Human *Fusobacterium necrophorum* strains have a leukotoxin gene and exhibit leukotoxic activity

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### INTRODUCTION

*Fusobacterium necrophorum*, a Gram-negative anaerobe, causes a variety of necrotic infections in humans and animals. There are two subspecies: subsp. *necrophorum* and subsp. *funduliforme*. In cattle, subsp. *necrophorum* is more prevalent and production of leukotoxin is a major virulence factor. The leukotoxin operon (*lktBAC*) consists of three genes, *lktB*, *lktA* and *lktC*, of which *lktA* is the structural toxin gene. The subspecies identity of human *F. necrophorum* is less certain and it is not known whether human strains possess the leukotoxin gene or leukotoxin activity. Therefore, the objective of this study was to identify the subspecies status of four human clinical strains of *F. necrophorum* and determine whether they have the leukotoxin gene or leukotoxin activity. Phenotypic and genotypic characteristics suggested that the four strains belonged to subsp. *funduliforme*, which was confirmed by sequencing the 16S rRNA gene. Analysis of the four strains by PCR revealed the presence of the leukotoxin operon. Partial DNA sequencing identified one human strain with full-length *lktA*, whereas the others exhibited considerable heterogeneity in size. All strains had a leukotoxin operon promoter-containing intergenic region similar to that of bovine subsp. *funduliforme* strains, which was confirmed by DNA sequencing and Southern blotting. Despite variations in the *lktA* gene, all strains secreted leukotoxin as demonstrated by Western blotting. Flow cytometry assays revealed that the leukotoxin was toxic to human white blood cells. In conclusion, the human strains examined contained a leukotoxin gene whose gene product was biologically active. The importance of leukotoxin as a virulence factor in human fusobacterial infections needs further evaluation.

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Abbreviations: PMN, polymorphonuclear leukocyte; PRAS, pre-reduced anaerobically sterilized.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of the human *Fusobacterium necrophorum* strains RMA10682 and RMA16505 reported in this paper are EF447426 and EF447427, respectively.
Historically, *F. necrophorum* has been shown to be associated with Lemierre’s syndrome in humans. However, recent studies indicate that it may also play an important role in persistent sore throat cases (Aliyu et al., 2004; Batty et al., 2005). Using real-time PCR methods, it has been reported that *F. necrophorum* strains isolated from persistent sore throat cases resembled subsp. *funduliforme*. However, in a recent study, Jensen et al. (2007) reported that *F. necrophorum* subsp. *funduliforme* is part of the normal flora of human tonsils. In view of these observations, it is essential to confirm the subspecies status of human strains by 16S rRNA gene sequencing.

In bovine *F. necrophorum*, production of leukotoxin is a major virulence factor (Narayanan et al., 1999a; Nagaraja et al., 2005). Leukotoxin is a large secreted protein that is cytotoxic to neutrophils and to a lesser extent to lymphocytes (Narayanan et al., 2001, 2002b). The leukotoxin operon (*lktBAC*) consists of three genes: *lktB*, *lktA* and *lktC*, of which *lktA* is the structural gene of leukotoxin (Narayanan et al., 2001; Oelke et al., 2005). The leukotoxin operon promoter-containing intergenic region varies in size and nucleotide sequence between the two subspecies (Zhang et al., 2006). Virulence factors implicated for human strains include the production of endotoxin and haemolysin (Brazier, 2006). Previous studies have shown that LPS from human strains resembles that of animal subsp. *funduliforme* in composition (being rich in amino sugars) (Brown et al., 1997). Studies to determine the biological activity of LPS have revealed that it is less toxic to rabbits (Hofstad & Kristoffersen, 1971). It is not known whether human *F. necrophorum* isolates have the *lktA* gene or leukotoxin activity. Our objectives were: (i) to subspeciate the human *F. necrophorum* strains based on their phenotypic and genotypic characteristics, and (ii) to determine whether human isolates contain the *lktBAC* operon and exhibit leukotoxic activity.

