OUTER MEMBRANE PROTEINS OF *FUSOBACTERIUM NECROPHORUM*

AND THEIR ROLE IN ADHESION TO BOVINE CELLS

by

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B.V.Sc. & A.H., A. N. G. R. Agricultural University, India, 2004
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Submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Diagnostic Medicine/Pathobiology
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Abstract

Fusobacterium necrophorum is a Gram-negative, anaerobic, and rod-shaped to pleomorphic bacterium. It is frequently associated with necrotic infections of animals and humans. It is a major bovine pathogen and causes hepatic abscesses, foot rot, and necrotic laryngitis (calf-diphtheria). Liver abscesses in feedlot cattle and foot rot in beef and dairy cattle are of significant economic importance to the cattle industry. *Fusobacterium necrophorum* is classified into two subspecies, subsp. *necrophorum* and subsp. *funduliforme.* The subsp. *necrophorum* is more virulent and isolated more frequently from bovine hepatic abscesses than subsp. *funduliforme*.

Outer membrane proteins (OMPs) of Gram-negative bacteria play an important role in their adhesion to host eukaryotic cells and hence, help in the establishment of infection and disease. Our objectives were to characterize OMPs of the two subspecies of *F. necrophorum* and assess their role in adhesion to bovine cells. Electrophoretic separation of extracted OMPs of subsp. *necrophorum* showed a total of 19 bands. Four bands of 38, 40, 60 and 74 kDa were more prominent than others. The OMP of subsp. *funduliforme* showed a total of 20 proteins bands, of which, five were prominent (37.5, 58, 70, 140 and 150 kDa). The 40 kDa band was prominent in subsp. *necrophorum* while 37.5 kDa band was prominent in subsp. *funduliforme.* The human strains of *F. necrophorum* subsp. *funduliforme* had more heterogeneous banding patterns than the bovine strains of subsp. *funduliforme.*

The role of OMPs in adhesion was studied using bovine endothelial cell line (EJG cells). A significant decrease in the attachment of subsp. *necrophorum* and subsp. *funduliforme* to bovine endothelial cell line (EJG cells) was observed when the cell line was preincubated with the OMPs of each subspecies. Treatment of the bacterial cells with trypsin also decreased their binding. In addition, when each subspecies was incubated with the polyclonal antibody

produced against their OMPs before adding them to endothelial cells, there was a significant reduction in the bacterial attachment and the inhibition was subspecies specific.

A 40 kDa OMP of subsp. *necrophorum* was identified that binds to the bovine endothelial cells with high affinity. The protein when preincubated with the endothelial cells, lead to a significant decrease in the bacterial binding to the endothelial cells. The N-terminal sequencing of the protein indicated similarity to FomA, an outer membrane protein of *Fusobacterium nucleatum*, an oral pathogen of humans.

In summary, OMPs of *F. necrophorum* subsp. *necrophorum* and subsp. *funduliforme* differ from each other and they play a significant role in binding to bovine endothelial cells. We identified a 40 kDa OMP in subsp. *necrophorum* that binds to the bovine endothelial cells with high affinity and have a potential role as adhesin.

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Dedication

I dedicate my doctoral work to my family and all pervasive, the most powerful, and omnipresent Lord,

SRI KRISHNA

Chapter 1 - Outer membrane proteins of Gram-negative, non-spore forming anaerobic rods as adhesins

1. Introduction

Gram-negative, non-spore forming anaerobic bacteria are widespread and cause a variety of infections in animals and humans (24). These represent a very diverse group of bacteria, but their study has been difficult due to requirement for anaerobic growth conditions and specialized media. Most of the anaerobes of clinical importance are part of normal microflora found in mouth, intestine, upper respiratory, urinary and genital tracts of animals and humans (26).

One of the initial and critical events in bacterial infections is the adhesion of pathogens to host cells, which is important for either extracellular colonization or internalization into the cells (78),(3). Bacteria use different mechanisms for successful attachment. A number of bacterial structures such as flagella, pili or fimbriae and outer membrane proteins can be involved in bacterial adhesion (3). These complex bacterial antigens used for adhesion and invasion can range from single monomeric proteins to multimeric macromolecules performing sophisticated tasks that can be compared to nanomachines (61). Fimbriae are adhesive organelles which protrude from the surface of bacteria and help in attachment such as P pilus, type I pili, and Afa/Dr adhesins of uropathogenic *E. coli* (61). In Gram-negative bacteria, there are adhesins other than pili or fimbriae. These are the outer membrane proteins (OMPs) which have been shown to play an important role in bacterial adhesion. Autotransporters are proteins located on the bacterial surface and represent a simplest form of protein export in bacteria secreted through type V secretion system and help in adhesion in addition to the transport; for example, antigen 43, AIDA-A of *E. coli*, and BabA for *Helicoabacter pylori* (61). Attachment of the bacteria to the epithelial cell surface is crucial to the pathogenesis of infectious disease in numerous Gram-

negative bacterial species such as, *Actinobacillus actinomycetemcomitans* (25), *Aeromonas caviae* (65), and *Francisella tularensis* (32). Gram-negative anaerobes such as *Fusobacterium nucleatum* and *Porphyromonas gingivalis* have been shown to contain different OMPs that play important role in their adhesion to host cells and establish infections.

In this review, the focus is on the OMPs of gram-negative anaerobic rods that play an important role in bacterial adhesion and disease pathogenesis. There are more than two dozen genera of Gram-negative anaerobic bacilli. However, in most clinical infections, only the genera *Bacteroides*, *Prevotella*, and *Fusobacterium,* which are prevalent in the body as normal flora, are need to be considered. These three genera constitute one-third of the total anaerobic isolates from clinical specimens (17). In addition, *Dichelobacter* and *Porphyromonas* are two other Gram-negative anaerobes of veterinary importance.

2. Bacteroides

Among *Bacteroides* group, the most common is *B. fragilis* followed by *B. thetaiotaomicron* among members of the *Bacteroides* group (17). *B. fragilis* is a part of normal human intestinal microbiota and is isolated from opportunistic infections such as peritonitis, soft tissue abscesses and bacteremia. *B. fragilis* cause diarrhea in lambs, calves, piglets, foals, and infant rabbits (26). Fetal infection with *B. fragilis* leads to abortions in bovine (41). The mortality rate can go up to 19% (31). *B. fragilis* has a capsule and fimbriae or pili that aid help in its attachment to host cells (63). In addition, there are some OMPs that have been implicated in the bacterial attachment to host cells. Lectin like adhesin was found in *B. fragilis* that showed binding affinity towards D-glucosamine, D-galactosamine (66), and sialic acid (13). OmpA is major OMP of *B. fragilis* and is considered important to maintain cell structure. OmpA is shown to be important for adhesion in *E. coli* but no study supports the same function in *B. fragilis*

(83). Experiments have shown that when *B. fragilis* is treated with trypsin, it inhibited both

haemagglutination and adhesion to human intestinal cell lines indicating that the adhesin

responsible is protein (16). Although, pili also have been suggested to play a role in

haemagglutination (62), appendages removal using a protease, did not prevent adhesion to a

human intestinal cell line or haemagglutination (55). A plasminogen binding protein Bfp60 was

identified by Sijbrandi *et al.* in 2005 that also bound to extracellular matrix protein, laminin-

1(15, 75). Recently, a TonB dependent OMP has been identified in *B. fragilis* that binds human

fibronectin, a component of extracellular matrix component (56). *B. fragilis* also showed a

neuraminidase-dependent adhesive property to RBC and epithelial cells, present on both

encapsulated and /or piliated strains and unencapsulated strains, indicating that adhesion is

possibly associated with outer membrane structure that is able to span out to capsular layer when

it is present however, there is no identification for this adhesion yet (23). As mentioned before,

B. fragilis is involved in peritonitis and soft tissue infections; the intra-abdominal abscess during

peritonitis is composed of fibrin wall formed from conversion of fibrinogen. A surface

associated protein of 54 kDa size has been found recently which binds to fibrinogen (31). *B.

thetaiotamicron* a member of *Bacteroides* group shown to have three OMPs, SusC, SusD and

SusE that binds polysaccharide (63). These OMPs belong to members of eight gene cluster

involved in starch utilization. SusC and SusD act as major starch binding proteins while SusE

enhance the binding (74). CsuF is another OMP found in *B. thetaiotamicron* that is found to be

important for the bacterium to grow on chondroitin sulfate. However, it is not clear whether

CsuF is a chondroitin sulfate binding protein (9).

3. Porphyromonas

The genus *Porphyromonas* is characterized by the production of porphyrin pigments and there are 12 species that have been isolated from the oral cavities of dogs, cats, humans and nonhuman primates (20). The members of this genus produce large amount of cell associated protoheme. Porphyromonas are normal inhabitants of oropharynx, nose, gastrointestinal and geniotourinary tract and have been isolated from different clinical specimens during infection from nearly all anatomical sites including central nervous system, sinusitis, osteomyelitis, bacteremia, pleuropulmonary, dental-oral, genitourinary, and soft tissue infections (35). There are various species under genus *Porphyromonas* that cause disease conditions in humans and animals. *P. gingivalis* and *P. endodontalis* are associated with periodontitis, *P. levii* cause bacterial vaginosis in humans (30), foot rot (82) and bovine necrotic vulvovaginitis (14) in cattle. *P. asaccharolytica* cause liver abscess in humans (35), *Porphyromonas catoniae* is associated with oral infections in humans (34), *Porphyromonas gulae* is common in oral cavities of dogs and cats and is found commonly in infected bite wounds of humans (1). Except in *P. gingivalis*, no studies are available on the OMPs as adhesin in other spceis of *Porphyromonas*.

