

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

Sambasivarao Tadepalli,^{1†} George C. Stewart,² T. G. Nagaraja¹
and Sanjeev K. Narayanan¹

Correspondence
Sanjeev K. Narayanan
sanjeev@vet.k-state.edu

¹Department of Diagnostic Medicine and Pathobiology, Kansas State University, Manhattan, KS 66506-5606, USA

²Department of Veterinary Pathobiology and Bond Life Sciences Center, University of Missouri, Columbia, MO 65211, USA

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.

Received 29 August 2007

Accepted 19 October 2007

INTRODUCTION

Fusobacterium necrophorum, a Gram-negative, rod-shaped obligate anaerobe, is a normal inhabitant of the gastrointestinal, respiratory and genitourinary tracts of animals and humans (Hagelskjaer & Prag, 2000; Nagaraja *et al.*, 2005), and is associated with a variety of necrotic infections (Brazier *et al.*, 2002; Navas *et al.*, 1994; Reasonable *et al.*, 2003; Tan *et al.*, 1996). In cattle, the organism is the primary causative agent of calf diphtheria, liver abscesses and foot rot (Nagaraja & Chengappa, 1998; Nagaraja *et al.*, 2005; Tan *et al.*, 1996). In humans, *F. necrophorum*

primarily causes acute pharyngitis, thrombophlebitis and abscessation of the internal jugular vein, termed Lemierre's syndrome (Brazier, 2006; Hagelskjaer & Prag, 2000; Navas *et al.*, 1994; Riordan & Wilson, 2004).

F. necrophorum has two major subspecies: subsp. *necrophorum* and subsp. *funduliforme* (Shinjo *et al.*, 1991). In cattle, subsp. *necrophorum* is the most prevalent subspecies associated with infections and these strains generally produce significantly higher levels of leukotoxin than strains of subsp. *funduliforme* isolated from animals (Nagaraja *et al.*, 2005; Scanlan *et al.*, 1982; Tan *et al.*, 1992). Based on biochemical tests, SDS-PAGE and pyrolysis MS, Hall *et al.* (1997) found that some human strains resembled animal subsp. *funduliforme*, whilst others resembled neither subspecies. Smith & Thornton (1997) also reported similar observations in their studies determining the pathogenic effects of animal and human strains in mice. Therefore, the synonymy of human strains with animal subsp. *funduliforme* cannot be established in the absence of 16S rRNA gene sequence data (Smith & Thornton, 1997).

[†]Present address: Novartis Animal Health US, Inc., Larchwood, IA 51241, USA.

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.*, 2002a; 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 rRNA 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 rRNA 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 μ g ml⁻¹) and Pronase (50 μ g 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 μ g 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 μ m 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 electrotransferred onto nitrocellulose membranes (Millipore). The membranes were blocked with 0.2% skimmed milk overnight at

Table 1. Primers used in this study

Primer	Sequence (5'→3')	Reference
5'lktpXmXh	tctccgggctcgagGAAATCTTTAAAGCAC*	Zhang <i>et al.</i> (2006)
3'lktpXm	tctccgggCATAATTTCTCCCAATTTTATT*	Zhang <i>et al.</i> (2006)
Fund5p	CTCAATTTTTGTGGAAGCGAG	Zhang <i>et al.</i> (2006)
Fund3p	CATTATCAAAATAACATATTTCTCAC	Zhang <i>et al.</i> (2006)
UPS-START	ATCAATATGGCTCCGGAAAAAG	Narayanan <i>et al.</i> (2001)
UPS-END	CCCTTTAAAATTCATGATTTATTG	Narayanan <i>et al.</i> (2001)
BSBSE-START	AAATGAGYGGCATCAAAAAT	This study
BSBSE-END	TCCATCTGCTTCCAARACYGCAT	This study
SX-START	ATTAGAACTTTTAAAGAGAGCTT	This study
SX-END	CTTGCTCCTCCACTTTCTTTAA	This study
GAS-START	GCTTCTGGAAGTGTTTC	This study
GAS-END	CTATTTTTTATATGTGC	This study
GAS-Downstream	GTGCTTTCCGGAGCGAG	This study
FINAL-Upstream	AACCACAGTAGAAGTG	This study
FINAL-START	TAAAGCCATTGTGAAG	This study
FINAL-END	TTTTCCCTTTTCTCC	This study
RpoB forward	TCTCTACGTATGCCTCACGGATC	Aliyu <i>et al.</i> (2004)
RpoB reverse	CGATATTCATCCGAGAGGGTCTCC	Aliyu <i>et al.</i> (2004)
Haem forward	CATTGGGTTGGATAACGACTCCTAC	Aliyu <i>et al.</i> (2004)
Haem reverse	CAATTCCTTGTCTAAGATGGAAGCGG	Aliyu <i>et al.</i> (2004)
LktC forward	TGTTTTGAAAGAGAAGAAGTAAAAGA	This study
LktC reverse	TTTTGTAATATGCTCCAAGATCACA	This study
16S forward	CGCGTAAAGAACTTGCCTCT	This study
16S reverse	CTTCAGGTGCAACCCACTCT	This study

