Real-time PCR investigation into the importance of *Fusobacterium necrophorum* as a cause of acute pharyngitis in general practice

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*Fusobacterium necrophorum* is recognized as the cause of a severe life-threatening illness characterized by bacteremia with metastatic abscesses following an acute sore throat (Lemierre’s disease). However, the importance of *F. necrophorum* as a cause of simple sore throat in the community is unknown. Using quantitative real-time PCR with primers targeting the rpoB gene, 100 routine throat swabs collected from patients presenting to general practitioners with pharyngitis were analysed for the presence of *F. necrophorum*-specific DNA. The results were compared with those obtained from throat swabs collected from 100 healthy subjects. Ten clinical samples were positive for *F. necrophorum* DNA, identified as *F. necrophorum* subspecies *funduliforme*, using a haemagglutinin-related protein gene-specific PCR assay. All the healthy controls were negative (two-tailed *P* value = 0.0015; Fisher exact test). These findings suggest that *F. necrophorum* may play a more important role as a cause of simple sore throat in the community than has been previously appreciated.

INTRODUCTION

*Fusobacterium necrophorum* is recognized as the cause of Lemierre’s disease, a life-threatening septicemic illness with metastatic abscesses secondary to a septic thrombophlebitis of the internal jugular vein following an initial acute sore throat (Lemierre, 1936; Hagelskjaer Kristensen & Prag, 2000). The organism is an obligate anaerobic Gram-negative pleomorphic bacillus, believed to be a member of the normal human oropharyngeal, gastrointestinal and urogenital tract flora (Hagelskjaer et al., 1998). The species *F. necrophorum* is divided into two subspecies, *F. necrophorum* subsp. *necrophorum* (biovar A) and *F. necrophorum* subsp. *funduliforme* (biovar B), with the former considered to be the main pathogen in animals, and the latter associated with infection in humans (Beerens et al., 1971; Smith & Thornton, 1993). The invasive potential of *F. necrophorum* has been attributed to the production of a specific leukotoxin, proteolytic enzymes, lipopolysaccharide endotoxin and haemagglutinin (Tan et al., 1994; Hagelskjaer Kristensen & Prag, 2000).

Lemierre’s disease usually affects previously healthy young adults who lack any identified risk factor for an invasive bacterial infection, and the disease has been described as unmistakable when the full gamut of symptoms and signs are present (Lemierre, 1936). It has been uncommon in the ‘antibiotic era’, presumably due to empirical antibiotic therapy of acute pharyngitis preventing many cases progressing to invasive disease. An exhaustive review of anaerobic pleuropulmonary infections seen between 1958 and 1971 failed to identify a single case of Lemierre’s disease (Bartlett & Finegold, 1972), leading to its description as ‘the forgotten disease’ (Weesner & Cisek, 1993; Moore-Gillon et al., 1984) and unfamiliarity with the condition has been associated with misdiagnosis and inadequate antibiotic therapy (Hagelskjaer Kristensen & Prag, 2000; Baddour et al., 1986; Sherer et al., 2000).

There is evidence that invasive infection caused by *F. necrophorum* infection is increasing in the UK (Brazier et al., 2002; Brazier, 2002; Jones et al., 2001). This increase has been attributed to increasing awareness of the disease, improved anaerobic diagnostic facilities and, latterly, the recent advice to general practitioners (GPs) not to prescribe empiric antibiotics for simple sore throats (Jones et al., 2001; Hagelskjaer et al., 1998; Brazier et al., 2002; Department of Health Standing Medical Advisory Committee, 1998).

Surprisingly, although an initial sore throat is a presenting feature of Lemierre’s disease, the importance of *F. necrophor-
um as a cause of sore throat in general practice is unknown. The aim of this study was to investigate the importance of *F. necrophorum* as a cause of sore throat in domiciliary practice using quantitative real-time PCR. PCR was chosen as a diagnostic tool as it is less laborious than recovery of this fastidious obligate anaerobe by culture on selective media with subsequent phenotypic characterization. Throat swabs taken from patients presenting with pharyngitis in general practice and submitted for routine bacterial culture were analysed for the presence of *F. necrophorum* DNA. In order to distinguish harmless colonization from active infection we also examined throat swabs from adults without pharyngitis, to establish base-line rates for the extent of carriage in health. It was anticipated that the carriage rate in patients presenting with pharyngitis would be the same as the rate in healthy adults without pharyngitis, and that if the organism was responsible for invasive disease, i.e. pharyngitis, an additional proportion of patients with sore throats would be found to harbour the organism. In addition, real-time PCR is a quantitative technique, and it was also anticipated that patients with active infection due to this organism would be distinguishable from those with harmless colonization due to the larger numbers of organisms recovered in acute infections. Ethical approval for this study was obtained from the local research ethics committee.

