varium*. Of most clinical significance are *F. necrophorum* and *F. nucleatum*, where the former is responsible for Lemierre's disease/syndrome, post-anginal sepsis and necrobacillosis,[1] and the latter is significant in periodontal disease,[2] in brain[3] and lung abcesses,[4] and is the cause of bacteraemia[5] in immunocompromised patients.

As these and other species within this genus are anaerobes, their routine isolation and subsequent identification can be problematic when employing conventional clinical microbiological techniques.[3] Thus, several groups have examined the use of molecular tools to aid the detection and identification of such organisms, whereby individual species have been targeted in specific disease states (e.g., *F. nucleatum* in root canal abcesses).[7,8] To date, however, there has been no report of a genus-specific polymerase chain reaction (PCR) assay for the cumulative detection of all species within this genus.

The aim of the current study is to develop a molecular PCR assay based on 16S ribosomal DNA (rDNA) that is genus-specific for fusobacteria and has the flexibility to identify species within this genus through subsequent downstream sequencing of positive PCR amplicons.

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Materials and methods

Fusobacterium genus-specific 16S rDNA oligonucleotide primers

Sequence data for the 16S rDNA gene were obtained from GenBank (www.ncbi.nlm.nih.gov/entrez), and conserved and variable regions were identified subsequently by the clustal alignment method employing a sequence alignment software package (DNASTAR, Wisconsin, USA), where the published 16S rDNA sequences of *F. canifelinum, F. equinum, F. gonidiaformans, F. mortiferum, F. naviforme, F. necrogenes, F. necrophorum, F. nucleatum, F. perfoetens, F. periodonticum, F. russii, F. simiae, F. ulcerans* and *F. varium* were aligned, for which sequence data existed in GenBank. In addition, 19 non-fusobacteria 16S rDNA genes from the following bacteria were also aligned and these included *Alcaligenes xylosoxidans, Bacillus* sp., *Burkholderia cepacia, Escherichia coli, Haemophilus influenzae, Klebsiella pneumoniae, Listeria monocytogenes, Micrococcus* sp., *Mycobacterium tuberculosis, Neisseria gonorrhoeae, N. meningitidis, Nocardia* sp., *Pseudomonas aeruginosa, P. putida, Salmonella enteriditis, Staphylococcus aureus, S. epidermidis, Stenotrophomonas maltophilia* and *Streptococcus pneumoniae*.

The primer pair FUSO1 (forward primer, 5′-GAG AGA GCT TTG CGT CC-3′ [17-mer]) and FUSO2 (reverse promer,

5′-TGG GCG CTG AGG TTC GAC -3′ [18-mer]) was designed to target conserved regions of the 16S rDNA gene for *Fusobacterium* spp., as detailed in Figure 1. The binding sites of the FUSO1 and FUSO2 primers in *F. nucleatum* (GenBank Accession Number AJ133496) relate to positions 212–228 and 821–804, respectively, yielding a fragment of 610 bp.

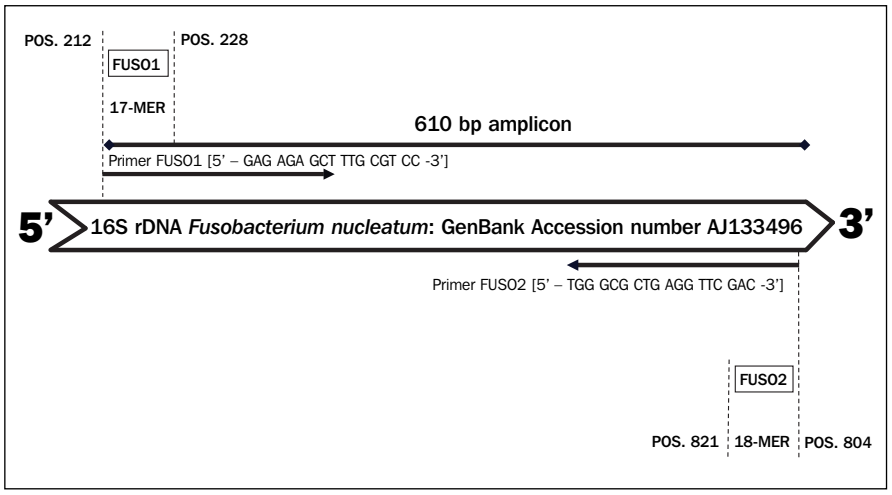

Fig. 1. Location of primers and description of the PCR assay in relation to *Fusobacterium nucleatum* AJ 133496.