*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

FACScan 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,

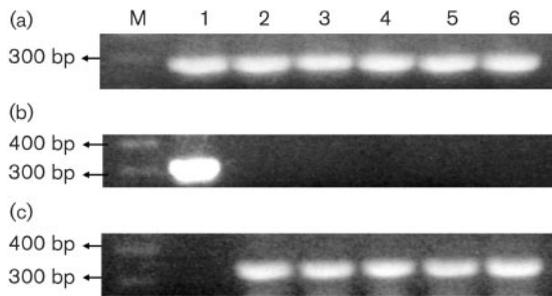


Fig. 1. PCR amplification of the *rpoB* (RNA polymerase β subunit) gene (a), the haemagglutinin gene (b) and the leukotoxin operon promoter-containing intergenic region (c) from four human and two bovine strains of *F. necrophorum*. Lanes: M, 100 bp DNA ladder; 1, bovine *F. necrophorum* subsp. *necrophorum* strain A25; 2, bovine *F. necrophorum* subsp. *funduliforme* strain B35; 3–6, human *F. necrophorum* strains RMA10682, RMA14786, RMA16505 and RMA16539, respectively.

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*.

lktB gene

PCR analysis of human strains using primers of sequences conserved in the *lktB* gene yielded products of the expected

size from all four strains. Nucleotide sequence analyses revealed greater sequence identity to subsp. *funduliforme lktB* than to subsp. *necrophorum lktB*. Southern blot hybridization with the subsp. *necrophorum lktB* probe resulted in a weak signal (data not shown) in comparison with the subsp. *funduliforme lktB* probe (Fig. 2b). Among the four human strains, there were differences in the size and intensity of the signals. Strains RMA14786 and B35 had similar-sized bands (~8.0 kb), whereas subsp. *necrophorum lktB* had a faintly hybridizing band of ~10.0 kb. Strains RMA10682, RMA16505 and RMA16539 had a smaller band of ~5.1 kb. A BLAST search of the partial *lktB* sequence of the human strains revealed 90–96% identity with the bovine subsp. *funduliforme lktB* and 80–86% identity with subsp. *necrophorum lktB*. The biological function of the LktB protein has not yet been determined.