**METHODS**

**Specimen collection and preparation.** One hundred throat swabs collected from patients presenting to their GP with sore throat were studied. They had been submitted to our laboratory for routine bacteriological examination and were selected by us at random, but avoiding duplicate patient swabs. They were first cultured in the routine bacteriological examination and were selected by us at random, but collected from patients presenting to their GP with sore throat were (Probact Transport Swab; Technical Service Consultants), refrigerated weeks. All swabs were held in Amies transport media with charcoal not had sore throat or been on antibiotic therapy in the preceding 2 weeks. Ethical approval for this study was obtained from the larger numbers of organisms recovered in acute infections. It was anticipated that the carriage rate in patients presenting with pharyngitis would be the same as the rate in healthy adults without pharyngitis, and that if the organism was responsible for invasive disease, i.e. pharyngitis, an additional proportion of patients with sore throats would be found to harbour the organism. In addition, real-time PCR is a quantitative technique.

**DNA extraction.** Nucleic acid was extracted from 200 μl of the PBS supernatant with a Total nucleic acid isolation kit (Roche Molecular Biochemicals) using an automated MagNA Pure extraction system (Roche Molecular Biochemicals) according to the manufacturer’s instructions. The DNA was eluted in 60 μl and held at –20 °C until use. As a positive control, and to provide the quantitative standards, *F. necrophorum* subsp. necrophorum NCTC 10576 (Shinjo et al., 1991) was grown on non-selective Fastidious Anaerobe agar media (Oxoid) and the chromosomal DNA isolated from the cells using the extraction method described by Narayanan et al. (2003). Clinical isolates of *Fusobacterium* spp., type and reference strains of *Fusobacterium* spp., oropharyngeal pathogenic and commensal micro-organisms (Table 1) were subcultured according to the instructions provided for each organism, and the DNA was purified from the cultures using either the method described by Narayanan et al. (2001) for the Gram-negative bacteria, or the Boom extraction method (Boom et al., 1990) for the Gram-positive bacteria incorporating three rapid freeze-thaw cycles prior to extraction.

**Design of primers and probes and PCR amplification.** The *F. necrophorum*-specific PCR targeted the rpoB gene sequence (accession no. AF527637) and was a modification of the forward primer (TP1) reported to be specific to the two subspecies of *F. necrophorum* by Narongwanichgarn et al. (2003): RPO forward 5’-TCTCTAGCT ATGCCTCAAGGATC-3’ (position 171–193) and a newly designed reverse primer RPO reverse 5’-GCATTTCATCCAGAGGCTGTCG-3’ (447–462) (see Fig. 2). BLAST analysis of the PCR primer sequences revealed a single 100 % identity match with the *F. necrophorum* rpoB gene sequence and no significant matches of concern with other sequences in the database were found.

LightCycler FRET hybridization probes were designed within target region rpoB, close to the reverse primer: rpoB probe 1 (5’-GAAGACATGGCTTCTTAGAGGAC–Pho-3’) and rpoB probe 2 (5’-GAGCTCGGATGAGGAC–Pho-3’) (see Fig. 2). The PCR reaction was performed in a total volume of 20 μl containing 4 μl DNA extract, 2 μl LightCycler FastStart DNA master hybridization mixture (Roche Diagnostics), 3.2 μl 25 mM MgCl2 (5 mM), 0.5 μl each of 20 pmol μl–1 RPO primers, 0.2 μl μl each of the 10 pmol μl–1 probes and 9.4 μl RNase free water.

The PCR LightCycler reaction was carried out as follows: one cycle of denaturation at 95 °C for 10 min followed by 50 cycles of amplification at 95 °C for 0 s, 60 °C for 2 s and 72 °C for 15 s with a single transition rate of 20 °C s–1 and a single fluorescence acquisition at 60 °C. On completion of amplification, a single melt cycle was produced by holding the reaction at 95 °C for 0 s, then at 45 °C for 30 s, followed by slow heating at a transition rate of 0.1 °C s–1 to 85 °C and continuous fluorescence acquisition.

