

LEUKOTOXIN GENE AND ACTIVITY IN ANIMAL AND HUMAN STRAINS OF
FUSOBACTERIUM SPECIES

by

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B. V. Sc. & A. H., A. N. G. R. AGRICULTURAL UNIVERSITY, INDIA, 1999

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DOCTOR OF PHILOSOPHY

Department of Diagnostic Medicine/ Pathobiology
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KANSAS STATE UNIVERSITY
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Abstract

Fusobacterium necrophorum, a gram negative anaerobe and an opportunistic pathogen, causes necrotic infections in humans and animals. Two subspecies of *F. necrophorum*, subsp. *necrophorum* and subsp. *funduliforme* are described. Leukotoxin (Lkt), a secreted protein encoded by a tricistronic operon (*lktBAC*), is the major virulence factor of *F. necrophorum*. The concentration of Lkt produced by subsp. *necrophorum* is higher than that of subsp. *funduliforme*. Quantitative-PCR was used to determine the relative expression of *lktA* by the two subspecies of bovine origin. The mRNA transcript of *lktA* was detectable in early-log phase of growth in subsp. *necrophorum*, whereas in subsp. *funduliforme*, the *lktA* transcript was detected only in the mid-log phase. Q-PCR analysis revealed that subsp. *necrophorum* had 20-fold more *lktA* transcript than subsp. *funduliforme*. The amount of *lktA* transcript declined by late-log phase in both subspecies; but *lktA* mRNA levels in subsp. *necrophorum* was 8-fold higher than in subsp. *funduliforme*. Leukotoxin protein stability assays showed the Lkt to be stable in both subspecies despite the decrease in the concentration of the protein during late-log phase.

The subspecies identity of human *F. necrophorum* strains and whether they possess *lktA* and leukotoxin activity are not known. Human clinical isolates (n = 4) of *F. necrophorum* were identified as subsp. *funduliforme* based on 16S rRNA sequence and absence of hemagglutinin gene. Four human strains had the *lkt* promoter, *lktB*, and *lktC* similar to that of subsp. *funduliforme*. One strain had full length *lktA*, while other three strains exhibited considerable heterogeneity. All four strains secreted Lkt that was toxic to human leukocytes.

Fusobacterium equinum, formerly *F. necrophorum*, is a newly recognized species. It is associated with infections of the respiratory tract in horses. Little is known about the virulence factors of *F. equinum*. Southern hybridization revealed that *F. equinum* strains had *lktA* gene with greater similarities to *F. necrophorum* subsp. *necrophorum*. The toxicity of culture supernatants of isolates to equine leukocytes was variable. Our data indicate that *F. equinum* isolates possess *lktA* gene and exhibit leukotoxin activity. The importance of leukotoxin as a virulence factor in human and equine fusobacterial infections needs to be investigated.

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Dedication

I dedicate my doctoral work to my family and the Lord of the Seven Hills,

LORD VENKATESWARA

Chapter 1 -
Differential Expression of the *lktA* leukotoxin gene in *Fusobacterium*
***necrophorum* subspecies**

Abstract

Liver abscesses in cattle are associated with *Fusobacterium necrophorum*, a gram negative, pleomorphic, rod-shaped, non-sporulating obligate anaerobe. In cattle there are two subspecies: subsp. *necrophorum* and subsp. *funduliforme*. Leukotoxin (LktA), an exotoxin encoded by the tricistronic operon (*lktBAC*) is a major virulence factor in subsp. *necrophorum*, causing apoptosis and necrosis of bovine leukocytes in a dose-dependent manner. The amount of leukotoxin secreted by subsp. *funduliforme* is considerably less compared to subsp. *necrophorum*. However, not much is known about the leukotoxin operon in subsp. *funduliforme*. In this study, we sequenced the subsp. *funduliforme* leukotoxin operon and found that a similar organization of the three genes was present as in subsp. *necrophorum* *lkt* operon. Significant differences were present in the N-terminus region of LktA despite high similarities (88%) to subsp. *necrophorum* LktA. The relative expression profiles of the *lktA* gene at various growth phases in both subspecies were determined by quantitative PCR (Q-PCR). Our results revealed that subsp. *funduliforme* had a 20-fold lower level of *lktA* specific mRNA transcript in mid-log phase than subsp. *necrophorum*. The amounts of leukotoxin protein detected in the mid-log growth phase were in agreement with the transcription profiles. The culture supernatant from mid-log phase was highly toxic to bovine PMNs than from either early- or late-log phases of growth. Therefore, the low toxicity associated with subsp. *funduliforme* leukotoxin may be due to the lower expression of *lktA* gene.

Introduction

Fusobacterium necrophorum is a normal inhabitant of the gastrointestinal and genitourinary tract of cattle (Nagaraja et al., 2005). The organism is an opportunistic pathogen and causes liver abscesses in feedlot cattle, diphtheria in calves and foot-rot in sheep and cattle (Tan et al., 1992). In cattle, there are two subspecies of *F. necrophorum*, subsp. *necrophorum* and subsp. *funduliforme* (Shinjo et al., 1991). The two subspecies can be distinguished by their growth, morphological, biochemical and molecular characteristics (Nagaraja et al., 2005; Aliyu et al., 2004; Zhang et al., 2006).

Several virulence factors are associated with *F. necrophorum* infections (Nagaraja et al., 2005; Tan et al., 1996). Of those, leukotoxin (Lkt), a secreted protein, is a major virulence factor in subsp. *necrophorum* (Tan et al., 1992). It was also observed that strains of subsp. *funduliforme* had lower levels of leukotoxin and therefore were believed to be less virulent (Scanlan et al., 1982, 1986; Tan et al., 1992, 1994). This may be one of the reasons for less frequent isolation of subsp. *funduliforme* from cattle liver abscesses (Langworth, 1977; Lechtenberg et al., 1988). Tan et al. (1992) observed that higher amounts of leukotoxin were achieved when *F. necrophorum* was grown in anaerobic BHI broth with pH ranging from 6.6 to 7.7 and the concentration, determined by MTT dye reduction assay, peaked at late-log or early-stationary phase. Also, strains of subsp. *funduliforme* had 18-fold less leukotoxin than subsp. *necrophorum*.

The leukotoxin gene (*lktA*) in subsp. *necrophorum* is encoded by the tricistronic *lktBAC* operon (Narayanan et al. 2001). The *lktA* determinant is ~10 kb, encoding a protein of ~336 kDa in size. LktA induces apoptosis and necrosis of bovine leukocytes in a dose-dependent manner (Narayanan et al., 2002). Very little is known about the *lktA* gene sequence or the leukotoxin operon in subsp. *funduliforme*. The difference in *lktA* gene sequence in subsp. *funduliforme* may explain the differences in the leukotoxin production and activity.

Zhang et al. (2006) showed that the *lkt* promoter-containing fragment in both subspecies was variable in size (548 bp in subsp. *necrophorum*; 338 bp in subsp. *funduliforme*) and activity.

The subsp. *necrophorum* promoter activity was 4-fold higher than subsp. *funduliforme* in β -galactosidase assays using a reporter plasmid in *E. coli* background. The results suggested that the differences in the leukotoxin expression may be in part due to the differences in the promoter strength.

The objectives of this study were to (1) determine the leukotoxin operon sequence in subsp. *funduliforme*, and (2) determine the transcriptional profiles of *lktA* specific mRNA, at various stages in the logarithmic phase of growth, in the two subspecies of *F. necrophorum*.

Materials and Methods

2.1. Bacterial strains

Fusobacterium necrophorum subsp *necrophorum* strains A4, A8, A21 and A25 and *F. necrophorum* subsp *funduliforme* strains B15, B19, B35 and B47 were previously isolated from bovine liver abscesses (Tan et al. 1992). The bacterial strains were grown overnight on blood agar (Remel Inc, Lenexa, KS, USA) at 39°C in an anaerobic glove box (Forma Scientific, Marietta, OH, USA) and subcultured in pre-reduced, anaerobically sterilized (PRAS) BHI broth. Both subspecies were grown to early-log ($OD_{600nm} = 0.3$), mid-log ($OD_{600nm} = 0.6 - 0.7$) and late-log phases ($OD_{600nm} = 1.0$) for total RNA isolation, Q-PCR and cytotoxicity assays.

2.2. RNA isolation

Bacterial cells were pelleted by centrifugation at 6,000 x g for 5 min. at 4°C. Total RNA was isolated from the harvested cells by TRIzol Max Bacterial RNA Isolation Kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. The RNA was resuspended in DEPC-treated water (Ambion Inc., Austin, TX, USA) by heating to 60°C for 10 min. Contaminating DNA was removed by treatment with the TURBO DNA-free kit (Ambion Inc., USA) following the manufacturer's directions. The absence of genomic DNA contamination was determined by PCR. Total RNA was quantified spectrophotometrically (Nanodrop Technology, Wilmington, DE, USA) and stored in aliquots at -80°C until used.

2.3. Reverse transcription and real-time PCR

Total RNA free of contaminating genomic DNA was used for the synthesis of complementary DNA (cDNA) followed by real-time PCR (RT-PCR). Complementary DNA synthesis and RT-PCR reactions were carried out on a Smart Cycler (Cepheid, CA, USA) using the SuperScript III Platinum SYBR Green One Step qRT-PCR Kit (Invitrogen, Carlsbad, CA, USA) following the manufacturers instructions. To 12.5 μl SYBR Green Supermix, 1.0 μl ROX reference dye (25 μM), 1.0 μl of each primer at 10.0 pmol μl^{-1} , 1.0 μl SS III enzyme mix, and 4.0 μl of total RNA were added and the final reaction volume was made to 25 μl with 4.5 μl of RNase- free water. All the reactions were performed in duplicate and in each experiment a no template RNA negative control was included. The RT-PCR reactions were set using 16S rDNA (reference) and *lktA* (target) primers (Table 1.1). Thermal cycling conditions were as follows: cDNA synthesis was performed at 50°C for 30 min followed by RT-PCR: initial denaturation at 95°C for 2 min, followed by 40 cycles of 95°C for 15s, annealing at 60°C for 30s and extension at 72°C for 30s. Fluorescence measurements were recorded during each annealing step. An additional step starting at 90°C to 60°C (0.05 °C s⁻¹) was performed to establish a melting curve. This was to verify the specificity of the real-time PCR reaction for each primer pair. The 16S rRNA was used as internal control (reference) for quantifying the relative expression of leukotoxin gene (target). The relative expression levels of leukotoxin were determined by the 2^{- $\Delta\Delta\text{Ct}$} method as described previously (Livak and Schmittgen, 2001; Desroche et al., 2005).

2.4. Western blot analysis

Fusobacterium necrophorum subsp. were grown to early-, mid- and late-log phases of growth. The culture supernatant (CS) was collected after harvesting the cells by centrifugation at 6,000 x g for 10 min at 4°C. The supernatants were filtered with 0.22 μm filter (Millipore, USA.) and concentrated 60x by centrifugation with 100, 000 kDa MW cut off filters (Centricon, USA). The concentrated CS was stored at -80°C until used. Equal volumes of concentrated CS (20 μl) was denatured in Laemmli's buffer in boiling water for 5 min (Laemmli, 1970) and resolved on a 4-20 % SDS –PAGE gel (Pierce, USA) under denaturing conditions. Proteins were transferred to nitro-cellulose membranes (Millipore, USA) and blocked overnight with 0.2% skim milk in phosphate buffered saline (PBS) at 4°C. The membranes were then incubated with anti-LktA rabbit polyclonal serum followed by incubation with secondary goat anti-rabbit

antibody conjugated with alkaline phosphatase (Sigma, St. Louis, MO). The colorimetric signal was visualized with the substrate BCIP/NBT.

2.5. Preparation of white blood cells

The bovine PMNs were prepared as per the protocol of Tan et al. (1992). Briefly, bovine whole blood was collected (BD Vacutainer, Becton Dickinson, NJ, USA) from healthy cattle by venipuncture of the internal jugular vein. The collected blood was transported on ice, centrifuged at 700 x g for 10 min at 4°C, and the buffy coat removed to another sterile tube. The residual RBCs were subjected to osmotic lysis with sterile distilled water. Purified WBCs were resuspended in complete RPMI 1640 media. The number of viable leukocytes was determined by a 0.4 % trypan blue dye exclusion assay (Narayanan et al., 2002).

2.6. Cell viability assay

The cytotoxic effects of *F. necrophorum* culture supernatants were determined by a cell viability assay using propidium iodide (Narayanan et al., 2002). Briefly, 1×10^6 viable white blood cells were treated with culture supernatants for 45 min at 37°C and 5% CO₂, washed twice and resuspended in 0.01M PBS and stained with 10 µl of propidium iodide (50 µg/ml stock) in the dark for 5 min. Untreated cells in complete RPMI 1640 were used as a negative (no toxin) control. The samples were processed on a FACScan flow cytometer using an argon ion laser (Becton Dickinson, San Jose, CA). Data were analyzed using Cell Quest analysis software (Becton Dickinson).

2.7. PCR analysis

Specific primers targeting the *lkt* operon constituent genes were designed. The amplified products were electrophoresed in 1-1.2 % agarose gels, purified (Wizard SV gel and PCR clean up system, Promega Corp. Madison, WI), and sequenced (Beckman Coulter CEQ 8000 Genetic Analysis System, Fullerton, CA, USA).

2.8. Primer efficiency

The efficiency of the primers to be used in the RT-PCR method was determined as described previously (Wong and Medrano, 2005). Briefly, 1 ng of total RNA was diluted 2-fold

(up to 1 pg) to set RT-PCR using *Fusobacterium* genus specific 16S rDNA primers and *Fusobacterium necrophorum lktA* specific primers (Table 1.1). The efficiency was determined by plotting a graph with log ng total RNA on the x-axis and the difference in C_t (target-reference) for each dilution on the y-axis. Only if the absolute value of the slope of the line was less than 0.1, then those primers were used for RT-PCR assays by comparative ($2^{-\Delta\Delta C_t}$) method.

2.9. Nucleotide sequence accession number

The subsp. *funduliforme* leukotoxin operon complete sequence has been deposited with GenBank and assigned the accession number AY972049.

3. Results

3.1. *lkt* operon sequence

The *lkt* operon of *F. necrophorum* subsp. *funduliforme* had the same tricistronic organization as subsp. *necrophorum* (Table 1.2 and Fig. 1.1). The first gene, *lktB*, is 650 bp in size. The structural gene, *lktA*, is 9,732 bp and the last gene *lktC* is 438 bp in size. The operon sequence was deposited with GenBank (Acc. number **AY972049**).

3.2. Relative expression of the leukotoxin genes

The primers (16S rDNA and *lktA*) used in Q-PCR analysis were efficient in both subspecies (Fig.1.2) and the absolute value of the slope of the line was less than 0.1. In the early-log phase, subsp. *necrophorum* leukotoxin specific mRNA was higher than in subsp. *funduliforme* (Table 1.3). Subspecies *necrophorum* had 19.7-fold more *lktA* specific mRNA in early-log phase than subsp. *funduliforme*. In the early-log phase, among the subsp. *funduliforme* strains tested, strain B35 had higher levels of *lktA* transcript than others. As the bacteria grew to mid-log phase, the *lktA* specific mRNA levels in subsp. *funduliforme* increased compared to early-log phase; but this increase was not sufficient to reach the mRNA levels in subsp. *necrophorum*, which was 20.4-fold higher than subsp. *funduliforme* in mid-log phase. By late-log/early-stationary phase of growth the *lktA* transcript declined in both the subspecies; but the levels in subsp. *necrophorum* were 7.4 fold higher than those of subsp. *funduliforme*.

3.3. Western blot analysis

The amounts of leukotoxin synthesized in subsp. *necrophorum* strains varied with the stages in log phase of growth (Fig 1.3). The leukotoxin levels were lowest in the early-log phase and gradually increased reaching the highest levels by mid-log phase. Leukotoxin production pattern in subsp. *funduliforme* resembled subsp. *necrophorum* with highest levels in mid-log phase.

3.4. Cytotoxicity assays

The mid-log phase culture supernatants from subsp. *necrophorum* were highly toxic to bovine PMNs (Fig. 1.4, lanes 2 to 5). The late-log phase culture supernatant of subsp. *necrophorum* was more toxic than early-log phase culture supernatant (data not shown). Subsp. *funduliforme* mid-log phase culture supernatants were less toxic than subsp. *necrophorum* (Fig.1.5, lanes 6 to 9). There was no significant decrease in the activity of the culture supernatants after removing the contaminated LPS in a polymyxin B column. Thus, the effects observed were due to leukotoxin alone and not due to LPS.

4. Discussion

The leukotoxin of *F. necrophorum* subsp. *necrophorum* was shown to be more toxic to bovine PMNs than subsp. *funduliforme* (Tan et al. 1994). This was further confirmed by Narayanan et al. (2001) in their study on determining the leukotoxin gene sequence and activity in subsp. *necrophorum*. It was not known whether the differences observed in toxic activities were due to differences in the leukotoxin protein sequences or resulted from expression level differences in the two subspecies.

DNA sequence analysis of the leukotoxin operon in subsp. *funduliforme* revealed that it consists of 3 genes: *lktB*, *lktA* and *lktC*. The toxin structural gene, *lktA* encoded a protein of 336 kDa in size that was toxic to bovine PMNs. The functions of the LktB and LktC proteins have not yet been determined. Strain-to-strain variations in leukotoxin activity among subsp. *funduliforme* were reported by Tan et al. (1992). In our study, we observed similar differences in *lktA* transcripts among subsp. *funduliforme* strains in early-log phase growth. Highest amounts

of LktA protein were detected during the mid-log phase in both the subspecies, in agreement with their mRNA levels. The toxic effects of culture supernatants from mid-log phase were highest in both the subspecies which was retained through the late-log phase. Our attempts at determining the half-life of *lktA* mRNA using well known transcription inhibitors thiolutin and rifampicin were unsuccessful as *F. necrophorum* subspecies were not susceptible to these well known transcription inhibitors (data not shown).

In conclusion, subsp. *funduliforme* has the tricistronic leukotoxin operon similar to subsp. *necrophorum*. The differences in the leukotoxin activity among the subsp. appear to be due to differences in the sequences and the differential transcription of the *lktA* gene.

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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.
- Desroche, N., Beltramo, C., Guzzo, J., 2005. Determination of an internal control to apply reverse transcription quantitative PCR to study stress response in the lactic acid bacterium *Oenococcus oeni* *J. Microbiol. Methods.* 60, 325-333.
- Langworth, B.F., 1977. *Fusobacterium necrophorum*: its characteristics and role as an animal pathogen. *Bacteriol. Rev.* 41, 373-390.
- Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* 227, 680-685.
- Lechtenberg, K.F., Nagaraja, T.G., Leipold, H.W., Chengappa, M.M., 1988. Bacteriologic and histologic studies of hepatic abscesses in cattle. *Am. J. Vet. Res.* 49, 58-62.
- Livak, K.J., and Schmittgen, T.D. 2001. Analysis of relative gene expression data using Real time Quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. *Methods* 25, 402-408.
- 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., Stewart, G.C., Chengappa, M.M., Willard, L., Shuman, W., Wilkerson, M., Nagaraja, T.G., 2002. *Fusobacterium necrophorum* Leukotoxin Induces Activation and Apoptosis of Bovine Leukocytes. *Infect. Immun.* 70, 4609-4620.
- 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.
- Scanlan, C.M., Berg, J.N., Campbell, F.F., 1986. Biochemical characterization of the leukotoxins of three bovine strains of *Fusobacterium necrophorum*. *Am. J. Vet. Res.* 47, 1422-1425.

- 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.
- 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., Smith, J.S., 1994. Biological and biochemical characterization of *Fusobacterium necrophorum* leukotoxin. Am. J. Vet. Res. 55, 515-521.
- Tan, Z.L., Nagaraja, T.G., and Chengappa, M.M. 1996. *Fusobacterium necrophorum* infections: virulence factors, pathogenic mechanisms and control measures. Vet. Res. Communi. 20: 113-140.
- Wong, M.L., Medrano, J.F., 2005. Real time PCR for mRNA quantification. Review Biotechniques. 39, 1-11.
- 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.

Table 1.1 Primers used to determine the relative expression of leukotoxin (*lktA* gene) in *Fusobacterium necrophorum* subspecies

Primer	Oligonucleotide sequence (5'→3')
16s-forward	AGCCGCGGTAATTACGTATG
16s-reverse	GAGTTGAGCTCCGCATTTTC
LktA-forward	AAATGGTGAAAGAATGACAA
LktA-reverse	TGCATAATTCCTACTCCTG

Table 1.2 Leukotoxin operon in *Fusobacterium* subsp. *funduliforme*

Gene (bp)	<i>necrophorum</i>	<i>funduliforme</i>	% similarity(aa)
<i>lktB</i>	1653	1650	87
<i>lktA</i>	9726	9732	88
<i>lktC</i>	438	438	96

Table 1.3 Relative expression of *lktA* as determined by Q-PCR

Growth phase	Subspecies	<i>lktA</i> C _T	16S C _T	ΔC_T <i>lktA</i> – 16S	$\Delta\Delta C_T$ $\Delta C_T - \Delta C_T$ <i>funduliforme</i>	Fold change $2^{-\Delta\Delta C_T}$
Early	<i>necrophorum</i>	27.47	9.9	17.2	-4.3	19.7
	<i>funduliforme</i>	34.0	12.1	21.9	0.00	1
Mid	<i>necrophorum</i>	18.77	9.77	9.0	-4.35	20.4
	<i>funduliforme</i>	24.9	11.55	13.35	0.00	1
Late	<i>necrophorum</i>	21.77	9.65	12.12	-2.88	7.36
	<i>funduliforme</i>	25.9	10.9	15.0	0.00	1

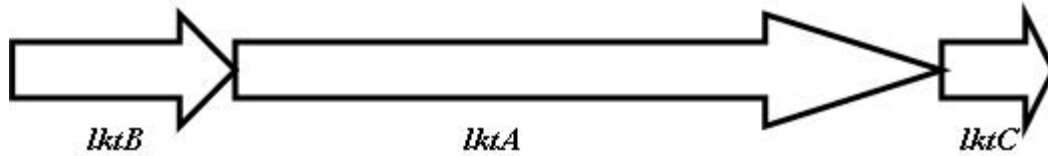
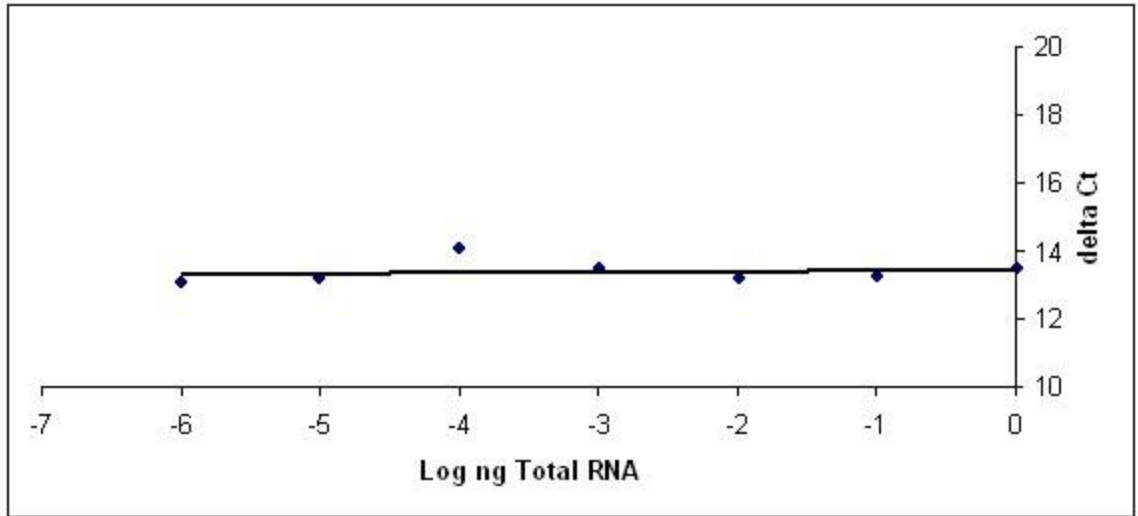
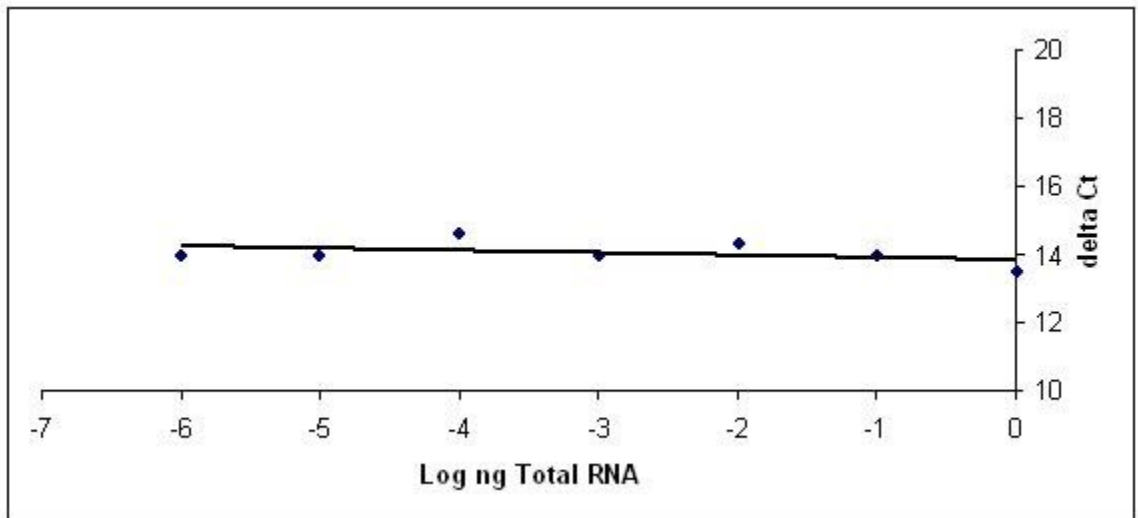


Figure 1.1. Leukotoxin operon in subsp. *funduliforme*

The leukotoxin operon in *Fusobacterium necrophorum* subsp. *funduliforme* consists of three genes, *lktB*, *lktA*, and *lktC*. The *lktB* determinant is 1,650 bp, *lktA* 9,732 bp, and *lktC* 438 bp in length and have 87%, 88%, and 96% similarities in amino acid sequences to the corresponding leukotoxin operon proteins in subsp. *necrophorum*.