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

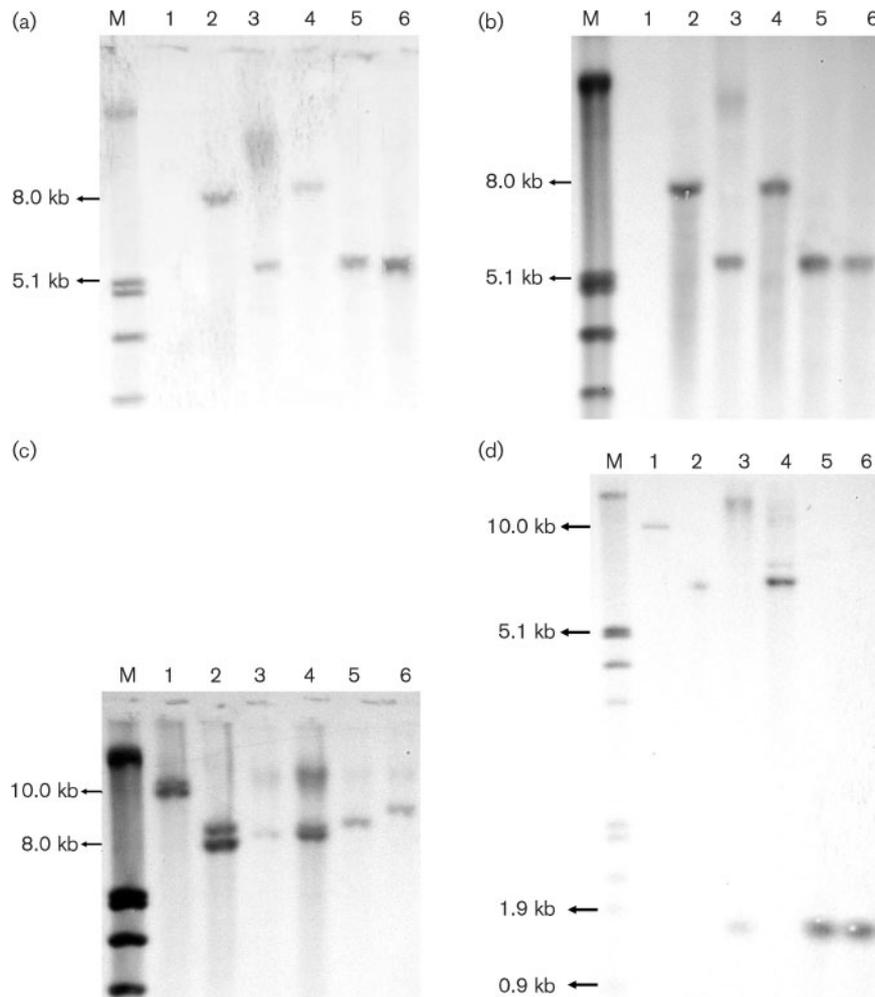


Fig. 2. Southern blot of *HaellI*-digested genomic DNA hybridized with the DIG-labelled *F. necrophorum* subsp. *funduliforme* leukotoxin operon promoter-containing intergenic region (a), or with the *F. necrophorum* subsp. *funduliforme* *lktB* probe (b), *lktA* probe (c) or *lktC* probe (d). Lanes: 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.

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

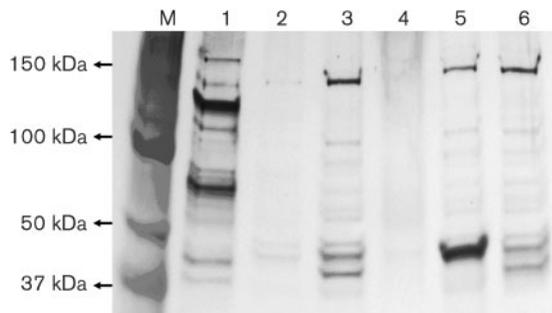


Fig. 3. Western blot of concentrated culture supernatants probed with rabbit polyclonal serum raised against the LktA protein from bovine *F. necrophorum* subsp. *necrophorum*. Lanes: M, pre-stained protein marker; 1, bovine *F. necrophorum* subsp. *necrophorum* strain A25; 2, bovine *F. necrophorum* subsp. *funduliforme* strain B35; 3–6, human 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

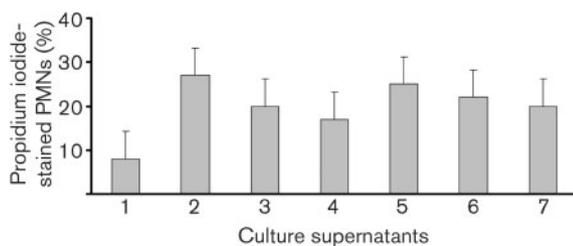


Fig. 4. Cell viability assay. Human PMNs were treated with culture supernatants at 37 °C and 5% CO₂ for 45 min, washed and then incubated with propidium iodide. PMNs were analysed by flow cytometry and 10 000 events were recorded. Culture supernatants: 1, RPMI 1640 only (no toxin); 2, bovine *F. necrophorum* subsp. *necrophorum* strain A25; 3, bovine *F. necrophorum* subsp. *funduliforme* strain B35; 4–7, human *F. necrophorum* strains RMA10682, RMA14786, RMA16505 and RMA16539, respectively. Results are shown as means ± SD of three independent assays.