**METHODS**

*F. necrophorum* strains and subspeciation. Four human clinical isolates (RMA10682, RMA14786, RMA16505 and RMA16539) of *F. necrophorum* (kindly provided by Dr Diane Citron, R. M. Alden Research Laboratory, Santa Monica, CA, USA) were confirmed by growth, morphological and biochemical methods (Tan et al., 1994); the strains were isolated from a liver abscess, tonsil biopsy, tonsil swab and neck wound, respectively. Two bovine strains, *F. necrophorum* subsp. *necrophorum* A25 and subsp. *funduliforme* B35, previously isolated from bovine liver abscesses (Lechtenberg et al., 1988), were also included in the study. Isolates were grown overnight on blood agar (Remel) at 39 °C in an anaerobic glove box (Forma Scientific) and subcultured in pre-reduced anaerobically sterilized (PRAS) brain heart infusion (BHI) broth (Tan et al., 1992). Identification as *F. necrophorum* and subspeciation of the human strains were performed using a RapID ANA II system (Remel) and by PCR amplification of the *rpoB* gene (RNA polymerase β-subunit; Aliyu et al., 2004), haemagglutinin gene (Aliyu et al., 2004) and the leukotoxin operon promoter-containing intergenic region (Zhang et al., 2006). The partial 16S RNA gene sequences of two human strains (RMA10682 and RMA16505), representative of the four, were determined using specific 16S forward and reverse primers (Table 1) that were designed based on the complete 16S RNA gene sequence (GenBank accession no. AJ867038) of bovine *F. necrophorum* subsp. *necrophorum*. The obtained sequences were then aligned with the 16S rRNA gene sequences of 12 other species of the genus *Fusobacterium* using CLUSTAL_X (opening gap penalty of 10 and extension penalty of 0.1), and a phylogenetic tree (Dorsch et al., 2001) was constructed using a distance matrix algorithm and the neighbour-joining method using PAUP for Windows (Sinauer Associates).

**Primers and PCR conditions.** Specific primers targeting the *rpoB* and haemagglutinin genes have been designed previously (Aliyu et al., 2004). All primers used in this study are listed in Table 1. The leukotoxin operon promoter-containing intergenic regions in subsp. *necrophorum* and subsp. *funduliforme* were amplified using Ex Taq (Takara Bio) as described previously (Zhang et al., 2006). The PCR-amplified products were electrophoresed in 1–1.2 % agarose gels, purified and sequenced using a Beckman Coulter CEQ 8000 genetic analysis system.

**Isolation of chromosomal DNA.** Bacterial chromosomal DNA was prepared according to a procedure described previously (Narayanan et al., 2001). Briefly, isolates were grown overnight in anaerobic BHI broth. The cells were then pelleted by centrifugation at 5000 g for 10 min at 4 °C. The pelleted cells were washed and resuspended in 100 mM Tris/HCl (pH 8.0), 10 mM EDTA. The cell wall was digested with lysozyme (1 mg ml⁻¹) and the resulting lysate was treated with 1 % Sarkosyl followed by RNase A (20 μg ml⁻¹) and Pronase (50 μg ml⁻¹). The samples were incubated on ice for 20 min, and then sequentially extracted twice with phenol and chloroform. The DNA from the extract was precipitated in 0.1 vol. 3 M sodium acetate (pH 5.2) and an equal volume of 2-propanol. The DNA was resuspended in 10 mM Tris/HCl (pH 8.0), 1 mM EDTA and stored at 4 °C until use. The concentration of extracted DNA was determined spectrophotometrically (Nanodrop Technologies).

**Southern blot hybridization.** Southern blot hybridization was performed according to a procedure described previously (Narayanan et al., 2001). Briefly, 3 μg chromosomal DNA was digested to completion using *Hae*III (Promega) and electrophoresed in a 1 % agarose gel overnight at 20 V at room temperature. The resolved and denatured DNA fragments were transferred to a positively charged nylon membrane (Roche Diagnostics) by a capillary mechanism, and the DNA immobilized by UV cross-linking. DNA probes were synthesized by random-labelling with DIG–dUTP (Roche Diagnostics) following the manufacturer’s directions. The immobilized DNA was probed with cloned full-length bovine subsp. *necrophorum* A25 *lktA* at 54 °C (Narayanan et al., 2001), whilst the PCR fragments (produced in this study) of subsp. *funduliforme* B35 leukotoxin operon promoter-containing intergenic region (at 42 °C), subsp. *funduliforme* B35 *lktB*, subsp. *necrophorum* A25 *lktB* (at 50 °C) or subsp. *necrophorum* A25 *lktC* (at 44 °C) were used to probe the membrane. Colorimetric detection of hybridized signals was carried out following the manufacturer’s instructions (Roche Diagnostics).