3.1. Porphyromonas gingivalis

This Gram-negative bacterium is often associated with periodontitis. Numerous studies showed that *P. gingivalis* binds to different epithelial cells (2). The molecular mechanism that lead to colonization of gingival sulcus epithelium is not well understood (8). Gingipains have been investigated extensively among a variety of virulence factors responsible for binding of *P. gingivalis* to host cells. These are also responsible for most of the extracellular and cell bound proteolytic activities produced by *P. gingivalis* (2). Gingipains are outer membrane-associated proteins (19) and are trypsin-like cysteine proteinases of *P. gingivalis* that have been implicated

in tissue colonization. Rgp A and B are Arg-gingipains while Kgp is Lys-gingipain. RgpA and Kgp contain a highly homologus adhesion domain at their carboxy terminus designated as adhesin-hemagglutinin (54). The RgpA-Kgp complex was formerly called as PrtR-PrtK complex. Another study by Bhogal *et al.* (1997), showed a 300 kDa cell-associated trypsin-like proteinases, PrtR-PrtK (RgpA-Kgp) complex, was composed of 48, 45, two 44, 39, 27, 17 and 15 kDa proteins (4). The 48 kDa was a Lys-specific proteinase while the 45 kDa was an Arg-specific proteinase which was complexed with the other proteins. The 45, 27, 17, 15 kDa and one of the 44 kDa proteins are encoded by the PrtR gene which is similar to *rgp1* gene of *P. gingivalis* H66 (58). These proteins are designated as PrtR45, PrtR44, PrtR27, PrtR17, and PrtR15. PrtR44 has also been termed as culture fluid hemagglutinin/adhesin (11, 59) while PrtR15 has been reported to bind to hemoglobin (52). This proteinase adhesin complex also binds to fibrinogen, fibronectin and laminin (60). The homology of PrtR44 with other non-proteinases components of the complex suggests a similar role for PrtR27, 17 and 15 in interaction with hemagglutination or adhesion (4). The 48 kDa Lys-specific proteinase is encoded by prtK gene which remains associated with other adhesins of 39, 15 and 44 kDa and is termed as PrtK39, PrtK15 and PrtK44. The PrtK48 showed same enzyme characteristics as of 48 kDa Lys-specific proteinase purified from the culture fluid of *P. gingivalis* 33277 by (18) and had same N-terminal sequence of 60 kDa Lys-specific proteinase isolated from culture fluid of *P. gingivalis* H66 by Pike *et al.* (1994). The difference in the size was reasoned to be due to C-terminal truncation (59). Immunization with PrtK-PrtK (RgpA-Kgp) complex has shown to protect against the periodontal bone loss in rat periodontitis model (64). Adhesin encoded by the gingipain genes are responsible for coaggregation with *Prevotella intermedia* (36). Studies have shown that antibodies raised against recombinant Arg-gingipain block the attachment of native

gingipain to epithelial cells (HEp-2) and inhibit adherence of *P. gingivalis* to epithelial cell monolayer (7). These proteins are incorporated into the outer membrane of the bacterium and have been shown to play a role in bacterial adhesion to oral epithelial cells (8, 54, 57). In addition to the hemagglutination domains found in cystine protenases, *P. gingivalis* also have multiple hemagglutinin genes (27). These hemagglutinin genes are encoded by several *hag* genes (77). HagA is one such hemagglutinin which contain four direct repeats and several regions of *hagA* have homology to different cystine proteinases such as *rgp1* (the arginine-specific cysteine protease/hemagglutinin gene cloned from strain H66), *prtR*, *prtP* (cysteine protease/hemagglutinin gene cloned from strain W12). HagE is another hemagglutinin found in *P. gingivalis* which is encoded by *hagE* gene and shows homology to *hagA* (27). Hemagglutinin B has been reported to be important for adherence to human coronary artery endothelial cells (77). Another *P. gingivalis* vesicle associated hemagglutining has been reported to be of 130 kDa which is responsible for hemagglutination mediated by *P. gingivalis* vesicles. The study also suggested a hemagglutination motif PVQNLT to be associated with hemagglutinin and that there could be many gene families which can encode this motif, and hence, it is possible to have multiple hemagglutinin or hemagglutinin related protease present in *P. gingivalis* (72). A 40 kDa outer membrane protein of *P. gingivalis* has been shown to be responsible for bacterial coaggregation (67). Outer membrane protein, Omp28, of *P. gingivalis* has also been suspected to be an adhesin due to its similarity in sequence with fimbrial protein of *Dichelobacter nodosus* that helps in attachment (76).

4. Prevotella group

Among *Prevotella*, *P. intermedia* and *P. loescheii* are of clinical importance and are part of the oral flora.

4.1. Prevotella intermedia group

The *P. intermedia* group consists of *P. intermedia*, *P. nigrescens* and *P. pallans. P. intermedia* and *P. nigrescens* are biochemically similar. *P. intermedia* and *P. nigrescens* are associated with periodontitis and also endodontic and odontogenic infections (22). However, *P. intermedia* is mostly associated with the infectious conditions whereas *P. nigrescens* is found both in healthy and in periodontitis. *P. pallans* does not have any clear disease association (22).

Prevotella intermedia has been associated with different forms of periodontal disease, such as pregnancy gingivitis, acute necrotizing ulcerative gingivitis, and adult peritonitis. The bacterium has also been found to be involved in pulmonary infections and cancrum oris (21). In addition, bacterium has also been isolated from atherosclerotic plaques (29). Although, the bacterium is of clinical importance, its virulence mechanism is not well understood. A 29 kDa surface adhesin has been identified in *P. intermedia* that mediates attachment to fibronectin, laminin and fibrinogen (86). An OMP, AdpC is shown to bind to fibrinogen as well as help the bacterium to invade the host cells (33). The protein contains leucine rich repeats which is present in variety of organisms and has been shown to play important role in immune response, apoptosis, adhesion, invasion, and signal transduction (33).

No study is available on the outer membrane proteins of *P. nigrescens* acting as adhesins except one where Bowden et al. (1991) reported that a 40 kDa outer membrane protein increases in expression during biofilm formation (5).

There are other *Prevotella species* such as *P. melaninogenica, P. loeschii, P. denticola, P. buccalis, P. veroralis* that reside in the oral flora and may cause opportunistic infections in mouth such as periodontitis (84). *P. loeschii* has also been reported in septic arthritis due to hematogenous spread of the bacterium (46). There are reports of lectin-like adhesin in *P.*

loeschii present on the fimbriae (45). *P. melaninogenica* has also been associated with footrot and foot abscesses of cattle (44).

5. Dichelobacter

Dichelobacter nodosus is the only species under the genus *Dichelobacter*. This bacterium is considered a principal causative agent of footrot in ruminants (48). In sheep, the disease is highly infectious and debilitating resulting in lameness, weight loss and poor wool growth (70). Genome sequencing of *D. nodosus* followed by analysis of atypical regions of nucleotide revealed a putative large, repetitive secreted protein (DNO_0690) which is suspected to mediate adhesion to the extracellular matrix. However, it does not state clearly if the protein is also present on the outer membrane of *D. nodosus* (48). Further studies are needed to determine the OMPs of *D. nodosus* that may act as an adhesins.

6. Fusobacterium

Genus *Fusobacterium* includes several Gram-negative bacilli with pointed or fusiform ends and produce butyric acid as major end product of the metabolism (10). The genus has a total of 13 species of which three species are associated with disease conditions. *F. nucleatum* is associated with oral infections in humans, *F. necrophorum* is associated with necrotic infections in animals and humans, and *F. equinum* is associated with oral, paraoral, and lower respiratory tract infections in horses (79). Different OMPs have been reported as adhesins in *F. nucleatum* and a hemagglutinin protein in *F. necrophorum* while there is no report on any OMP that serve as adhesin in *F. equinum*.

6.1. Fusobacterium nucleatum

Fusobacterum nucleatum is a human oral pathogen which is associated with a variety of oral infections. The bacterium is present in normal oral microflora and has been found to act as

bridging organism between early (Gram-positive species) and later colonizers (Gram-negative species) of oral biofilm formation as it is frequently found to be associated with both groups and coaggregates with many species present in the biofilm (39). Initially, a galactose binding protein (lectin) of 300 to 330 kDa was reported in *F. nucleatum* FN22 and was suggested to help the bacterium in binding to galactosyl residues which are commonly found as terminal sugars on salivary glycoconjugates (47). Later, a 30 kDa galactose binding adhesin was reported in *F. nucleatum* PK1594 that helps in hemagglutination and coaggregation with *P. gingivalis*. These galactose binding adhesins help them to attach to the various oral surfaces, as well as erythrocytes. These lectins have also been suggested to play a role in interactions with epithelial cells, fibroblasts and lymphocytes (71). A 39.5 kDa membrane protein was identified by Kaufman et al (1989) that help the bacterium to coaggregate with *Streptococcus sanguis* (40). FadA is a membrane protein adhesin that was found to help the bacterium in binding to oral mucosal KB cells. The adhesin is composed of 129 amino acids with 18 amino acid signal peptide corresponding to 13.6 kDa as intact and 12.6 kDa in secreted form. A deletion mutant of *fadA* showed 70-80% decrease in the bacterial binding to KB and Chinese hamster ovarian cells indicating a important role of this adhesin in binding to host cells (28). FadA also helps *F. nucleatum* to invade the host cells. However, it is suggested that FadA is anchored in the inner membrane and protrude through the outer membrane of *F. nucleatum* (85). An arginin-inhibitable outer membrane protein RadD is shown in *F. nucleatum* to be important for inte-species adherence and structured architecture of multispecies biofilm. FomA is another outer membrane protein adhesin that is shown to help in bacterial coaggregation and that the protein binds to statherin, a human salivary protein (43, 51). The protein has also been used a vaccine

candidate to prevent bacterial coaggregation for treatment of periodontal infection and halitosis with promising results in mice model (43).