DNA extraction

All DNA isolation procedures were carried out in a class II biological safety cabinet (MicroFlow, England) in a room physically separated from that used to set up nucleic acid amplification reaction mixes and also from the 'post-PCR' room, in accordance with the good molecular diagnostic procedures (GMDP) guidelines of Millar *et al.*,[9] in order to minimise contamination and hence the possibility of false-positive results.

Bacterial genomic DNA was extracted from a well-characterised wild-type strain of *F. nucleatum* previously obtained from the oral cavity of a patient, and DNA was extracted using a high-purity PCR template preparation kit (Roche, England), in accordance with the manufacturer's instructions.

Genomic DNA was also extracted from well-characterised isolates of *P. putida, P. fluorescens, P. stutzeri* and from *Asaia* sp., *Alcaligenes xylosoxidans* NIPHL 96/01, *Bacillus licheniformis, Burkholderia cepacia* genomovar IIIa NIPHL CF/97/58, *B. cepacia* genomovar IIIa NIPHL CF/96/27, *B. cepacia* genomovar IIIa NIPHL CF/96/02, *B. multivorans, Campylobacter jejuni, Chromobacter violaceum* NIPHL 96/51, *Curtobacterium* sp., *Haemophilus influenzae, Inquilinus limosus, Lactobacillus gasseri, Mycobacterium malmoense, Pandoraea apista* NIPHL 02/02, *Ralstonia paucula, Serratia marcescens* NIPHL CF/97/27, *Sphingomonas paucimobilis* NIPHL CF/97/40, *Staphylococcus aureus, Stenotrophomonas maltophilia* NIPHL 97/23 and *Thermoactinomyces* sp.

Extracted DNA was stored at –80°C prior to PCR amplification. For each batch of extractions, a negative extraction control containing all reagents but no organism was performed, as well as an extraction positive control from *F. nucleatum*.

PCR amplification

Amplification reactions were set up in accordance with GMDP, as detailed in the guidelines of Millar *et al.*[9] All reaction mixes were set up in a PCR hood in a room separate from that used to extract DNA and from the amplification and post-PCR room in order to minimise contamination. Initially, PCR amplification conditions were optimised by separately varying $MgCl_2$ concentration, annealing temperature, primer concentration and DNA template concentration. Following optimisation, reaction mixes (100 µL) were set up as follows: 10 mmol/L Tris–HCl (pH 8.3), 50 mmol/L KCl, 2.5 mmol/L $MgCl_2$, 200 µmol (each) dATP, dCTP, dGTP and dTTP, 1.25 units of *Thermus aquaticus* (*Taq*) DNA polymerase (Amplitaq, Perkin Elmer), 20 pmol (each) of the primers FUSO1 and FUSO2, and 4 µL DNA template. Following a 'hot start', the reaction mixtures were subjected to the following empirically optimised thermal cycling parameters in a Perkin Elmer 2400 thermocycler: 94°C for 5 min, followed by 30 cycles of 94°C for 30 sec, 60°C for 30 sec, 72°C for 30 sec, followed by a final extension at 72°C