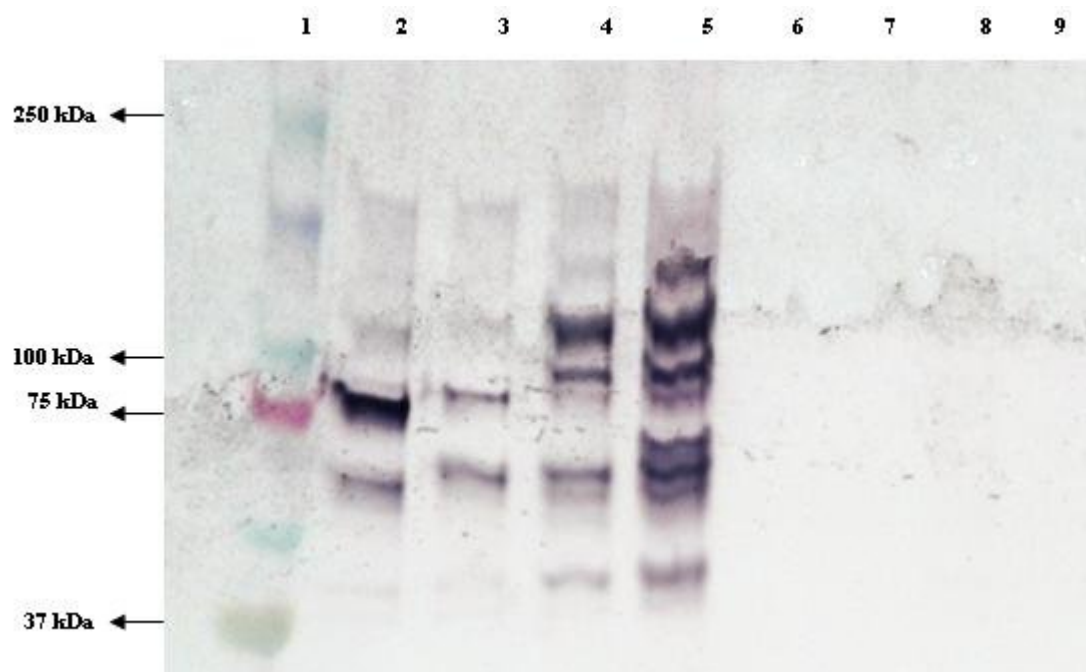
[A]



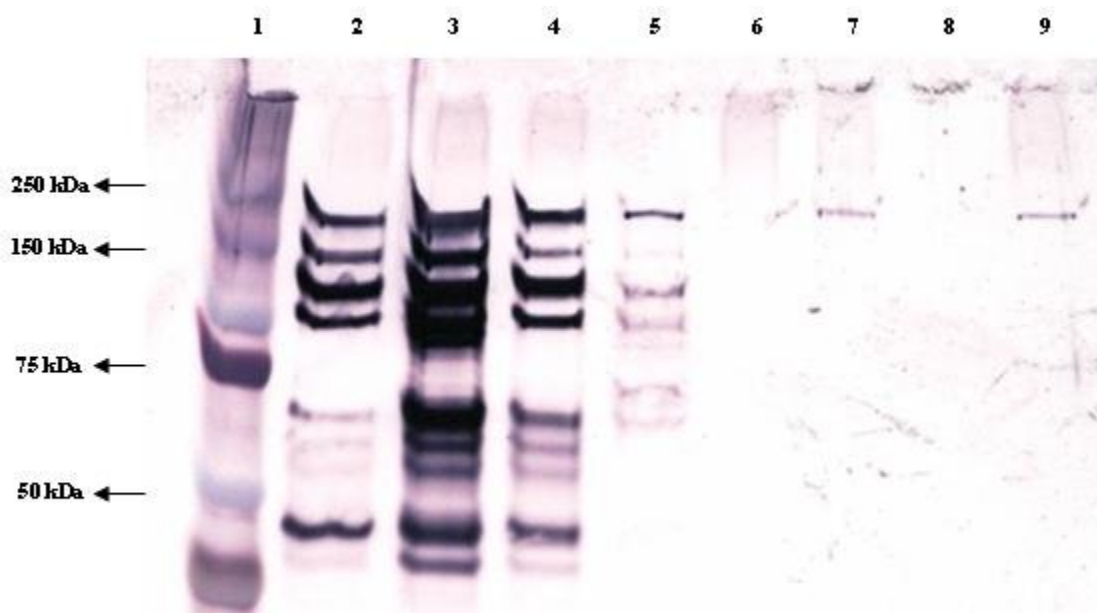
[B]

Figure 1.2. Primer efficiency

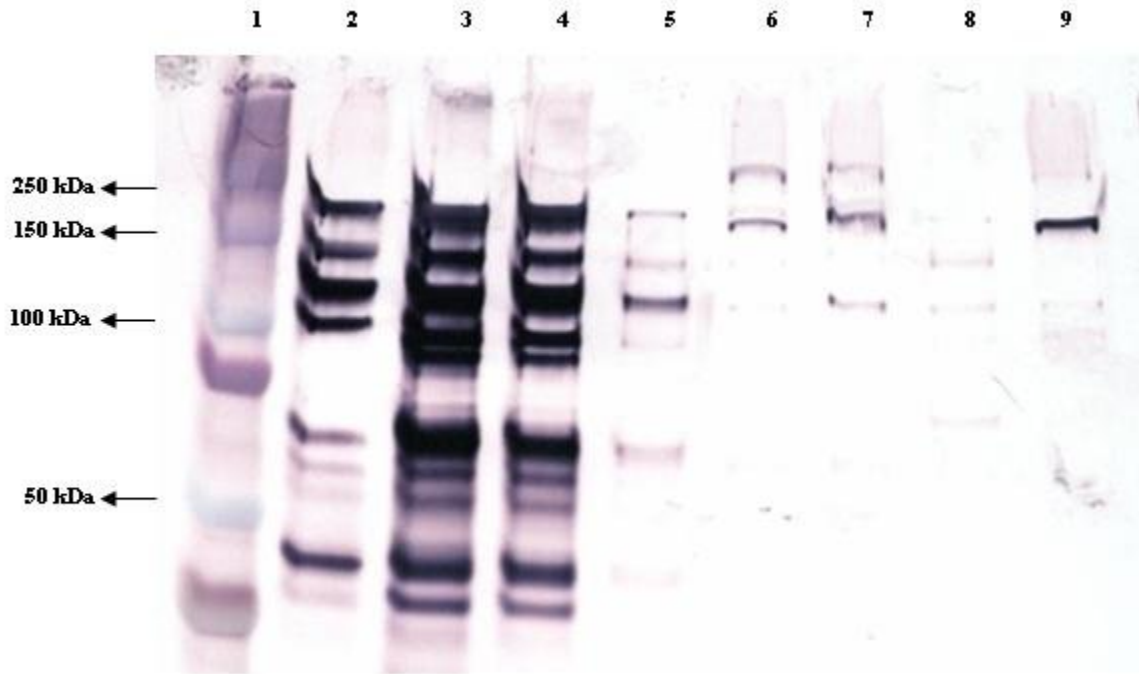
The efficiencies of primer sets used in Q-PCR analysis were determined by 2- fold dilution of DNA-free total RNA (1µg) in *Fusobacterium necrophorum* subspecies (A) *necrophorum* and (B) *funduliforme*.



(a)



(b)



(c)

Figure 1.3. Western blot analysis

The concentrated culture supernatants from various stages (a) early-log, (b) mid-log and (c) late-log phases of growth probed with anti-*Fusobacterium necrophorum* leukotoxin rabbit serum. Lane description: 1, Pre-stained protein marker; lanes 2- 5 represent subsp. *necrophorum* strains (A4, A8, A21 and A25) and lanes 6-9 represent subsp. *funduliforme* strains (B15, B19, B35 and B47), respectively.

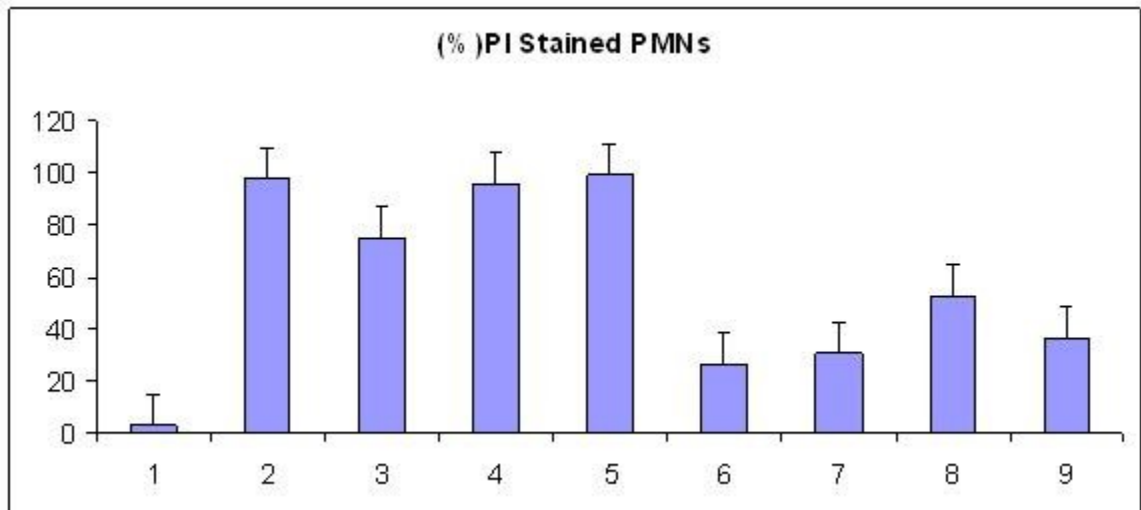


Figure 1.4. Cytotoxicity assay

The bovine peripheral blood polymorphonuclear cells (PMNs) were treated with mid-log phase culture supernatants of *Fusobacterium necrophorum* subspecies and the percentage of viable cells was determined by staining with fluorescent dye, propidium iodide by flow cytometry. Error bars represent standard error of (n=3) independent experiments. Lane description: 1, RMPI medium alone; 2 to 5 represent subsp. *necrophorum* strains (A4, A8, A21 and A25) and 6 to 9 represent subsp. *funduliforme* strains (B15, B19, B35 and B47) respectively.

Chapter 2 -
Human *Fusobacterium necrophorum* Strains Are Leukotoxin-
Positive

Abstract

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 leukotoxin has been shown to be a major virulence factor. The leukotoxin operon consists of three genes, *lktB*, *lktA*, and *lktC*, of which *lktA* is the leukotoxin structural gene. The subspecies identity of human *F. necrophorum* is less certain and it's not known whether human strains possess the leukotoxin gene or leukotoxic activity. Therefore, our objective was to identify the subspecies of four human clinical strains of *F. necrophorum* and determine whether the strains have the leukotoxin gene and leukotoxin activity. Phenotypic and genotypic characteristics suggested that the strains belong to subsp. *funduliforme* which was verified by sequencing the 16S rDNA. PCR analysis of four strains revealed the presence of the leukotoxin operon. All four human strains had a leukotoxin operon promoter similar to that of bovine subsp. *funduliforme* strains, which was confirmed by DNA sequencing and Southern blot analysis. Partial DNA sequencing had identified that one human strain had the full length *lktA* and others exhibited considerable heterogeneity in size. Despite variations in the *lktA* gene, all strains secreted leukotoxin as demonstrated by Western blot analysis. The secreted leukotoxin was toxic to the human white blood cells as determined by flow cytometry. In conclusion, the human strains examined contained the leukotoxin gene and produced biologically active leukotoxin. The importance of leukotoxin as a virulence factor in human fusobacterial infections is not known.

Fusobacterium necrophorum, a gram-negative, rod-shaped obligate anaerobe, is a normal inhabitant of the gastrointestinal, respiratory, and genitourinary tracts of animals and humans (10, 13, 33). It is an opportunistic pathogen associated with various necrotic infections (necrobacillosis) in animals and humans (2, 25, 33). The organism is an important cattle pathogen and is the primary causative agent of calf diphtheria (necrotic laryngitis), liver abscesses, and foot rot (13, 33, 16). In humans, *F. necrophorum* causes abscesses, acute pharyngitis, thrombophlebitis and abscessation of the internal jugular vein, termed Lemierre's syndrome (2, 26). Children and young adults (16-19 yr) are more susceptible to *F. necrophorum* infections than adults (26). In the last ten years, there has been a rise in the incidence of *F. necrophorum* infections in children (3, 11, 24).

Fusobacterium necrophorum has two major subspecies: subsp. *necrophorum* and subsp. *funduliforme* (28). In the bovine, subsp. *necrophorum* is the most prevalent subspecies associated with infections and produces more leukotoxin than subsp. *funduliforme* (16). Strains of *F. necrophorum* causing human infections are clearly distinct from subsp. *necrophorum* of animal origin and identity with subsp. *funduliforme* is also less certain (12). The pathogenic effects observed in mice when experimentally challenged with some of the human strains of *F. necrophorum* were similar to subsp. *funduliforme*; however, other strains induced lesions that resembled neither subspecies (29, 30). Therefore, the subspecies status of the human strains is unclear.

In bovine *F. necrophorum*, leukotoxin is a major virulence factor (16). The leukotoxin is a large secreted protein (336 kDa) that is cytotoxic in a dose dependent manner to neutrophils and to a lesser extent to lymphocytes (17, 20). The leukotoxin operon consists of three genes; *lktB*, *lktA*, and *lktC*, of which *lktA* is the structural gene for leukotoxin (18, 23). The *lkt* operon promoter varies in nucleotide sequence and length between the two subspecies of bovine *F. necrophorum* (35). Virulence factors implicated for human strains include endotoxin and hemolysin (2, 10). However, there is no evidence that human strains of *F. necrophorum* have leukotoxin activity or contain the *lktA* gene. Therefore, our objectives were to subspeciate the human strains and determine whether *F. necrophorum* isolates from human clinical cases contain the *lkt* operon and exhibit leukotoxin activity.

Materials and Methods

Bacterial strains and subspeciation. Four strains (RMA10682, RMA14786, RMA16505, and RMA16539) of human clinical isolates of *F. necrophorum* (kindly provided by Dr. Diane Citron, R. M. Alden Research Laboratory, Santa Monica, CA.) were used in the study. The strains were isolated from a liver abscess, tonsil biopsy, tonsil swab, and a neck wound, respectively (D. M. Citron, Personal communication). Two bovine strains, *F. necrophorum* subsp. *necrophorum* A25 and subsp. *funduliforme* B35, previously isolated from bovine liver abscesses (31) were also included in the study. Isolates were grown overnight on blood agar (Remel Inc, Lenexa, KS, USA) at 39°C in an anaerobic glove box (Forma Scientific, Marietta, OH, USA) and subcultured in pre-reduced, anaerobically sterilized (PRAS) BHI broth. Subspeciation of the human strains was performed by using the RapID Ana II system (Remel Inc., Lenexa, KS, USA) and by PCR amplifications of the RNA polymerase gene (β -subunit; *rpoB*), the haemagglutinin gene (*HA*) (1), and the *lkt* operon promoter region sequence (35). The partial 16s rDNA sequence was amplified from the human strains (RMA10682, RMA16505) with the following: forward primer 5'- CGCGTAAAGAAC TTGCC TCT-3' and reverse primer 5'- CTTCAGGTGCAACCCACTCT -3'. The obtained sequences were then aligned with 16S rDNA sequences of 12 other species of the genus *Fusobacterium* using ClustalX program (opening gap penalty of 10 and extension penalty of 0.1) and a phylogenetic tree was constructed using distance matrix algorithm and a neighbor-joining method (7) using the PAUP program (Sinauer Associates Inc. Publishers, Sunderland, MA).

Primer design and PCR conditions. Specific primers targeting *rpoB* and hemagglutinin (*HA*) genes were previously designed by Aliyu et al., (1). All other primers used in this study are listed in Table 2.1. The *lkt* operon promoter regions in subsp. *necrophorum* and subsp. *funduliforme* were amplified using Ex-Taq (Takara Bio Inc., Japan) as previously described (35). The PCR amplified products were electrophoresed in 1-1.2 % agarose gels, purified (Wizard SV gel and PCR clean up system, Promega Corp. Madison, WI), and sequenced (Beckman Coulter CEQ 8000 Genetic Analysis System, Fullerton, CA, USA).

Isolation of chromosomal DNA. Bacterial chromosomal DNA was prepared according to the procedure described by Narayanan et al., (18). Briefly, isolates were grown overnight in anaerobic BHI broth. The cells were then pelleted by centrifugation at 5,000 x g for 10 min. at 4°C. The pelleted cells were washed and resuspended in TE buffer (Tris 100 mM; EDTA 10.0 mM; pH 8.0). The cell wall was digested with lysozyme (1 mg/ml), and the resulting lysate was treated with Sarkosyl (1%) followed by RNaseA (20 µg/ml) and pronase (50 µg/ml). The samples were incubated on ice for 20 min. and then sequentially extracted with phenol and chloroform twice. The DNA from the extract was then precipitated in 1/10 vol. 3M sodium acetate (pH 5.2) and an equal volume of 2-propanol. The DNA was resuspended in TE (10 mM Tris-HCl, 1mM EDTA, pH 8.0) and stored at 4°C until used. The concentration of extracted DNA was determined spectrophotometrically (Nanodrop Technologies Inc., Wilmington, DE).

Preparation of culture supernatants. *F. necrophorum* bovine and human strains were grown in PRAS BHI to an absorbance of 0.6-0.7 at OD_{600nm} and pelleted by centrifugation at 5,000 x g for 20 min. at 4°C. The supernatant was collected, filtered through a 0.22 µm filter (Millipore, MA, USA), and aliquots were stored at -80° C until used.

Southern hybridization. Southern hybridization was performed according to the procedure of Narayanan et al., (18). Briefly, 3 µg of chromosomal DNA were digested to completion by the restriction endonuclease *Hae III* (Promega Corp. Madison, WI) and electrophoresed in a 1% agarose gel overnight at 20V at room temperature. The resolved and denatured DNA fragments were transferred to a positively-charged nylon membrane (Roche Diagnostics Corporation, Indianapolis, IN) by a capillary mechanism and the DNA immobilized by UV cross-linking. DNA probes were synthesized by random-labeling with DIG-dUTP (Roche Diagnostics, Indianapolis, IN, USA) according to the manufacturer's directions. The immobilized DNA was probed with full-length bovine subsp. *necrophorum* A25 *lktA* (at 54°C); subsp. *funduliforme* B35 leukotoxin operon promoter (at 42°C), subsp. *funduliforme* B35 *lktB* (at 50°C) or subsp. *necrophorum* A25 *lktC* (at 44°C). Colorimetric detection of hybridized signals was carried out as per the manufacturer's instructions. (Roche Diagnostics, Indianapolis, IN, USA).

Western-blot analysis. Culture supernatants from all strains were concentrated (60x) by centrifugation with 100,000 kDa MW cut off filters (Centricon, USA) and were resolved on 4-20% Tris-glycine polyacrylamide gels (Pierce Biotechnology, Rockford, IL) under denaturing conditions. Proteins were then electro-transferred onto nitrocellulose membranes (Millipore Corp., MA). The membranes were blocked with 0.2% skim milk overnight at 4°C. Rabbit polyclonal antiserum raised against the strain A25 native leukotoxin (18) was used as primary antibody. Goat anti-rabbit immunoglobulin conjugated with alkaline phosphatase (Sigma-Aldrich, St. Louis, MO, USA) was used as the secondary antibody, and the immunoreactive proteins were detected using 5-bromo-4-chloro-3-indolylphosphate/nitro blue tetrazolium (BCIP/NBT) as substrate.

Preparation of white blood cells. The polymorphonuclear leukocytes from peripheral blood (PMNs) were prepared as described (31). 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 x g for 10 min at 4°C, and the buffy coat removed to another sterile tube. The residual erythrocytes (RBCs) were subjected to osmotic lysis by sterile distilled water. Purified leukocytes (WBCs) were resuspended in a complete RPMI 1640 (5% fetal bovine serum; penicillin and streptomycin antibiotics) media. Human whole blood was collected by venipuncture from a healthy donor, erythrocytes were lysed with 6x volume of RBC lysis buffer (Sigma-Aldrich, St. Louis, MO, USA) at room temperature (5-10 min.) and resuspended in complete RPMI 1640 (23). The final concentration of viable cells was determined by a 0.4 % trypan blue dye exclusion assay (20).

Cell viability assay. The cytotoxic effects of *F. necrophorum* culture supernatants were determined by a cell viability assay using propidium iodide (20). Briefly, 1×10^6 viable white blood cells were treated with culture supernatants for 45 min at 37°C and 5% CO₂, washed twice and resuspended in 0.01M PBS and stained with 10 µl of propidium iodide (50 µg/ml stock) in dark for 5 min. Untreated cells in complete RPMI 1640 were used as a negative control. The samples were processed on a FACScan flow cytometer using an Argon ion laser (Becton Dickinson, San Jose, CA). Data were analyzed using Cell Quest analysis software (Becton Dickinson).

Nucleotide sequence accession numbers. The nucleotide sequences of the human strains RMA10682 and RMA16505 16S rDNA have been deposited in GenBank and assigned accession numbers EF447426 and EF447427 respectively.

Results

Subspeciation. All four human strains formed a flocculent button after overnight growth in PRAS BHI broth and were negative for alkaline phosphatase based on the RapId ANA II test. The *rpoB* gene (Fig. 2.1A) specific for *F. necrophorum* was present in all four strains, but all were PCR-negative for the hemagglutinin gene (Fig. 2.1B).

The PCR amplification of the *lkt* promoter region of the human isolate strains revealed a subsp. *funduliforme* type product (Fig. 2.1C). The results from the Southern hybridization of *Hae*III digested genomic DNA probed with the DIG labeled subsp. *funduliforme lkt* operon promoter DNA revealed that each of the human strains possessed a subsp. *funduliforme*-type promoter fragment (Fig. 2.2) and no signal was detected when probed with the labeled subsp. *necrophorum* promoter. While the band size for strain RMA14786 was similar to bovine subsp. *funduliforme* (strain B35), strains RMA10682, RMA16505, and RMA16539 had smaller sized bands. Nucleotide sequence analyses of the *lkt* promoter regions were identical to the subsp. *funduliforme lkt* promoter with only a few minor base pair changes (data not shown). The aligned 16S rDNA sequences of the two strains were nearly identical and had greater identity (99%) to subsp. *funduliforme* (Fig 2.3)

The *lktB* gene. PCR reaction using primers conserved in the *lktB* gene yielded products of the expected size from all four strains. Partial DNA sequencing revealed greater sequence identity to subsp. *funduliforme lktB* than subsp. *necrophorum*. Southern hybridization using a subsp. *necrophorum lktB* probe resulted in a weak signal (data not shown) in comparison to subsp. *funduliforme lktB* probe (Fig. 2.4). There were differences in size and intensity of the signal among the four human strains. Strains RMA14786 and B35 had a similar sized band (~8.0 kb) whereas the subsp. *necrophorum lktB* had faintly hybridized with a ~10.0 kb band. Strains RMA10682, RMA16505, and RMA16539 had a single band at ~5.1 kb.

The *lktA* gene. The presence of the *lktA* gene was determined by digesting the genomic DNA to completion with the restriction endonuclease *Hae*III and probing with the subsp. *necrophorum lktA* open reading frame in a southern hybridization reaction (Fig. 2.5). Genomic DNA digestion from subsp. *necrophorum* produced two hybridizing bands (10 kb and 11 kb) in Southern hybridizations whereas subsp. *funduliforme* DNA yielded three bands (Fig 5; the smallest band is approximately 375 bp). The bovine subsp. *necrophorum lktA* has a single recognition site for enzyme *Hae*III whereas subsp. *funduliforme* has two *Hae*III sites. Among the human strains, unique hybridization patterns were observed. Strain RMA14786 DNA gave rise to a doublet similar in size to the subsp. *funduliforme* fragments but a higher molecular weight hybridizing band was also evident. Strains RMA10682, RMA16505, and RMA16539 gave rise to a single strongly hybridizing band that differs in size and a faintly hybridizing larger molecular weight band similar to that obtained with the other human strain RMA14786.

The *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 hybridization with subsp. *necrophorum lktC* probe (Fig. 2.6). The positive controls, bovine subsp. *necrophorum* and subsp. *funduliforme*, had the expected band sizes (10 kb and 7 kb) respectively. Two hybridization patterns were observed among the human strains. The first pattern (~1.5 kb) was similar among the three strains, RMA10682, RMA16505, and RMA16539, and was different from the two bovine subspecies. The second pattern (~6.0 kb) was present only in strain RMA14786, and resembled hybridization pattern of subsp. *funduliforme*.

Western blot analysis. The leukotoxin from *F. necrophorum* was unstable (Fig. 2.7) and appeared in the culture supernatant as multiple breakdown products. Supernatant from subsp. *necrophorum* had all the expected bands (~250 kDa, 150 kDa, 130 kDa and 110 kDa) (lane 1). The subspecies *funduliforme* supernatant contained less toxin and the antibodies react less well to *funduliforme* leukotoxin (lane 2). Reactive species of 150 kDa and ~40 kDa sizes were observed. 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 few bands and a poorer affinity. The other three strains gave rise to strongly reactive bands whose patterns were similar to each other, but were distinct from that of subsp. *necrophorum*. Strains RMA10682 and RMA16539 had an intense ~150 kDa size band and a variably expressed 40 kDa band.

Leukotoxin activity. Viability assays using human peripheral PMNs indicated that all the human strains culture supernatants were toxic (Fig. 2.8). Subsp. *necrophorum* and *funduliforme* culture supernatants were also toxic to human PMNs. In order to confirm that the observed toxicity was not due to contaminating LPS, we tested the toxic effects of the culture supernatant after passing the supernatants over a polymyxin B column. No significant decrease in the toxic activities was observed.