Positive controls were included in each PCR run (*F. necrophorum* subsp. necrophorum ATCC 25286). A contamination control was also included in the form of sterile water, i.e. a no template control. In addition, sterile swabs were included as extraction controls and processed as above in each run at a frequency of one extraction control for every tenth patient’s throat swab. PCR quantification standards (10–4 down to 10–9, see below) were also included in every run of 32 samples to facilitate quantification of the bacterial load in positive samples.

**Inhibition study.** As the swabs were collected in charcoal transport media, a protocol was devised to check for PCR inhibition and enable quantification of *F. necrophorum* DNA in positive samples. A suspension of *F. necrophorum* subsp. necrophorum ATCC 25286 matching 0.5 McFarland standards was prepared and 10-fold serial dilutions were made from neat to 10–4. Twenty microlitres of each dilution were added in parallel to a swab with Amies transport media with charcoal and a swab with Amies transport media without charcoal. These were held at 4 °C for 24 h and the swabs subsequently treated as described in specimen preparation. Subsequent extraction and PCR was as described above.

**Sensitivity and specificity.** The lower levels of detection for the assay were determined by making serial dilutions of purified chromosomal DNA obtained from *F. necrophorum* subsp. necrophorum NCTC 10576 (Shinjo et al., 1991) and performing PCR as described (see Quantification, below). The specificity of the rpoB assay was established by testing the chromosomal DNA extracted from a panel of micro-organisms comprising *F. necrophorum* type and reference strains, and 14 clinical isolates of *F. necrophorum*, *Fusobacterium varium* and *Fusobacterium necrophorum* subsp. necrophorum.
Table 1. Result of specificity tests for rpoB primers