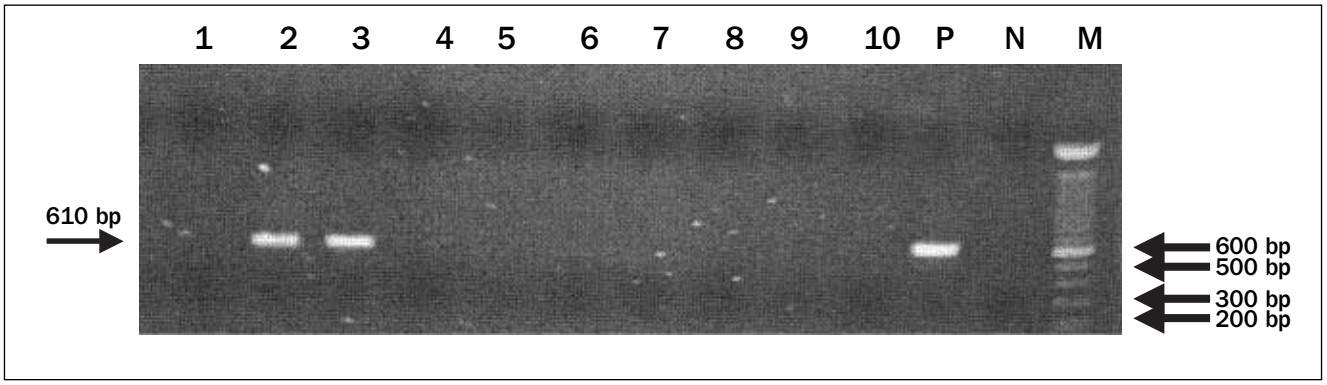

Fig. 2. PCR amplification of 16S rDNA from genomic DNA extracted from faeces of patients with suspected gastrointestinal infections, employing the novel primer pair FUSO1 and FUSO2 and demonstrating the presence of a 610 bp PCR amplicon, specific for *Fusobacterium* spp. Lanes 1, 4–10: patients whose faecal DNA did not demonstrate the presence of *Fusobacterium* sp.; Lane 2: patient B (*F. varium*); Lane 3: patient C (*F. varium*); Lane P: positive control (*F. nucleatum* DQ490532); Lane N: negative control (LAL molecular grade water; Biowhittaker, USA); Lane M: molecular weight marker (100 bp molecular weight marker, Gibco Life Technologies, Paisley, Scotland, UK).

Table 1. Description of patients from whom *Fusobacterium* spp. were detected and identified, including GenBank Accession Numbers of resulting 16S rDNA sequences.

Patient	Sex	Conventional faecal bacterial isolation	Source of specimen	PCR detection of *Fusobacterium* sp.	Number of bases called	Identification	Submitted GenBank Accession Number
A	M	*E. coli*	Hospital	+	561	*F. nucleatum*/ *F. canifelinum*	DQ490523
B	F[2]	No significant growth	Community	+	556	*F. varium*	DQ490524
C	F	No significant growth	Community	+	554	*F. varium*	DQ490525
D	M	*E. coli*	Hospital	+	550	*F. nucleatum*	DQ490526
E	M	No significant growth	Community	+	555	*F. varium*	DQ490527
F	F	*E. coli* O55 and *Campylobacter* sp.	Community	+	554	*F. nucleatum*/ *F. canifelinum*	DQ490528
G	M	No significant growth	Community	+	564	*F. mortiferum*/ *F. varium*	DQ490529
H	F	No significant growth	Community	+	550	*F. mortiferum*/ *F. necrogenes*	DQ490530

for 7 min. Positive (*F. nucleatum* DNA) and multiple negative (water) amplification controls were included with each set of PCR reactions.

Amplicon detection

Following amplification, samples (10 µL) were removed from each reaction mixture and examined by electrophoresis (80 V, 45 min) in gels composed of 2% (w/v) agarose (Gibco, UK) in TAE buffer (40 mmol/L Tris, 20 mmol/L acetic acid, 1 mmol/L EDTA [pH 8.3]) and stained with ethidium bromide (5 µg/100 mL). Gels were visualised under ultraviolet (UV) illumination using a gel image analysis system (UVP Products, England) and all images were archived as digital graphic files (*.bmp).

Sequencing of PCR amplicons and analysis of sequence data

All *Fusobacterium* PCR amplicons were purified using a QIAquick PCR purification kit (Qiagen, UK) and were eluted in Tris–HCl (10 mmol/L [pH 8.5]) prior to sequencing, particularly to remove dNTPS, polymerases, salts and primers. The amplicons were sequenced in both directions on the ALF II Express automated sequencer using the forward and reverse primers FUSO1 and FUSO2, which were labelled with Cy-5 fluorescent dye (Oligosynthesis Unit, The Queen's University of Belfast, UK) and used in conjunction with the Thermo Sequenase fluorescence-labelled primer cycle sequencing kit (Amersham, UK). The sequences obtained were aligned using the MegAlign software (DNAStar, Wisconsin, USA) and compared with those stored in the Genbank data system using the BLAST alignment software (www.blast.genome.ad.jp/).

Screening faeces for the presence of fusobacteria: proof of principle

Eighty faecal samples, obtained from GP and hospital patients, were collected from routine specimens submitted for the detection of bacterial and parasitic pathogens. Faecal specimens were chosen randomly, regardless of the patient's illness. Fresh faeces (1 g) was taken and suspended in 9 mL 1% (w/v) saline to obtain a 1 in 10 (w/v) faecal dilution for DNA extraction. The faecal-saline suspension (100 µL) was mixed with 40 µL diatomaceous earth (DE, Sigma) and 900 µL lysis buffer, and incubated at room temperature for 10 min. Lysis buffer contained 120 mg guanidine thiocyanate (Sigma) in 100 mL 0.1 mol/L Tris–HCl (pH 6.4; Sigma), 22 mL 0.2 mol/L EDTA solution (pH 8.3; Prolabo) and 2.6 g Triton X-100 (Sigma).