6.2. *Fusobacterium necrophorum*

Fusobacterium necrophorum is a normal inhabitant of the rumen of cattle and is frequently associated with a variety of necrotic infections of animals, and is a major pathogen of cattle (50). The organism is also a human pathogen, causes abscesses in internal organs and is also the etiological agent of acute pharyngitis, which in young adults leads to a syndrome called, Lemierre's syndrome (6).

Fusobacterium necrophorum is classified into subsp. *necrophorum* and subsp. *funduliforme*, the two subspecies differ in morphological, biochemical, molecular, and virulence characteristics (73, 80). Of the two subspecies, subsp. *necrophorum* is more frequently encountered in fusobacterial infections than subsp. *funduliforme* in animals. The subsp. *necrophorum* is more virulent than subsp. *funduliforme* due to presence of a potent leukotoxin. Also, subsp. *necrophorum* produces more leukotoxin than subsp. *funduliforme* (50).

Fusobacterial infections in cattle include hepatic abscesses, foot rot, and necrotic laryngitis (calf-diphtheria) (42, 49, 68). Decreased pH in the rumen (ruminal acidosis) causes irritation, erosion, and ulceration of the ruminal wall. *F. necrophorum* by an unknown mechanism binds to the epithelium lining the rumen (81), causes abscesses in the ruminal wall and eventually reaches liver via portal circulation to cause hepatic abscesses (49, 69). The bacterial invasion through the ruminal wall and spread via circulation needs attachment to vascular endothelium, a critical step for vascular invasion and spread (50)..

Fusobacterium necrophorum is not capsulated and fimbriae have not been demonstrated (12). Earlier studies have implicated a putative outer membrane protein hemagglutinin of 19

kDa, to mediate attachment of *F. necrophorum* to ruminal epithelial cells and the antiserum developed against this protein inhibited the attachment of *F. necrophorum* to bovine ruminal cells (37, 38).   However, there is no further study to support this result. Furthermore, there is controversy about hemagglutinin protein as in another study, a partial DNA sequence of suggested hemagglutinin protein encodes for 89.65 kDa protein which is much larger than previously reported 19 kDa protein (53).

7. Conclusion

Outer membrane proteins of many Gram negative bacteria play an important role in its establishment and infection in the host. Similarly, Gram negative, non-spore forming anaerobic rods have been shown to have OMPs that play a role in bacterial attachment. However, only a few bacterial species, such as *Porphyromonas gingivalis* and *Fusobacterium nucleatum*, that are important in causing disease in humans, have been investigated. Further studies are warranted on OMPs of other Gram-negative, non-spore forming anaerobic rods in order to identify mechanisms of host cell attachment.

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Chapter 2 - Outer membrane proteins of *Fusobacterium necrophorum* subsp. *necrophorum* and subsp. *funduliforme*

Abstract

Fusobacterium necrophorum, a Gram-negative, rod-shaped anaerobic bacterium, is frequently associated with necrotic infections of animals and in humans. It is a major pathogen of cattle, in which it causes liver abscess, foot rot, and necrotic laryngitis. *Fusobacterium necrophorum* is classified into subsp. *necrophorum* and subsp. *funduliforme*, which differ in morphological, biochemical, molecular, and virulence characteristics. The outer membrane proteins (OMPs) of many Gram-negative bacteria play an important role in bacterial adhesion and establishment of infection. The OMPs of *F. necrophorum* have not been characterized. Hence, we analyzed OMP profiles of bovine strains of subsp. *necrophorum* and subsp. *funduliforme,* and human strains of *F. necrophorum*. Electrophoretic separation of extracted OMP of subsp. *necrophorum* showed a total of 19 bands. Four bands of 38, 40, 60 and 74 kDa were more prominent than the other bands. The OMP of subsp. *funduliforme* showed a total of 20 bands, and of which, five were prominent (37.5, 58, 70, 140 and 150 kDa). The most prominent bands were 40 and 37.5 kDa in subsp. *necrophorum* and subsp. *funduliforme,* respectively. The human strains of *F. necrophorum* examined had more heterogeneous banding patterns and had a different profile than the bovine subsp. *funduliforme*. A total of 11 protein bands in subsp. *necrophorum* and 13 protein bands in subsp. *funduliforme* were recognized by sera from cattle with liver abscess. The intensities of many of the bands in subsp. *necrophorum* were of higher intensity than that of subsp. *funduliforme*. The two subspecies of *F. necrophorum* differ in their OMP profiles and the relevance of the difference in relation to the virulence and pathogenesis needs to be investigated.

1. Introduction

   Fusobacterium necrophorum, a Gram-negative, rod-shaped, anaerobic bacterium, is associated with a variety of necrotic infections in animals and humans (3, 22). The organism is a major pathogen of cattle in which it causes hepatic abscesses, foot rot, and necrotic laryngitis or calf-diphtheria (10). In humans, *F. necrophorum* cause a condition called Lemierre's syndrome that primarily affects young and healthy persons. The disease is characterized by initial pharyngotonsillitis or peritonsillar abscess followed by swelling and tenderness near sternocleidomastoid muscle due to septic thrombophlebitis of ipsilateral internal jugular vein. Later, the patient develops high fever and disseminated metastatic abscesses (4). The organism is also associated with abscesses of internal organs in humans (3).

   Fusobacterium necrophorum is classified into subsp. *necrophorum* and subsp. *funduliforme* (16) and the two subspecies differ in cell morphology, colony characteristics, growth pattern in broth, extracellular enzymes, hemagglutination, hemolytic and leukotoxic activities, chemical composition of LPS, and virulence in mice (21). While subsp. *necrophorum* agglutinates chicken erythrocytes, and has phosphatase and lipase activities, subsp. *funduliforme* has no or weak hemagglutinating ability, or phosphatase and lipase activities. In liver abscesses of cattle, subsp *necrophorum* is found more frequently (71% to 95% of liver abscesses) and most often in pure culture (up to 75%) compared to subsp. *funduliforme,* which is recovered from 5% to 29% of liver abscess and rarely as a pure culture (up to 22%) (8). The higher prevalence of subsp. *necrophorum* is because of the increased virulence attributed to its higher leukotoxin production. Interestingly, clinical strains associated with human infections mostly belong to subsp. *funduliforme* (20).

The OMPs of many Gram negative bacteria play an important role in bacterial attachment to the host cells and in the establishment of infection. Not much is known about the OMPs of *F. necrophorum*. Ainsworth *et al*. (1993) reported on electrophoretic comparison of OMP of *F. necrophorum* subsp. *necrophorum* and subsp. *funduliforme* and described differences in the protein bands between 38 to 45 kDa (1). In this report, we describe OMP profiles of *F. necrophorum* subsp. *necrophorum* and subsp. *funduliforme*. We compare OMP profiles of subsp. *funduliforme* of bovine and human origin. Additionally, we identify OMPs of bovine strains that were immunodominant with sera obtained from blood of cattle with naturally occurring or with experimentally induced liver abscesses.

2. Material and Methods

2.1. Bacterial strains and culture

Six strains of *F. necrophorum* subsp. *necrophorum* (RA15, RA16, RA17, 8L1, A50 and A25) and five strains of subsp. *funduliforme* (B17, B30, B35, B36 and B47), previously isolated from liver abscesses or ruminal contents of cattle were used (11, 22). Four human clinical isolates (RMA10682, RMA14786, RMA16505 and RMA16539) of *F. necrophorum* provided by Dr. Diane Citron (R. M. Alden Laboratory, Santa Monica, CA) were also included in the study. Frozen cultures were thawed, streaked on blood agar plates, incubated in an anaerobic glove box (Forma Scientific, Marietta, OH) and single colonies were then grown in pre-reduced anaerobically sterilized brain heart infusion (PRAS-BHI) broth (22). Overnight cultures were used to inoculate PRAS-BHI and grown for 6-8 h ($OD_{600} = 0.6$).

2.2. Extraction of OMPs

The OMPs from *F. necrophorum* subsp. *necrophorum* and subsp. *funduliforme* were isolated according to the method described by Osborn and Munson (1974) with slight

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modifications (13). Briefly, cultures were grown in 1 liter of anaerobic BHI broth for 12 to 14 h.

The cells were pelleted by centrifugation, resuspended in 20 ml of cold 0.75M sucrose-10 mM

Tris buffer at pH 7.8 with lysozyme (2 mg/ml of cell suspension) and incubated on ice for 20

min. Formation of spheroplast form was achieved by diluting the suspension with two volumes

of cold 1.5 mM EDTA at constant rate of delivery. The spheroplasts were lysed by

ultrasonication in an ice-water bath (3 mm microtip at 20W output pulse setting). The cell debris

was removed by centrifugation at 6,500 g for 15 min at 4 °C. The supernatant was then

ultracentrifuged in Beckman Coulter centrifuge with 70.1 Ti rotor at 386,984 g at 2-4°C for 2 h,

and the supernatant was discarded. The pellet was resuspended 2 ml of 20 mg/ml Triton X-100

and 10 ml of cold 0.25 M sucrose, 3.3 mM Tris-1 mM EDTA, pH 7.8 (STE buffer) and

incubated at room temperature for 45 min to dissolve the inner membrane and the volume was

adjusted to that of the original sonicate suspension and the ultracentrifugation was repeated at

125,392 g for 2 h at 2-4 °C and the pellet was collected in cold STE buffer and stored at -80° C

until use.