<table>
<thead>
<tr>
<th>Organism</th>
<th>Result</th>
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<tbody>
<tr>
<td><strong>Type/reference strains of <em>Fusobacterium</em> spp.</strong></td>
<td></td>
</tr>
<tr>
<td>ATCC 25286&lt;sup&gt;T&lt;/sup&gt; <em>F. necrophorum</em> subsp. necrophorum</td>
<td>+</td>
</tr>
<tr>
<td>NCTC 10576 <em>F. necrophorum</em> subsp. necrophorum</td>
<td>+</td>
</tr>
<tr>
<td>ATCC 51357&lt;sup&gt;T&lt;/sup&gt; <em>F. necrophorum</em> subsp. funduliforme</td>
<td>+</td>
</tr>
<tr>
<td>NCTC 12111&lt;sup&gt;T&lt;/sup&gt; <em>Fusobacterium ulcerans</em></td>
<td>–</td>
</tr>
<tr>
<td>NCTC 10723&lt;sup&gt;T&lt;/sup&gt; <em>Fusobacterium necrogens</em></td>
<td>–</td>
</tr>
<tr>
<td>NCTC 10560&lt;sup&gt;T&lt;/sup&gt; <em>F. varium</em></td>
<td>–</td>
</tr>
<tr>
<td>NCTC 11326&lt;sup&gt;T&lt;/sup&gt; <em>F. nucleatum</em> subsp. fusiforme</td>
<td>–</td>
</tr>
<tr>
<td>NCTC 12276&lt;sup&gt;T&lt;/sup&gt; <em>F. nucleatum</em> subsp. animalis</td>
<td>–</td>
</tr>
<tr>
<td>ATCC 49256&lt;sup&gt;T&lt;/sup&gt; <em>F. nucleatum</em> subsp. vincenti</td>
<td>–</td>
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<tr>
<td>ATCC 10953&lt;sup&gt;T&lt;/sup&gt; <em>F. nucleatum</em> subsp. polymorphum</td>
<td>–</td>
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<tr>
<td>ATCC 25586&lt;sup&gt;T&lt;/sup&gt; <em>F. nucleatum</em> subsp. nucleatum</td>
<td>–</td>
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<tr>
<td>NCTC 11464 <em>Fusobacterium naviforme</em></td>
<td>–</td>
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<tr>
<td>ATCC 33568&lt;sup&gt;T&lt;/sup&gt; <em>Fusobacterium simiae</em></td>
<td>–</td>
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<tr>
<td>ATCC 33693&lt;sup&gt;T&lt;/sup&gt; <em>Fusobacterium periodonticum</em></td>
<td>–</td>
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<tr>
<td>ATCC 25533&lt;sup&gt;T&lt;/sup&gt; <em>Fusobacterium russii</em></td>
<td>–</td>
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<tr>
<td>ATCC 25557&lt;sup&gt;T&lt;/sup&gt; <em>Fusobacterium mortiferum</em></td>
<td>–</td>
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<tr>
<td>ATCC 29250&lt;sup&gt;T&lt;/sup&gt; <em>Fusobacterium perfecans</em></td>
<td>–</td>
</tr>
<tr>
<td>ATCC 27768&lt;sup&gt;T&lt;/sup&gt; <em>Fusobacterium praunztzii</em></td>
<td>–</td>
</tr>
<tr>
<td>ATCC 25563&lt;sup&gt;T&lt;/sup&gt; <em>Fusobacterium gonidiformans</em></td>
<td>–</td>
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<tr>
<td><strong>Pharyngeal pathogens</strong></td>
<td></td>
</tr>
<tr>
<td>NCTC 8198&lt;sup&gt;T&lt;/sup&gt; <em>Streptococcus pyogenes</em></td>
<td>–</td>
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<tr>
<td>ATCC 43078&lt;sup&gt;T&lt;/sup&gt; <em>Streptococcus dysgalactiae</em> subsp. dysgalactiae</td>
<td>–</td>
</tr>
<tr>
<td>NCTC 7907 <em>Corynebacterium ulcerans</em></td>
<td>–</td>
</tr>
<tr>
<td>ATCC 11913 <em>Corynebacterium diphtheriae</em></td>
<td>–</td>
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<tr>
<td>NCTC 8452&lt;sup&gt;T&lt;/sup&gt; <em>Arcanobacterium haemolyticum</em></td>
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<tr>
<td>NCTC 12700 <em>Neisseria gonorrhoeae</em></td>
<td>–</td>
</tr>
<tr>
<td>NCTC 14027 <em>Mycoplasma hominis</em></td>
<td>–</td>
</tr>
<tr>
<td>ATCC 15293 <em>Mycoplasma pneumoniae</em></td>
<td>–</td>
</tr>
<tr>
<td><strong>Commensal flora of the pharynx</strong></td>
<td></td>
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<tr>
<td>ATCC 25923 <em>Staphylococcus aureus</em> subsp. aureus</td>
<td>–</td>
</tr>
<tr>
<td>ATCC 12228 <em>Staphylococcus epidermidis</em></td>
<td>–</td>
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<tr>
<td>ATCC 10556&lt;sup&gt;T&lt;/sup&gt; <em>Streptococcus sanguis</em></td>
<td>–</td>
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<tr>
<td>ATCC 25175&lt;sup&gt;T&lt;/sup&gt; <em>Streptococcus mutans</em></td>
<td>–</td>
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<tr>
<td>ATCC 49124&lt;sup&gt;T&lt;/sup&gt; <em>Streptococcus vestibularis</em></td>
<td>–</td>
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<tr>
<td>ATCC 13419 <em>Streptococcus salivarius</em></td>
<td>–</td>
</tr>
<tr>
<td>ATCC 43497 <em>Streptococcus canis</em></td>
<td>–</td>
</tr>
<tr>
<td>NCTC 11460&lt;sup&gt;T&lt;/sup&gt; <em>Peptostreptococcus anaerobius</em></td>
<td>–</td>
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<tr>
<td>ATCC 29743 <em>Peptinophilus asaccharolyticus</em></td>
<td>–</td>
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<tr>
<td>ATCC 10790&lt;sup&gt;T&lt;/sup&gt; <em>Veillonella parvula</em></td>
<td>–</td>
</tr>
<tr>
<td>ATCC 29522 <em>Haemophilus actinomycetemcomitans</em></td>
<td>–</td>
</tr>
<tr>
<td>ATCC 10049 <em>Actinomyces israelii</em></td>
<td>–</td>
</tr>
<tr>
<td>ATCC 27853 <em>Pseudomonas aeruginosa</em></td>
<td>–</td>
</tr>
<tr>
<td>ATCC 33574&lt;sup&gt;T&lt;/sup&gt; <em>Prevotella buccae</em></td>
<td>–</td>
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<tr>
<td>ATCC 25611&lt;sup&gt;T&lt;/sup&gt; <em>Prevotella intermedia</em></td>
<td>–</td>
</tr>
<tr>
<td>ATCC 25845&lt;sup&gt;T&lt;/sup&gt; <em>Prevotella melanogenica</em></td>
<td>–</td>
</tr>
<tr>
<td>ATCC 25260&lt;sup&gt;T&lt;/sup&gt; <em>Porphyromonas asaccharolytica</em></td>
<td>–</td>
</tr>
<tr>
<td>ATCC 23715 <em>Yersinia enterocolitica</em></td>
<td>–</td>
</tr>
<tr>
<td>NCTC 10596&lt;sup&gt;T&lt;/sup&gt; <em>Eikenella corroden</em></td>
<td>–</td>
</tr>
<tr>
<td>NCTC 9380&lt;sup&gt;T&lt;/sup&gt; <em>Pasteurella haemolytica</em></td>
<td>–</td>
</tr>
<tr>
<td>NCTC 9343&lt;sup&gt;T&lt;/sup&gt; <em>Bacteroides fragilis</em></td>
<td>–</td>
</tr>
<tr>
<td>ATCC 27087 <em>Treponema phagedenis</em></td>
<td>–</td>
</tr>
<tr>
<td><strong>Clinical isolates</strong></td>
<td></td>
</tr>
<tr>
<td>R11113 <em>F. necrophorum</em></td>
<td>+</td>
</tr>
</tbody>
</table>