Discussion

In cattle, the two subspecies of *F. necrophorum* can be easily distinguished by their morphological, biochemical, and virulence characteristics (16). Smith and Thornton (30) classified the human strains based on their pathogenic effects in mice. It was observed that pathogenic effects of most human strains resembled subsp. *funduliforme* in producing mild, localized lesions in mice and none of them behaved like the subsp. *necrophorum*. Hall et al. (12) concluded that human strains were clearly distinct from subsp. *necrophorum* of animal origin based on phenotypic reactions, pyrolysis mass spectrometry and SDS PAGE analysis, but similarity with subsp. *funduliforme* was less certain. Aliyu et al., (1) using a hemagglutinin gene specific PCR assay (21) reported that throat swabs from acute pharyngitis cases in humans were identified as subsp. *funduliforme*.

In our study, microscopic morphology (gram negative short rods) and the biochemical profile based on the RapId ANA test suggested that all four human strains were of the subsp. *funduliforme*. The presence of the hemagglutinin gene, a characteristic feature of subsp. *necrophorum* (21) was consistent with their subspecies *funduliforme* classification. Additionally, the human strains had the size and sequence of the *lkt* operon promoter similar to that of subsp.

funduliforme. The phylogenetic analysis of the 16S rDNA confirmed that human strains belong to subsp. *funduliforme* (Fig 2.3).

Although *F. necrophorum* causes acute and sometimes fatal infections in humans very little is known about the virulence factors of human strains (2, 3, 10). Endotoxin from human strains was shown to be toxic to mice and was not different from that of animal strains (4). Hemolysin is the other putative virulence factor, but its role in pathogenicity is not known (2). In the bovine *F. necrophorum* strains, leukotoxin is a primary virulence factor (16, 17, 19). The importance of leukotoxin as a virulence factor in *F. necrophorum* infections is indicated by a correlation between toxin production and ability to induce abscesses in laboratory animals (6), and the inability of leukotoxin non-producing strains to induce foot abscesses in cattle following intra-dermal inoculation (8), and a relationship between anti-leukotoxin antibody titers and protection against infection in experimental challenge studies (27).

In this study, we demonstrated the presence of the leukotoxin operon, *lktBAC* in all the human strains. A BLAST search analysis of partial sequence of the *lktB* genes of the human strains revealed a 90-96% identity with the bovine subsp. *funduliforme lktB*. The function of the LktB protein is not yet determined.

Data from Southern hybridization indicated that strain RMA 14786 leukotoxin structural gene *lktA* may be more similar to subsp. *funduliforme lktA*. However, the other three strains *lktA* gene indicated fewer similarities to both subspecies. Instead of the doublet, a single band was observed in the other three strains. This might be due to the absence of a recognition site for the enzyme *Hae III* in the proximity of the *lktA* gene in their genome. Hence, a single band of high molecular weight 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. The partial *lktC* nucleotide sequence of strain RMA14786 was similar (95%) to *lktC* of either bovine subspecies. The strains RMA10682, 16505, and 16539 *lktC* resembled each other but were different from both the bovine subspecies.

There were differences in the cytotoxic effects of the culture supernatants among the human strains. Similar strain-to-strain variations in the cytotoxic effects of leukotoxin among the bovine strains of *F. necrophorum* were reported earlier (31). This may be due to the inactivation of leukotoxin by proteolytic enzymes. Coyle-Dennis and Lauerma (5, 6) reported that pretreatment of culture supernatant with proteolytic enzymes (trypsin and protease) had decreased leukotoxin activity. Hence, the differences in cytotoxic activity among human strains might be related to the various proteolytic enzyme(s) or yet to be identified factor(s) secreted by individual strains. In conclusion, our data showed that the human strains belong to subsp. *funduliforme*, had the leukotoxin operon *lktBAC* and exhibited leukotoxic activity.

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References

1. **Aliyu, S. H., R. K. Marriott, M. D. Curran, S. Parmar, N. Bentley, N. M. Brown, J. S. Brazier, and H. Ludlam.** 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.
2. **Brazier, J. S.** 2006. Human infections with *Fusobacterium necrophorum*. *Anaerobe.* **12**:165-172.
3. **Brazier, J. S., V. Hall, E. Yusuf, and B. I. Duerden.** 2002. *Fusobacterium necrophorum* infections in England and Wales 1990-2000. *J. Med. Microbiol.* **51**:269-272.
4. **Brown, R., H. G. Lough, and I. R. Poxton.** 1997. Phenotypic characteristics and lipopolysaccharides of human and animal isolates of *Fusobacterium necrophorum*. *J. Med. Microbiol.* **46**:873-878.
5. **Coyle-Dennis, J. E., and L. H. Lauerma.** 1978. Biological and biochemical characteristics of *Fusobacterium necrophorum* leukotoxin. *Am. J. Vet. Res.* **39**: 1790-1793.
6. **Coyle-Dennis, J. E., and L. H. Lauerma.**1979. Correlations between leukocidin production and virulence of two isolates of *Fusobacterium necrophorum*. *Am. J. Vet. Res.* **40**:274-276.
7. **Dorsch, M., D. N. Love, and G. D. Bailey.** 2001. *Fusobacterium equinum* sp. nov., from the oral cavity of horses. *Int. J. Syst. Bacteriol.* **51**:1959-1963.
8. **Emery, D. L., J. A. Vaughan, B. L. Clark, and D. J. Stewart.** 1986. Virulence determinants of *Fusobacterium necrophorum* and their prophylactic potential in animals. p. 267-274. *In* D. J. Stewart, J. E. Peterson, N.M. McKern, and D.L. Emery (ed.), Foot rot in Ruminants. Proceedings of a workshop, Melbourne: CSIRO Division of Animal Health, Australian Wool Corporation, Glebe, New South Wales, Australia.
9. **Garcia, M. M., K. M. Charlton, and K. A. McKay.** 1975. Characterization of endotoxin from *Fusobacterium necrophorum*. *Infect. Immun.* **11**: 371-379.
10. **Hagelskjaer, K. L., and J. Prag.** 2000. Human necrobacillosis, with emphasis on Lemierre's syndrome. *Clin. Infec. Dis.* **31**: 524-532.

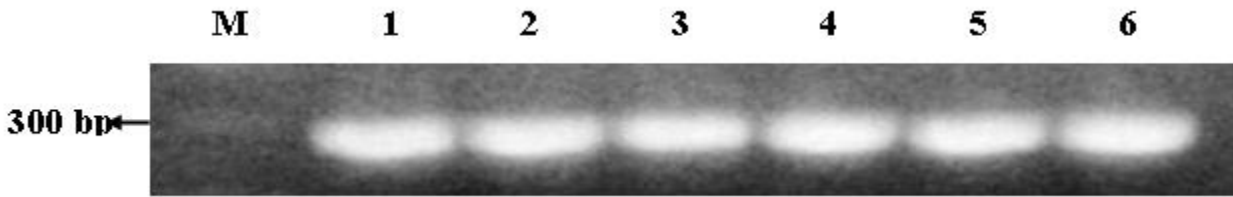
11. **Hagelskjaer, L. H., J. Prag, J. Malczynski, and J. H. Kristensen.** 1998. Incidence and clinical epidemiology of necrobacillosis, including Lemierre's syndrome, in Denmark 1990-1995. *Eur. J. Clin. Microbiol. Infect. Dis.* **17**: 561-565.
12. **Hall, V., B. I. Duerden, J. T. Magee, H. C. Ryley, and J. S. Brazier.** 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.
13. **Langworth, B. F.** 1977. *Fusobacterium necrophorum*: Its Characteristics and role as animal pathogen. *Bacteriol. Rev.* **41**:373-390.
14. **Lechtenberg, K. F., T. G. Nagaraja, H. W. Leipold, and M. M. Chengappa.** 1988. Bacteriological and histological studies of hepatic abscesses in cattle. *Am. J. Vet. Res.* **49**:58-62.
15. **Nagaraja, T.G., and M. M. Chengappa.** 1998. Liver abscesses in feedlot cattle: a review. *J. Anim. Sci.* **76**:287-298.
16. **Nagaraja, T. G., S. K. Narayanan, G. C. Stewart, and M. M. Chengappa.** 2005. *Fusobacterium necrophorum* infections in animals: Pathogenesis and pathogenic mechanisms. *Anaerobe.* **11**:239-246.
17. **Narayanan, S. K., M. M. Chengappa, G. C. Stewart, and T. G. Nagaraja.** 2003. Immunogenicity and protective effects of truncated recombinant leukotoxin proteins of *Fusobacterium necrophorum* in mice. *Vet. Microbiol.* **93**:335-347.
18. **Narayanan, S. K., T. G. Nagaraja, M. M. Chengappa, and G. C. Stewart.** 2001. Cloning, sequencing, and expression of the leukotoxin gene from *Fusobacterium necrophorum*. *Infect. Immun.* **69**:5447-5455.
19. **Narayanan, S. K., T. G. Nagaraja, M. M. Chengappa, and G. C. Stewart.** 2002. Leukotoxins of gram-negative bacteria. *Vet. Microbiol.* **84**:337-356.
20. **Narayanan, S. K., G. C. Stewart, M. M. Chengappa, L. Willard, W. Shuman, M. Wilkerson, and T. G. Nagaraja.** 2002. *Fusobacterium necrophorum* Leukotoxin Induces Activation and Apoptosis of *Bovine* Leukocytes. *Infect. Immun.* **70**:4609-4620.
21. **Narongwanichgarn, W., N. Misawa, J. H. Jin, K. K. Amoako, E. Kawaguchi, T. Shinjo, T. Haga, and Y. Goto.** 2003. Specific detection and differentiation of two subspecies of *Fusobacterium necrophorum* by PCR. *Vet. Microbiol.* **91**:183-195.

22. **Navas, E., J. M. Martinez-San, M. Garcia- Villanueva, and A. de Blas.** 1994. Brain abscess with intracranial gas formation: case report [letter]. *Clin. Infect. Dis.* **19**:219-220.
23. **Oelke, A. M., T. G. Nagaraja, M. J. Wilkerson, and G. C. Stewart.** 2005. The leukotoxin operon of *Fusobacterium necrophorum* is not present in other species of *Fusobacterium*. **11**:123-129.
24. **Ramirez, S., T. G. Hild, C. N. Rudolph, J. R. Sty, S. C. Kehl, P. Havens, K. Henrickson, and M. J. Chusid.** 2003. Increased diagnosis of Lemierre syndrome and other *Fusobacterium necrophorum* infections at a Children's Hospital. *Pediatrics.* **112**:e380.
25. **Raymund, R. R., E. R. Anne, and R. W. Walter.** 2003. Lemierre syndrome variant: Necrobacillosis associated with inferior vena cava thrombosis and pulmonary abscesses after trauma-induced leg abscesses. *Mayo Clinic Proc.* **78**:1153-1156.
26. **Riordan, T., and M. Wilson.** 2004. Lemierre's syndrome: more than a historical curiosa. *Postgrad. Med. J.* **80**:328-334.
27. **Saginala S., T. G. Nagaraja, Z. L. Tan, K. F. Lechtenberg, M. M. Chengappa, K. E. Kemp and P. M. Hine.** 1997. Serum neutralizing antibody response and protection against experimentally induced liver abscesses in steers vaccinated with *Fusobacterium necrophorum*. *Am. J. Vet. Res.* **57**:483-488.
28. **Shinjo, T., T. Fujisawa, and T. Mitsuoka.** 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.
29. **Smith, G. R., and E. A. Thornton.** 1993. Pathogenicity of *Fusobacterium necrophorum* strains from man and animals. *Epidemiol. Infect.* **110**:499-506.
30. **Smith, G. R., and E. A. Thornton.** 1997. Classification of human and animal strains of *Fusobacterium necrophorum* by their pathogenic effects in mice. *J. Med. Microbiol.* **46**: 879-882.
31. **Tan, Z. L., T. G. Nagaraja, and M. M. Chengappa.** 1992. Factors affecting the leukotoxin activity of *Fusobacterium necrophorum*. *Vet. Microbiol.* **32**:15-28.

32. **Tan, Z. L., T. G. Nagaraja, and M. M. Chengappa.** 1994. Biochemical and biological characterization of ruminal *Fusobacterium necrophorum*. FEMS Microbiol. Lett. **120**:81-86.
33. **Tan, Z.L., T. G. Nagaraja, and M. M. Chengappa.** 1996. *Fusobacterium necrophorum* infections: virulence factors, pathogenic mechanism and control measures. Vet. Res. Commun. **20**:113-140.
34. **Tan, Z. L., T. G. Nagaraja, M. M. Chengappa, and J. J. Staats.** 1994. Purification and quantification of *Fusobacterium necrophorum* leukotoxin by using monoclonal antibodies. Vet. Microbiol. **42**:121-133.
35. **Zhang, F., T. G. Nagaraja, D. George, G. C. Stewart.** 2006. The two major subspecies of *Fusobacterium necrophorum* have distinct leukotoxin operon promoter regions. Vet. Microbiol. **112**:73-78.

Table 2.1. Primers used in this study

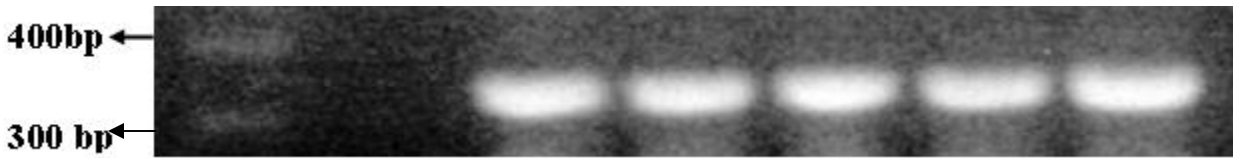
Primer	Oligonucleotide Sequence(5'→3')	Reference
5'lktpXmXh	tctcccgggctcgagGAAATCTTTAAAGCAC	Zhang et al., 2006
3'lktpXm	tctcccgggCATAATTTCTCCCAATTTTATT	Zhang et al., 2006
fund5p	CTCAATTTTTGTTGGAAGCGAG	Zhang et al., 2006
fund3p	CATTATCAAAATAACATATTTCTCAC	Zhang et al., 2006
UPS-START	ATCAATATGGCTTCCGGAAAAG	Narayanan et al., 2001
UPS-END	CCCTTTAAAATTCATGATTTATTG	Narayanan et al., 2001
BSBSE-START	AAATGAGYGGCATCAAAAAT	This study
BSBSE-END	TCCATCTGCTTCCAARACYGCAT	This study
SX-START	ATTAGAACTTTTAAAGAGAGCTT	This study
SX-END	CTTGTCCTCCACTTTCTTTAA	This study
GAS-START	GCTTCTGGAAGTGTTTC	This study
GAS-END	CTATTTTTTATATGTGC	This study
GAS-Downstream	GTGCTTTCGGAGCGAG	This study
FINAL-Upstream	AACCACCAGTAGAAGTG	This study
FINAL-START	TTAAAGCCATTGTGAAG	This study
FINAL-END	TTTTTCCCTTTTTCTCC	This study
RpoB-forward	TCTCTACGTATGCCTCACGGATC	Aliyu et al., 2004
RpoB-reverse	CGATATTCATCCGAGAGGGTCTCC	Aliyu et al., 2004
Haem-forward	CATTGGGTTGGATAACGACTCCTAC	Aliyu et al., 2004
Haem-reverse	CAATTCTTTGTCTAAGATGGAAGCGG	Aliyu et al., 2004
LktC forward	TGTTTTGAAAGAGAAGAAGTGAAAGA	This study
LktC reverse	TTTTGTAATATGCTCCAAGATCACA	This study
16s-forward	CGCGTAAAGAACTTGCCTCT	This study
16s-reverse	CTTCAGGTGCAACCCACTCT	This study



[A]



[B]



[C]

Figure 2.1. PCR analysis

PCR amplification of *rpoB* (RNA polymerase β subunit) gene (A), *HA* (hemagglutinin) gene (B), and *lkt* (leukotoxin) operon promoter (C) of four human strains and two bovine strains. Lane Description: M, 100bp DNA ladder; 1, bovine subsp. *necrophorum* strain A25; 2, bovine subsp. *funduliforme* strain B35; lanes 3 to 6 are human strains RMA10682, 14786, 16505, and 16539, respectively.

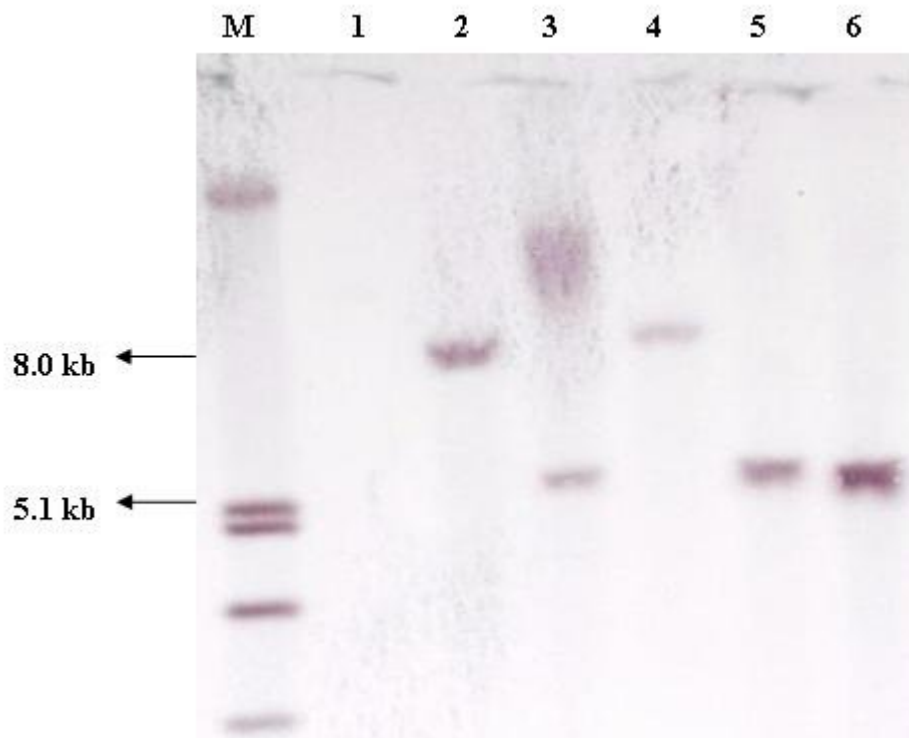


Figure 2.2. Southern hybridization using leukotoxin operon promoter probe

The *Hae*III digested genomic DNA with DIG labeled subsp. *funduliforme* leukotoxin operon promoter. Lane description: M, DIG labeled marker; 1, bovine subsp. *necrophorum* strain A25; 2, bovine subsp. *funduliforme* strain B35; lanes 3 to 6 are human strains RMA10682, 14786, 16505, and 16539, respectively.

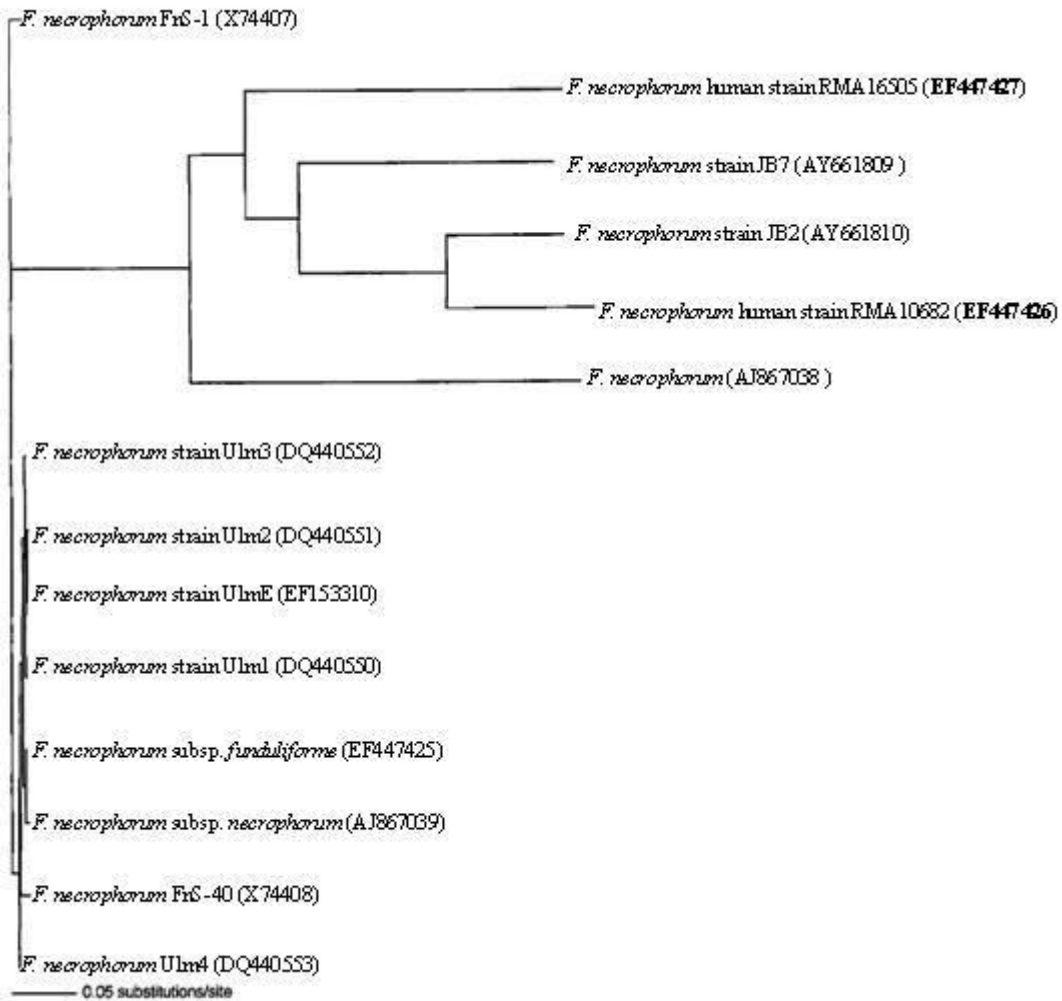


Figure 2.3. Phylogenetic analysis of human strains

Phylogenetic tree of 16S rDNA gene sequences of genus *Fusobacterium* (*F*) aligned with the sequences from *F. necrophorum* human clinical cases. Sequence accession numbers are represented in parentheses.

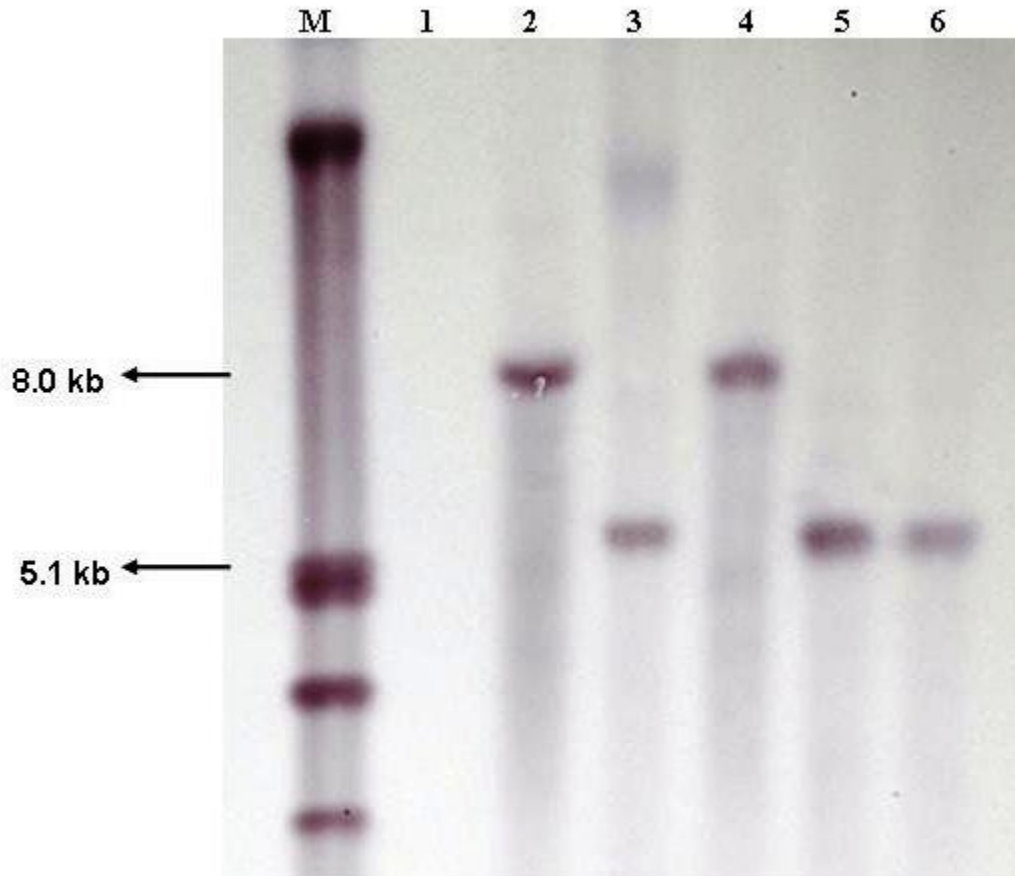


Figure 2.4. Southern hybridization using *lktB* probe

The *Hae III* digested genomic DNA with DIG labeled subsp. *funduliforme lktB*. Lane description: M, DIG labeled marker; 1, bovine subsp. *necrophorum* strain A25; 2, bovine subsp. *funduliforme* strain B35; lanes 3 to 6 human strains RMA10682, 14786, 16505, and 16539, respectively.