2.3. SDS-PAGE analysis of OMPs

The protein concentrations of extracts were determined by BCA protein assay (Bio-Rad

Inc., Hercules, CA). The extracted proteins were suspended in SDS-PAGE sample loading

buffer by adjusting the protein concentration to 1 µg/µl. A 20 µg of the extracted protein

fraction was loaded on to SDS-PAGE with 4% stacking and 10% separation gels and

electrophoresed. The protein bands were stained with colloidal coomassie blue (Thermo

Scientific, Rockford, IL).

2.4. Effects of growth media on OMPs of bovine strains

Bacteria were grown on blood agar plates and a single colony was inoculated into 10 ml of either anaerobic pre-reduced BHI broth or lactate broth containing lactic acid as the major energy source (23). The overnight culture of bacteria were then inoculated into larger volumes of BHI or lactate medium and incubated for 12 h. Cells were pelleted and OMPs extracted as described before. The OMPs (20 μg) were loaded on SDS-PAGE gel and stained by silver staining or coomassie blue.

2.5. Western blot analyses of OMPs using sera from cattle with naturally-occurring or experimentally-induced liver abscesses

Blood from cattle (n=4) with naturally-occurring liver abscesses were obtained from a local abattoir. Blood from cattle (n=5) with experimentally-induced abscesses were obtained on days 0 (before inoculation), 5, and 10 after intraportal inoculation of *F. necrophorum* subsp. *necrophorum*. The steers with experimentally-induced liver abscesses were part of an unrelated study. Steers were inoculated with *F. necrophorum* subsp. *necrophorum,* strain 8L1 via ultrasound guided, intraportal catheter to induce liver abscesses (7). Each steer was inoculated with a 10 ml of *F. necrophorum* subsp. *necrophorum* culture containing 1 x 10^9 CFU/ml. Steers were euthanized on day 10 and livers were examined for abscesses. Sera were separated from blood after centrifugation and stored at -20° C until use.

The OMP bands of were run on SDS-PAGE gel and transferred to nitrocellulose membrane. The immunoblot was performed with antisera from cattle with liver abscesses collected at slaughter or cattle with experimentally-induced liver abscesses as the primary antibody and mouse anti-bovine antibody conjugated with alkaline phosphatase as the secondary antibody (Sigma-Aldrich, St. Louis, MO). The increase in protein band densities of outer

membrane proteins were measured by a scanning densitometry (Total lab TL120, Nonlinear Dynamics Ltd, Newcastle, UK).

3. Results

3.1. OMP profiles of subsp. necrophorum and subsp. funduliforme

Initially, we compared the OMP profile of subsp. *necrophorum* strain A25 and subsp. *funduliforme* strain B35. A number of major and minor protein bands were visualized following electrophoretic separations of OMP extracts of both subspecies of *F. necrophorum* on the SDS-PAGE (Fig. 2.1). Subsp. *necrophorum* OMPs showed a total of 19 protein bands of which four bands of sizes 38, 40, 60 and 74 kDa were more prominent than the other bands (Fig. 2.1). The OMPs of subsp. *funduliforme* showed a total of 20 proteins bands of which five bands of sizes 37.5, 58, 70, 140 and 150 kDa were more prominent than other bands (Fig. 2.1). Among the prominent bands of subsp. *funduliforme*, 140 kDa was also present in subsp. *necrophorum* but the band intensity was higher in subsp. *funduliforme* than in subsp. *necrophorum*. All other less prominent bands present in both subspecies have been listed in Table 2.1. Of all the bands displayed, the most prominent band was 40 and 37.5 kDa in subsp. *necrophorum* and *funduliforme* respectively.

We also analyzed OMP profiles of six different bovine strains of subsp. *necrophorum* and five strains of subsp. *funduliforme*. Protein bands of 28, 38, 40, 60, and 74 kDa were specific and consistently present in the six strains of subsp. *necrophorum*, and protein bands of 37.5, 49, 58, 70, 150 and 200 kDa OMP bands were specific and consistently present in the five strains of subsp. *funduliforme* specifically (Table 2.2). As seen before with strain A25 and B35, all six strains of subsp. *necrophorum* and five strains of subsp. *funduliforme* showed 40 and 37.5 kDa as the most prominent band respectively. There was no difference in the banding patterns of the

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different strains in each subspecies. We compared the OMP profiles of subsp. *necrophorum*

isolated from liver and from ruminal contents and observed no difference in banding patterns

(Fig. 2.2).

The four human strains examined did not have uniform banding patterns compared to the

bovine strains (Fig. 2.3). Two bands of 37 and 37.5 kDa were present in RMA 10682 and RMA

14786 and a 100 kDa band was present in RMA 16505 and RMA 16539. A 40 kDa was present

in RMA 16505 and a 49 kDa band was present in RMA 16539 and these bands were specific to

these strains. The four human strains had prominent 140 and 70 kDa protein bands similar to

subsp. *funduliforme.* The 37.5 kDa protein band which is prominent in all the bovine

funduliforme strains was only prominent in strain RMA 10682 and RMA 14786 among human

strains.

3.2. Effects of growth media on outer membrane proteins profile

The OMP profiles of *F. necrophorum* subsp. *necrophorum* grown in two different growth

media (BHI and lactate broth) were not different, based on Coomassie blue staining (data not

shown). However, when gels were stained with silver staining, three protein bands of 19, 24 and

75 kDa were only present in cells grown in BHI medium (Fig. 2.4).

3.3. Western blot analyses of OMPs of **subsp**. *necrophorum* and **subsp**. *funduliforme*

with sera from cattle with liver abscess and cattle challenged with subsp. *necrophorum*

Antisera from cattle with liver abscesses recognized 13 OMPs (15, 17, 20, 24, 26, 28, 40, 52,

54, 60, 62, 74 and 100 kDa) in subsp. *necrophorum* and 15 OMPs (15, 17, 20, 24, 37.5, 42, 52,

54, 60, 72, 100, 140, 150, and 250 kDa) in subsp. *funduliforme* (Fig. 2.5a). Many of the bands in

subsp. *necrophorum* were of higher intensities than observed with subsp. *funduliforme.* The

band of 40 kDa in subsp. *necrophorum* was the most dominant while in subsp. *funduliforme*, the

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band of 37.5 kDa was the most dominant. In subsp. *necrophorum,* even though the 38 kDa band that was prominent in SDS-PAGE gel analysis did not appear immunodominant in western blot. Similarly, the 70, 140 and 150 kDa bands that were prominent in SDS-PAGE gel analysis of subsp. *funduliforme* were less immunodominant in western blot analysis.

Of the five steers intraportally inoculated with subsp. *necrophorum*, four developed liver abscesses and one steer had no liver abscesses. Western blot assay with sera from blood collected on 0, 5 and 10 days post-inoculation from steers that developed liver abscesses showed a gradual increase in the band intensities of 17, 40 and 74 kDa proteins consistently while a 24 kDa protein showed increase in intensity with serum from only one of the four steers. There was no increase in the band intensities from days 0 to 10 when serum from the steer that did not develop abscesses was used (Figs. 2.6 and 2.7).

Discussion

The two subspecies of *F. necrophorum,* subsp. *necrophorum* and subsp. *funduliforme* differ in their colony appearances, microscopic morphology, biochemical and molecular characteristics, and in virulence (10). The SDS-PAGE analyses of OMP of strains within each subspecies, whether of ruminal or hepatic abscess origin, showed remarkably uniform banding patterns, which is in agreement with the observation reported by Ainsworth and Scanlan (1993). However, the OMP profiles of the two subspecies differed in the total number of bands and in the number and intensities of the bands. Each subspecies had few prominent bands that were absent in the other subspecies. The subsp. *necrophorum* had a band size of 40 kDa*,* which was also most immunodominant and the subsp. *funduliforme* had a band of 37.5 kDa that was most immunodominant. Ainsworth and Scanlan (1993) have reported that subsp. *necrophorum* had a major protein band of 44.5 kDa and the subsp. *funduliforme* had a major band of 38.4 kDa. We

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also observed a difference in banding patterns between the two subsp. between 140 to 200 kDa where subsp. *funduliforme* showed a presence of 200, 190, and 150 kDa band that were absent in subsp. *necrophorum.*