Table 1. Cont.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Result</th>
</tr>
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<tbody>
<tr>
<td>R11759 <em>F. necrophorum</em></td>
<td>+</td>
</tr>
<tr>
<td>R12351 <em>F. necrophorum</em></td>
<td>+</td>
</tr>
<tr>
<td>R18063 <em>F. necrophorum</em></td>
<td>+</td>
</tr>
<tr>
<td>R14824 <em>F. necrophorum</em></td>
<td>+</td>
</tr>
<tr>
<td>R17802 <em>F. necrophorum</em></td>
<td>+</td>
</tr>
<tr>
<td>R18196 <em>F. necrophorum</em></td>
<td>+</td>
</tr>
<tr>
<td>R18216 <em>F. necrophorum</em></td>
<td>+</td>
</tr>
<tr>
<td>R18126 <em>F. necrophorum</em></td>
<td>+</td>
</tr>
<tr>
<td>R15531 <em>F. necrophorum</em></td>
<td>+</td>
</tr>
<tr>
<td>R17708 <em>F. necrophorum</em></td>
<td>+</td>
</tr>
<tr>
<td>R17662 <em>F. necrophorum</em></td>
<td>+</td>
</tr>
<tr>
<td>R10861 <em>F. necrophorum</em></td>
<td>+</td>
</tr>
<tr>
<td>R17753 <em>F. necrophorum</em></td>
<td>+</td>
</tr>
<tr>
<td>R17443 <em>F. varium</em></td>
<td>–</td>
</tr>
<tr>
<td>R18217 <em>F. nucleatum</em></td>
<td>–</td>
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</tbody>
</table>

Real-time PCR investigation of *Fusobacterium necrophorum*

Quantification of bacterial load in positive throat swabs. Quantification of *F. necrophorum* DNA in positive samples was performed by including 10-fold serial dilutions of known concentration standards of *F. necrophorum* subsp. necrophorum NCTC 10576 chromosomal DNA from neat (90 ng µl<sup>–1</sup>, equivalent to ~37 million organisms µl<sup>–1</sup>) down to 10<sup>–9</sup> (90 attograms µl<sup>–1</sup>, equivalent to 0·037 organisms µl<sup>–1</sup>) in our rpoB analysis. The *F. necrophorum* genome has not been fully sequenced, and the calculation rests on the assumption that it is of similar molecular mass to the recently fully sequenced *F. nucleatum* subsp. nucleatum genome (accession no. NC_003454), and that *F. necrophorum* also possesses a single gene copy of rpoB per bacterial cell.

PCR laboratory conditions and control measures. PCR was carried out under stringent conditions in a CPA-accredited hospital diagnostic laboratory. All DNA manipulations pre- and post-PCR were performed in separate designated rooms with separate pipetting devices to avoid contamination of the samples with foreign DNA. Master mixture water controls and DNA extraction controls were used for every batch of samples processed.

DNA sequence analysis. Purified PCR products (QIAquick PCR purification kit; Qiagen) of all positive samples by real-time PCR retrieved from the LightCycler glass capillaries were sequenced on a Beckman Coulter CEQ 2000XL DNA analysis system. Sequencing reactions were carried out according to the manufacturer’s instructions supplied with the CEQ DTCS-Quick Start kit and loaded on to a capillary sequencer. PCR products were sequenced in both directions using the forward/reverse rpoB PCR primers at a concentration of 2 pmol per reaction. The sequence of the amplicons was then subjected to a BLAST search analysis to confirm the source as *F. necrophorum*.