**Preparation of culture supernatants.** Bovine and human strains of *F. necrophorum* were grown in PRAS BHI to an OD₆₀₀ value of 0.6–0.7 and pelleted by centrifugation at 5000 g for 20 min at 4 °C. The supernatant was collected, filtered through a 0.22 μm filter (Millipore) and concentrated 60-fold with 100 kDa molecular mass cut-off filters (Millipore). Aliquots were stored at −80 °C until use.

**Western blotting.** The concentrated culture supernatants from all strains were resolved by SDS-PAGE in 4–20 % Tris/glycine polyacrylamide gels (Pierce Biotechnology). Proteins were then electro-transferred onto nitrocellulose membranes (Millipore). The membranes were blocked with 0.2 % skimmed milk overnight at
Table 1. Primers used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’→3’)</th>
<th>Reference</th>
</tr>
</thead>
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<tr>
<td>5’lktpXmXh</td>
<td>ttccgggtgcgagGAATCTTTTAAAGCAC</td>
<td>Zhang et al. (2006)</td>
</tr>
<tr>
<td>3’lktpXm</td>
<td>tttcgggATCAATTCCCTGAATTATTTAT*</td>
<td>Zhang et al. (2006)</td>
</tr>
<tr>
<td>Fund5p</td>
<td>CTCAATTTTTGTGGAGAGCAG</td>
<td>Zhang et al. (2006)</td>
</tr>
<tr>
<td>Fund3p</td>
<td>CATTATGAATAACATATTCTCAC</td>
<td>Zhang et al. (2006)</td>
</tr>
<tr>
<td>UPS-START</td>
<td>ATCAATGAGCCGGAGAAAG</td>
<td>Narayanan et al. (2001)</td>
</tr>
<tr>
<td>UPS-END</td>
<td>CCCCCTAAAATTCATGATTTTATG</td>
<td>Narayanan et al. (2001)</td>
</tr>
<tr>
<td>BSBSE-START</td>
<td>AATTAGYGGCGATCAAAT</td>
<td>This study</td>
</tr>
<tr>
<td>BSBSE-END</td>
<td>TCCATCGCTGTTCAARAGGYCAT</td>
<td>This study</td>
</tr>
<tr>
<td>SX-START</td>
<td>ATTAGAATTTTTAAAGAGAGCTT</td>
<td>This study</td>
</tr>
<tr>
<td>SX-END</td>
<td>CTGTGCTTCACCTTTCTTTAA</td>
<td>This study</td>
</tr>
<tr>
<td>GAS-START</td>
<td>GCCCTCTGAAGAGTTTCC</td>
<td>This study</td>
</tr>
<tr>
<td>GAS-END</td>
<td>CTATTTTTATATGIGC</td>
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</tr>
<tr>
<td>GAS-Downstream</td>
<td>GTGCTTTCGGAGGACGAG</td>
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<tr>
<td>FINAL-Upstream</td>
<td>AACCACGATAGAAGTG</td>
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</tr>
<tr>
<td>FINAL-END</td>
<td>TTTTTCCTTTTTCC</td>
<td>This study</td>
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<td>RpoB forward</td>
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</tr>
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<td>RpoB reverse</td>
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<td>Aliyu et al. (2004)</td>
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<tr>
<td>16S forward</td>
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</tr>
<tr>
<td>16S reverse</td>
<td>CTTCAGGTGCACACCTC</td>
<td>This study</td>
</tr>
</tbody>
</table>

*Lower-case letters represent restriction endonuclease sites incorporated into the primers for ease of cloning.

4 °C. Rabbit polyclonal antiserum raised against strain A25 native leukotoxin (Narayanan et al., 2001) was used to probe the membrane. Goat anti-rabbit immunoglobulin conjugated to alkaline phosphatase (Sigma-Aldrich) was used as the secondary antibody, and the immunoreactive proteins were detected using 5-bromo-4-chloro-3-indolylphosphate/nitro blue tetrazolium as the substrate.

Preparation of white blood cells. Polymorphonuclear leukocytes (PMNs) from peripheral blood were prepared as described previously (Tan et al., 1992). Briefly, bovine whole blood was collected from healthy cattle by venipuncture of the internal jugular vein. The collected blood was transported on ice, centrifuged at 700 g for 10 min at 4 °C and the buffy coat removed to another sterile tube. The residual erythrocytes were subjected to osmotic lysis by sterile distilled water. Purified leukocytes were resuspended in complete RPMI 1640 containing 5% fetal bovine serum, 100 IU penicillin l⁻¹ and 100 mg streptomycin l⁻¹ (Sigma-Aldrich). Human whole blood was collected by venipuncture from a healthy donor, erythrocytes were lysed with 6 vols RBC lysis buffer (Sigma-Aldrich) at room temperature for 5–10 min and the purified leukocytes were resuspended in complete RPMI 1640 (Oelke et al., 2005). The final concentration of viable cells was determined by a 0.4% trypan blue dye exclusion assay (Narayanan et al., 2002b).