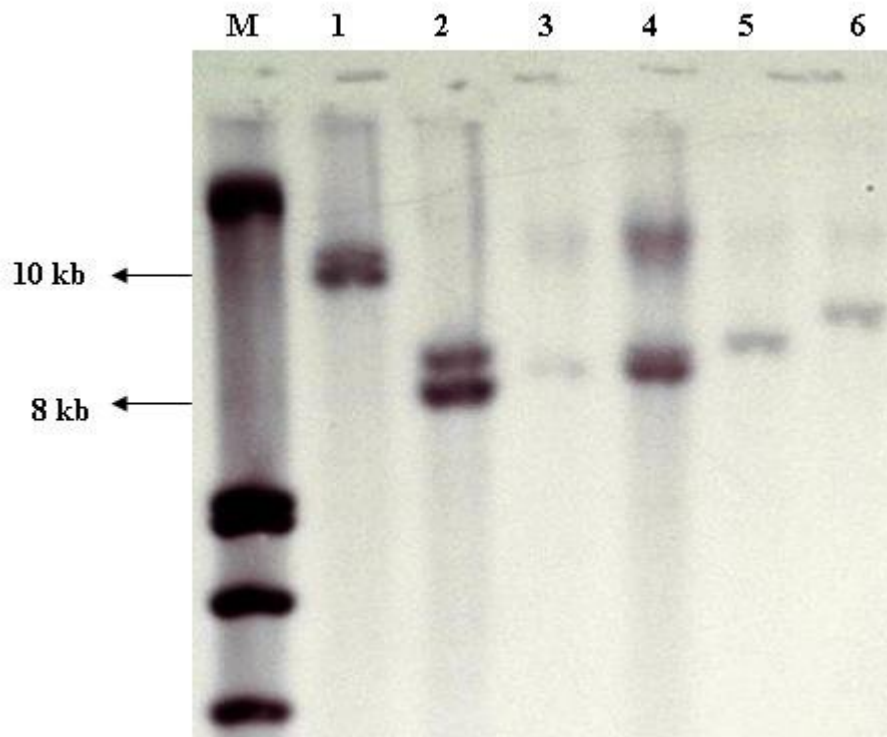


Figure 2.5. Southern hybridization using *lktA* probe

Southern hybridization of *Hae III* digested genomic DNA probed with DIG labeled subsp. *necrophorum lktA* probe. Lane description: M, DIG labeled marker; 1, bovine subsp. *necrophorum* strain A25; 2, bovine subsp. *funduliforme* strain B35; lanes 3 to 6 human strains RMA10682, 14786, 16505, and 16539, respectively.

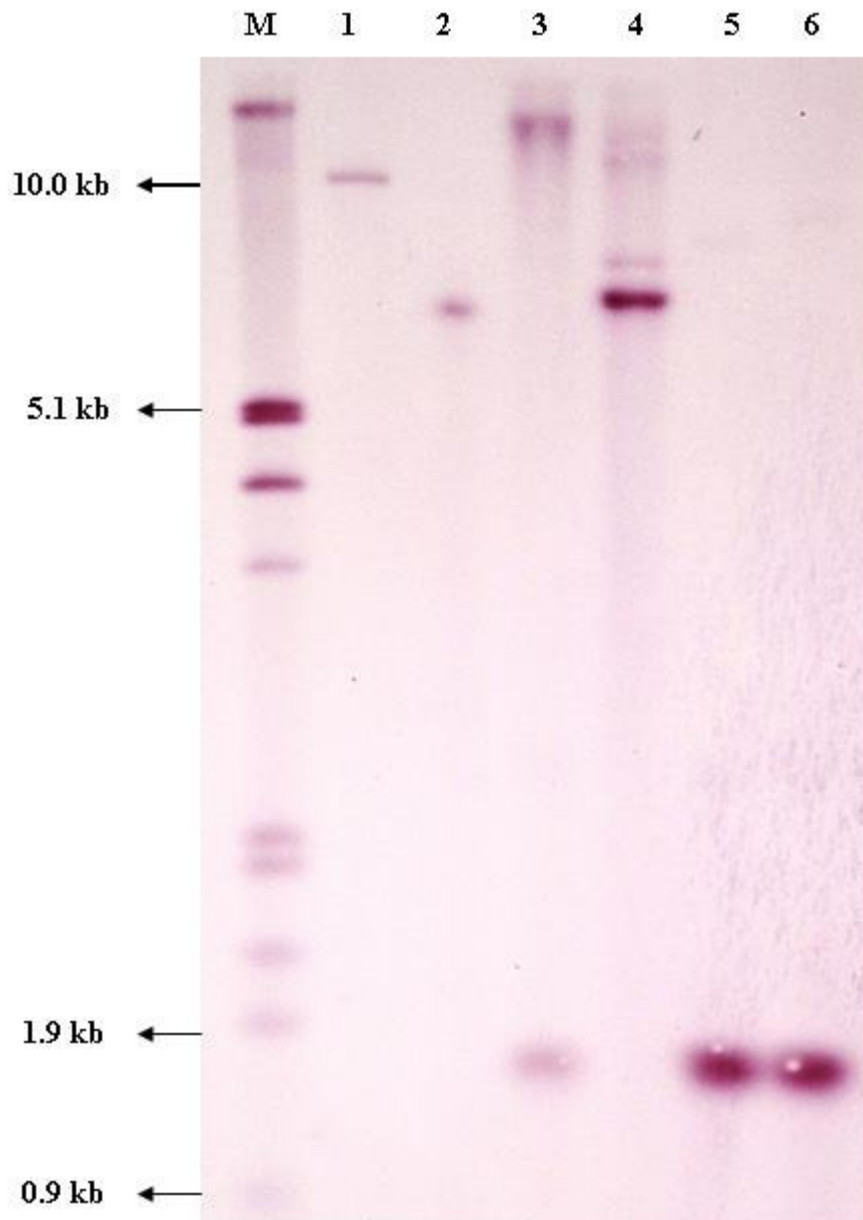


Figure 2.6. Southern hybridization using *lktC* probe

The *Hae III* digested genomic DNA probed with DIG labeled subsp. *necrophorum lktC*. Lane description: M, DIG labeled marker; 1, bovine subsp. *necrophorum* strain A25; 2, bovine subsp. *funduliforme* strain B35; lanes 3 to 6 human strains RMA10682, 14786, 16505, and 16539, respectively.

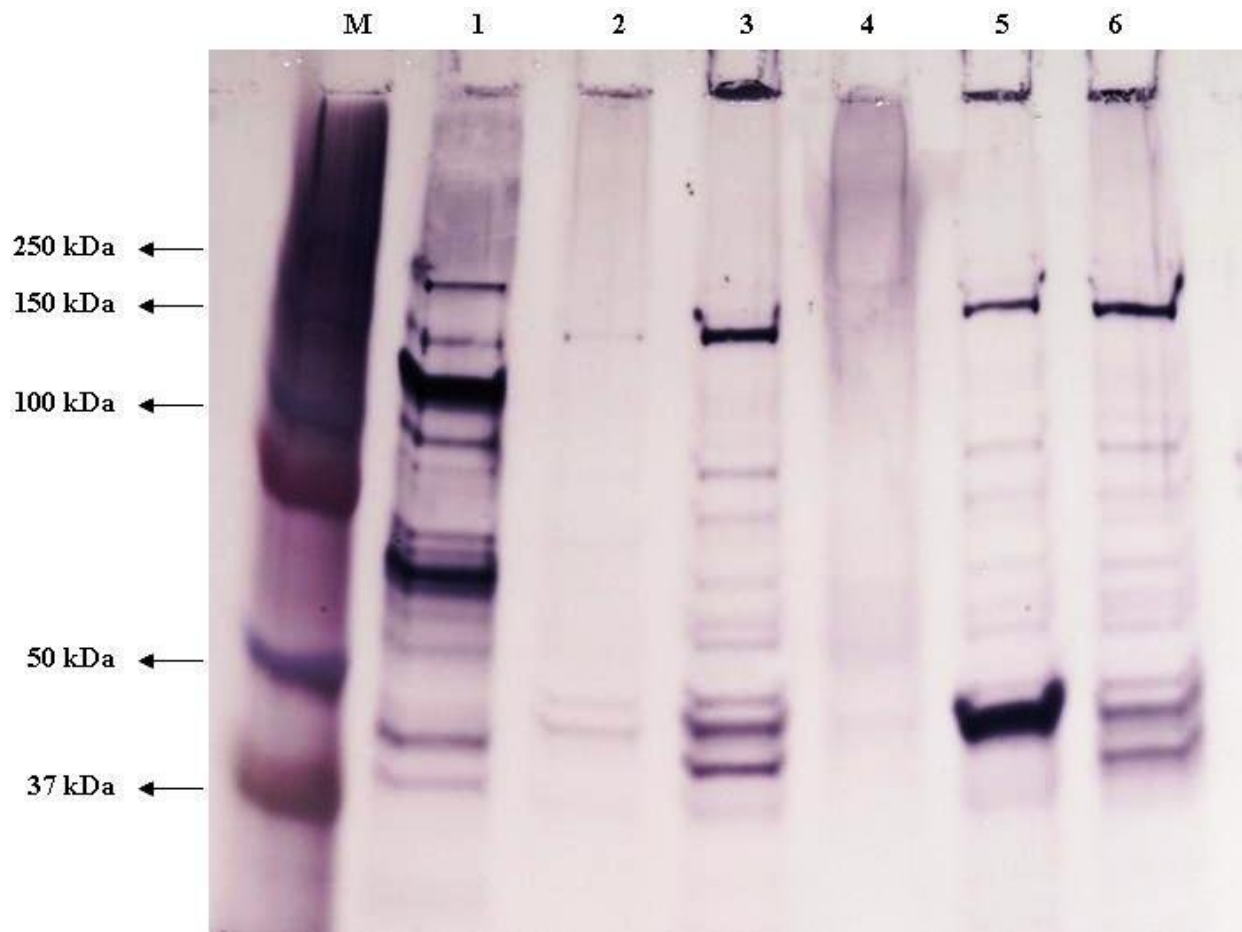


Figure 2.7. Western blot analysis

Concentrated culture supernatants probed with rabbit polyclonal serum raised against the LktA protein from bovine subsp. *necrophorum*. Lane description: M, prestained protein marker; 1, bovine subsp. *necrophorum* strain A25; 2, bovine subsp. *funduliforme* strain B35; lanes 3 to 6 human strains RMA10682, 14786, 16505, and 16539, respectively.

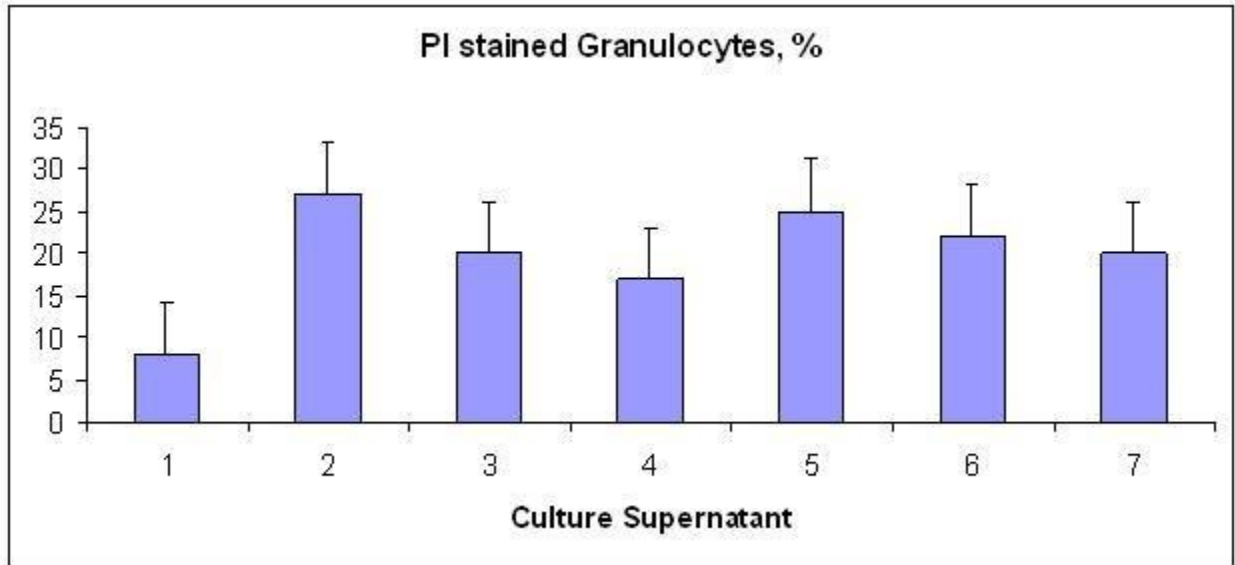


Figure 2.8. Cell viability assay

Human PMNs in complete RPMI were treated with respective culture supernatants at 37°C and 5% CO₂ for 45 min, washed and incubated with propidium iodide (PI). The PMNs were analyzed by flow cytometry and 10,000 events were recorded. Lanes: (1) PMNs in RPMI alone(no toxin), (2) bovine subsp. *necrophorum* strain A25, (3) bovine subsp. *funduliforme* strain B35, and lanes 4 to 7 human strains RMA10682, 14786, 16505, and 16539 respectively. Error bars represent the standard deviation of the average of three independent assays.

Chapter 3 -
***Fusobacterium equinum* possesses a leukotoxin gene and exhibits**
leukotoxin activity

Abstract

Fusobacterium equinum, a gram negative, rod-shaped and obligate anaerobic bacterium is a newly described species. The organism is associated with necrotic infections of the respiratory tract in horses that include necrotizing pneumonia, pleuritis and paraoral infections. The species is closely related to *F. necrophorum* that causes liver abscesses in cattle and sheep, calf-diphtheria in cattle, and foot-rot in sheep and cattle. Leukotoxin, an exotoxin is an important virulence factor in bovine strains of *F. necrophorum*. Our objective was to examine strains (n=10) of *F. equinum* for the leukotoxin (*lktA*) gene and its toxic effects on equine leukocytes. Southern hybridization and partial DNA sequencing revealed that all ten strains had the *lktA* gene with greater similarities to *F. necrophorum* subsp. *necrophorum*. The secreted leukotoxin was detected in the culture supernatant and its biological activity was determined by viability assays with equine leukocytes (PMNs) using flow cytometry. Culture supernatants of four strains (E1, E7, E9, and E10) were highly toxic to equine PMNs; one strain (E5) was moderately toxic. However, the remaining strains (E2, E3, E4, E6, and E8) culture supernatants were only mildly toxic to equine PMNs. In conclusion, our data indicate that *F. equinum* isolates possess *lktA* gene and exhibit leukotoxin activity, which may be a potential virulence factor.

1. Introduction

Fusobacterium equinum, a gram negative, rod-shaped, obligate anaerobe is a newly recognized species of bacteria. The taxonomic status of *Fusobacterium* spp. isolated from horses is not clearly identified in the literature (Citron, 2002). Bailey and Love (1991) were the first to report that the *Fusobacterium* spp. isolates were different from *F. necrophorum* based on phenotypic characteristics and DNA-DNA hybridization. Their observations were confirmed by Racklyeft and Love (2000) in their study on bacterial association with lower respiratory tract (LRT) infections of horses. Based on 16S rDNA gene sequence, Dorsch et al. (2001) reported that equine isolates formed a separate genetic cluster within the genus *Fusobacterium*.

Fusobacterium equinum is a normal inhabitant of the gastrointestinal, respiratory, and genitourinary tracts of horses (Bailey and Love, 1991; Jang and Hirsh, 1994; Racklyeft and Love, 1999). It is an opportunistic pathogen and is generally associated with abscesses and various necrotic (necrobacillosis) infections in horses (Zicker et al., 1990; Bailey and Love, 1991; Moore, 1993; Jang and Hirsh, 1994; Trevillian et al., 1998; Racklyeft and Love, 2000). More commonly, the organism is associated with oral and paraoral (PO) infections, and LRT infections like pleuro-pneumonia and pleuritis (Bailey and Love, 1991; Jang and Hirsh, 1994; Racklyeft and Love, 2000).

No virulence factors have been identified for *F. equinum*. In *F. necrophorum*, a primary etiological agent of liver abscesses in feedlot cattle, leukotoxin is a major virulence factor (Nagaraja et al., 2005). The leukotoxin gene *lktA* is a member of the tricistronic *lktBAC* operon and the complete sequence of the *lkt* operon has been determined (Narayanan et al., 2001; Oelke et al., 2005). The LktA leukotoxin protein is a high molecular weight (336 kDa) exotoxin that causes apoptosis and necrosis of bovine PMNs in a dose-dependent manner (Narayanan et al., 2002). Antibodies generated against purified native or recombinant leukotoxin protected mice against experimental challenge with *F. necrophorum* (Narayanan et al., 2003). The purpose of this study was to determine whether *F. equinum* clinical isolates have the leukotoxin (*lktA*) gene and exhibit leukotoxin activity.

2. Materials and Methods

2.1. Identification of Isolates

Ten *F. equinum* strains, isolated from horses, were used in this study. Strains E1 and E2 were isolated from lungs, E3 from sinus, E4 and E5 from the guttural pouch, and strains E6 to E10 were isolated from the tracheal washings of horses diagnosed with pneumonia. Two bovine strains, *F. necrophorum* subsp *necrophorum* A25 and subsp *funduliforme* B35, included in this study were previously isolated from bovine liver abscesses (Tan et al., 1992). Isolates were grown overnight on blood agar (Remel, Lenexa, KS, USA) at 39°C in an anaerobic glove box (Forma Scientific, Marietta, OH, USA) and subcultured in pre-reduced, anaerobically sterilized BHI broth (PRAS BHI).

2.2. Primer design and PCR conditions

Fusobacterium equinum 16S rDNA sequence (GenBank No. AJ295750) was aligned with all *F. necrophorum* 16S rDNA complete sequences deposited with NCBI database (ClustalW program) to identify a region specific to *F. equinum* 16S rDNA gene and primers were designed to amplify that region. The forward primer 5'-TTTCAGTCGGGAAGAAGAA – 3' (position 418- 436 bp) had two mismatches at the 3'- terminus whereas the reverse primer 5'-TAAGGCAGTTTCCAACGCA -3' (position 608-590 bp) had a single mismatch at 3'- terminus. The partial *F. equinum* 16S rDNA sequence deposited (GenBank No. AJ295750) was used to design the primers-forward primer 5'- CGCGTAAAGA AACTTGCCTCT -3' and reverse primer 5'- CTTCAGGTGC AACCCACTCT -3' to PCR amplify the 16S rDNA sequence from *F. equinum* clinical isolates. Specific primers used for hemagglutinin (*HA*) gene were previously designed by Aliyu et al. (2004). The PCR conditions were as follows : 1 cycle 94°C for 3 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at $T_m -5^\circ\text{C}$ for 30 seconds, and extension at 72°C (1 min per kb of expected product). The amplified products were electrophoresed in 1-1.2 % agarose; gel purified using Wizard SV gel and PCR clean up system (Promega Corp. Madison, WI) and sequenced (Beckman Coulter CEQ 8000 Genetic Analysis System, Fullerton, CA, USA). The analyzed sequences were then aligned with 16S rDNA sequences of 13 other species of the genus *Fusobacterium* using ClustalX program (opening gap penalty of 10 and extension penalty of 0.1) and a phylogenetic tree was constructed using

distance matrix algorithm and a neighbor-joining method (Dorsch et al., 2001) using the PAUP program (Sinauer Associates Inc. Publishers, Sunderland, MA).

2.3. Extraction of chromosomal DNA

Bacterial chromosomal DNA was prepared according to the procedure described by Narayanan et al. (2001). Briefly, isolates were grown overnight in anaerobic BHI broth. The cells were then pelleted by centrifugation at 5,000 x g for 10 min. at 4°C. The pelleted cells were washed and resuspended in TE buffer (Tris 100 mM; EDTA 10 mM; pH 8.0). The cell wall was digested with lysozyme (1 mg/ml), and the resulting lysate was treated with Sarkosyl (1%) followed by RNaseA (20 µg/ml) and pronase (50 µg/ml). The samples were incubated on ice for 20 min and then sequentially extracted with phenol and chloroform twice. The DNA from the extract was then precipitated in 1/10 vol. 3M sodium acetate (pH 5.2) and equal vol. 2-propanol. The DNA was resuspended in TE (Tris-HCl 10 mM, EDTA 1 mM; pH 8.0) and stored at 4°C until used. Nanodrop spectrophotometer readings (Nanodrop Technologies Inc., Wilmington, DE, USA) were used to determine the concentration of DNA.

2.4. Preparation of culture supernatants

Fusobacterium equinum and *F. necrophorum* strains were grown in PRAS BHI to an absorbance of 0.6-0.7 at OD_{600nm} and pelleted by centrifugation at 5,000 x g for 20 min. at 4°C (Tan et al., 1994). The supernatants were filtered through a 0.22 µm filter (Millipore, MA, USA) and aliquots were stored at -80° C until used.

2.5. Southern hybridization

Southern hybridization was performed according to the procedure of Narayanan et al. (2001). Briefly, 3 µg of chromosomal DNA was digested to completion by the restriction endonuclease *Hae III* (Promega Corp. Madison, WI, USA) and electrophoresed in a 1% agarose gel overnight at 20V at room temperature. The resolved DNA fragments were transferred to a positively-charged nylon membrane (Roche Diagnostics Corporation, Indianapolis, IN) by capillary mechanism and the DNA immobilized by UV cross-linking. DNA probes were synthesized by random-labeling method with DIG-dUTP (Roche Diagnostics) according to manufacturer's instructions. The immobilized DNA was probed with full-length bovine subsp.

necrophorum A25 *lktA* (at 54°C); subsp. *necrophorum* A25 and subsp. *funduliforme* B35 *lktB* (at 50°C); and subsp. *necrophorum* subsp. *necrophorum* A25 *lktC* (at 44°C). Colorimetric detection of hybridized signals was carried out as per manufacturer's instructions. (Roche Diagnostics, Indianapolis, USA).

2.6. Western-blot analysis

Protein profiles from concentrated (60 x) culture supernatants from all strains were resolved on 4-20% Tris-glycine gels for protein electrophoresis under denaturing conditions (Pierce, Rockford, IL) and electro-transferred onto nitrocellulose membrane (Millipore Corp., MA). The membrane was blocked with 0.2% skim milk overnight at 4°C. Rabbit polyclonal antisera raised against the bovine subsp. *necrophorum* strain A25 native leukotoxin (Narayanan et al., 2002) was used as the primary antibody. Goat anti-rabbit immunoglobulin conjugated with alkaline phosphatase (Sigma-Aldrich, St.Louis, MO) was used as the secondary antibody, and the immunoreactive proteins were detected using 5-bromo-4-chloro-3-indolylphosphate/nitro blue tetrazolium (BCIP/ NBT) as substrate.

2.7. Preparation of white blood cells

The equine leukocytes were prepared as per the protocol described by Yoshiaki et al. (1979). Briefly, whole blood was collected from a healthy horse by venipuncture of the jugular vein. After sedimentation of erythrocytes for 30 min at room temperature, the plasma was then transferred to another sterile tube. The remaining erythrocytes in plasma were lysed by RBC lysis buffer (Sigma-Aldrich, St. Louis, MO), washed twice in PBS and finally resuspended in complete RPMI 1640 (containing penicillin and streptomycin antibiotics). The number of viable leukocytes in the blood was determined by a 0.4 % trypan blue dye exclusion assay (Narayanan et al., 2002).

2.8. Cell viability assay

The cytotoxic effects of *F. equinum* and *F. necrophorum* culture supernatants on horse leukocytes were determined by a cell viability assay using propidium iodide (Narayanan et al., 2002). Briefly, 1×10^6 viable white blood cells were treated with culture supernatants for 45 min at 37°C and 5% CO₂, washed twice, resuspended in PBS and stained with 10 µl of propidium

iodide (50 µg/ml stock) in the dark for 5 min. Untreated cells in complete RPMI 1640 were used as a negative control. The samples were processed on a FACScan flow cytometer using an Argon ion laser (Becton Dickinson, San Jose, CA). Data were analyzed using Cell Quest analysis software (Becton Dickinson, San Jose, CA).

2.9. Nucleotide sequence accession numbers

The partial 16S rDNA sequence of *F. equinum* strains (E1 and E2) have been deposited in GenBank and assigned the accession numbers EF447428 and EF447429 respectively.

3. Results

3.1. Species identification of the isolates

The PCR analysis with *Fusobacterium equinum* 16S rDNA gene specific primers amplified the expected size (103 bp) product in all the equine isolates but not in either subspecies of bovine *F. necrophorum* (Fig. 3.1A). The partial 16S rDNA sequences of isolates E1 and E2 were identical to each other and to the sequence deposited with GenBank (AJ295750) (Fig. 3.2). *Fusobacterium equinum* strains formed sediment after overnight growth in BHI broth and were negative for alkaline phosphatase activity when tested by biochemical methods. All ten clinical isolates were PCR negative for hemagglutinin (*HA*) gene (Fig. 3.1B).

3.2. The *lktA* gene

The presence of the *lktA* gene in the clinical isolates was determined by Southern hybridization reactions (Fig. 3.3). In the bovine subsp. *necrophorum lktA*, there is a single recognition site for *Hae*III, whereas subsp. *funduliforme* has two sites. Therefore, digestion of subsp. *necrophorum* DNA produced two hybridizing bands whereas subsp. *funduliforme* DNA yielded three bands (one being too small to be visualized in Fig. 3.3). Among equine strains, four distinct hybridization patterns were observed that resembled neither bovine subspecies. In the first pattern, a less intense 8 kb and an intense ~3.0 kb bands were observed in strains E1, E3, E4, E6, and E7. The second pattern was observed in strains E2 and E5 that had a 10 kb and <3.0 kb bands. The third pattern, a single ~9.0 kb band was seen only in strains E8 and E9. The

fourth pattern, a single ~11.0 kb band (corresponded in size to one of subsp. *necrophorum* bands), was observed only in strain E10.

3.3. Western blot analysis

Proteins from concentrated culture supernatants were resolved by SDS-PAGE and probed with rabbit polyclonal sera raised against affinity purified leukotoxin from subsp. *necrophorum* (Fig. 3.4). Because of the known instability of the leukotoxin, a number of breakdown products are revealed by Western blot analysis (Tan et al. 1994). The protein species recognized by the antiserum were significantly different in subsp. *necrophorum*, subsp. *funduliforme* and *F. equinum* isolates. Supernatant from subsp. *necrophorum* (lane 1) had all the expected bands (~250 kDa, 150 kDa, 130 kDa and 110 kDa). Only two bands (lane 2) were detected in subsp. *funduliforme* (150 kDa and ~40 kDa size). A single 150 kDa size band corresponding in size to that of subsp. *necrophorum* strain A25 was observed in all of the *F. equinum* isolates.