Speciation of *F. necrophorum* using PCR targeting the haemagglutinin-related protein gene. A primer pair targeting the haemagglutinin-related protein gene (HAEM), which has been reported as...
unique to F. necrophorum subsp. necrophorum (Narongwanichgarn et al., 2003), was designed from the available sequence (accession no. AF529887): HAEMF 5’-CATTTGGGTGATAACGACTCCTAC-3’ (position 1786–1810) and HAEMR 5’-CAATCTTTGTCTAAGATGAGGAAGC-3’ (position 2096–2071). The ideal amplification conditions were experimentally determined on the LightCycler using FastStart DNA Master SYBR-green 1 kit and DNA obtained from the reference strain F. necrophorum subsp. necrophorum NCTC 10576. The PCR reaction was performed in a total volume of 20 µl containing 4 µl DNA extract, 2 µl of the LightCycler SYBRGreen kit, 2-4 µl 25 mM MgCl₂ (4 mM), 0-5 µl each of 20 pmol µl⁻¹ HAEM primers, and 10-6 µl RNase free water.

The PCR LightCycler reaction was carried out as follows: one cycle of denaturation at 95 °C for 10 min followed by 50 cycles of amplification at 95 °C for 0 s, 65 °C for 2 s and 72 °C for 15 s with a single transition rate of 20 °C s⁻¹ and a single fluorescence acquisition at 72 °C. On completion of amplification, a single melt cycle was produced by holding the reaction at 95 °C for 0 s, then at 65 °C for 30 s, followed by slow heating at a transition rate of 0-1 °C s⁻¹ to 95 °C and continuous fluorescence acquisition.

**Statistical analysis.** Statistical analysis was performed using 2 × 2 contingency tables and the Fisher exact test to assess whether frequency differences observed between the two groups were statistically different, employing Analyse-it software version 1.69 (Analyse-It Software; Leeds, UK).

### RESULTS AND DISCUSSION

Throat swabs were collected between March and September 2003 from 100 patients (age range 5 months–79 years, mean 25 years, male: female ratio 42:58) and 100 healthy adults (age range 22–64 years, mean 40 years, male: female ratio 45:55).

The rpoB LightCycler assay was optimized using DNA obtained from the strains F. necrophorum subsp. funduliforme ATCC 51357 and F. necrophorum subsp. necrophorum NCTC 10576 and the limit of detection determined to be 1–10 genome equivalents per reaction, equivalent to 45–450 copies per throat swab (Figs 1a and b). The melting temperature (Tm) of the two rpoB-specific probes from the amplicons was 63-5 °C (data not shown).

Nucleotide sequence analysis from the two strains of F. necrophorum subsp. necrophorum and the type strain of F. necrophorum subsp. funduliforme confirmed the specificity of the PCR. The sequences, which were identical, scored the highest upon a BLAST search analysis, with the F. necrophorum subsp. necrophorum sequence for rpoB (AF529887) displaying just 6 nucleotide changes (Fig. 2). The second highest score was obtained with the rpoB sequence from F. nucleatum subsp. nucleatum (AE010506), displaying a major dissimilarity of 55 nucleotide differences within the target sequence. The specificity of the assay was further supported by the absence of cross-reactivity when the other species of fusobacteria and the commensal and pathogenic oropharyngeal micro-organisms were examined (Table 1); only the type species of F. necrophorum were amplified. Moreover, all 14 clinical patient isolates of F. necrophorum were PCR-positive, while the clinical isolates identified as F. nucleatum and F. varium failed to amplify, as expected.

![Fig. 1.](image)

In the HAEM PCR assay, positive amplification was obtained only for F. necrophorum subsp. necrophorum strains (NCTC 10576 and ATCC 25286), while F. necrophorum subsp. funduliforme and all the other Fusobacterium type species and isolates were PCR-negative. The HAEM PCR assay sensitivity was equivalent to that obtained for the rpoB assay.

The possibility that the Amies charcoal transport medium might contain PCR inhibitors which could affect the performance of the assay was excluded by running a number of spiked experiments. These demonstrated no interference with the PCR for F. necrophorum DNA due to the presence of inhibitors in this transport medium, as an identical detection limit was observed with or without charcoal (data not shown).