Cell viability assay. The cytotoxic effects of F. necrophorum culture supernatants were determined by cell viability assays using propidium iodide (Narayanan et al., 2002b). Briefly, 1×10⁶ viable white blood cells were treated with culture supernatants for 45 min at 37 °C and 5% CO₂, washed twice and resuspended in 0.01 M PBS, and stained with 10 µl propidium iodide (50 µg ml⁻¹) stock in the dark for 5 min. White blood cells in complete RPMI 1640 treated similarly were used as a negative control. The samples were processed on a FACScam flow cytometer using an Argon ion laser (Becton Dickinson). Data were analysed using Cell Quest analysis software (Becton Dickinson).

RESULTS AND DISCUSSION

Subspeciation of F. necrophorum isolates

We employed growth, morphological and molecular methods to subspeciate the human strains of F. necrophorum. All four human strains were Gram-negative short rods, formed a flocculent button after overnight growth in PRAS BHI broth and were negative for alkaline phosphatase activity based on the RapID ANA II test. These results were indicative of the four strains belonging to subsp. funduliforme (Amoako et al., 1993; Tan et al., 1994). The rpoB gene (Fig. 1a) specific for F. necrophorum (Aliyu et al., 2004) was present in all four strains; however, all were PCR-negative for the haemagglutinin gene (Fig. 1b). The absence of the haemagglutinin gene was consistent with their subsp. funduliforme classification (Narongwanichgarn et al., 2003).

In cattle, the two subspecies of F. necrophorum can easily be distinguished by their growth, morphological and biochemical characteristics (Nagaraja et al., 2005). Previous studies to determine the subspecies of F. necrophorum human strains were inconclusive. Smith & Thornton (1993,
1997) attempted to classify human strains based on their pathogenic effects in mice. The pathogenic effects of most human strains resembled subsp. funduliforme, producing mild, localized lesions in mice, and none of them behaved like subsp. necrophorum. Hall et al. (1997) reported that human strains were clearly distinct from subsp. necrophorum of animal origin based on phenotypic reactions, pyrolysis MS and SDS-PAGE analysis, but similarity with subsp. funduliforme was less certain.

PCR amplification of the leukotoxin promoter-containing intergenic region of the human isolates revealed a subsp. funduliforme-type product (Fig. 1c). Results from Southern blot hybridization of HaeIII-digested genomic DNA probed with the DIG-labelled subsp. funduliforme leukotoxin operon promoter-containing intergenic region revealed that each of the human strains possessed a subsp. funduliforme-type promoter-containing intergenic region (Fig. 2a) and no signal was detected when probed with the labelled subsp. necrophorum promoter-containing intergenic region. Whilst the band size for strain RMA14786 was similar to that of bovine subsp. funduliforme (strain B35), strains RMA10682, RMA16505 and RMA16539 had smaller-sized bands. Nucleotide sequence analyses of the leukotoxin promoter-containing intergenic regions were identical to the subsp. funduliforme leukotoxin promoter-containing intergenic region (data not shown). Additionally, the aligned 16S rRNA gene sequences of the two strains were identical to each other and had greater identity (99%) to subsp. funduliforme (data not shown). Therefore, the four human strains of F. necrophorum belonged to subsp. funduliforme.

**lktA gene**

The presence of the lktA gene was determined by digesting the genomic DNA to completion with HaeIII and probing with the subsp. necrophorum lktA in a Southern blot hybridization reaction (Fig. 2c). Genomic DNA digestion from subsp. necrophorum produced two hybridizing bands (~11 and ~10 kb) in Southern blot hybridizations, whereas subsp. funduliforme DNA yielded three bands (~9 kb, ~8 kb and a faint band of 375 bp). The bovine subsp. necrophorum lktA has a single recognition site (GenBank accession no. AF312861) for enzyme HaeIII, whereas subsp. funduliforme has two HaeIII sites (GenBank accession no. AY972049). Among the human strains, two unique hybridization patterns were observed. Strains RMA10682, RMA16505 and RMA16539 gave rise to a single strongly hybridizing band that varied in size from 8 to 9 kb, and a faintly hybridizing larger 10 kb band. Strain RMA14786 DNA gave rise to a doublet similar in size to that of subsp. funduliforme, but a larger hybridizing band similar to that observed in other human strains was also evident.