It has long been recognized that *F. necrophorum,* particularly subsp. *necrophorum,* agglutinates erythrocytes from chicken and other animal species (6). The agglutinating ability is either absent or weak in subsp. *funduliforme* (21).The ability to hemagglutinate erythrocytes was initially used to differentiate human and animal *F. necrophorum* isolates (2). The human isolates agglutinated human, guinea pig and rabbit erythrocytes; while animal isolates agglutinated chicken, sheep, human, bovine, rabbit and guinea pig erythrocytes. Shinjo *et al*. (1980) reported that among the animal isolates, both subspecies of *F. necrophorum* varied in their ability to agglutinate erythrocytes of different animal species (18). Animal isolates of subsp. *necrophorum* with hemagglutinating ability were more virulent than animal and human isolates of subsp. *funduliforme* lacking hemagglutinin (9, 19). Shinjo and Kiyoyama (1986) reported that a hemagglutinin-lacking mutant strain of *F. necrophorum* was not lethal to mice in comparison to the wild type strain (17). The agglutinin was identified as a heat-labile, low molecular weight surface protein (19 kDa), rich in amino acids alanine, glutamine and histidine and was called hemagglutinin (24). However, Narongwanichgarn *et al*. (2003) and Semenikhin *et al*. (2005) attributed hemagglutinating activity to a large size outer membrane protein of approximately 90 kDa (containing at least 814 amino acids as deduced from the partially sequenced gene (12, 15). The primers designed on the basis of sequence of hemagglutinin provided by Narongwanichgarn *et al* (2003) is able to clearly differentiate between subsp. *necrophorum* and subsp. *funduliforme* indicating that this gene is only present in subsp. *necrophorum* which supports the observation that hemagglutination property is only limited to subsp. *necrophorum*. We were unable to find

discernable bands of 19 or 90 kDa in any of the strains of *F. necrophorum* subsp. *necrophorum* except in one instance when we compared the OMP profile of subsp. *necrophorum* grown in different media. In contrast to the bovine strains, the four human strains of subsp. *funduliforme* that we analyzed showed considerable heterogeneity in the OMP profiles indicating a potential difference in genetic make-up of these strains compared to bovine strains. Studies have shown that the OMP profile of Gram-negative bacteria is influenced by the growth medium (5,14). As the differential expression of OMP of gram negative bacteria in response to external milieu may help them to survive better in a given complex environment, in this study we found that SDS-PAGE analysis of OMPs grown in different media showed a different expression profiles with three OMP of size 75, 24 and a 19 kDa appearing in BHI high nutrient medium compared to a ML medium which is not a nutrient rich medium.

Western blot analysis of outer membrane proteins with sera from cattle with liver abscesses showed that antibodies were able to identify several OMP bands of both the subsp. However, a few protein bands were more immunodominant in both the subsp. compared to other OMP protein bands indicating that those OMPs of *F. necrophorum* were immunogenic. Many gram negative bacteria have shown to have immunodominant OMPs, such as *P. gingivalis* where proteins of 115, 55 and 47 kDa were identified to be dominant with sera from humans with periodontal diseases (24). When we challenged steers with subsp. *necrophorum*, there was a gradual increase in the band intensity with different phases of sera with control sera as least reactive while the sera obtained at the end of challenge study as the most reactive. Moreover, the increase in the band intensity was limited to few bands indicating that these protein bands were immunodominant.

In conclusion, our study showed that the OMP profile of subsp. *necrophorum* is different from that of the subsp. *funduliforme*. The OMP profiles of human strains, which are more closely related to subsp. *funduliforme,* were also different from bovine strains of subsp. *funduliforme*. Our study also showed that there are immunodominant OMPs present in the both subspecies as detected with sera from cattle with liver abscess. Further studies are needed to understand the role of OMP in establishment of infection caused by *F. necrophorum*.

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Figure Legends

Figure 2.1: SDS-PAGE analysis of outer membrane proteins of *Fusobacterium necrophorum* subsp. *necrophorum,* strain A25 and subsp. *funduliforme,* strain B35 using Coomassie blue staining. Predominant bands are shown with arrow.

Figure 2.2: SDS-PAGE analysis of outer membrane proteins of ruminal (RA15, RA16 and RA17) and liver (8L1, A50 and A25) strains of subsp. *necrophorum* of bovine origin using Coomassie staining. Arrows indicate different protein bands.

Figure 2.3: Heterogeneity of outer membrane protein profiles of subsp. *funduliforme* strains isolated from humans compared to subsp. *funduliforme* strains of bovine origin.

Figure 2.4: Outer membrane protein profiles of *Fusobacterium necrophorum* subsp. *necrophorum* grown in PRAS-BHI and modified lactate media broth.

Figure 2.5: Western blot analysis of outer membrane proteins of *Fusobacterium necrophorum* subsp. *necrophorum* and subsp. *funduliforme* with sera obtained from cattle at slaughter with liver abscesses.

Figure 2.6: Western blot analysis of outer membrane proteins of *F. necrophorum* subsp. *necrophorum* with sera from steers intraportally inoculated with subsp. *necrophorum* strain 8L1 A) that did not develop liver abscesses and B) that developed liver abscesses. Lane 1 = Molecular marker; Lane 2 = day 0 before inoculation, Lane 3 = 5 days post-inoculation: Lane 4 = 10 days post-inoculation. The arrows indicate the OMP bands that increased in intensity.

Figure 2.7: Antibody titers against outer membrane proteins of *Fusobacterium necrophorum* subsp. *necrophorum,* as measured by scanning densitometry, in steers with (B) or without (A) experimentally-induced liver abscesses.

Tables

Table 2.1. SDS-PAGE analysis of OMPs of subsp. *necrophorum* strain A25 and subsp. *funduliforme* strain B35.

Protein bands (kDa)	*Fusobacterium necrophorum*	
	Subsp. *necrophorum*, strain A25	Subsp. *funduliforme,* strain B35
Prominent bands		
150	-	+
140	+	+
74	+	-
70	-	+
60	+	-
58	-	+
40	+	-
38	+	-
37.5	-	+
Non prominent bands		
200	-	+
100	+	-
60	+	+
54	+	+
52	+	+
49	-	+
32	+	+
30	+	+
28	+	-
24	+	+
22	+	+
17	+	+
15	+	+

Table 2.2. Different protein bands specific for subsp. *necrophorum* and subsp. *funduliforme* OMPs.

Protein bands, KDa	*Fusobacterium necrophorum*										
	Subsp. *necrophorum*						Subsp. *funduliforme*				
	RA15	RA16	RA17	8L1	A50	A25	B35	B36	B30	B17	B47
200	-	-	-	-	-	-	+	+	+	+	+
150	-	-	-	-	-	-	+	+	+	+	+
70	-	-	-	-	-	-	+	+	+	+	+
58	-	-	-	-	-	-	+	+	+	+	+
49	-	-	-	-	-	-	+	+	+	+	+
37.5	-	-	-	-	-	-	+	+	+	+	+
74	+	+	+	+	+	+	-	-	-	-	-
60	+	+	+	+	+	+	-	-	-	-	-
40	+	+	+	+	+	+	-	-	-	-	-
38	+	+	+	+	+	+	-	-	-	-	-
28	+	+	+	+	+	+	-	-	-	-	-

Table 2.3. Comparison of OMPs of subsp. *funduliforme* of human and bovine origin.

Protein bands, KDa	*Fusobacterium necrophorum*							
	Human clinical isolates, subsp. *funduliforme*				Bovine liver abscess isolates, subsp. *funduliforme*			
	RMA10682	RMA16505	RMA14786	RMA16539	B17	B30	B35	B36
200	+	+	+	+	+	+	+	+
150	-	-	-	-	+	+	+	+
140	+	+	+	+	+	+	+	+
100	-	+	-	+	-	-	-	-
70	+	+	+	+	+	+	+	+
58	+	+	+	+	+	+	+	+
50	+	+	+	+	+	+	+	+
49	-	-	-	+	+	+	+	+
40	-	+	-	-	-	-	-	-
37.5	+	-	+	-	+	+	+	+
37	+	-	+	-	-	-	-	-
26	+	+	+	+	+	+	+	+

Figure 2.1. SDS-PAGE analysis of subsp. *necrophorum* **and subsp.** *funduliforme*
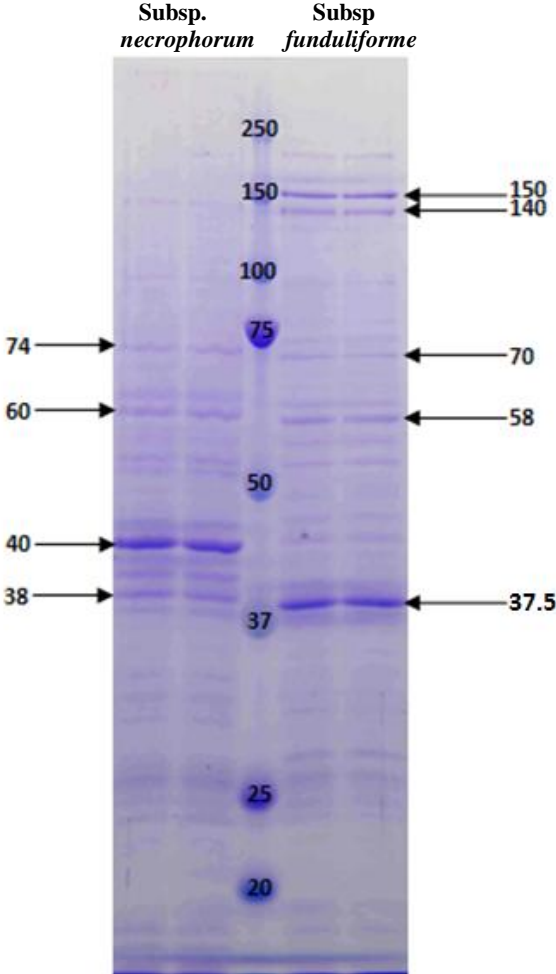
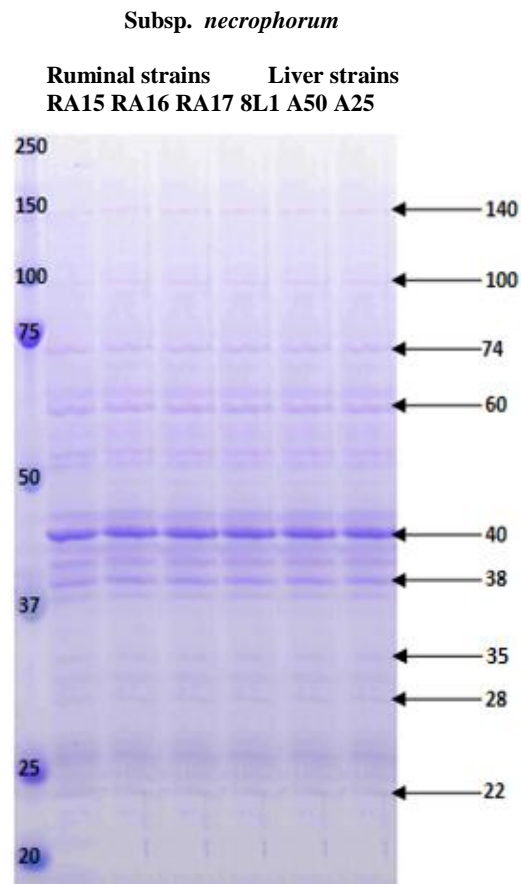**Figure 2.2 OMP analysis of subsp.** *necrophorum* **of ruminal and liver origin**