In the patients presenting with acute pharyngitis, ten clinical samples were positive for F. necrophorum DNA using the rpoB assay, while all the healthy controls were negative. The extraction and no-template controls included for each batch were negative. All positive results were confirmed at least twice alongside the quantification standards. The bacterial load estimate for each swab is given in Table 2, and represents...
the mean of the three independent results (none of which varied by more than 0.1 log or 1.5-fold) obtained for each sample. A broad range of detection from 8 to 10^4 genome equivalents per reaction was observed for the positive samples. When run on an agarose gel, all 10 samples displayed an amplicon of the expected molecular mass (C24: 276 bp). Seven of the positive samples with the highest bacterial load yielded sufficient nucleic acid to permit sequencing, and the sequences were again identical to those found in the type strains of *F. necrophorum* subspp. *necrophorum* and *funduliforme* (Fig. 2). Since the sequence of *F. necrophorum* subsp. *funduliforme* is exactly the same for this region (Fig. 2), we were unable to identify which subspecies was present in the clinical throat specimens. In order to address this, we subjected the ten rpoB-positive clinical samples to the HAEM LightCycler PCR assay and all were negative, thus confirming their identity as *F. necrophorum* subsp. *funduliforme*.

**Routine culture of clinical throat swabs**

Group A streptococcus was isolated from 16 swabs, Group C streptococcus from three swabs and Group G streptococcus from three swabs. Group A streptococcus was isolated from 16 swabs, Group C streptococcus from three swabs and Group G streptococcus from three swabs. A broad range of detection from 8 to 10^4 genome equivalents per reaction was observed for the positive samples. When run on an agarose gel, all 10 samples displayed an amplicon of the expected molecular mass (C24: 276 bp). Seven of the positive samples with the highest bacterial load yielded sufficient nucleic acid to permit sequencing, and the sequences were again identical to those found in the type strains of *F. necrophorum* subspp. *necrophorum* and *funduliforme* (Fig. 2). Since the sequence of *F. necrophorum* subsp. *funduliforme* is exactly the same for this region (Fig. 2), we were unable to identify which subspecies was present in the clinical throat specimens. In order to address this, we subjected the ten rpoB-positive clinical samples to the HAEM LightCycler PCR assay and all were negative, thus confirming their identity as *F. necrophorum* subsp. *funduliforme*.

**Table 2. Estimated bacterial load of positive clinical throat swabs**

<table>
<thead>
<tr>
<th>No.</th>
<th>Age (y)</th>
<th>No. DNA copies/reaction</th>
<th>No. DNA copies/swab*</th>
<th>Culture result</th>
<th>Clinical details</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>16</td>
<td>10 000</td>
<td>450 000</td>
<td>No growth</td>
<td>Recurrent pharyngitis</td>
</tr>
<tr>
<td>2</td>
<td>21</td>
<td>70</td>
<td>3150</td>
<td>No growth</td>
<td>Recurrent sore throat</td>
</tr>
<tr>
<td>3</td>
<td>30</td>
<td>25 000</td>
<td>1 125 000</td>
<td>No growth</td>
<td>Recurrent sore throat</td>
</tr>
<tr>
<td>4</td>
<td>21</td>
<td>350</td>
<td>15 750</td>
<td>No growth</td>
<td>Pharyngitis</td>
</tr>
<tr>
<td>5</td>
<td>26</td>
<td>250</td>
<td>11 250</td>
<td>No growth</td>
<td>Persistent sore throat</td>
</tr>
<tr>
<td>6</td>
<td>23</td>
<td>80</td>
<td>3600</td>
<td>No growth</td>
<td>Sore throat, on penicillin</td>
</tr>
<tr>
<td>7</td>
<td>21</td>
<td>7000</td>
<td>315 000</td>
<td>Group G strep.</td>
<td>Sore throat</td>
</tr>
<tr>
<td>8</td>
<td>18</td>
<td>8</td>
<td>360</td>
<td>Group A strep.</td>
<td>Sore throat</td>
</tr>
<tr>
<td>9</td>
<td>20</td>
<td>15 000</td>
<td>675 000</td>
<td>No growth</td>
<td>Sore throat, on penicillin</td>
</tr>
<tr>
<td>10</td>
<td>43</td>
<td>12</td>
<td>540</td>
<td>No growth</td>
<td>Sore throat</td>
</tr>
</tbody>
</table>

*Conversion from copies/reaction to copies/swab was achieved by applying the ×45 dilution factor.