3.4. Leukotoxin activity

The biological activity of the secreted leukotoxin was determined by viability assay using equine peripheral PMNs flow cytometry (Fig.3.5). Culture supernatants from strains E1, E7, E9 and E10 were highly toxic to equine PMNs. Strain E5 supernatant was moderately toxic while others were only mildly toxic to equine PMNs. In order to confirm that the observed toxicity was not due to contaminating LPS, we tested the toxic effects of the culture supernatant after passing supernatants over a polymyxin B column. No significant decrease in cytotoxic activity was observed. Interestingly, subspecies *funduliforme* culture supernatant was highly toxic while subsp. *necrophorum* culture supernatant was found to be only moderately toxic to equine PMNs.

4. Discussion

Fusobacterium equinum is indistinguishable from both the *F. necrophorum* subspecies based on morphological and biochemical characteristics. Dorsch et al. (2001) suggested certain characteristics to distinguish *F. equinum* from *F. necrophorum*. But in our study, we were unable to distinguish *F. equinum* from *F. necrophorum* based on those characteristics. For

example, growth of *F. equinum* resembled subsp. *funduliforme* by forming a flocculent deposit in overnight cultures grown in anaerobic BHI broth. The *F. equinum* isolates resembled bovine subsp. *funduliforme* by lacking enzyme alkaline phosphatase and hemagglutinin gene, which were the criteria suggested by Dorsch et al. (2001). The hemagglutinin gene (*HA*) is present only in bovine *F. necrophorum* subsp. *necrophorum* and absent in subsp. *funduliforme*. Therefore, the only available method to date for differentiating *F. equinum* from *F. necrophorum* is by sequencing the 16S rDNA gene.

To address this problem, we designed a new PCR based assay for identifying *Fusobacterium equinum*. In this assay, primers were designed with 3'- end mismatches to specifically amplify *F. equinum* 16S rDNA. No product was amplified in bovine subsp. *necrophorum* and subsp. *funduliforme* as well as *F. necrophorum* human strains (data not shown). However, the expected product was observed in equine isolates, thus confirming their identity as *F. equinum*. Hence, this PCR based assay can be used to successfully differentiate between *F. necrophorum* and *F. equinum*.

Fusobacterium necrophorum, a closely related species to *F. equinum*, is associated with various necrotic infections including hepatic abscesses in cattle, sheep and humans (Tan et al 1996). In horses, mostly race horses, intra-abdominal and hepatic abscesses are frequently caused by obligate anaerobic bacteria (Zicker et al. 1990). *Fusobacterium equinum* was identified as an etiological agent in approximately 33% of these cases. In addition, an unusual case of paracaecal abscess caused by *F. equinum* was identified by Trevillian et al. (1998).

Several virulence factors have been identified for *F. necrophorum* infections in cattle and humans (Nagaraja et al., 2005; Brazier, 2006). These include hemolysin, hemagglutinin, endotoxin and leukotoxin. These virulence factors aid the bacterium in colonizing the epithelium and thereby initiating the disease process. Very little is known about the virulence factors associated with *F. equinum* infections in horses. In bovine *F. necrophorum*, leukotoxin is the major virulence factor (Nagaraja et al., 2005). The leukotoxin operon consists of three genes, *lktB*, *lktA*, and *lktC*, of which *lktA* is the structural gene (Narayanan et al.2001; Oelke et al., 2005). The functions of *LktB* and *LktC* are not yet determined. The entire leukotoxin gene is

9,726 bp long, encoding a protein of 3,241 amino acids with a molecular weight of 336,956 daltons. The leukotoxin is a large secreted protein (336 kDa) that is cytotoxic, in a dose dependent manner, to neutrophils and to a lesser extent to lymphocytes (Narayanan et al., 2003). The *lkt* operon is under the control of a single promoter which differs in sequence and length between the two subspecies of bovine *F. necrophorum* (Zhang et al., 2006).

Data from our study revealed the presence of the *lktA* gene in all *F. equinum* isolates. In order to understand the various hybridization patterns observed, we set up PCR with primers targeting different regions of *lktA* gene. Interestingly, no region of *lktA* was amplified in isolate E2 by PCR using *F. necrophorum* – based primers, whereas only few regions were amplified in other isolates (data not shown). However our, Southern hybridization with the *lktA* probe revealed the presence of leukotoxin (*lktA*) gene in isolate E2, thus, suggesting that our inability to amplify the entire leukotoxin gene might be due to sequence differences at the primer annealing sites.

Our attempts at identifying the other two genes of the leukotoxin operon (*lktB* and *lktC*) were inconclusive. The Southern hybridization with both subsp. *necrophorum* and subsp. *funduliforme* *lktB* gene as probe produced faint bands. There was no significant enhancement in the band intensity despite the low stringent conditions employed in Southern hybridization. Therefore, it is not clear whether *lktB* is totally absent or has little identity to the *F. necrophorum* *lktB* gene. We were unsuccessful in identifying the *lktC* gene by either PCR or Southern hybridization technique, suggesting that the *lktC* gene might either be absent or sequence divergent.

The leukotoxin operon promoter was found to be unique and different in both the subspecies of bovine *F. necrophorum* (Zhang et al. 2006). The leukotoxin operon promoter in *F. equinum* was not detected by Southern hybridization with either bovine leukotoxin operon promoters as probe. The probable reason for the failure to detect the promoter element indicates that the *F. equinum* leukotoxin operon promoter may be different from either of the bovine leukotoxin operon promoters.

Fusobacterium necrophorum secretes various extracellular proteolytic enzymes that aid in the colonization and enhance the disease process by evading the host immune system (Wahren et al. 1971; Amoako et al 1993). Leukotoxin has been demonstrated to induce apoptosis in the host (bovine) immune effector cells (Narayanan et al., 2002) and hence an important virulence factor. There are differences in the type and amounts of proteolytic enzymes secreted by the two subspecies (Amoako et al., 1993).

There were differences in the cytotoxic effects of the culture supernatants among the *F. equinum* strains. Similar strain-to-strain variations in the cytotoxic effects of leukotoxin among the bovine strains of *F. necrophorum* were reported earlier (Tan et al, 1992). The reason for these variations is unclear. However, the role of proteolytic enzymes in the inactivation of leukotoxin has not been ruled out. The effects of proteolytic enzymes (trypsin and protease) on leukotoxin activity were studied by Coyle-Dennis and Lauerman (1978). It was observed that pretreatment of culture supernatant with proteolytic enzymes had lowered the leukotoxin activity. Therefore, the differences in cytotoxic activity among *F. equinum* strains may be related to the various proteolytic enzyme(s) or yet to be identified factor(s) produced or secreted by individual strains that need to be determined.

To our knowledge, this is the first report to document the presence of leukotoxin gene in *F. equinum* and its toxic effects on leukocytes. Further studies are needed to characterize the leukotoxin gene (*lktA*) and study the toxic effects of the purified leukotoxin on equine PMNs, and its role in the pathogenesis of paraoral and lower respiratory tract infections.

Acknowledgements

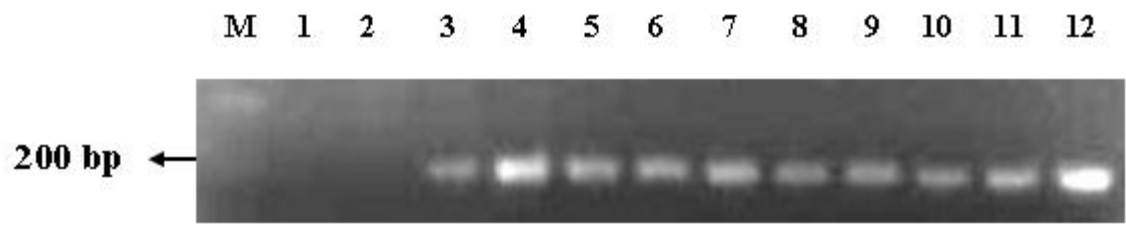
We thank David George for DNA sequencing, Tammy Koopman for assistance with the flow cytometry, and Ashvin Nagaraja and Leslie O’Conor for help in the laboratory.

References

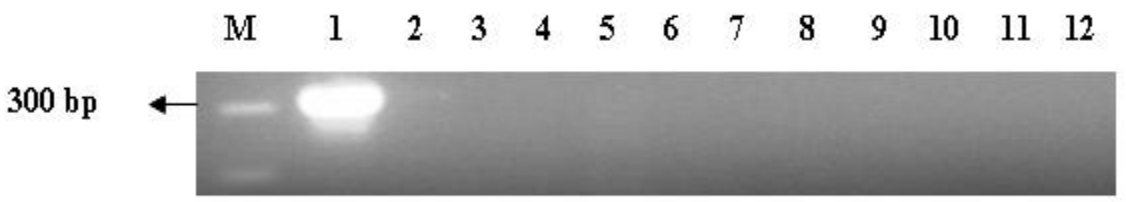
- Aliyu, S.H., Marriott, R.K., Curran, M.D., Parmar, S., Bentley, N., Brown, N.M., Brazier, J.S., Ludlam, H., 2004. Real-time PCR investigation into the importance of *Fusobacterium necrophorum* as a cause of acute pharyngitis in general practice. *J. Med. Microbiol.* 53, 1029-1035.
- Amoako, K.K., Goto, Y., Shinjo, T., 1993. Comparison of extracellular enzymes of *Fusobacterium necrophorum* subsp. *necrophorum* and *Fusobacterium necrophorum* subsp. *funduliforme*. *J. Clin. Microbiol.* 31, 2244-2247.
- Bailey, G.D., Love, D.N., 1991. Oral associated bacterial infection in horses: studies on the normal anaerobic flora from the pharyngeal tonsillar surface and its association with lower respiratory tract and paraoral infections. *Vet. Microbiol.* 26, 367-379.
- Citron, D.M., 2002. Update on the taxonomy and clinical aspects of the genus *Fusobacterium*. *Clin. Infect. Dis.* 35, S22-27.
- Coyle-Dennis, J.E., Lauerman, L.H., 1978. Biological and biochemical characteristics of *Fusobacterium necrophorum* leukotoxin. *Am. J. Vet. Res.* 39, 1790-1793.
- 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.
- Jang, S.S., Hirsh, D.C., 1994. Characterization, distribution, and microbiological associations of *Fusobacterium* spp. in clinical specimens of animal origin. *J. Clin. Microbiol.* 32, 384-387.
- Moore, R.M., 1993. Pathogenesis of obligate anaerobic bacterial infections in horses. *Compend. Cont. Educ. Pract. Vet.* 15, 278-286.
- 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., Stewart, G.C., Chengappa, M.M., Willard, L., Shuman, W., Wilkerson, M., Nagaraja, T.G., 2002. *Fusobacterium necrophorum* Leukotoxin Induces Activation and Apoptosis of *Bovine* Leukocytes. *Infect. Immun.* 70: 4609–4620.
- 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.
- Racklyeft, D., Love, D.N., 1999. Bacteriology of normal lower respiratory tract of horses and its significance for lower respiratory tract disease. *Aust. Equine Vet.* 17, 66-75.
- Racklyeft, D.J., Love, D.N., 2000. Bacterial infection of the lower respiratory tract in 34 horses. *Aust. Vet. J.* 78, 549-559.
- 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.
- Tan, Z.L., Nagaraja, T.G., Chengappa, M.M., Smith, J.S., 1994. Biological and biochemical characterization of *Fusobacterium necrophorum* leukotoxin. *Am. J. Vet. Res.* 55, 515-521.
- Trevillian, C.J., Anderson, B.H., Collett, M.G., 1998. An unusual paracecal abscess associated with *Fusobacterium necrophorum* in a horse. *Aust. Vet. J.* 76, 659-662.
- Wahren, A., Bornholm, K., Holme, T., 1971. Formation of proteolytic activity in continuous culture of *Sphaerophorus necrophorus*. *Acta. Path. Microbiol. Scand. Section B.* 79, 391-398.
- Yoshiaki, F., Perryman, L.E., Crawford, T.B., 1979. Leukocyte Cytotoxicity in a Persistent Virus Infection: Presence of Direct Cytotoxicity but Absence of Antibody- Dependent Cellular Cytotoxicity in Horses Infected with Equine Infectious Anemia Virus. *Infect. Immun.* 24, 628-636.
- 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.

Zicker, S.C., Wilson, W.D., Medearis, I., 1990. Differentiation between intra-abdominal neoplasms and abscesses in horses, using clinical and laboratory data: 40 cases (1973-1988). J. Am. Vet. Med. Assoc. 196, 1130-1134



[A]



[B]

Figure 3.1. PCR analysis

PCR amplification of 16s rDNA using *Fusobacterium equinum* specific primers (A) and hemagglutinin gene (B) in *F. equinum* strains and two bovine *F. necrophorum* strains.

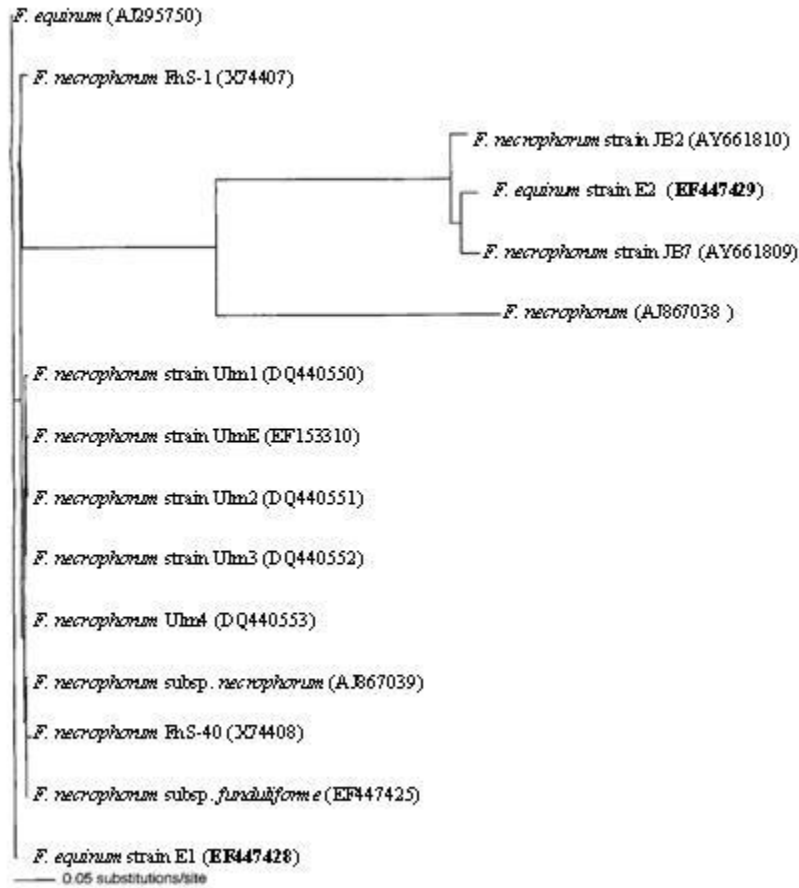


Figure 3.2. Phylogenetic analysis of *Fusobacterium equinum* isolates

Phylogenetic tree of 16S rDNA gene sequences of genus *Fusobacterium* (*F*) aligned with the sequences from *F. equinum* clinical cases. Sequence accession numbers are represented in parentheses.

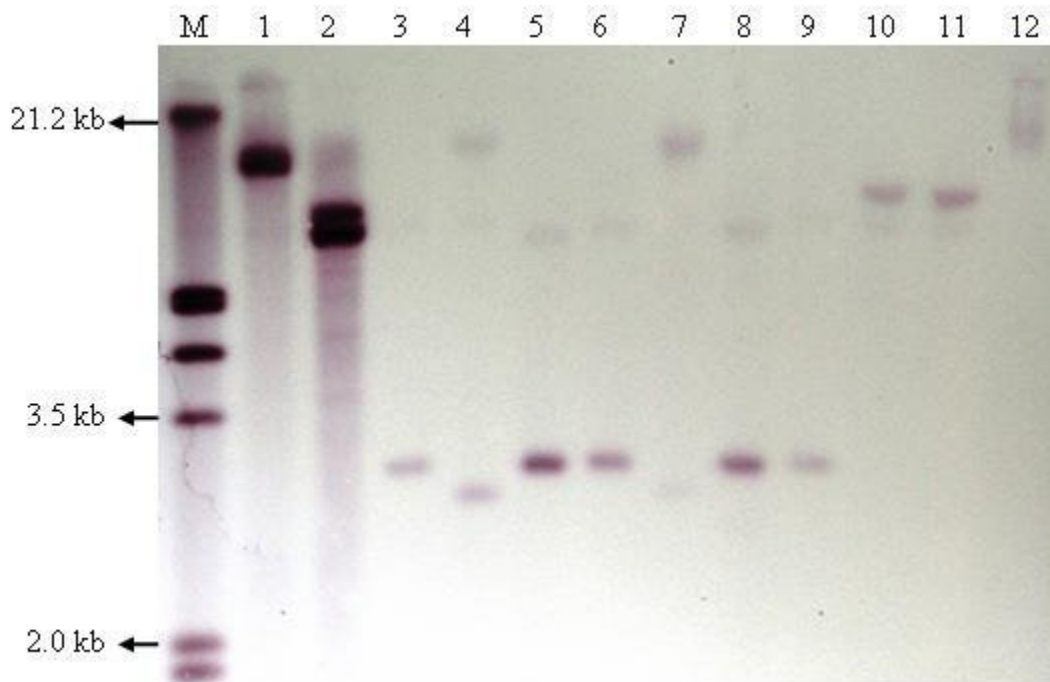


Figure 3.3. Southern hybridization using *lktA* probe

Southern hybridization patterns of *Hae III* digested genomic DNA probed with DIG labeled bovine subsp. *necrophorum lktA*. Lane description: M, DIG labeled marker; lane 1, bovine subsp. *necrophorum* strain A25; lane 2, bovine subsp. *funduliforme* strain B35; lanes 3 to 12 equine strains E1 to E10, respectively.

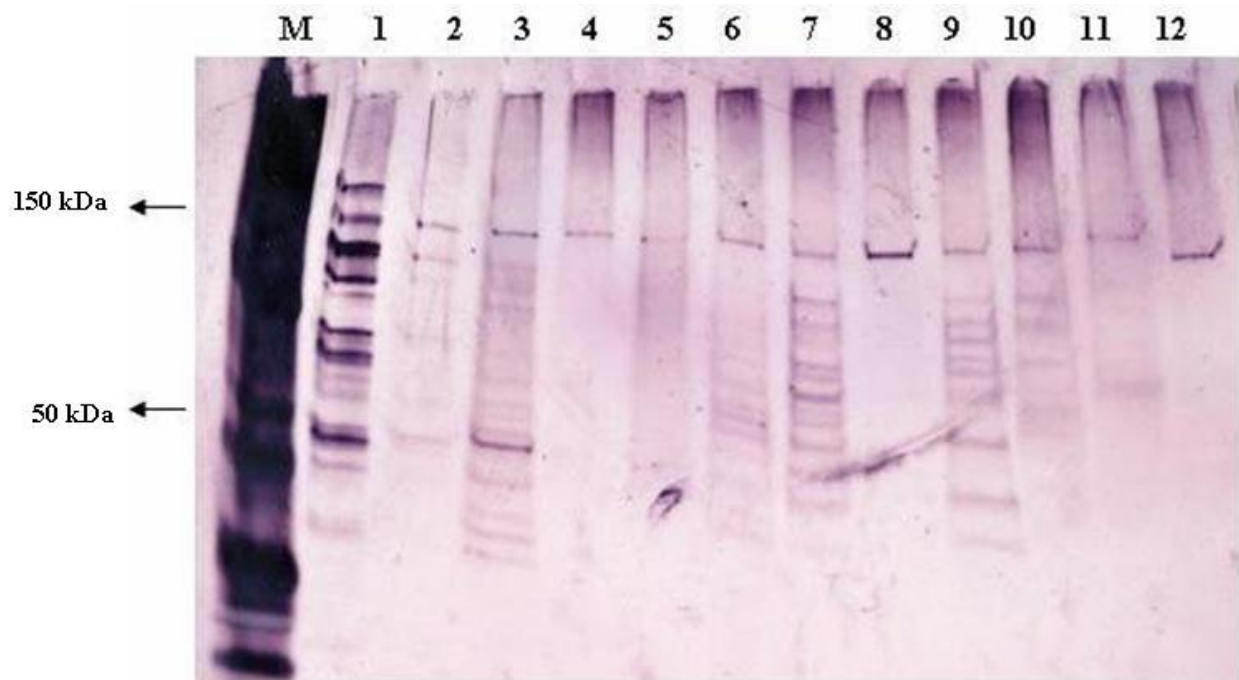


Figure 3.4. Western blot analysis

Concentrated culture supernatants were probed with rabbit polyclonal serum raised against the LktA protein from bovine subsp. *necrophorum*. Lane description: – M, prestained protein marker; lane 1, bovine subsp. *necrophorum* strain A25; lane 2, bovine subsp. *funduliforme* strain B35; lanes 3 to 12 equine strains E1 to E10 respectively.

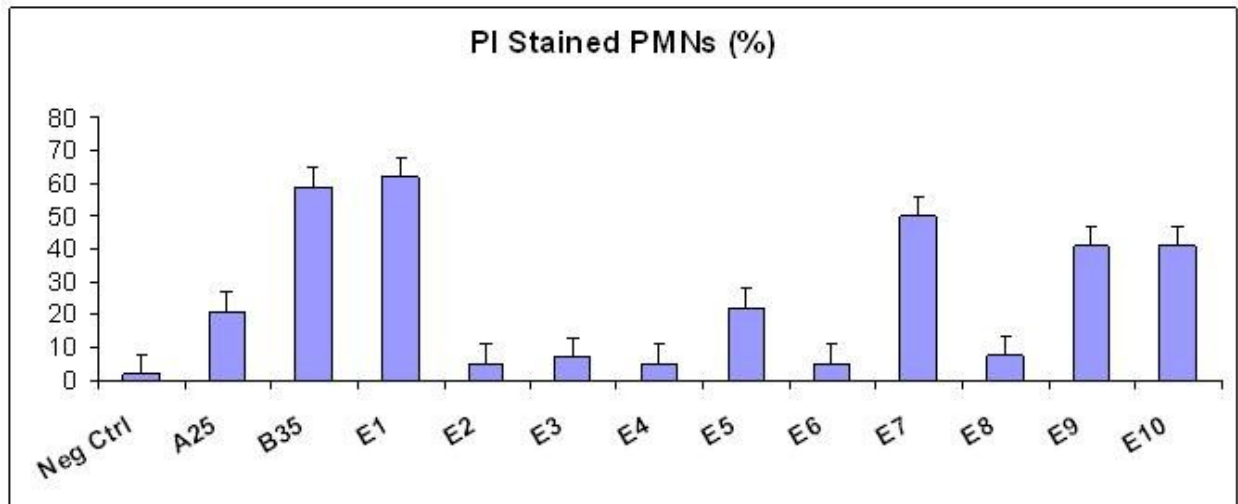


Figure 3.5. Cell viability assay

Equine leukocytes in RPMI treated with culture supernatants and stained with propidium iodide (PI) were analyzed by flow cytometry and 10,000 events were recorded. Leucocytes in medium alone (Neg ctrl), bovine subsp. *necrophorum* strain A25, bovine subsp. *funduliforme* strain B35, *F. equinum* isolates E1 to E10. (Error bars represent the S.E. of (n=3) independent experiments).

Chapter 4 -
***Fusobacterium necrophorum* Virulence Factors: A Review**

Introduction

Fusobacterium necrophorum is a gram negative, pleomorphically rod-shaped, non-spore forming and obligate anaerobic organism. As an opportunistic pathogen it is frequently associated with necrotic lesions in man and animals (Langworth, 1977; Hagelskjaer, 1998; Kristensen, 2000; Nagaraja et al., 2005). In animals, *Fusobacterium necrophorum* causes abscesses in domestic, wild and captive animals. Edwards et al. (2001) reported *F. necrophorum* as the causative agent of necrotic stomatitis in wild-caught pronghorns (*Antilocapra americana*) that later progressed to fatal septicemia. *Fusobacterium necrophorum* associated necrotic infections were also detected in other wild animals like blue duiker (*Cephalophus monticola fuscicolor*), white-tailed deer (*Odocoileus virginianus*), and captive wallabies (*Macropus rufogriseus*) (Wobeser et al., 1975; Oliphant et al., 1984; Smith et al., 1986; Roeder et al., 1989).

In domestic animals, *F. necrophorum* is associated with liver abscesses in feed lot cattle, calf-diphtheria (necrotic laryngitis) and foot rot in sheep and cattle (Nagaraja et al., 2005), necrotic rhinitis and neonatal septicemic conditions in swine (Ramos-Vara et al., 1997). In humans, *F. necrophorum* is the etiological agent of Lemierre's syndrome (Lemierre, 1936; Riordan, 2004). Lemierre's syndrome is characterized initially by fever which leads to thrombophlebitis of the internal jugular vein followed by abscessation that metastasizes to various internal organs. Falkler et al. (1999) have isolated *F. necrophorum* from 87.5% of NOMA (Cancrum oris) lesions in malnourished children from developing countries in Africa.

Fusobacterium necrophorum is a normal inhabitant of the respiratory, gastrointestinal and genitourinary tracts of animals and humans (Langworth, 1977; Tan et al., 1996; Nagaraja et al., 2005). *F. necrophorum* is the primary etiological agent of bovine hepatic abscesses, from which it is frequently isolated in pure culture (Scanlan and Hathcock, 1983; Lechtenberg et al., 1988). In cattle, there are two subspecies of *F. necrophorum*, subsp. *necrophorum* and subsp. *funduliforme* that are distinguished by growth, colonial and morphological characteristics and biochemical features ((Table 4.1; Shinjo et al., 1991; Nagaraja et al., 2005). The virulence factors and pathogenic mechanisms that contribute to the transition of this otherwise commensal

organism to a pathogen are poorly understood. In this paper, we have reviewed the various virulence factors and their role in initiating the disease process.

Virulence Factors

For successful colonization and initiation of the disease process, pathogenic bacteria employ various strategies to evade the host immune responses, proliferate in tissue, and cause damage to the host. In this endeavor, bacteria produce several toxins that cause various pathologies in the host, which sometimes may be fatal to the host. Several virulence factors (toxins) have been identified in the diseases associated with *F. necrophorum*. These include endotoxin, haemagglutinin, hemolysin, leukotoxin, capsule, dermonecrotin, platelet aggregation factor, collagenolytic cell wall component and proteases. The role of individual factors in the pathogenesis of *F. necrophorum* infections is described below.