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.

ACKNOWLEDGEMENTS

We thank David George for DNA sequencing, Tammy Koopman for assistance with the flow cytometer and Ashvin Nagaraja for help in the laboratory. This paper is contribution no.08-00-j from the Kansas Agricultural Experiment Station.

REFERENCES

- Aliyu, S. H., Marriott, R. K., Curran, M. D., Parmar, S., Bentley, N., Brown, N. M., Brazier, J. S. & Ludlam, H. (2004). Real-time PCR investigation into the importance of *Fusobacterium necrophorum* as a cause of acute pharyngitis in general practice. *J Med Microbiol* **53**, 1029–1035.
- Amoako, K. K., Goto, Y. & Shinjo, T. (1993). Comparison of extracellular enzyme of *Fusobacterium necrophorum* subsp. *necrophorum* and *Fusobacterium necrophorum* subsp. *funduliforme*. *J Clin Microbiol* **31**, 2244–2247.
- Batty, A., Wren, M. W. D. & Gal, M. (2005). *Fusobacterium necrophorum* as the cause of recurrent sore throat: comparison of isolates from persistent sore throat syndrome and Lemierre's disease. *J Infect* **51**, 299–306.
- Brazier, J. S. (2006). Human infections with *Fusobacterium necrophorum*. *Anaerobe* **12**, 165–172.
- Brazier, J. S., Hall, V., Yusuf, E. & Duerden, B. I. (2002). *Fusobacterium necrophorum* infections in England and Wales 1990–2000. *J Med Microbiol* **51**, 269–272.
- Brown, R., Lough, H. G. & Poxton, I. R. (1997). Phenotypic characteristics and lipopolysaccharides of human and animal isolates of *Fusobacterium necrophorum*. *J Med Microbiol* **46**, 873–878.
- Coyle-Dennis, J. E. & Lauerman, L. H. (1978). Biological and biochemical characteristics of *Fusobacterium necrophorum* leukotoxin. *Am J Vet Res* **39**, 1790–1793.
- Coyle-Dennis, J. E. & Lauerman, L. H. (1979). Correlations between leukocidin production and virulence of two isolates of *Fusobacterium necrophorum*. *Am J Vet Res* **40**, 274–276.
- Dorsch, M., Love, D. N. & Bailey, G. D. (2001). *Fusobacterium equinum* sp. nov., from the oral cavity of horses. *Int J Syst Evol Microbiol* **51**, 1959–1963.
- Hagelskjaer, K. L. & Prag, J. (2000). Human necrobacillosis, with emphasis on Lemierre's syndrome. *Clin Infect Dis* **31**, 524–532.