Subsp. *necrophorum*

Ruminal strains **Liver strains**
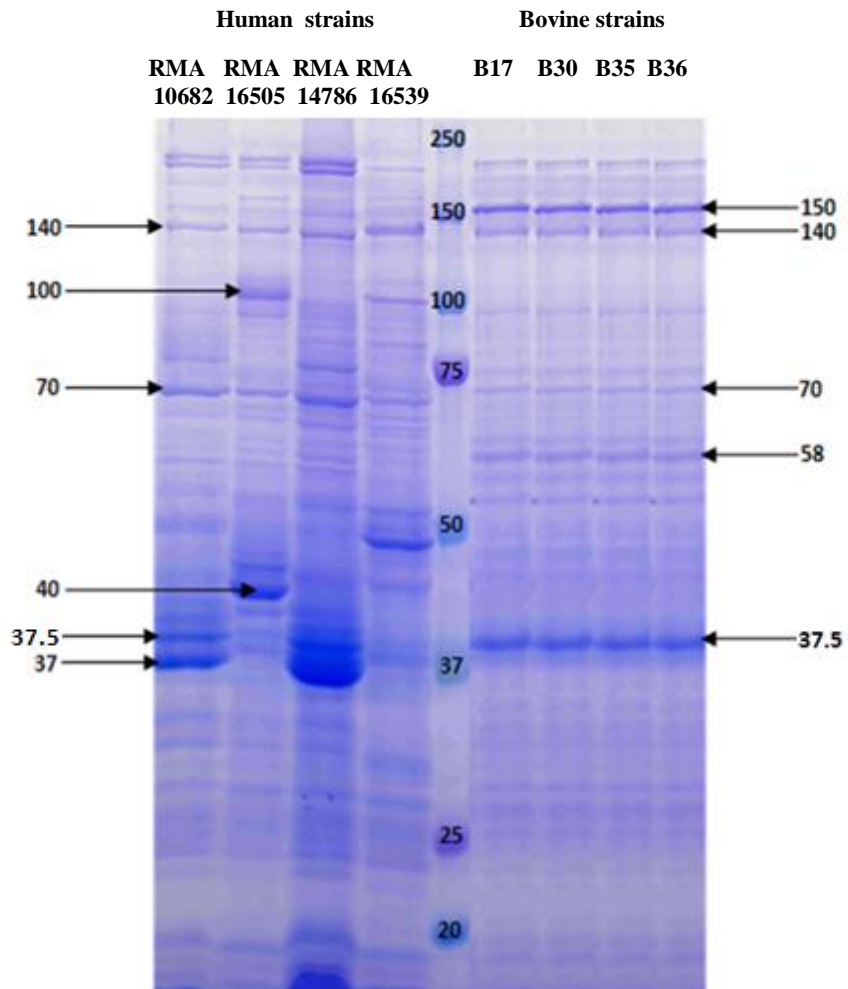**RA15 RA16 RA17 8L1 A50 A25**

Figure 2.4. OMP analysis of subsp. *necrophorum* grown in LB and PRAS-BHI medium
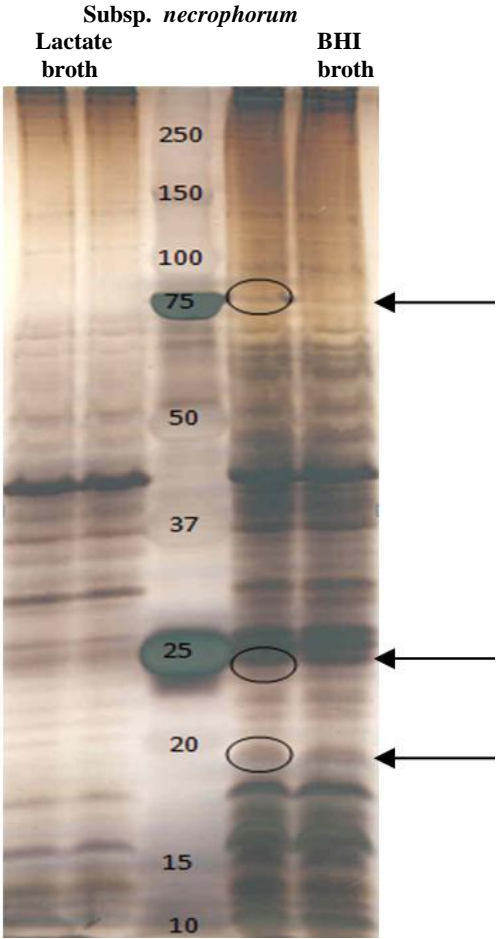
Figure 2.5. Western blot analysis of OMPs of subsp. *necrophorum* **and subsp.** *funduliforme* **with sera from cattle with liver abscesses**
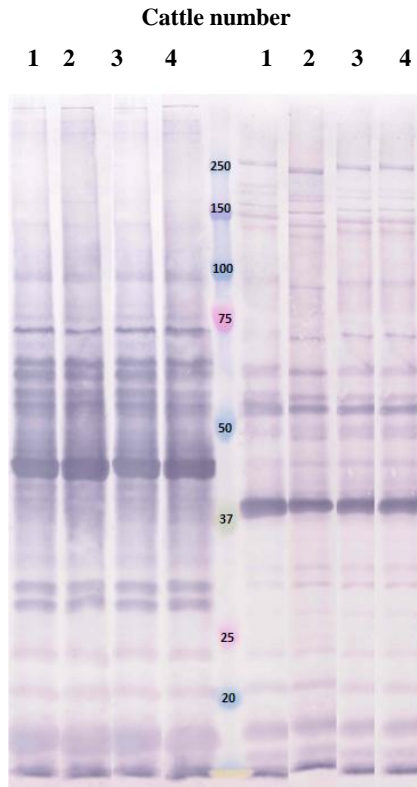
Figure 2.6. Western blot analysis of OMPs of subsp. *necrophorum* with sera from steer infected with subsp. *necrophorum*
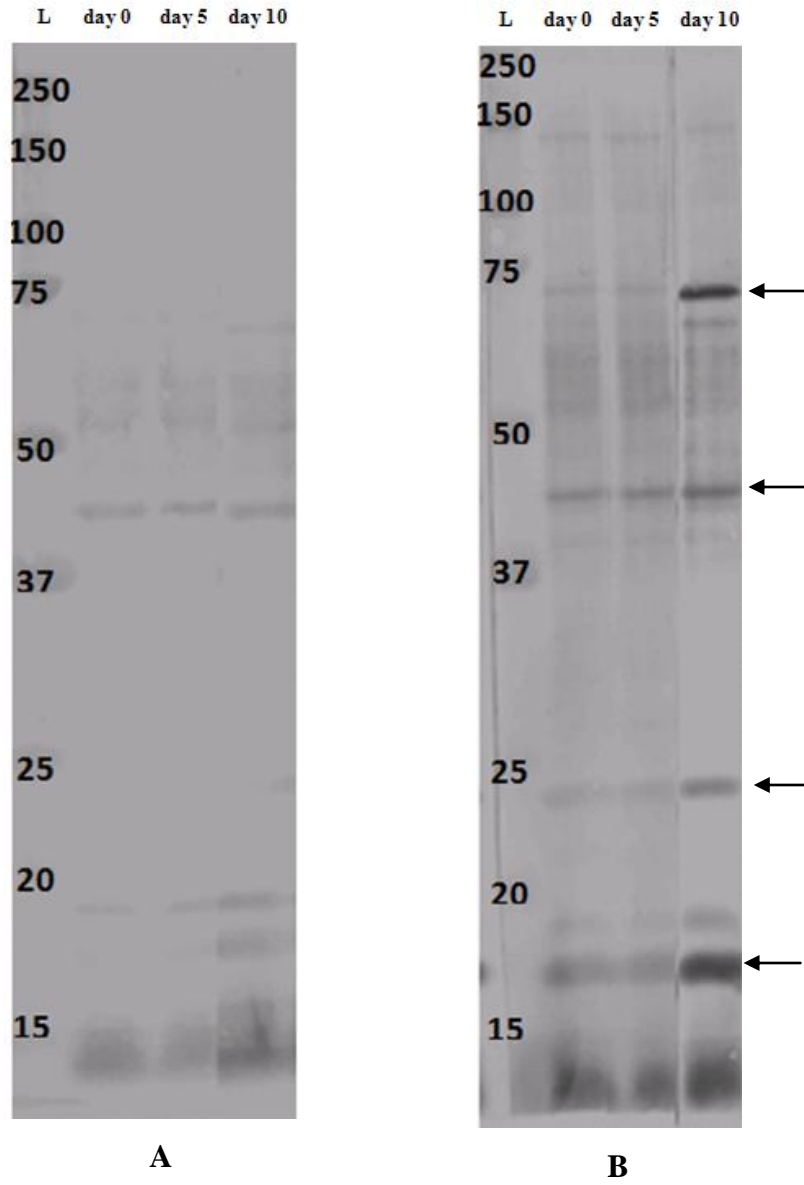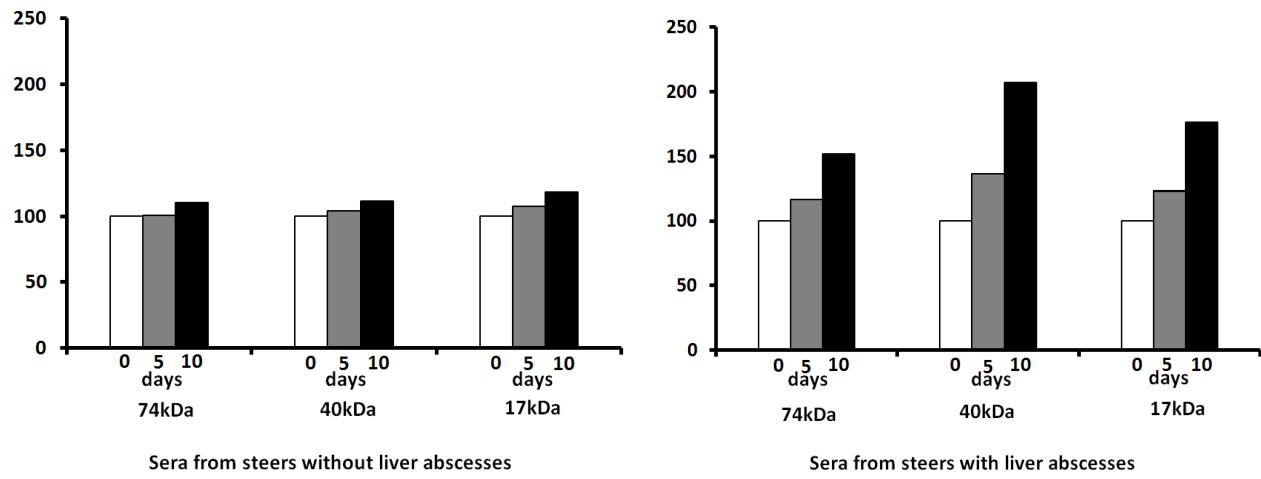