Endotoxin

There was little evidence to support either the presence of or biological activity of LPS in *F. necrophorum* until the beginning of 1900s. Césari (1912) was the first to report the presence of endotoxic activity in *F. necrophorum* cultures, which was later supported by Kirchheiner (1940), by extracting lipopolysaccharide (LPS) using trichloroacetic acid method and evaluating the toxicity in mice. These observations were further strengthened by Sonnenwirth et al. (1972) with the detection of endotoxin in *Fusobacterium necrophorum* by a rapid and sensitive Limulus assay.

The chemical composition of *F. necrophorum* LPS was analyzed by several investigators (Hofstad and Kristoffersen, 1971; Garcia et al., 1975; Okahashi et al., 1988). The composition of the LPS varied depending on the subspecies, purity of LPS and the analytical techniques employed. On average, *F. necrophorum* LPS has reducing and neutral sugars (50.5%), hexosamines (8.5%), 2-keto-3-deoxy-octonate (KDO) (0.8%), lipid A (21.0%), protein (2.4%), nitrogen (2.1%) and phosphorus (1.7%), similar to a ‘classical’ LPS seen in other gram negative

bacteria. The two most abundant sugar types identified in *F. necrophorum* LPS were hexoses and heptoses (Inoue et al., 1985).

Previous studies indicated that subsp. *necrophorum* LPS was more toxic than subsp. *funduliforme* (Kanoë, et al., 1976, Langworth, 1977; Kanoë, et al., 1979). To understand the factors responsible for the difference in toxicity, studies were conducted to compare the composition of LPS in both subspecies. Inoue et al. (1985) reported that subsp. *necrophorum* (2.57 %) had higher amounts of LPS than subsp. *funduliforme* (1.24 %) based on the dry cell weight. The LPS from subsp. *funduliforme* had higher concentration of glucose (66.2 %) compared to subsp. *necrophorum* (21.5 %). There were differences (Table 4.2) in the amounts of aminosugar (12.9 % in subsp. *necrophorum* and 0.9 % in subsp. *funduliforme*) and Lipid A (34.0 % and 4.2 % in subsp. *necrophorum* and subsp. *funduliforme*, respectively) composition of the LPS. The subsp. *necrophorum* LPS lacks the O-antigen making it smooth, thereby can evade the host immune system, whereas rough LPS makes subsp. *funduliforme* an easy target for clearance by the host defense mechanisms (Brown et al., 1997). However, Berg and Scanlan (1982) did not observe any functional differences in the toxicity of endotoxin in rabbits between the two subspecies. These conflicting observations may be due to different bacterial strains and techniques used. In addition, Garcia et al. (1999) reported that the subsp. *funduliforme* strain used by Inoue et al. (1985) was identified to be a new species, *F. pseudonecrophorum* by Shinjo et al. (1990). Therefore, additional studies are needed to precisely determine the quality and biological activity of LPS in *F. necrophorum* subspecies.

Meisel-Mikolajczyk and Dobrowolska (1974) isolated endotoxin from human strains using Westphal method and compared the composition with similarly isolated endotoxin from animal strains (Table 4.3). The human strains had low amounts of amino sugars, amino acids, and heptoses compared to endotoxin from animal strains. Brown *et al.* (1997) reported that endotoxin from human strains closely resembled bovine *F. necrophorum* subsp. *funduliforme* endotoxin in composition.

The biological activity of *F. necrophorum* LPS from animal and human strains (Hill *et al.*, 1974) was studied both *in vitro* and *in vivo* models. *In vitro*, *F. necrophorum* LPS (>100

µg/ml) was shown to be highly cytotoxic to mouse peritoneal macrophages (Garman et al., 1980). The in vivo effects of *F. necrophorum* LPS in laboratory animals were studied by several investigators. The LPS was lethal to rabbits, mice and chicken embryos; caused localized and generalized shwartzman reactions and induced classic 'biphasic' febrile reaction in rabbits when given intravenously (Warner et al., 1974; Garcia et al., 1975; Langworth, 1977; Inoue et al., 1985). *Fusobacterium necrophorum* LPS also caused erythema and edema in rabbit skin (Berg and Scanlan, 1982) and hepatic degeneration and necrosis in mice (Nakajima et al., 1985). The role of *F. necrophorum* LPS in inducing local skin reaction in mice and guinea pigs was determined (Kanoë et al., 1995). Intradermal injection of LPS elicited inflammatory, hemorrhagic lesions and increased vascular permeability. These effects were dose dependent in guinea pigs; while only certain mouse strains were sensitive to *F. necrophorum* LPS.

The effect of LPS on the genesis of hepatic abscesses in laboratory animals was studied by several investigators. Scanlan et al. (1983) evaluated certain biological features in liver abscess formation in rats. The rats were inoculated with both subspecies intraportally. There was a significant increase in serum alanine aminotransferase (ALT) levels in rats inoculated with subsp. *necrophorum*. The duration of bacteremia, weight loss and leukocytosis were longer in rats given subsp. *necrophorum* than subsp. *funduliforme*. The leukocytosis was characterized by severe neutrophilia with a left shift. The plasma endotoxin from subsp. *necrophorum* was easily detectable; subsp. *funduliforme* endotoxin was not detected anytime during the test period. Hence, subsp. *necrophorum* endotoxin was shown to be more toxigenic than subsp. *funduliforme* endotoxin in causing liver abscesses in rats.

The pathogenesis of *F. necrophorum* induced liver abscesses in mice and acute hepatic necrosis in rabbits were studied by Nakajima et al. (1985, 1987, 1988). Injection of a mixture of *F. necrophorum* and LPS induced hepatic necrosis and abscess formation in mice (Nakajima, 1985). The role of cytoplasmic and cell wall fractions in inducing liver abscesses in mice was also investigated. Cytoplasmic fractions (rich in sugar and protein) were found to be more potent in causing hepatic necrosis than cell wall fractions. It was believed that the cytoplasmic fractions may inhibit phagocytosis by macrophages in the sinusoids, which may be responsible for the degeneration of the hepatocytes. Endotoxin from *F. necrophorum* or *E. coli* promoted the

pathogenic effects of cytoplasmic or supernatant fractions of *F. necrophorum* in the formation of hepatic necrosis and suppurative meningitis in rabbits (Nakajima, 1988).

The immunobiological properties of *F. necrophorum* LPS were investigated by several researchers. The antigenic nature of LPS from both subsp. was determined by intraperitoneal injections in mice and rabbits. The antibody titers against the respective LPS were determined by a ring test (Inoue et al., 1985); thus, confirming the antigenic nature of *F. necrophorum* LPS. Garcia et al. (2000) demonstrated that the differences in toxicity associated with *F. necrophorum* subspecies' endotoxin was due to the changes in leukocyte trafficking and endotoxin content in mice. The subsp. *necrophorum* endotoxin caused an initial neutrophilia, followed by progressive monocytosis and severe lymphopenia leading to liver abscess formation. Sustained monocytosis and neutropenia were prominently observed, and no liver abscesses developed with subsp. *funduliforme* endotoxin. Though both endotoxins activated PMNs chemotactically, subsp. *necrophorum* endotoxin activated PMNs at much lower doses (1 µg) than subsp. *funduliforme* endotoxin. It was suggested that the intense neutrophilia observed with subsp. *necrophorum* endotoxin might be a terminal host defense mechanism activated by inflammatory cytokines. Failure to cause liver abscesses by subsp. *funduliforme* endotoxin may be due to the differences in the cellular pathways (monocytosis) leading to clearance of infection.

It was assumed that LPS induced immune responses were T-cell independent; and hence, no memory T-cells were present to trigger antibody responses upon subsequent exposure to LPS. To determine this, Hodges et al. (1982) studied the role of trichloro acetic acid (TCA) extracted lipopolysaccharide-protein (LPS-P) complex from *F. necrophorum* in inducing the immunologic memory both at the cellular and humoral levels. At the cellular level, the response to LPS-P was evaluated by a direct and indirect spleen plaque forming cell (PFC) count. In the direct method, the number of antibody producing B-cells was measured; whereas the indirect PFC counts of IgG were measured. The primary PFC counts reached a maximum by 14-days after IV injection and 28 days for IM injection of LPS-P. The higher secondary PFC response counts indicated the increased number of IgM and IgG synthesizing cells after the booster dose. A booster dose at post-infection day 105 resulted in an increased PFC counts involving IgM and IgG. At the humoral level, the concentrations of specific serum immunoglobulins were measured by ELISA.

This concentration was in agreement with the PFC counts measured at the cellular level indicating the presence of LPS-P sensitive memory cells. Thus, the LPS-P stimulated helper T-cell antibody responses and its role as a sole B-cell stimulant needs to be evaluated.

Okahashi et al. (1988) studied the mitogenic activities of purified *F. necrophorum* LPS in mice. It was observed that the partially purified LPS was more mitogenic than purified LPS to mouse spleen cells, which may be due to the presence of contaminating endotoxic proteins. The purified *F. necrophorum* LPS was able to activate peritoneal macrophages in synthesizing the pro-inflammatory cytokine, interleukin-1 (IL-1). The role of these cytokines stimulated by LPS in the development of liver abscesses needs to be further evaluated for determining the importance of endotoxin as a virulence factor for *F. necrophorum*.

Haemagglutinin

It has long been known that *F. necrophorum* subsp. *necrophorum* agglutinates erythrocytes from chicken and other animal species (Langworth, 1977). This ability to haemagglutinate erythrocytes was initially used to differentiate human and animal *F. necrophorum* isolates (Beerens, 1954). The human isolates agglutinated human, guinea pig and rabbit erythrocytes; while animal isolates agglutinated chicken, sheep, human, bovine, rabbit and guinea pig erythrocytes. Among the animal isolates, Shinjo et al. (1980) determined that both subspecies of *F. necrophorum* vary in their ability to agglutinate erythrocytes from different animal species. Subsp. *funduliforme* had relatively low hemagglutination titer for chicken and cattle erythrocytes compared to subsp. *necrophorum*.

The macromolecular nature of haemagglutinin (HA) in *F. necrophorum* subsp. *necrophorum* was determined by Nagai et al. (1980). Haemagglutinin was shown to be a heat labile, low molecular weight protein (19 kDa), rich in alanine, glutamine and histidine amino acids. Electron microscopy studies revealed that HA formed a filamentous rod (0.5-1.0 nm) whose subunits polymerized to form a large molecule. It was not known whether HA was an outer membrane protein (OMP) or a secreted protein that agglutinates erythrocytes. Recently, Kanoe et al. (1998) demonstrated that *F. necrophorum* subsp. *necrophorum* HA was present on

the cell surface and suggested HA might be a bacterial appendage. Miyazato et al. (1978) reported the presence of structures that resembled *Enterobacteriaceae* fimbriae on the *F. necrophorum* subspecies' cell surface. However, no further studies were undertaken to confirm the above observations. Therefore, it is not known whether HA and fimbriae are synonymous.

The relationship between the presence of HA and pathogenicity of *F. necrophorum* to mice has been studied (Langworth, 1977). Animal isolates of subsp. *necrophorum* having HA were found to be more virulent than animal and human isolates of subsp. *funduliforme* lacking HA (Smith, 1975). Shinjo and Kiyoyama (1986) also reported that a mutant strain of *F. necrophorum* lacking HA failed to kill mice upon challenge in comparison to the wild type strain.

Higher virulence of subsp. *necrophorum* compared to subsp. *funduliforme* may be due to its ability to adhere to the epithelial cells on mucosal surfaces. *Fusobacterium necrophorum* was shown to adhere to Vero cells (Kanoë et al., 1985), bovine ruminal cells (Kanoë and Iwaki, 1987), bovine portal cells (Yamaguchi et al., 1999), and mice, rabbit, and goat cheek cells (Okada et al., 1999). The adherence was inhibited by pre-treatment with anti-hemagglutinin serum (Kanoë et al., 1985; Kanoë and Iwaki, 1987). Okada et al. (1999) showed by scanning electron microscopy that fusobacterial cells were able to penetrate mice and rabbit cheek cells indicating that *F. necrophorum* infections are highly invasive, which is mediated by hemagglutinin. Trypsin and pepsin pretreatment of cells reduced the adherence of bacterial cells, whereas no significant decreases in adherence was observed upon pretreatment with lipase and neuraminidase (Kanoë et al., 1985). Takayama et al. (2000) demonstrated that adherence of subsp. *necrophorum* to ruminal cells was mediated by cellular type 1 collagen. Degradation of cellular actin was observed in bovine portal cells after treatment with *F. necrophorum* (Yamaguchi et al., 1999). Thus, hemagglutinin aids in adherence to ruminal cells leading to actin degradation, and therefore appears to be important in initial stages of infection.

Dermonecrotic toxin

The toxic effects of *F. necrophorum* cell fractions on the skin were observed by Garcia et al. (1975). Intradermal injections of intact cells, sonic extracts and cell wall fractions in rabbits and guinea pigs caused erythema and necrosis of the skin. However, the cytoplasmic fractions were unable to induce necrosis in guinea pig skin. Recently Kanoe et al. (1995b) isolated a dermonecrotic toxin from the cell wall preparations of *F. necrophorum* that induced hemorrhagic necrosis in the skins of guinea pigs and rabbits. The importance of dermonecrotoxin in the development of *F. necrophorum* infections (including foot rot) needs to be evaluated.

Platelet aggregation

Platelet aggregation is suggested to be an important virulence factor for microbial pathogenesis, because of its involvement in the development of thrombocytopenia, disseminated intravascular coagulation, fibrin deposition, and other coagulative effects (Kurpiewski et al., 1983). The ability of *F. necrophorum* subsp. *necrophorum* alone to aggregate human platelets (Forrester et al., 1985) and bovine platelets (Kanoe et al., 1989; Horose et al., 1992) has been demonstrated. The release of serotonin from aggregated platelets was not due to platelet lysis, but was a typical aggregation-degranulation reaction (Forrester et al., 1985). The absence of platelet lysis was confirmed by cytosolic lactic acid dehydrogenase (Forrester, et al., 1985) and scanning electron microscopy technique (Kanoe et al., 1989). The platelet aggregation was ion-dependent (Ca^{2+}) and a cyclooxygenase sensitive event. It was also observed that platelet aggregation was fibrinogen dependent when human platelets were used compared to bovine platelets whose aggregation was independent of fibrinogen. Both endotoxin and hemagglutinin were shown to be associated with bovine platelet aggregation (Kanoe et al., 1989; Horose et al., 1992). The aggregation of bovine platelets was slower than that of human platelets. The intravascular coagulation associated with platelet aggregation may help the growth of *F. necrophorum* by establishing an anaerobic microenvironment. Hence, platelet aggregation appears to play an important role in the early stages of infection.

Hemolysin

It is important to establish an anaerobic environment for growth and initiation of the disease process by *F. necrophorum*. Also, like many other pathogenic bacteria, *F. necrophorum* requires iron for its growth (Tan et al., 1992). Hence, lysis of erythrocytes by *F. necrophorum* will not only provide iron but also create anaerobic environment by reducing the oxygen supply to the site of infection (Tan et al., 1996). It was also observed that hemolytic strains of *F. necrophorum* were more pathogenic than non-hemolytic strains (Fievez, 1963).

Garcia et al. (1975) demonstrated that cytoplasm and intracytoplasmic extract of *F. necrophorum* were hemolytic to chicken, rabbit, sheep, bovine, and human erythrocytes. The culture supernatant of *F. necrophorum* was hemolytic to rabbit, human, and dog erythrocytes while trace hemolysis was seen with goat, sheep and bovine erythrocytes (Abe et al., 1979). However, Garcia et al. (1975) were unable to observe hemolytic activity with *F. necrophorum* culture filtrate. The variable sensitivities of erythrocytes to *F. necrophorum* hemolysin were determined (Amoako et al., 1994). Horse, cat, dog, rabbit, quail, pigeon, human erythrocytes were highly sensitive to subsp. *necrophorum* hemolysin than subsp. *funduliforme*. Cattle, sheep, and chicken erythrocytes were found to be insensitive to hemolysin from both the subspecies. However, Tan et al. (1994b) reported that sheep erythrocytes were equally sensitive to hemolysin from both the subspecies. Cat erythrocytes were refractory to subsp. *funduliforme* hemolysin (Amoako et al., 1994).

Hemolysin is an unstable and heat labile exotoxin, inactivated by heat treatment at 56°C for 30 min (Roberts, 1967). Amoako et al. (1996b) observed that Tween-80 was able to stabilize hemolysin without affecting the hemolytic activity. Thus, it was made easy to determine the role of hemolysin in the pathogenesis of *F. necrophorum* infections. Several growth, physical and chemical factors were shown to influence the secretion and hemolytic activity. The subsp. *necrophorum* grown in a medium rich in proteins was demonstrated to be highly hemolytic than when grown in medium rich in easily fermentable sugars (Amoako et al., 1994), thus indicating a possible difference in the nature of hemolysin of the two subspecies. Decreased activity was observed when *F. necrophorum* was grown in either acidic or basic medium. Hemolysin

production was seen from early log phase of growth and continued until 18 hr without losing the activity. The increased hemolytic activity observed upon inclusion of Tween-80 in the culture medium was due to increased extraction of hemolysin from the cell wall. Complete inhibition of hemolysin production was achieved by treatment with sodium azide (Amoako et al., 1996a). Inclusion of hemoglobin into culture medium increased hemolysin activity at lower concentration ($0.1 \mu\text{g ml}^{-1}$) whereas higher concentration of hemoglobin lowered the hemolysin activity. Addition of antibiotics like chloramphenicol had no effect on the hemolytic activity. In conclusion, the secretion of hemolysin by *F. necrophorum* may be energy, temperature and pH dependent.

The differences in the sensitivity of various animal species erythrocytes to *F. necrophorum* hemolysin may be due to its interactions with erythrocyte membranes (Abe et al., 1979; Amoako et al., 1997; 1998). Hemolysin was readily bound to horse and dog erythrocyte membranes, but not cattle and sheep. The binding was temperature independent, whereas hemolysis was dependent on temperature reaching maximal titers at 37°C. Pretreatment of erythrocytes membranes with pronase, proteinase K, trypsin or neuraminidase did not alter hemolysin binding, suggesting that protein or sialyl residues are not involved as receptors. The hemolysin binding was significantly reduced by prior treatment with phospholipase A₂ but not with phospholipase C (Amoako et al., 1998). Thin layer and gas-liquid chromatography showed that fatty acid profile from hydrolysis of bovine liver phosphatidylcholine by hemolysin and phospholipase A₂ were similar (Abe et al., 1979; Amoako et al., 1998). Therefore, phosphatidylcholine might be the possible receptor for *F. necrophorum* hemolysin. Cattle, sheep, and goat erythrocytes membranes have a lower concentrations of phosphatidylcholine which might explain their insensitivity to hemolysin. Kanoe et al. (1999) demonstrated that guinea pig ileal longitudinal muscle stimulation by *F. necrophorum* hemolysin was dependant on Ca²⁺ flux, thus suggesting that hemolysin increases the probability of contact of *F. necrophorum* and ileal mucosa, and increases the chances of colonization by *F. necrophorum*.

Leukotoxin

The cytotoxic effects of *F. necrophorum* cultures and culture supernatant on rabbit and sheep leukocytes were first observed by Roberts (1967). The emigration of leukocytes from the vessels near the injection site was inhibited, which were later invaded by *F. necrophorum*. The macromolecular nature of the toxin was determined to be a high molecular weight protein, termed leucocidin. Roberts (1967) also determined that leucocidin had no similarity with the previously described toxin, hemolysin. Hence, it was thought that leucocidin might be an important virulence factor for *F. necrophorum*. The leukotoxin destroyed rabbit peritoneal macrophages in vitro (Fales et al., 1977), confirming the cytotoxic effects observed by Roberts previously. Coyle-Dennis and Lauerman (1979) observed that non-leukotoxin producing strains of *F. necrophorum* were less pathogenic to mice in causing abscesses. In another study, Emery et al. (1986) observed that leukotoxin producing strains of *F. necrophorum* needed fewer cells to induce infection and mortality in mice. Hence, leukotoxin was shown to be an important virulence factor in *F. necrophorum* infections.

F. necrophorum subsp. *necrophorum* was shown to produce more leukotoxin than subsp. *funduliforme* (Scanlan et al., 1982, 1986; Tan et al., 1992, 1994a, b, 1996). It was demonstrated that subsp. *necrophorum* produced about 18-fold more leukotoxin than subsp. *funduliforme* (Tan et al., 1992). The differences in leukotoxin production observed may be responsible for the variations in virulence associated with the two subspecies (Shinjo et al., 1981a; Berg and Scanlan, 1982; Smith, 1992; Tan et al., 1994b, 1996) and explain why subsp. *necrophorum* was most frequently isolated from bovine hepatic abscesses (Scanlan and Hathcock, 1983; Lechtenberg et al, 1988). Tan et al (1994a) also observed that subsp. *necrophorum* strains isolated from the rumen were less leukotoxic than those isolated from bovine hepatic abscesses. The lower leukotoxic activity of ruminal strains may be due to the inhibition of the expression of the leukotoxin gene in the rumen environment compared to the liver.

Various factors (growth phase, composition of medium, culture pH, incubation temperature, redox potential, and iron concentration) affecting leukotoxin production were studied (Tan et al., 1992). Maximal leukotoxic activities were observed during late log and early

stationary phases of growth in both subspecies. Brain-heart infusion broth at pH 6.6-7.7 and low redox potential favored maximal growth and leukotoxin production. The leukotoxic activity was not affected by iron concentration. The specificity of *F. necrophorum* leukotoxin was previously reported (Emery et al., 1984; Tan et al., 1994b). Leukocytes (PMN) from cattle and sheep were highly susceptible, whereas leukocytes from rabbits and swine were not susceptible to the leukotoxin.

Several attempts to characterize *F. necrophorum* leukotoxin yielded conflicting data regarding its biochemical characteristics, such as the molecular weight and stability to heat (Fales et al., 1977; Coyle-Dennis and Lauerman, 1978; Emery et al., 1984; Kanoe et al., 1986; Scanlan et al., 1986). These discrepancies may be due to the subspecies and strains used, culture conditions, or the techniques employed to characterize the leukotoxin. In general, *F. necrophorum* leukotoxin is considered to be a soluble, proteinaceous and heat labile exotoxin with specificity for ruminant neutrophils (Coyle-Dennis and Lauerman, 1978; Emery et al., 1984, 1985, 1986a; Kanoe et al., 1986; Tan et al., 1992, 1994d).

Narayanan et al (2001) determined the precise nature of *F. necrophorum* subsp. *necrophorum* leukotoxin by sequencing and expressing the full length leukotoxin in *E. coli*. The leukotoxin operon consists of three genes, *lktB*, *lktA*, and *lktC*, of which the second gene is the leukotoxin structural gene (Fig.4.1). The deduced amino acid sequence of the open reading frame (ORF) of *lktB* had sequence identity to heme-hemopexin utilization protein (HxuB) of *Haemophilus influenzae*. The biological function of *lktC* is not yet determined. The entire leukotoxin gene (*lktA*) ORF is 9,726 bp long, encoding a protein of 3,241 amino acids with a molecular weight of 336, 956 daltons. The *F. necrophorum* leukotoxin was unique in lacking the amino acid cysteine, and has no similarity to any other bacterial toxins described. The recombinant leukotoxin was expressed as five truncated polypeptides with overlapping amino acids, and was found to be active against bovine leukocytes. The truncated polypeptides were named, BSBSE, SX, GAS, SH, and FINAL starting from the N-terminus of leukotoxin respectively.

The cytotoxicity of the *F. necrophorum* subsp. *necrophorum* leukotoxin was demonstrated by Narayanan et al. (2002). The recombinant leukotoxin induced apoptosis of bovine leukocytes in a dose dependent manner as shown by electron microscopy and flow cytometer studies. At higher concentrations, the leukotoxin caused necrotic cell death of bovine peripheral leukocytes. At moderate concentrations, bovine mononuclear cells were also induced to undergo apoptosis. However, the subsp. *funduliforme* leukotoxin was moderately toxic to bovine peripheral leukocytes as compared to subsp. *necrophorum* leukotoxin. The immunogenic effects of *F. necrophorum* subsp. *necrophorum* leukotoxin were studied in mice (Narayanan et al., 2003). All five truncated polypeptides were found to be immunogenic, with GAS polypeptide eliciting the least immune response. The polypeptides, BSBSE and GAS afforded significant protection against challenge with *F. necrophorum* in mice. Thus, the ability of the *F. necrophorum* subsp. *necrophorum* leukotoxin to modulate the host immune system by its toxicity and toxin induced apoptosis of immune effector cells helps it to initiate the disease process and hence may be an important virulence factor.

The promoter sequence elements were not observed in the intergenic regions of the leukotoxin operon, suggesting that the operon might be transcribed by an upstream promoter. Identifying the promoter might explain the differences observed in the leukotoxic activities among the two subspecies. Zhang et al. (2006) determined that the leukotoxin promoter was unique to each subspecies; 548 bp in subsp. *necrophorum* and 337 bp in subsp. *funduliforme*. They observed that the subsp. *funduliforme* promoter activity was weak compared to subsp. *necrophorum*. It was indicated that these differences in promoter activities may be due to unknown transcription regulators. Analysis of the *lktA* gene transcript profile during various growth phases revealed that subsp. *necrophorum* had 19-fold more leukotoxin specific mRNA transcript in the early-log phase and 2.1 fold more in mid-log phase of growth than subsp. *funduliforme*. Interestingly, leukotoxin specific mRNA transcript was detected only in subsp. *funduliforme* in late-log or early stationary phase of growth. But the leukotoxic activity was lower than subsp. *necrophorum*. The protein profiles from the respective phases of growth indicated rapid degradation of leukotoxin in subsp. *funduliforme*, which might be responsible for the low leukotoxic activity observed (Tadepalli et al., unpublished data).

The human strains of *F. necrophorum* isolated from clinical cases were evaluated for the presence of leukotoxin gene (Tadepalli et al., unpublished). Southern hybridization revealed the presence of leukotoxin gene in human strains. In vitro cytotoxicity assays indicated the toxic effects of secreted leukotoxin on human peripheral leukocytes. Hence, leukotoxin might be an important virulence factor in *F. necrophorum* human strains.