Thus, data from our Southern blot hybridization studies suggested that the strain RMA 14786 leukotoxin structural gene lktA may be more similar to subsp. funduliforme lktA, whereas, the lktA gene of the other three strains indicated fewer similarities to either subspecies. Instead of the doublet, a single band was observed in these three strains. This may be due to the absence of a recognition site for HaeIII in the proximity of the lktA gene in their genome. Hence, a single band of high molecular mass was observed in strains RMA16505 and RMA16539, and a distinct band equivalent to the lower-sized band in subsp. funduliforme was observed in strain RMA10682.

**lktC gene**

The lktC gene was identified by PCR using primers common to both subspecies. Sequencing of the PCR-amplified product revealed the presence of lktC in all four
human strains. The sequencing results were further confirmed by Southern blot hybridization with a subsp. necrophorum lktC probe (Fig. 2d). The positive controls, bovine subsp. necrophorum and subsp. funduliforme, had the expected band sizes (~10 and ~7 kb, respectively). Two hybridization patterns were observed among the human strains. The first pattern (a band of ~1.5 kb) observed in strains RMA10682, RMA16505 and RMA16539 was different from either of the two bovine subspecies. The second pattern (a band of ~6.0 kb) was present only in strain RMA14786 and resembled the hybridization pattern of bovine subsp. funduliforme. The partial lktC nucleotide sequence of strain RMA14786 was similar (95%) to the lktC sequence of both bovine subspecies. The lktC sequences of strains RMA10682, RMA16505 and RMA16539 resembled each other but were different from the two bovine subspecies. The biological function of the LktC protein has yet to be determined.

Western blotting

The leukotoxin from F. necrophorum is highly unstable and breaks down into multiple products under denaturing conditions (Tan et al., 1994; Fig. 3). Culture supernatant from subsp. necrophorum had all the expected intense bands (~250, 150, 130 and 110 kDa) (Fig. 3, lane 1), whereas the supernatant from subsp. funduliforme produced less-intense bands (Fig. 3, lane 2). Two patterns of hybridization were observed in the culture supernatants of human clinical isolates. The first hybridization pattern seen in the culture supernatant of strain RMA14786 resembled that of subsp. funduliforme with fewer bands and a weaker

Fig. 2. Southern blot of Haelll-digested genomic DNA hybridized with the DIG-labelled F. necrophorum subsp. funduliforme leukotoxin operon promoter-containing intergenic region (a), or with the F. necrophorum subsp. funduliforme lktB probe (b), lktA probe (c) or lktC probe (d). Lanes: M, DIG-labelled marker; 1, bovine F. necrophorum subsp. necrophorum strain A25; 2, bovine F. necrophorum subsp. funduliforme strain B35; 3–6, human F. necrophorum strains RMA10682, RMA14786, RMA16505 and RMA16539, respectively.
affinity. The other three strains gave rise to variably intense bands of 150 and 40 kDa, which were similar to each other but distinct from that of subsp. *necrophorum*.

### Leukotoxin activity

Viability assays using human peripheral PMNs indicated that all of the culture supernatants from the human strains were toxic (Fig. 4); subsp. *necrophorum* and *funduliforme* culture supernatants were also toxic to human PMNs. In order to confirm that the observed toxicities were not due to contaminating LPS, we tested the toxic effects of the culture supernatants after passing them over a polymyxin B column. No significant decrease in toxic activity was observed (data not shown). Similar strain-to-strain variations in the cytotoxic effects of leukotoxin among the bovine strains of *F. necrophorum* have been reported previously (Tan et al., 1992). Significant decreases in leukotoxin activity were observed when culture supernatants were pre-treated with the proteolytic enzymes trypsin, chymotrypsin and protease (Coyle-Dennis & Lauerman, 1978, 1979; Tan et al., 1994). Hence, the differences in cytotoxic activity among human strains may be related to the various proteolytic enzyme(s) secreted by individual strains, or to yet-to-be identified factors.

### Conclusions

In conclusion, our study showed that the four human strains belonged to subsp. *funduliforme* and contained the leukotoxin operon *lktBAC*. The secreted leukotoxin was toxic to human PMNs. It is possible that leukotoxin may be an important virulence factor and may have a role in human *F. necrophorum* infections. Further research on the role of the leukotoxin of *F. necrophorum* is warranted.

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### REFERENCES