A

B

Figure 2.7. Scanning densitometer analysis

Sera from steers without liver abscesses

Sera from steers with liver abscesses

Chapter 3 - Outer membrane proteins of *Fusobacterium necrophorum* mediate attachment to bovine endothelial cells

Abstract

Fusobacterium necrophorum, a Gram-negative anaerobe, is frequently associated with necrotic infections of animals and humans. Fusobacterial infections in cattle include hepatic abscesses, foot rot, and necrotic laryngitis. *F. necrophorum* is classified into subsp. *necrophorum* and subsp. *funduliforme*, and the two subspecies differ in morphological, biochemical, molecular, and virulence characteristics. Attachment of bacteria to the host cell surface is critical to the pathogenesis in several Gram-negative bacterial species**.** Outer membrane proteins (OMPs) have been shown to play an important role in bacterial adhesion and establishment of infection. However, there is no information available on the role of OMPs in the attachment of *F. necrophorum* to host cells. We evaluated whether OMPs of *F. necrophorum* mediate attachment to bovine endothelial cells as pathogenesis of liver abscess formation involves travel of *F. necrophorum* from rumen to liver via portal circulation. The bacterium also needs to interact with endothelial cells of liver sinuses before establishing infection in liver. The binding of subsp. *necrophorum* to the bovine endothelial cells was higher than that of subsp. *funduliforme.* Trypsin treatment of bacterial cells decreased their binding to endothelial cells indicating the proteinaceous nature of potential adhesins of *F. necrophorum.* Preincubation of *F. necrophorum* with extracted OMPs decreased the binding of bacterial cells to the endothelial cells. In addition, binding of each subspecies to the endothelial cells were inhibited by the polyclonal antibody raised against their OMPs. Also, OMPs of *F. necrophorum* bound with high affinity to the bovine fibronectin, an extracellular matrix protein which may be

a ligand for bacterial adherence. We concluded that OMPs of *F. necrophorum* mediate

attachment of bacterial cells to the host cells.

1. Introduction

Fusobacterium necrophorum is a Gram-negative, anaerobic, and rod-shaped to

pleomorphic bacterium. It is frequently associated with necrotic infections of animals and

humans (15). It is a major pathogen of cattle and the infections in cattle include hepatic

abscesses, foot rot, and necrotic laryngitis (calf-diphtheria) (9, 19). Liver abscesses in feedlot

cattle and foot rot in beef and dairy cattle are of significant economic importance to the cattle

industry (3, 7, 12). *F. necrophorum* is classified into subsp. *necrophorum* and subsp.

funduliforme, and the two subspecies differ in morphological, biochemical, molecular, and

virulence characteristics (13, 16). Of the two subspecies, subsp. *necrophorum* is more frequently

encountered in fusobacterial infections than subsp. *funduliforme* (6, 7). The subsp. *necrophorum*

is more virulent than subsp. *funduliforme* because of more potent or increased production of

virulence factors, particularly the leukotoxin (9).

The outer membrane proteins (OMPs) of Gram-negative bacteria play an important role

bacterial adherence to the host cell. Generally, bacterial cell attachment to the host eukaryotic

cells is mediated by capsule or proteins on fimbriae or OMPs (2, 14). *F. necrophorum* is not

capsulated and fimbriae have not been demonstrated (4). The initial attachment of *F.*

necrophorum to rumen epithelial cells and subsequent attachment to endothelial cells of blood

vessels in ruminal wall or in liver sinusoids are essential steps in the cause of ruminal and liver

abscesses. However, the means by which *F. necrophorum* attaches to epithelial or endothelial

cells has not been determined. Outer membrane proteins mediating attachment to epithelial cells

have been characterized in *F. nucleatum,* a human oral pathogen (5). We conducted studies to determine whether OMP of *F. necrophorum* mediate attachment to bovine endothelial cells.

2. Materials and Methods

2.1 Bacterial strains and culturing for attachment assay

Fusobacterium necrophorum subsp. *necrophorum,* strain A25 and subsp. *funduliforme,* strain B35, previously isolated from liver abscesses of cattle (6) were used in this study. The organisms were cultured in brain heart infusion broth (BHI, Becton-Dickinson, Franklin Lakes, NJ) that was pre-reduced with the addition of 0.05% cysteine hydrochloride and anaerobically sterilized (PRAS-BHI) (18). Bacterial cells for attachment assay were prepared by inoculating 0.1 ml of an overnight culture derived from a single colony into a 10-ml PRAS-BHI broth and grown to an OD_{600} to 0.6. One ml of the bacterial culture was then pelleted by centrifuging at 6500 g for 20 min and resuspended in anaerobic EMEM medium (Thermo Scientific, Rockford, IL).

2.2 Extraction of OMPs

The OMPs from strain A25 and B35 were extracted according to the method described by Osborn and Munson (11) with slight modifications. Briefly, cultures were grown in 1 liter of PRAS- BHI broth for 12 to 14 h. The cells were pelleted by centrifugation, resuspended in 20 ml of cold 0.75M sucrose-10 mM Tris buffer at pH 7.8 with lysozyme (2 mg/ml of cell suspension) and incubated on ice for 20 min. Formation of spheroplasts was achieved by diluting the suspension with two volumes of cold 1.5 mM EDTA at constant rate of delivery. The spheroplasts were lysed by ultrasonication in an ice-water bath with a 3 mm microtip at 20W output pulse setting. The cell debris was removed by centrifugation at 6,500 g for 15 min at 4 °C. The supernatant was then centrifuged at 386,984 g at 2-4°C for 2 h, and the supernatant was

discarded. The pellet was resuspended 2 ml of 20 mg/ml Triton X-100 and 10 ml of cold 0.25 M

sucrose, 3.3 mM Tris-1 mM EDTA, pH 7.8 (STE buffer) and incubated at room temperature for

45 min to dissolve the inner membrane and the volume was adjusted to that of the original

sonicate suspension and the ultracentrifugation was repeated at 125,392 g for 2 h at 2-4 °C and

the pellet was collected in cold STE buffer and stored at -80° C until use.

2.3. Culture of endothelial cells

Bovine adrenal gland capillary endothelial cell line (EJG cells CRL-8659, ATCC,

Manassus, VA) was used in this study. Cells were grown in EMEM medium with 10 % fetal calf

serum and 1% solution of streptomycin and penicillin (Invitrogen Inc., Carlsbad, CA). The

medium was changed every 3 or 4 days until the cells became a monolayer. The cells were then

trypsinized and subcultured to maintain the cell line for use in experiments.

2.4. Trypsin *treatment of F. necrophorum subsp. necrophorum for attachment assay*
with EJG cells

The EJG cells were seeded in wells of a 6-well plate as mentioned before.