Capsule

The bacterial capsules play an important role in the development of the disease process by suppressing the host immunological responses and by enhancing adherence of the pathogen to the host cells (Brook, 1994; Iida et al., 2006). The presence of a mucopolysaccharide in *F. necrophorum* was first detected by Brook and Walker (1986). Garcia et al. (1992) reported the presence acid polysaccharide capsule in *F. necrophorum* subsp. *necrophorum*. They observed that the clinical isolates had very thick capsules compared to laboratory subcultured *F. necrophorum* subsp. *necrophorum*. The importance of the capsule as a virulence factor is not clear. Subcutaneous abscesses were induced by capsulated strains of *F. necrophorum* in mice, whereas non-capsulated strains failed to induce the abscesses (Brook and Walker, 1986). Capsule was not detected in subsp. *funduliforme* isolates (Emery, 1989; Garcia et al., 1992).

Collagenolytic cell wall component (CCWC)

Interactions between the extracellular matrix (ECM) of bovine cells and attached *F. necrophorum* are critical during the early stages of infection. Okamoto et al. (2001) purified a protein from the cell wall fractions of *F. necrophorum* that hydrolyzed bovine type 1 collagen (Okada et al., 2000). This collagenolytic protein was heat labile and its activity was inhibited at acidic pH (4.0). The cytotoxic effects of CCWC were studied on bovine kidney cells and rabbit granulocytes and hepatocytes. The bovine kidney and rabbit granulocytes appeared smooth and were morphologically irregular with partial loss of microvilli after treatment with the toxin. However, the rabbit hepatocytes looked rough and had tiny holes in the cell membranes with loss of cytoplasm. The CCWC also produced dermal ischemia and hemorrhagic lesions when given as intracutaneous injection in guinea pigs (Okamoto et al., 2005) exhibiting dermatotoxic activity.

Thus CCWC might aid in the spread of *F. necrophorum* to viscera once it's attached to bovine ruminal epithelium and initiate the disease process.

Extracellular enzymes

The secreted bacterial proteins with enzymatic activity (protease, proteinase, elastase, collagenase, hyaluronidase, and neuraminidase) facilitate local tissue spread (Gibbons and McDonald, 1961; Deiner et al., 1973; Blackwood et al., 1983; Shah and Williams, 1989; Andreas et al., 2002) and help in penetrating the host mucosal surfaces (eg. *Shigella flexneri*, *Salmonella typhimurium*, *Listeria monocytogenes*, *Yersinia enterocolitica*, etc.) (Jensen et al., 1998). By promoting bacterial colonization on the mucosal surfaces of alimentary tract and genitourinary tract, extracellular enzymes may help in the spread of the bacterium into host tissues.

The proteolytic activity of *F. necrophorum* was first observed in the soluble fraction following sonication of the bacterial cells by Wahren et al. (1971). The protease was partially purified by Nakagaki et al. (1991) and was found to be heat labile and its activity was inhibited by para-hydromercuribenzoate (Nakagaki et al., 1991). The culture supernatants of both subspecies of *F. necrophorum* were analyzed by Amoako et al. (1993) for the presence of 13 extracellular enzymes. Lipase, alkaline phosphatase and DNase productions were predominantly seen in subsp. *necrophorum* alone. Some strains of subsp. *necrophorum* and subsp. *funduliforme* had collagenase activity. The hydrolysis of gelatin by *F. necrophorum* is controversial (Amoako et al., 1993; Scanlan et al., 1992; Tan et al., 1994a). The activity of leukotoxin protein produced by *Fusobacterium necrophorum* was decreased by treatment with proteolytic enzymes, trypsin and protease (Coyle-Dennis and Lauerman, 1978).

Conclusion

The virulence factors described assist *Fusobacterium necrophorum* to adhere to the host epithelium, penetrate and reach the viscera, colonize and initiate the disease process. One of the factors, haemagglutinin helps in adhering to host epithelial cells. Hemolysin may aid in creating an anaerobic environment for growth of *F. necrophorum* by lysis of erythrocytes and thereby

reduce oxygen transport to the site of infection. The platelet aggregation factor also creates an anaerobic microenvironment by disseminated intravascular coagulation for growth of *F. necrophorum*. The secreted extracellular enzymes may aid in the spread of *F. necrophorum* to various tissues. The capsule might help in evading the host immune system especially by preventing macrophage mediated phagocytosis. During the spread and colonization process, the host immune effector cells might be induced to undergo apoptosis and necrosis by leukotoxin, which is pivotal for the establishment of the disease process.

References

- Abe, P.M., Kendall, C.J., Stauffer, L.R., Holland, J.W., 1979. Hemolytic activity of *Fusobacterium necrophorum* culture supernatants due to presence of Phospholipase A and Lysophospholipase. Am. J. Vet. Res. 40, 92-96.
- Amoako, K.K., Goto, Y., Shinjo, T., 1993. Comparison of extracellular enzymes of *Fusobacterium necrophorum* subsp. *necrophorum* and *Fusobacterium necrophorum* subsp. *funduliforme*. J. Clin. Microbiol. 31, 2244-2247.
- Amoako, K.K., Goto, Y., Shinjo, T., 1994. Studies on the factors affecting the hemolytic activity of *Fusobacterium necrophorum*. Vet. Microbiol. 41, 11-18.
- Amoako, K.K., Goto, Y., Misawa, N., Xu, D.L., Shinjo, T., 1996a. Stability and stabilization of *Fusobacterium necrophorum* hemolysin. Vet. Microbiol. 50, 149-153.
- Amoako, K.K., Goto, Y., Xu, D.L., Shinjo, T., 1996b. The effects of physical and chemical agents on the secretion and stability of a *Fusobacterium necrophorum* hemolysin. Vet. Microbiol. 51, 115-124.
- Amoako, K.K., Goto, Y., Misawa, N., Xu, D.L., Shinjo, T., 1997. Interactions between *Fusobacterium necrophorum* hemolysin, erythrocytes and erythrocyte membranes. FEMS Microbiol. Lett. 150, 101-106.
- Amoako, K.K., Goto, Y., Misawa, N., Xu, DL., Shinjo, T., 1998. The erythrocyte receptor for *Fusobacterium necrophorum* hemolysin: phosphatidylcholine as a possible candidate. FEMS Microbiol. Lett. 168, 65-70.
- Andreas, W., Gregor, Z., Joachim, G., Tammo, K., Matthias von, M., Stefanie, B., Helmut, E., Roland, N., 2002. Decreased virulence of a pneumolysin-deficient strain of *Streptococcus pneumoniae* in murine meningitis. Infect. Immun. 70, 6504-6508.
- Berg, J.N., Scanlan, C.M. 1982. Studies of *Fusobacterium necrophorum* from bovine hepatic abscesses: Biotypes, quantitation, virulence, and antibiotic susceptibility. Am. J. Vet. Res. 43, 1580-1586.
- Blackwood, L.L., Stone, R.M., Iglewshi, B.H., Pennington, J.E., 1983. Evaluation of *Pseudomonas aeruginosa* exotoxin A and elastase as virulence factors in acute lung infection. Infect. Immun. 39, 198-201.

- 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.
- Brook, I., Walker, R.I., 1986. The relationship between *Fusobacterium* species and other flora in mixed infection. J. Med. Microbiol. 21, 93-100.
- Brook, I., 1994. Role of encapsulated bacteria in synergistic infections. FEMS Microbiol. Rev. 13, 65-74.
- Césari, E., 1912. Étude sur le bacilli de Schmorl. Ann. Inst. Pasteur. 26, 802-816.
- 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.
- Deiner, B., Carrick, L., Berk, R.S., 1973. In vivo studies with collagenase from *Pseudomonas aeruginosa*. Infect. Immun. 7, 212-217.
- Edwards, J.F., Davis, D.S., Roffe, T.J., Ramiro-Ibanez, F., Elzer, P.H., 2001. Fusobacteriosis in captive wild-caught pronghorns (*Antilocapra americana*). Vet. Pathol. 38, 549-552.
- Emery, D.L., Vaughan, J.A., 1986a. Generation of immunity against *Fusobacterium necrophorum* in mice inoculated with extracts containing leucocidin. Vet. Microbiol. 12, 255-268.
- Emery, D.L., Vaughan, J.A., Clark, B.L., Stewart, D.J., 1986b. Virulence determinants of *Fusobacterium necrophorum* and their prophylactic potential in animals. In: D.J. Stewart, J.E. Peterson, N.M. McKern and D.L. Emery (eds), *Foot rot in Ruminants*. Proceedings of a workshop, Melbourne (CSIRO Division of Animal Health, Australian Wool Corporation, Australia), 267-274.
- Emery, D.L., 1989. Antigens of *Fusobacterium necrophorum*. In: J.R. Egerton, W.K. Yong and G.G. Riffkin (eds), *Foot rot and Foot Abscess of Ruminants*, (CRC Press, Boca Raton, FL), 135-140.
- Fales, W.H., Warner, J.F., Teresa, G.W., 1977. Effects of *Fusobacterium necrophorum* leukotoxin on rabbit peritoneal macrophages in vitro. Am. J. Vet. Res. 38, 491-495.
- Falkler, W.A., Enwonwu, C.O., Idigbe, E.O., 1999. Isolation of *Fusobacterium necrophorum* from cancrum oris (NOMA). Am. J. Trop. Med. Hyg. 60, 150-156.

- Forrester, L.J., Campbell, B.J., Berg, J.N., Barrett, J.T., 1985. Aggregation of platelets by *Fusobacterium necrophorum*. J. Clin. Microbiol. 22, 245-249.
- Garcia, M.M., Alexander, D.C., McKay, K.A. 1975a. Biological characterization of *Fusobacterium necrophorum* cell fractions in preparations for toxin and immunization studies. Infect. Immun. 11, 609-616.
- Garcia, M.M., Charlton, K.M., McKay, K.A. 1975b. Characterization of endotoxin from *Fusobacterium necrophorum*. Infect. Immun. 11, 371-379.
- Garcia, M.M., Becker, S.A.W.E., Brooks, B.W., Berg, J.N., Finegold, S.M., 1992. Ultrastructure and molecular characterization of *Fusobacterium necrophorum* biovars. Can. J. Vet. Res. 56, 318-325.
- Garcia, G.G., Amoako, K.K., Xu, D.L., Inoue, T., Goto, Y., Shinjo, T. 1999. Chemical composition of endotoxins produced by *Fusobacterium necrophorum* subsp. *necrophorum* and *F. necrophorum* subsp. *funduliforme*. Microbios 100, 175-179.
- Garcia, G.G., Goto, Y., Shinjo, T. 2000. Endotoxin-triggered hematological interactions in *Fusobacterium necrophorum* infections. Microbios 102, 39-44.
- Garman, R.D., Lyons, M.F., Hepper, K.P., Teresa, G.W. 1980. The in vitro cytotoxicity of native and modified trichloroacetic acid-extracted antigens from *Fusobacterium necrophorum* for mouse peritoneal macrophages. Comp. Immun. Microbiol. Infect. Dis. 2, 531-543.
- Gibbons, R.J., Macdonald, J.B., 1961. Degradation of collagenous substrates by *Bacteroides melaninogenicus*. J. Bacteriol. 81, 614-621.
- Hagelskjaer, L.H., Prag, J., Malczynski, J., Kristensen, J.H., 1998. Incidence and clinical epidemiology of necrobacillosis, including Lemierre's syndrome, in Denmark 1990-1995. Eur. J. Clin. Microbiol. Infect. Dis. 17, 561-565.
- Hill, G.B., Osterhout, S., Pratt, P.C. 1974. Liver abscess production by non-spore forming anaerobic bacteria in a mouse model. Infect. Immun. 9, 599-603.
- Hodges, G.F., Regan, K.M., Foss, C.L., Teresa, G.W. 1982a. Induction of immunologic memory by a lipopolysaccharide-protein complex isolated from *Fusobacterium necrophorum*: cellular response. Am. J. Vet. Res. 43, 117-121.
- Hodges, G.F., Regan, K.M., Foss, C.L., Teresa, G.W. 1982b. Induction of immunologic memory by a lipopolysaccharide-protein complex isolated from *Fusobacterium necrophorum*: humoral response. Am. J. Vet. Res. 43, 122-129.

- 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.
- Hofstad, T. 1989. Virulence determinants in nonsporeforming anaerobic bacteria. Scand. J. Infect. Dis. suppl. 62, 15-24.
- Horose, M., Kiyoyama, H., Ogawa, H., Shinjo, T., 1992. Aggregation of bovine platelets by *Fusobacterium necrophorum*. Vet. Microbiol. 32, 343-350.
- Iida, K., Seki, M., Saito, M., Kawamara, Y., Kajiwara, H., Yoshida, S., 2006. Capsule of *Streptococcus pyogenes* is essential for delayed death of mice in a model of streptococcal toxic shock syndrome. Microbiol. Immunol. 50, 127-130.
- Inoue, T., Kanoe, M., Goto, N., Matsumura, K., Nakano, K. 1985. Chemical and biological properties of lipopolysaccharides from *Fusobacterium necrophorum* biovar A and biovar B strains. Jpn. J. Vet. Sci. 47, 639-645.
- Ishii, T., Kanoe, M., Inoue, T., Kai, K., Blobel, H., 1988. Cytotoxic effects of a leukocidin from *Fusobacterium necrophorum* on bovine hepatic cells. Med. Microbiol. Immunol. 177, 27-32.
- Jensen, V.B., Harty, J.T., Bradley, D. J., 1998. Interactions of the Invasive Pathogens *Salmonella typhimurium*, *Listeria monocytogenes*, and *Shigella flexneri* with M Cells and Murine Peyer's Patches. Infect. Immun. 66, 3758-3766.
- Kanoe, M., Nagai, S., Toda, M., 1985. Adherence of *Fusobacterium necrophorum* to Vero cells. Zentralbl. Bakteriologie. Mikrobiol. Hyg. A. 260, 100-107.
- Kanoe, M., Iwaki, K., 1987. Adherence of *Fusobacterium necrophorum* to bovine ruminal cells. J. Med. Microbiol. 23, 69-73.
- Kanoe, M., Yamanaka, M., 1989. Bovine platelet aggregation by *Fusobacterium necrophorum*. J. Med. Microbiol. 29, 13-17.
- Kanoe, M., 1990. *Fusobacterium necrophorum* hemolysin in bovine hepatic abscess. J. Vet. Med. B. 37, 770-773.
- Kanoe, M., Kiritani, M., Inoue, M. 1995a. Local skin reaction in mice and guinea pigs induced by a single intradermal inoculation of *Fusobacterium necrophorum* lipopolysaccharide. Microbios 81, 93-101.

- Kanoe, M., Abe, K., Kai, K., Blobel, H., 1995b. Dermonecrotic activity of a cell wall preparation from *Fusobacterium necrophorum*. Lett. Appl. Microbiol. 20, 145-147.
- Kanoe, M., Koyanagi, Y., Kondo, C., Mamba, K., Makita, T., Kai, K., 1998. Location of hemagglutinin in bacterial cells of *Fusobacterium necrophorum* subsp. *necrophorum*. Microbios 96, 33-38.
- Kanoe, M., Toyoda, Y., Shibata, H., Nasu, T., 1999. *Fusobacterium necrophorum* hemolysin stimulates motility of ileal longitudinal smooth muscle of the guinea-pig. Fundam. Clin. Pharmacol. 13, 547-554.
- Kirchheiner, E., 1940. Recherches biochimiques et immunologiques compares sur *Sphaerophorus necrophorus* et *Sphaerophorus funduliformis*. Ann. Inst. Pasteur. 64, 238-254.
- Kristensen, L.H., Prag, J., 2000. Human necrobacillosis, with emphasis on Lemierre's syndrome. Clin. Infect. Dis. 31, 524-532.
- Kurpiewski, G.E., Forrester, L.J., Campbell, B.J., Barrett, J.T., 1983. Platelet aggregation by *Streptococcus pyogenes*. Infect. Immun. 39, 704-708.
- Langworth, B.F. 1977. *Fusobacterium necrophorum*: Its characteristics and role as an animal pathogen. Bact. Rev. 41, 373-390.
- Lemierre, A., 1936. On certain septicemias due to anaerobic organisms. Lancet. I, 701-703.
- Meisel-Milolajczyk, F., Dobrowolska, T. 1974a. Comparative immunological studies on the endotoxins of two *Fusobacterium necrophorum* strains. Bull. Acad. Pol. Sci. Biol. 22, 555-62.
- Meisel-Milolajczyk, F., Dworzczynski, A. 1974b. Serological investigations on endotoxins isolated from *Sphaerophorus funduliformis* (syn. *Fusobacterium necrophorum*) strains. Bull. Acad. Pol. Sci. Biol. 22, 549-54.
- Mikolajczyk E, Meisel-Mikolajczyk F. 1978. Toxicity of endotoxin from *Fusobacterium necrophorum* strains to chick embryos. Bull. Acad. Pol. Sci. Biol. 26, 15-18.
- Miyazato, S., Shinjo, T., Yago, H., Nakamura, N., 1978. Fimbriae (Pili) detected in *Fusobacterium necrophorum*. Jpn. J. Vet. Sci. 40, 619-621.
- Nagai, S., Kanoe, M., Toda, M., 1984. Purification and partial characterization of *Fusobacterium necrophorum* hemagglutinin. Zentralbl. Bakteriologie. Mikrobiol. Hyg. A. 258, 232-241.
- 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.
- Nakagaki, M., Fukuchi, M., Kanoe, M., 1991. Partial characterization of *Fusobacterium necrophorum* protease. *Microbios* 66, 117-123.
- Nakajima, Y., Nakamura, K., Takeuchi, S. 1985. Effects of the components of *Fusobacterium necrophorum* in experimental liver abscess formation in mice. *Jpn. J. Vet. Sci.* 47, 589-595.
- Nakajima, Y., Ueda, H., Yagi, Y., Nakamura, K., Motoi, Y., Takeuchi, S. 1986. Hepatic lesions in cattle caused by experimental infection of *Fusobacterium necrophorum*. *Jpn. J. Vet. Sci.* 48, 509-515.
- Nakajima, Y., Ueda, H., Takeuchi, S., Fujimoto, Y. 1987. The effects of *Escherichia coli* as a trigger for hepatic infection of rabbits with *Fusobacterium necrophorum*. *J. Comp. Path.* 97, 207-215.
- Nakajima, Y., Ueda, H., Takeuchi, S. 1988. Synergistic effects of *Fusobacterium necrophorum* lipopolysaccharide, cytoplasmic, and culture supernatant fractions on induction of acute hepatic necrosis in rabbits. *Am. J. Vet. Res.* 49, 125-129.
- Nakajima, Y. 1988. Shwartzman reaction in the brain induced by fractions of *Fusobacterium necrophorum* and *Escherichia coli* lipopolysaccharide in rabbits. *Acta. Pathol. Jpn.* 38, 541-547.
- 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.
- Narayanan, S.K., Chengappa, M.M., Stewart, G.C., Nagaraja, T.G., 2003. Immunogenicity and protective effects of truncated recombinant leukotoxin proteins of *Fusobacterium necrophorum* in mice. *Vet. Microbiol.* 93, 335-347.

- 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.
- Okada, Y., Kanoe, M., Yaguchi, Y., Watanabe, T., Ohmi, H., Okamoto, K., 1999. Adherence of *Fusobacterium necrophorum* subspecies *necrophorum* to different animal cells. *Microbios* 99, 95-104.
- Okahashi, N., Koga, T., Nishihara, T., Fujiwara, T., Hamada, S. 1988. Immunobiological properties of lipopolysaccharides isolated from *Fusobacterium nucleatum* and *F. necrophorum*. *J. Gen. Microbiol.* 134, 1707-1715.
- Okamoto, Y., Kanoe, M., Okamoto, K., Sakamoto, K., Yaguchi, Y., Watanabe, T., 2000. Effects of *Fusobacterium necrophorum* subspecies *necrophorum* on extracellular matrix of tissue-cultured bovine kidney cells. *Microbios* 101, 147-156.
- Okamoto, K., Kanoe, M., Watanabe, T., 2001. Collagenolytic activity of a cell wall preparation from *Fusobacterium necrophorum* subsp. *necrophorum*. *Microbios* 106, 89-95.
- Okamoto, K., Kanoe, M., Inoue, M., Watanabe, T., Inoue, T., 2005. Dermotoxic activity of a collagenolytic cell wall component from *Fusobacterium necrophorum* subsp. *necrophorum*. *Vet. J.* 169, 308-310.
- Okamoto, K., Kanoe, M., Yaguchi, Y., Inoue, T., Watanabe, T., 2006. Effects of a collagenolytic cell wall component from *Fusobacterium necrophorum* subsp. *necrophorum* on rabbit tissue-culture cells. *Vet. J.* 171, 380-382.
- Oliphant, J.C., Parsons, R., Smith, G.R., 1984. Aetiological agents of necrobacillosis in captive wallabies. *Res. Vet. Sci.* 36, 382-384.
- Orcutt, J. 1930. A study of *Bacillus necrophorus* obtained from cows. *J. Bacteriol.* 20, 343-360.
- Ramos-Vara, J.A., Rook, J., Scanlan, C.M., Mugli, F., Yamini, B., 1997. *Fusobacterium necrophorum* septicemia in a lamb: pathologic and microbiologic characterization. *J. Vet. Diagn. Invest.* 9, 79-82.
- Riordan, T., Wilson, M., 2004. Lemierre's syndrome: more than a historical curiosa. *Postgrad. Med. J.* 80, 328-334.
- Roberts, D.S., 1967. The pathogenic synergy of *Fusiformis necrophorus* and *Corynebacterium pyogenes*. I. Influence of the leucocidal exotoxin of *F. necrophorus*. *Br. J. Exp. Pathol.* 48, 665-673.

- Roeder, B.L., Chengappa, M.M., Lechtenberg, K.F., Nagaraja, T.G., Varga, G.A., 1989. *Fusobacterium necrophorum* and *Actinomyces pyogenes* associated facial and mandibular abscesses in blue duiker. *J. Wildl. Dis.* 25, 370-377.
- 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.
- Scanlan, C.M., Berg, J.N., and Lairemore, M.D. 1983. Comparative biological features of a rat liver abscess model induced with three *Fusobacterium necrophorum* strains. *Am. J. Vet. Res.* 44, 1789-1792.
- Scanlan, C.M., Hoyumpa, A.H., Ainsworth, P.C., 1992. A semiquantitative enzyme method for identifying *Fusobacterium necrophorum* biovars A and B. *J. Vet. Diagn. Invest.* 4, 86-87.
- Shah, H.N., Williams, R.A.D., 1987. Catabolism of aspartate and asparagines in *Bacteroides intermedius* and *Bacteroides gingivalis*. *Curr. Microbiol.* 15, 313-319.
- Shinjo, T., Shirakihara, M., Iwata, T., 1980. Hemagglutinin activity of *Fusobacterium necrophorum* with erythrocytes of various animals. *Bull. Facul. Agricul.* 27, 403-407.
- Shinjo, T., Hiraiwa, K., Miyazato, S. 1990. Recognition of Biovar C of *Fusobacterium necrophorum* (Flügge) Moore and Holdeman as *Fusobacterium necrophorum* sp. nov., nom., rev. (ex Prévot 1940). *Int. J. Syst. Bacteriol.* 40, 71-73.
- Smith, G.R., Turner, A., Cinderey, R. 1986. Susceptibility of wallabies to *Fusobacterium necrophorum*. *Vet. Rec.* 118, 691-693.
- Sonnenwirth, A.C., Yin, E.T., Sarmiento, E.M., Wessler, S. 1972. *Bacteroidaceae* endotoxin detection by *Limulus* assay. *Am. J. Clin. Nutr.* 25, 1452-1454.
- Takayama, Y., Kanoe, M., Maeda, K., Okada, Y., Kai, K., 2000. Adherence of *Fusobacterium necrophorum* subsp. *necrophorum* to ruminal cells derived from bovine rumenitis. *Lett. Appl. Microbiol.* 30, 308-311.
- 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., 1994a. Biochemical and biological characterization of *Fusobacterium necrophorum*. *FEMS Microbiol. Lett.* 120, 81-86.

- Tan, Z.L., Nagaraja, T.G., Chengappa, M.M., Staats, J.S., 1994b. Biological and biochemical characterization of *Fusobacterium necrophorum* leukotoxin. Am. J. Vet. Res. 55, 515-521.
- Tan, Z.L., Nagaraja, T.G., Chengappa, M.M., 1994c. Selective enumeration of *Fusobacterium necrophorum* from the bovine rumen. Appl. Environ. Microbiol. 60, 1387-1389.
- Tan, Z.L., Nagaraja, T.G., Chengappa, M.M., Staats, J.J., 1994d. Purification and quantification of *Fusobacterium necrophorum* leukotoxin by using monoclonal antibodies. Vet. Microbiol. 42, 121-133.
- 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.
- Wahren, A., Bernholm, K., Holme, T., 1971. Formation of proteolytic activity in continuous culture of *Sphaerophorus necrophorus*. Acta. Path. Microbiol. Scand. Section B. 79, 391-398.
- Warner, J.F., Fales, W.H., Sutherland, R.C., Teresa, G.W. 1974. Endotoxin from *Fusobacterium necrophorum* of bovine hepatic abscess origin. Am. J. Vet. Res. 36, 1015-1019.
- Wobeser, G., Runje, W., Noble, D., 1975. Necrobacillosis in deer and pronghorn antelope in Saskatchewan. Can. Vet. J. 16, 3-9.
- Yamaguchi, M., Kanoe, M., Kai, K., Okada, Y., 1999. Actin degradation concomitant with *Fusobacterium necrophorum* subsp. *necrophorum* adhesion to bovine portal cells. Microbios 98, 87-94.
- 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.