**Fig. 2. Alignment of the sequences determined for two *F. necrophorum* subspp. *necrophorum* and *funduliforme* with the published sequence for *F. necrophorum* subsp. *necrophorum*. Dots (.) indicate PCR primers sequences which were not determined in the sequencing analysis. Dashes (-) indicate complete identity with the previously published sequence (AF527637) while asterisks indicates no sequence, highlighting the observed insertion (AT) in our sequence data for both subspecies. Primer and probe sequences are highlighted in bold. The sequences determined herein for *F. necrophorum* subsp. *necrophorum* (NCTC 10576) and *funduliforme* (ATCC 51357^T^) are available in GenBank under accession numbers AY519655 and AY519656.
from two swabs. One swab positive for Group A and one positive for Group G streptococcus was also positive for F. necrophorum-specific DNA (Table 2).

Statistical analysis

Ten clinical samples were positive for F. necrophorum-specific DNA using the rpoB assay while all the healthy controls were negative. This represents a statistically significant finding (two-tailed P value = 0.0015). The result is still significant when the two samples positive for F. necrophorum-specific DNA but also positive for β-haemolytic streptococci are excluded (two-tailed P value = 0.0068).

This is the first study to report the prevalence of F. necrophorum in both community-acquired sore throat and as a member of the normal throat flora. This study also confirms the role of F. necrophorum subsp. funduliforme as the main subspecies recovered in human infection. Our results question the current dual assertions that F. necrophorum is a usual member of the normal human throat flora and that it is not a primary pathogen at this site (Smith & Thornton, 1993; Tan et al., 1996; Sinave et al., 1989).

Although recognized as a cause of a life-threatening illness starting with a sore throat, F. necrophorum has not been considered an important primary pathogen in the throat, and is rarely mentioned in literature on the subject (Bourbeau, 2003). In this study, however, we detected F. necrophorum in 10% of symptomatic patients, a frequency that was second only to Group A streptococcus. Group A streptococcus is considered to be the leading bacterial agent of sore throat in the community, recovered from up to 10% of adult and 30% of paediatric cases (Bisno, 2001), but our findings suggest that F. necrophorum may occupy just as prominent a role that has hitherto been overlooked. We did not detect F. necrophorum in healthy subjects and this supports the contention that all the isolates recovered from patients were playing a primary pathogenic role in the throat infection. Acute pharyngitis is one of the commonest illnesses seen by GPs, and sensitive technique in the examination of throat swabs for the presence of F. necrophorum and sensitive technique in the examination of throat swabs for the presence of F. necrophorum may be extremely uncommon in the UK (Brazier, 2002).

This study indicates that F. necrophorum has an important role in causing sore throat in the community. Further studies are required to confirm this suspicion. Completing the classical Koch’s postulates for proof of pathogenicity, by generating a sore throat in human volunteers, would present ethical problems; generating pharyngitis by inoculating experimental animals could be helpful, but the fact that a different subspecies is implicated in animal infection (Beeren et al., 1971; Smith & Thornton, 1993) may mean that an animal model studying human strains, possibly less capable of causing disease in animals (Brazier, 2002), may be misleading. The fact that we usually did not detect F. necrophorum with important bacterial pathogens strengthens the suspicion of a primary pathogenic role, but we did not test for the presence of all possible throat pathogens, for example, for Epstein–Barr virus, which has been linked anecdotally with Lemierre’s disease (Dagan & Powell, 1987; Alvarez & Schreiber, 1995). A further case-control study could address this issue. The ages of cases and controls were not perfectly matched in the current study and future studies should match ages more closely than was possible here. A placebo-controlled evaluation of the efficacy of a specific anti-anaerobic antibiotic, such as metronidazole, in patients with pharyngitis positive for F. necrophorum in throat swabs could also provide supportive evidence for a pathogenic role, as this antibiotic has no activity against the other recognized bacterial pathogens, which are aerobic. The information obtained from such studies will inform the debate on empirical antibiotic prescription for sore throat and offer the possibility of effective treatment for an apparently important cause of pharyngitis, with the opportunity to reduce the incidence of a life-threatening invasive disease through early diagnosis and treatment of the initial stage of infection in the throat.

In conclusion, this PCR-based approach proved to be a simple and sensitive technique in the examination of throat swabs for the presence of F. necrophorum. This organism appears to be an important cause of community-acquired sore throat. Further studies will be required to confirm this and establish the epidemiology of infection with this organism.

REFERENCES


Baddour, L. M., Land, M. A., Barrett, F. F., Rivara, F. P., Bruce, W. M. &