- Hall, V., Duerden, B. I., Magee, J. T., Ryley, H. C. & Brazier, J. S. (1997). A comparative study of *Fusobacterium necrophorum* strains from human and animal sources by phenotypic reactions, pyrolysis mass spectrometry and SDS-PAGE. *J Med Microbiol* **46**, 865–871.
- Hofstad, T. & Kristoffersen, T. (1971). Preparation and chemical characteristics of endotoxic lipopolysaccharide from three strains of *Sphaerophorus necrophorus*. *Acta Pathol Microbiol Scand B Microbiol Immunol* **79**, 385–390.
- Jensen, A., Hagelskjaer Kristensen, L. & Prag, J. (2007). Detection of *Fusobacterium necrophorum* subsp. *funduliforme* in tonsillitis in young adults by real-time PCR. *Clin Microbiol Infect* **13**, 695–701.
- Lechtenberg, K. F., Nagaraja, T. G., Leipold, H. W. & Chengappa, M. M. (1988). Bacteriological and histological studies of hepatic abscesses in cattle. *Am J Vet Res* **49**, 58–62.
- Nagaraja, T. G. & Chengappa, M. M. (1998). Liver abscesses in feedlot cattle: a review. *J Anim Sci* **76**, 287–298.
- Nagaraja, T. G., Narayanan, S. K., Stewart, G. C. & Chengappa, M. M. (2005). *Fusobacterium necrophorum* infections in animals: pathogenesis and pathogenic mechanisms. *Anaerobe* **11**, 239–246.
- Narayanan, S. K., Nagaraja, T. G., Chengappa, M. M. & Stewart, G. C. (2001). Cloning, sequencing, and expression of the leukotoxin gene from *Fusobacterium necrophorum*. *Infect Immun* **69**, 5447–5455.
- Narayanan, S. K., Nagaraja, T. G., Chengappa, M. M. & Stewart, G. C. (2002a). Leukotoxins of gram-negative bacteria. *Vet Microbiol* **84**, 337–356.
- Narayanan, S. K., Stewart, G. C., Chengappa, M. M., Willard, L., Shuman, W., Wilkerson, M. & Nagaraja, T. G. (2002b). *Fusobacterium necrophorum* leukotoxin induces activation and apoptosis of bovine leukocytes. *Infect Immun* **70**, 4609–4620.
- Narongwanichgarn, W., Misawa, N., Jin, J. H., Amoako, K. K., Kawaguchi, E., Shinjo, T., Haga, T. & Goto, Y. (2003). Specific detection and differentiation of two subspecies of *Fusobacterium necrophorum* by PCR. *Vet Microbiol* **91**, 183–195.
- Navas, E., Martinez-San, J. M., Garcia-Villanueva, M. & de Blas, A. (1994). Brain abscess with intracranial gas formation: case report. *Clin Infect Dis* **19**, 219–220.
- Oelke, A. M., Nagaraja, T. G., Wilkerson, M. J. & Stewart, G. C. (2005). The leukotoxin operon of *Fusobacterium necrophorum* is not present in other species of *Fusobacterium*. *Anaerobe* **11**, 123–129.
- Razonable, R. R., Rahman, A. E. & Wilson, W. R. (2003). Lemierre syndrome variant: necrobacillosis associated with inferior vena cava thrombosis and pulmonary abscesses after trauma-induced leg abscesses. *Mayo Clin Proc* **78**, 1153–1156.
- Riordan, T. & Wilson, M. (2004). Lemierre's syndrome: more than a historical curiosa. *Postgrad Med J* **80**, 328–334.
- Scanlan, C. M., Berg, J. N. & Fales, W. H. (1982). Comparative *in vitro* leukotoxin production of three bovine strains of *Fusobacterium necrophorum*. *Am J Vet Res* **43**, 1329–1333.
- Shinjo, T., Fujisawa, T. & Mitsuoka, T. (1991). Proposal of two subspecies of *Fusobacterium necrophorum* (Flugge) Moore and Holdeman: *Fusobacterium necrophorum* subsp. *necrophorum* subsp. nov., nom. rev. (ex Flugge 1886), and *Fusobacterium necrophorum* subsp. *funduliforme* subsp. nov., nom. rev. (ex Halle 1898). *Int J Syst Bacteriol* **41**, 395–397.
- Smith, G. R. & Thornton, E. A. (1993). Pathogenicity of *Fusobacterium necrophorum* strains from man and animals. *Epidemiol Infect* **110**, 499–506.
- Smith, G. R. & Thornton, E. A. (1997). Classification of human and animal strains of *Fusobacterium necrophorum* by their pathogenic effects in mice. *J Med Microbiol* **46**, 879–882.
- Tan, Z. L., Nagaraja, T. G. & Chengappa, M. M. (1992). Factors affecting the leukotoxin activity of *Fusobacterium necrophorum*. *Vet Microbiol* **32**, 15–28.
- Tan, Z. L., Nagaraja, T. G. & Chengappa, M. M. (1994). Biochemical and biological characterization of ruminal *Fusobacterium necrophorum*. *FEMS Microbiol Lett* **120**, 81–86.
- Tan, Z. L., Nagaraja, T. G. & Chengappa, M. M. (1996). *Fusobacterium necrophorum* infections: virulence factors, pathogenic mechanism and control measures. *Vet Res Commun* **20**, 113–140.
- Zhang, F., Nagaraja, T. G., George, D. & Stewart, G. C. (2006). The two major subspecies of *Fusobacterium necrophorum* have distinct leukotoxin operon promoter regions. *Vet Microbiol* **112**, 73–78.