Table 4.1. Distinguishing characteristics between subsp. *necrophorum* and subsp. *funduliforme*

Criterion	Subsp. <i>necrophorum</i>	Subsp. <i>funduliforme</i>
Phenotypic Characteristics		
Morphology	Pleomorphic filaments	Small rods
Colony morphology	Smooth, opaque, and umbonate with irregular edges	Small, waxy, yellowish, raised, and sticky
Sedimentation in broth	Absent	Present
Genotypic Characteristics		
G + C content (mol %)	28-32	27-33
DNA homology	53-76% to subsp. <i>funduliforme</i>	51-73% to subsp. <i>necrophorum</i>
Biochemical properties		
Proteolytic activity	++	+
Phosphatase	+	-
Lipase	+	-
DNAase	+	-
Biological activity		
Agglutination of chicken erythrocytes	+	-
Platelet aggregation	+	-
Adherence to mammalian cells	+	-
Pathogenicity to mice	+++	+
Cytotoxicity to bovine PMNs	+++	+
Leukotoxin operon	Present	Present
Promoter	+(548 bp)	+(337 bp)
<i>lktB</i>	+(1652 bp)	+(1649 bp)
<i>lktA</i>	+(9725 bp)	+(9731 bp)
<i>lktC</i>	+(437 bp)	+(437 bp)

Table 4.2. Chemical composition and biological activities of lipopolysaccharides from *F. necrophorum* subspecies

Item	Subsp. <i>necrophorum</i>	subsp. <i>funduliforme</i>
LPS (% of dry cell weight)	2.6	1.2
Composition (%)		
Protein	4.6	3.2
Hexose	21.5	66.2
Methylpentose	3.7	1.5
Aminosugar	12.9	0.9
Heptose	12.0	5.5
Keto-deoxy-octonate (KDO)	2.3	1.3
Total Phosphorus	1.3	0.7
Lipid A	34.0	4.2
Biological activity		
LD ₅₀ for 10 day-old chick embryos (μg)	79.4	365.3
ED ₅₀ for hemorrhagic necrosis of solid tumor (μg)	0.78	42.61

Adapted from Inoue et al. (1985)

Table 4.3. Chemical composition of endotoxin isolated from animal and human strains of *Fusobacterium necrophorum*

Composition (%)	Animal strains	Human Strains
Protein	12.5	7.0
Hexose	12.0	10.8
Methylpentoses	3.0	3.0
Aminosugar	20.0	5.7
Heptose	11.0	5.5
Keto-deoxy-octonate(KDO)	6.8	3.6

Adapted from Meisel-Mikolajczyk and Dobrowolska (1974).



Figure 4.1. Leukotoxin operon in *F. necrophorum* subspecies

The tricistronic leukotoxin operon of *Fusobacterium necrophorum* subspecies' showing the promoter element and the corresponding three genes (*lktB* 1650 bp, *lktA* 9732 bp and *lktC* 438 bp).

Appendix A

**Heterogeneity of the leukotoxin *lktA* gene in strains of human
Fusobacterium necrophorum and equine *Fusobacterium equinum***

1. Introduction

Fusobacterium necrophorum, a gram negative, non-sporulating, rod-shaped obligate anaerobe, is associated with various necrotic infections in animals and human. In cattle, *F. necrophorum* causes liver abscesses, calf-diphtheria and foot rot (Langworth, 1977; Tan et al. 1996). The infection in humans begins as acute pharyngitis that progresses to pyrexia, thrombophlebitis of the internal jugular vein leading to abscess formation, termed Lemierre's syndrome (Brazier, 2006). *Fusobacterium equinum*, formerly *F. necrophorum*, causes paraoral abscesses and lower respiratory tract infections, especially pneumonia and pleuritis in horses (Dorsch et al., 2001).

Leukotoxin is a major virulence factor in bovine strains of *F. necrophorum* (Tan, et al. 1994; Narayanan, et al., 2001) and causes apoptosis and necrosis of bovine leukocytes in a dose-dependent manner (Narayanan, et al. 2002). It was further shown that the leukotoxin gene was a member of the tricistronic operon *lktBAC*. The toxin structural gene, *lktA* is ~10 kb in size encoding a 336 kDa protein. The full length leukotoxin was truncated into five determinants with over-lapping sequence for ease of expression and further analysis. The five determinants were named BSBSE, SX, GAS, SH and FINAL from the 5'- to 3'- end of *lktA* gene respectively (Narayanan, et al., 2001). The functions of LktB and LktC are not yet determined. Significant differences in the biological activities among the bovine subspecies were observed (Tan et al. 1992). It was reported that on an average, subsp. *funduliforme* strains had a 18-fold lower leukotoxin titer compared to subsp. *necrophorum*. The leukotoxin produced by some strains of subsp. *funduliforme* was at undetectable levels (Tan et al. 1992). A similar strain-to-strain variation in leukotoxin biological activity was observed in human *F. necrophorum* and *F. equinum* strains (Tables A.1 and A.2). Our objective for this study was to determine the leukotoxin (*lktA*) gene sequence to explain the observed differences in biological activities.

2. Materials and Methods

2.1. Strains

Four strains (RMA10682, RMA14786, RMA16505, and RMA16539) of human clinical isolates of *F. necrophorum* (kindly provided by Dr. Diane Citron, R. M. Alden Research Laboratory, Santa Monica, CA.) were used in the study. The four strains were isolated from a liver abscess, tonsil biopsy, tonsil swab, and a neck wound, respectively (D. M. Citron, Personal communication). Ten *F. equinum* strains, isolated from horses, were used in this study. Strains E1 and E2 were isolated from lungs, E3 from sinus, E4 and E5 from the guttural pouch, and strains E6 to E10 were isolated from the tracheal washings of horses diagnosed with pneumonia. Two bovine strains, *F. necrophorum* subsp. *necrophorum* A25 and subsp. *funduliforme* B35, previously isolated from bovine liver abscesses (Tan et al., 1992) were also included in the study. Isolates were grown overnight on blood agar (Remel Inc, Lenexa, KS, USA) at 39°C in an anaerobic glove box (Forma Scientific, Marietta, OH, USA) and subcultured in pre-reduced, anaerobically sterilized (PRAS) BHI broth. Subspeciation of the human strains was determined by the RapID Ana II system (Remel Inc., Lenexa, KS, USA) and by PCR amplifications of *rpoB* and haemagglutinin (*HA*) gene (Aliyu et al. 2004), and the *lkt* operon promoter region sequence (Zhang et al., 2005). The *F. equinum* isolates were identified by PCR analysis of the 16S rDNA (Tadepalli et al. unpublished data).

2.2. Isolation of chromosomal DNA

Bacterial chromosomal DNA was prepared according to the procedure described by Narayanan et al. (2001). Briefly, isolates were grown overnight in anaerobic BHI broth. The cells were then pelleted by centrifugation at 5,000 x g for 10 min at 4°C. The pelleted cells were washed and resuspended in TE buffer (Tris 100 mM; EDTA 10 mM; pH 8.0). The cell wall was digested with lysozyme (1 mg/ml), and the resulting lysate was treated with Sarkosyl (1%) followed by RNaseA (20 µg/ml) and pronase (50 µg/ml). The samples were incubated on ice for 20 min and then sequentially extracted with phenol and chloroform twice. The DNA from the extract was then precipitated in 1/10 vol. 3 M sodium acetate (pH 5.2) and an equal volume of 2-propanol. The DNA was resuspended in TE (10 mM Tris-HCl, 1mM EDTA, pH 8.0) and stored

at 4°C until used. The quality and concentration of extracted DNA was determined spectrophotometrically (Nanodrop Technologies Inc., Wilmington, DE).

2.3. Southern hybridization

Southern hybridization was performed according to the procedure of Narayanan et al., (2001). Briefly, 3 µg of chromosomal DNA were digested to completion by the restriction endonuclease *Hae III* (Promega Corp. Madison, WI) and electrophoresed in a 1% agarose gel overnight at 20V at room temperature. Double digestion of the genomic DNA with restriction enzymes *Eco RI*, *Eco RV* and *Swa I* was performed according to the manufacturer's directions (Promega Corp. Madison, WI). The resolved and denatured DNA fragments were transferred to a positively-charged nylon membrane (Roche Diagnostics Corporation, Indianapolis, IN) by a capillary mechanism and the DNA immobilized by UV cross-linking. DNA probes were synthesized by random-labeling with DIG-dUTP (Roche Diagnostics, Indianapolis, IN, USA) as per the manufacturer's directions. The immobilized DNA was probed with full-length bovine subsp. *necrophorum* A25 *lktA* (at 54°C). Colorimetric detection of hybridized signals was carried out as per the manufacturer's instructions (Roche Diagnostics, Indianapolis, IN, USA).

2.4. Primer design and PCR conditions

Specific primers targeting the RNA polymerase β-subunit and the hemagglutinin gene were previously designed by Aliyu et al., (2004). All other primers used in this study are listed in Table A.3. The *lkt* operon promoter regions in subsp. *necrophorum* and subsp. *funduliforme* were amplified using Ex-Taq (Takara Bio Inc., Japan) as previously described (Zhang et al., 2006). *Fusobacterium equinum* 16S rDNA sequence (GenBank AJ295750) was aligned with all *F. necrophorum* 16S rDNA complete sequences deposited with NCBI database (ClustalW program) to identify a region specific to *F. equinum* 16S rDNA gene and primers were designed to amplify that region. The forward primer 5'-TTTCAGTCGGGAAGAAGAA – 3' (position 418-436 bp) had two mismatches at the 3'- terminus whereas the reverse primer 5'-TAAGGCAGTTTCCAA CGCA -3' (position 608-590 bp) had a single mismatch at the 3'-terminus. The *lktA* determinants were PCR amplified using specific primers (Table A.1). The PCR amplified products were electrophoresed in 1-1.2 % agarose gels, purified (Wizard SV gel

and PCR clean up system, Promega Corp. Madison, WI), and sequenced (Beckman Coulter CEQ 8000 Genetic Analysis System, Fullerton, CA, USA).

3. Results and Discussion

All four human strains formed a flocculent button after overnight growth in PRAS BHI broth and were negative for alkaline phosphatase based on the RapId ANA II test. The *rpoB* gene specific for *F. necrophorum* was present in all four strains, but all were PCR-negative for the hemagglutinin gene. The PCR amplification of the *lkt* promoter region of the human isolate strains revealed a subsp. *funduliforme* type product (data not shown). The PCR analysis with *Fusobacterium equinum* specific 16S rDNA gene targeting primers amplified the expected size (103 bp) product in all the ten equine isolates but not in either subsp. of bovine *F. necrophorum* (data not shown). *Fusobacterium equinum* strains formed sediment after over-night growth in BHI broth and were negative for alkaline phosphatase enzyme when tested by biochemical methods. All ten clinical isolates were PCR negative for hemagglutinin (*HA*) gene.

The restriction enzymes were selected such that upon complete digestion hybridization with *lktA* would result in at least one band that is approximately 2.0 – 4.0 kb (with in *lktA* gene) in size. Southern hybridization of *Eco RI* and *Eco RV* double digested genomic DNA of subsp. *necrophorum* had the expected bands (4.2 kb, 2.7 kb, 1.4 kb , 0.9 kb and 0.5 kb doublet) (Fig A.1A; lane 2). Similarly subsp. *funduliforme* had expected (3.8 kb, 3.0 kb, 2.7 kb, 1.3 kb, 1.1 kb and 0.9 kb) bands (Fig A.1A; lane 3). The hybridization pattern among the human strains genomic DNA was variable. Strain RMA10682 had a single band whereas strain RMA16539 had three (3.5–4.2 kb) faintly hybridized bands (Fig A.1A; lanes 4 and 7). Strain RMA14786 hybridization pattern closely resembled subsp. *funduliforme* except for the presence of an additional band at 2.5 kb instead of 1.3 kb band while Strain RMA16505 had only two (5.0 kb and 1.4 kb) bands (Fig A.1A; lanes 5 and 6).

Hybridization pattern for subsp. *necrophorum* genomic DNA digested with *EcoRI* and *Swa I* had six (5.2 kb, 3.6 kb, 2.2 kb, 2.0 kb, 1.6 kb and 0.8 kb) bands as expected (Fig A.1B; lane 2). Likewise subspecies *funduliforme* had (5.5 kb, 3.6 kb, 3.0 kb, 2.0 kb, 1.6 kb, 1.2 kb and

1.0 kb) bands (Fig A.1B; lane 3). Strain RMA14786 hybridization pattern was identical to that of subsp. *funduliforme* (Fig A.1B; lane 5). Single band (~5.0 kb) was observed in strain RMA16505 (Fig A.1B; lane 6) while strains RMA10682 and RMA16539 had two (~5.2 kb and 2.2 kb) and three (~3.5 kb, 3.7 kb and 2.2 kb) bands, respectively (Fig A.1B; lanes 4 and 7).

The hybridization pattern for subsp. *necrophorum* genomic DNA digested with *Eco RV* and *Swa I* had (~4.9 kb, 4.2 kb, 3.0 kb and 1.6 kb) bands (Fig A.1C; lane 2). Similarly subsp. *funduliforme* had (2.8 kb, 2.6 kb, 1.6 kb and 1.0 kb) bands (Fig A.1C; lane 3). Strain RMA14786 hybridization pattern was similar to subsp. *funduliforme* with the exception of an additional 3.5 kb band (Fig A.1C; lane 5). The *Eco RV* and *Swa I* double digested genomic DNA of other strains RMA10682, 16505, and 16539 had hybridized weakly; a single band (>5.1 kb) was observed in strains RMA10682 and 16505, whereas a ~ 4.2 kb band was seen in strain RMA16539.

The data from the above hybridizations indicate that the *lktA* gene has variations in the sequence leading to the different band patterns observed. To confirm this, a PCR assay was designed to amplify the various *lktA* determinants (Narayanan et al., 2001). Interestingly, only in strain RMA14786 all the *lktA* determinants were amplified (Table A.4). However, in other strains we were unable to amplify certain *lktA* determinants. Among these three strains, BSBSE was present in RMA10682 and 16505; GAS determinant was observed in strains RMA16505 and RMA16539; while FINAL determinant was present in strain RMA16539 alone (Table A.4). DNA sequencing of the amplified products revealed that human strains *lktA* was more similar to subsp. *funduliforme*.

Because no virulence factors have been identified in *Fusobacterium equinum*, we tested the clinical isolates for the presence of the *lktA* gene. Our PCR analysis followed by partial DNA sequencing revealed that the *lktA* gene among *F. equinum* isolates was similar to subsp. *necrophorum*. Interestingly, similar strain-to-strain variations observed in human *F. necrophorum* strains were also observed in *F. equinum* isolates. Among the *F. equinum* isolates, strains E3, E5 and E10 had all the *lktA* determinants (Table A.4). Strain E1 had BSBSE, SX and GAS determinants; strain E4 had amplified only the GAS determinant; strain E6 had GAS and

SH determinants; strain E7 had BSBSE, SX and GAS determinants; strain E8 had only SH determinant; strain E9 had BSBSE and SX determinants only. However, we were unable to PCR amplify any determinant of the *lktA* in strain E2 (Table A.4).

Our data show that there is considerable degree of heterogeneity in the *lktA* gene among the human clinical strains of *F. necrophorum* and *F. equinum* clinical strains and this could explain the differences in leukotoxin secretions and activities in different strains.

References

1. 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.
2. Brazier, J.S., 2006. Human infections with *Fusobacterium necrophorum*. *Anaerobe.* 12, 165-172.
3. 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.
4. Langworth, B.F., 1977. *Fusobacterium necrophorum*: Its characteristics and role as an animal pathogen. *Bact. Rev.* 41, 373-390.
5. 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.
6. Narayanan, S.K., Stewart, G.C., Chengappa, M.M., Willard, L., Shuman, W., Wilkerson, M., Nagaraja, T.G., 2002. *Fusobacterium necrophorum* leukotoxin induces activation and apoptosis of bovine leukocytes. *Infect. Immun.* 70, 4609-4620.
7. Tan, Z.L., Nagaraja, T.G., Chengappa, M.M., 1992. Factors affecting the leukotoxin activity of *Fusobacterium necrophorum*. *Vet. Microbiol.* 32, 15-28.
8. Tan, Z.L., Nagaraja, T.G., Chengappa, M.M., Staats, J.S., 1994. Biological and biochemical characterization of *Fusobacterium necrophorum* leukotoxin. *Am. J. Vet. Res.* 55, 515-521.
9. 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.
10. 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.

Table A.1. Toxicity of human *Fusobacterium necrophorum* strains culture supernatants

Strain	% Toxicity to human PMNs
Human subsp. <i>funduliforme</i>	
RMA10682	17
RMA14786	25
RMA16505	21
RMA16539	20

Table A.2. Toxicity of *Fusobacterium equinum* strains culture supernatants

Strain	% Toxicity to equine PMNs
E1	62.0
E2	5.0
E3	7.0
E4	5.0
E5	22.0
E6	5.0
E7	50.0
E8	7.5
E9	41.0
E10	41.0

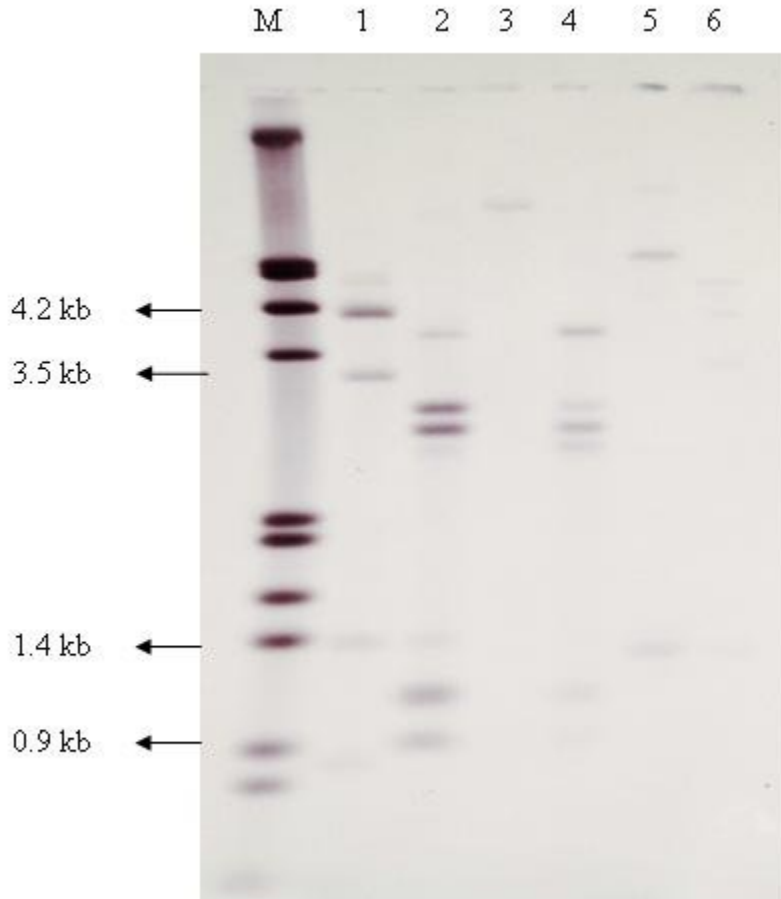
Table A.3. Primers used in this study

Primer	Oligonucleotide Sequence(5'→3')	Reference
BSBSE-START	AAATGAGYGGCATCAAAAAT	This study
BSBSE-END	TCCATCTGCTTCCAARACYGCAT	This study
SX-START	ATTAGAACTTTTAAAGAGAGCTT	This study
SX-END	CTTGCCTCCACTTTCTTTAA	This study
GAS-START	GCTTCTGGAAGTGTTTC	This study
GAS-END	CTATTTTTTATATGTGC	This study
GAS-Downstream	GTGCTTCCGGAGCGAG	This study
FINAL-Upstream	AACCACCAGTAGAAGTG	This study
FINAL-START	TTAAAGCCATTGTGAAG	This study
FINAL-END	TTTTTCCCTTTTTCTCC	This study
RpoB-forward	TCTCTACGTATGCCTCACGGATC	Aliyu et al., 2004
RpoB-reverse	CGATATTCATCCGAGAGGGTCTCC	Aliyu et al., 2004
Haem-forward	CATTGGGTTGGATAACGACTCCTAC	Aliyu et al., 2004
Haem-reverse	CAATTCTTTGTCTAAGATGGAAGCGG	Aliyu et al., 2004
fund5p	CTCAATTTTTGTTGGAAGCGAG	Zhang et al., 2006
fund3p	CATTATCAAATAACATATTTCTCAC	Zhang et al., 2006
16s-forward	TTTCAGTCGGAAGAAGAA	This study
16s-reverse	TAAGGCAGTTTCCAACGCA	This study

Table A.4. The PCR analysis of *lktA* determinants among the human *Fusobacterium necrophorum* and *Fusobacterium equinum* strains

Strain	BSBSE	SX	GAS	SH	FINAL
Bovine subsp. <i>funduliforme</i> , B35	+	+	+	+	+
Human subsp. <i>funduliforme</i>					
RMA10682	+	-	-	-	-
RMA14786	+	+	+	+	+
RMA16505	+	-	+	-	-
RMA16539	-	-	+	-	+
<i>F. equinum</i> strains					
E1	+	+	+	-	-
E2	-	-	-	-	-
E3	+	+	+	+	+
E4	-	-	+	-	-
E5	+	+	+	+	+
E6	-	-	+	+	-
E7	+	+	-	+	-
E8	-	+	-	-	-
E9	+	+	-	-	-
E10	+	+	+	+	+

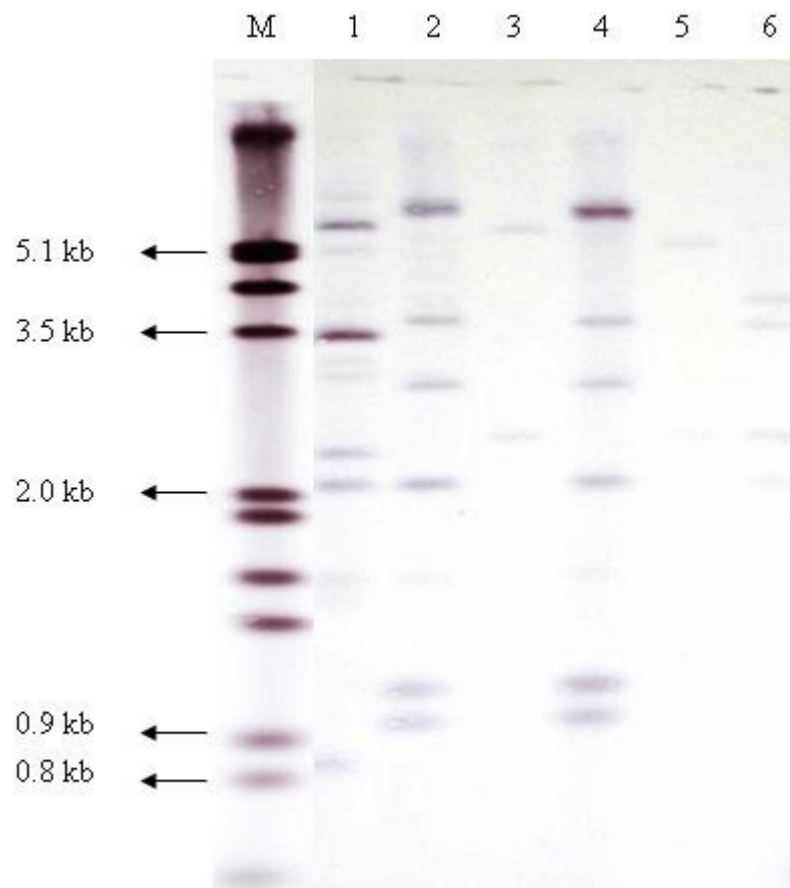
‘+’ = PCR product present; ‘-’ = PCR product absent



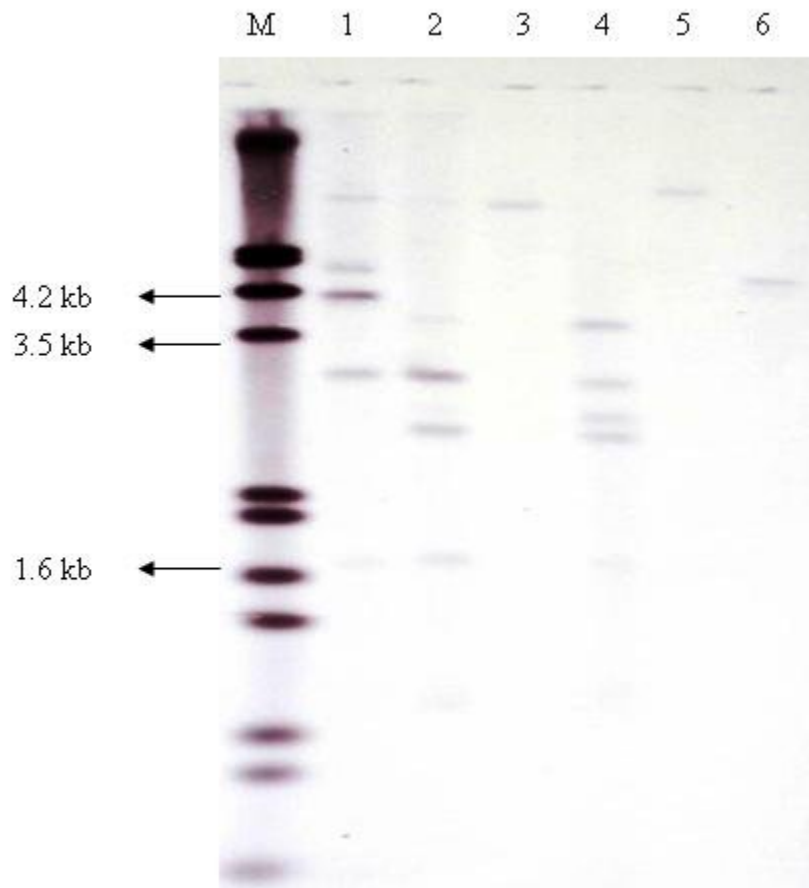
[A]

Figure A.1. Southern hybridization of double digested genomic DNA from *F. necrophorum* human strains

Southern hybridization of *Eco RI* and *Eco RV* (A), *Eco RI* and *Swa I* (B), and *Eco RV* and *Swa I* double digested genomic DNA with DIG labeled subsp. *necrophorum lktA* probe. Lane description: M, DIG labeled marker; 1, bovine subsp. *necrophorum* strain A25; 2, bovine subsp. *funduliforme* strain B35; lanes 3 to 6 human strains RMA10682, 14786, 16505, and 16539 respectively.



[B]
Figure A.1



[C]
Figure A.1