After incubation the preparation was centrifuged at 11,600 x*g* for 15 sec. The pellet was washed twice with washing buffer (120 mg guanidine thiocyanate [Sigma] in 100 mL 0.1 mol/L Tris–HCl [pH 6.4]), twice with 70% (v/v) ethanol and once with acetone. The washed pellet was dried on the heating block at 54°C for 10 min and then resuspended in 100 µL TE buffer (10 mmol/L Tris–HCl, 1 mmol/L EDTA [pH 8.0]), incubated at 54°C for 10 min and centrifuged at 11,600 x*g* for 2 min. The supernatant was removed to a fresh Eppendorf tube and stored at –80°C until required.

Universal 16S rDNA broad-range PCR was employed, as

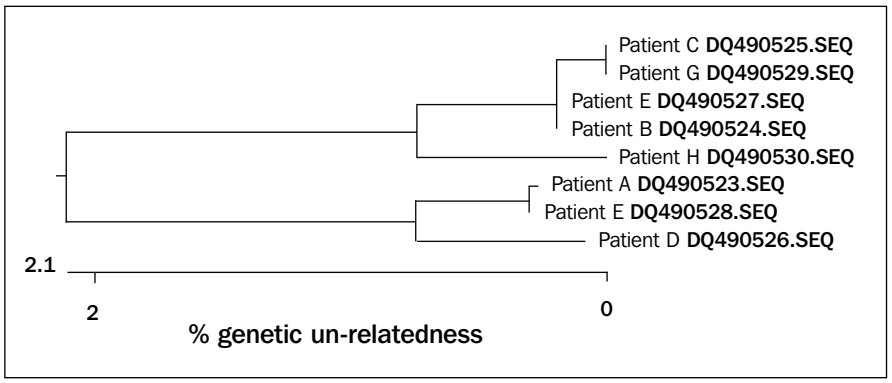

Fig. 3. A phylogenetic tree demonstrating the genetic relatedness among *Fusobacterium* spp. identified from faeces of eight patients with suspected gastrointestinal infections.

previously described,[10] to demonstrate the absence of PCR inhibition in extracted genomic DNA from faecal material.

Genus-specific *Fusobacterium* PCR was performed subsequently on all extracts, as described above, using the primer pair FUSO1 and FUSO2, and the resulting presumptive positive extracts generating a PCR amplicon of the expected size (i.e., 610 bp) were confirmed by sequence analysis.

Results and discussion

In silico analysis allowed the identification of two primer regions on the 16S rDNA gene locus, which had 100% homology with all *Fusobacterium* spp., but showed variation with the aligned non-*Fusobacterium* spp. BLAST searches of these primers demonstrated, *in silico*, that all *Fusobacterium* spp. could be amplified using the FUSO1 and FUSO2 primer pair. Subsequent wet PCR amplification of the 610-bp fragment was successful for the *F. nucleatum* isolate tested, giving a PCR fragment of the expected size (approximately 610 bp), whereas none of the non-*Fusobacterium* organisms tested produced PCR amplicons of the expected size.

Following demonstration of the ability to generate a PCR amplicon of the expected size using the FUSO1 and FUSO2 primers, the authors wished to prove the principle of the assay by examining a cohort of 80 patients for the presence of *Fusobacterium* spp. in faecal samples. From these 80 patients, eight (10%) generated an amplicon of the correct size (approximately 610 bp; Fig. 2) and these patients were considered presumptively positive for *Fusobacterium* sp. (Table 1). The positive control organism was confirmed as *F. nucleatum* by sequence analysis and the sequence was deposited in GenBank, with accession number DQ490532.

Subsequent sequence analysis of the 16S rDNA amplicons demonstrated that all were *Fusobacterium* spp. (Table 1). All 16S rDNA resulting sequences from the patients were deposited in GenBank (Table 1). Subsequently, a phylogenetic tree was constructed to examine the genetic relatedness of the *Fusobacterium* organisms from the eight patients (Fig. 3).

Overall, this study developed a simple PCR- and sequenced-based assay for the detection and identification of *Fusobacterium* spp., which was applied to a cohort of patients with suspected gastrointestinal infection. Specificity of the assay was demonstrated by the inability to generate a PCR amplicon when challenged with other bacterial genera that might be present with *Fusobacterium* spp., either from respiratory/salivary specimens or from faecal specimens.

In conclusion, this simple assay can be used for the genus-specific detection of *Fusobacterium* spp. from clinical specimens, and for subsequent species identification, which may aid routine diagnostic clinical bacteriology.

This work was supported by a kind charitable donation given by the wife and family of the late Mr. John (Jackie) McCaughern, in his memory, for the purposes of pursuing microbiological investigations into the sources and transmission of bacteriological aetiological agents of infection.

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