Fusobacterium necrophorum subsp. *necrophorum* was grown to an absorbance of 0.6 at 600 nm

and 1 ml of the culture was pelleted by centrifugation at 6,500 g rpm for 10 min. The pelleted

cells were treated with 1 ml of anaerobic EMEM cell culture medium containing 300 μg

trypsin/ml and incubated for 1 h at 37 °C. After incubation, the bacterial cells were again

pelleted down by centrifugation, washed with anaerobic EMEM medium containing 10% heat

inactivated FBS to neutralize any trypsin left, resuspended in anaerobic EMEM medium and

added to EJG cells. Bacterial cells treated with only anaerobic EMEM medium and endothelial

cells not incubated with bacteria served as controls. This experiment was done to examine if any

protein component on the outer membrane of *F. necrophorum* is responsible for binding to EJG cells.

2.5. Inhibition assay with OMPs of Fusobacterium necrophorum

The EJG cells were plated in 6-well plates as mentioned before and incubated with 400 µg per well of OMP of subsp. *necrophorum* for 1 h at 37 °C. One ml of subsp. *necrophorum* culture grown to 0.6 absorbance at 600 nm was pelleted by centrifugation at 6,500 g for 10 min and mixed with 1 ml anaerobic EMEM cell culture medium and added to the EJG cells and incubated for 1 h at 37 °C. The EJG cells were then washed with PBS 3 or 4 times vigorously to remove unbound bacteria. Finally, the EJG cells along with attached bacteria were scraped using 500 µl of trypsin-versen (Lonza Walkersville Inc., Walkerville, MD), and neutralized quickly with 1.5 ml of anaerobic EMEM containing heat inactivated 10% fetal bovine serum. The collected cells were serially diluted and spread-plated on blood agar plate. The colonies were counted after incubating the plates anaerobically for two days. The EJG cells without incubation with OMPs served as control.

2.6. Production of polyclonal antisera against OMPs of two subspecies

Polyclonal antisera against the outer membrane proteins of *F. necrophorum* were produced in rabbits. The total outer membrane proteins were extracted as mentioned above and sent to Genscript Inc., (Piscataway, NJ) for the antibody production in rabbits.

2.7. Antibody based inhibition of F. necrophorum binding to EJG cells

The cultured EJG cells were seeded in a 6-well plate (Corning Inc., Lowell, MA) by adding 3 ml of cells at a concentration of 1 x 10^5cells/ml and incubated in EMEM medium for 48 h to remove any effects of trypsinization on membrane proteins. After 48 h, the cells were washed with sterile PBS for 3 or 4 times. The EJG cells were then incubated with subsp.

necrophorum or subsp. *funduliforme* either pretreated with or without 1:100 dilution of polyclonal antiserum for 1 h at multiplicity of infection of 100:1 and incubated for 1 h. Cells were washed 4 times with sterile PBS and the EJG cells along with attached bacteria were scraped using 500 µl of trypsin-versen (Lonza Walkersville Inc., Walkersvile, MD), and neutralizing it quickly with 1.5 ml of anaerobic EMEM containing 10% heat inactivated fetal bovine serum The collected cells were serially diluted and spread-plated on blood agar plate. The colonies were counted after incubating the plates anaerobically for two days. To visualize bacterial attachment, the cells that were washed 3 to 4 times with sterile PBS were fixed by methanol for 1 min, followed by staining with Wright-Giemsa stain for 1 min and incubating with phosphate buffer for another 2 min. The cells were then washed with distilled water and examined under 20X magnification and photographed (Figure 3.1).

2.8. Identification of EJG cells bound OMPs of Fusobacterium necrophorum subsp. necrophorum

Outer membrane proteins of subsp. *necrophorum* were extracted as mentioned above and the EJG cells were seeded in a 6-well plate as before. The EJG cells were incubated with the OMP of subsp. *necrophorum* (400 µg) for 1 h. at 37 ℃. The unbound OMPs were removed by vigorous washing 4 or 5 times using sterile PBS. The EJG cells with the bound OMPs were taken out by scraping and heated at 95 ℃ for 10 min. in SDS-PAGE buffer. The proteins were run on 4% stacking and 10% separation SDS-PAGE gel and transferred to nitrocellulose membrane overnight at 25 mA current using transfer buffer (25mM Tris, 192mM glycine, 20% v/v methanol, pH 8.3) by western blot method using transblot apparatus (Biorad Inc., Hercules, CA) The blotted proteins were probed with polyclonal antibody raised against the OMPs of subsp. *necrophorum* followed by incubation with alkaline phosphatase conjugated anti-rabbit

antibody and detected by colorimetric reaction using BCIP/NBT premixed solution (Biorad Inc.). The experiment was repeated multiple times with consistent result showing that the band isolated were binding with higher affinity to the endothelial cells.

2.9. Detection of fibronectin on EJG cell culture

The EJG cells were grown in a 24-wells plate and fixed with 10% methanol for 10 min., followed by blocking with 2% non-fat dry milk in phosphate buffered saline containing 0.05% of Tween-20 (PBST) for 30 min. The cells were then incubated with DAPI (Vector Laboratories, Burlingam, CA) and mouse anti-fibronectin antibody (Sigma-Aldrich Inc., St. Louis, MO), followed by washing and incubation with rhodamine conjugated Affinipure goat anti-mouse IgG antibody (Jackson Immunoresearch Laboratories Inc., West Grove, PA). The stained cells were visualized using an immunofluorescence microscope (Nikon Eclipse TE-2000-U, Nikon Instruments Inc., Melville, NY).

2.10. Far-western analysis of OMPs with fibronectin

Bovine fibronectin (1 mg; Invitrogen Inc., Carlsbad, CA) was dissolved 1 ml of PBS (final concentration 1 µg/µl) and 250 µl was incubated with 250 µl of 10 mM EZ-Link Sulfo-NHS-LC-Biotin solution (Thermo Scientific, Rockford, IL) in PBS for 30 min. at room temperature with constant shaking. The excess biotin was neutralized by adding 500 µl of 1 M Tris-HCl and incubating for additional 15 min. The neutralized mixture was further washed 3 to 4 times with 5 ml PBS using 100 kDa filters (Millipore, Billerica, MA) and concentrated to a final volume of 200 µl. The biotinylated sample was then incubated with OMPs of subsp. *necrophorum* blotted on nitrocellulose membrane for 2 h at room temperature in PBST (PBS + 0.05% Tween-20) followed by washing with PBST three times for 10 min each. The biotinylated fibronectin bound to OMPs was detected using Avidin-HRP conjugate (BD

biosciences Pharmingen, San Diego, CA). The protein incubated only with Avidin-HRP served as the control.

2.11. *Statistical Analysis*:

All attachment and inhibition assays of *F. necrophorum* to EJG cells in triplicates, were performed a minimum of three replications. The data were analyzed using SAS software and a *P* value < 0.05 was considered significant. Each observation in a replication was converted to its log10 value and a mean log value was obtained from all the observation. The difference in the mean log value was analyzed using SAS software.

3. Results

3.1 Attachment of F. necrophorum to EJG cells

F. necrophorum attaches to the EJG cells (Fig. 3.1). Attachment assay with subsp. *necrophorum* and subsp. *funduliforme* showed that the number of subsp. *necrophorum* cells bound to EJG cells was higher ($P < 0.0001$) than subsp. *funduliforme* (Fig. 3.2).

3.2. Inhibition assay with after trypsin treatment of F. necrophorum and OMPs of F. necrophorum

The treatment of *F. necrophorum* subsp. *necrophorum* with trypsin also led to 25% reduction in the bacterial binding compared to control EJG cells (Fig. 3.3). Preincubation of EJG cells with OMPs of subsp. *necrophorum* caused significant reduction ($P<0.0038$) in the binding of subsp. *necrophorum* to EJG cells (Fig. 3.4). Similar reduction in binding of subsp. *funduliforme* to EJG cells was observed when the cells were preincubated with OMPs extracted from subsp. *funduliforme.*

3.3. Inhibition assay using polyclonal antibody raised against OMPs of F. necrophorum to EJG cells

When subsp. *necrophorum* and subsp. *funduliforme* were preincubated with the antibodies raised against their OMPs, the bacterial binding to EJG cells decreased significantly (P<0.0001). However, when either of the subspecies was preincubated with the antibody raised against the other subspecies, there was not a significant inhibition in the bacterial binding. The antibody raised against the OMPs of subsp. *necrophorum* reduced the binding of subsp. *necrophorum* cells by 82%, compared to a modest reduction (30%) in binding of subsp. *funduliforme* (Fig.3.5A). Similarly, the antibody raised against the OMPs of subsp. *funduliforme* reduced the binding of subsp. *funduliforme* cells by 83%, while the binding of subsp. *necrophorum* cells decreased only by 12 % (Fig 3.5B).

3.3. Identification of bound OMPs of F. necrophorum subsp. necrophorum

When OMPs that bound to endothelial cells were electrophoresed, 4 bands of 17, 24, 40 and 74 kDa were detected by western blot using polyclonal antisera raised against the OMPs of subsp. *necrophorum* (Fig. 3.6).

3.4. Far-western analysis of F. necrophorum subsp. necrophorum with fibronectin and detection of avidin binding proteins of F. necrophorum

Immunohistochemistry of EJG cells showed that the cells produce fibronectin in the cell culture (Fig. 3.7). The far-western analysis with biotinylated bovine fibronectin showed that the outer membrane proteins of *F. necrophorum* subsp. *necrophorum* had strong affinity for bovine fibronectin. Two OMPs of approximately 40 and 74 kDa size, bound with high affinity to the bovine fibronectin (Fig. 3.8).

In addition, an outer membrane protein of approximately 15 kDa bound with very high affinity to avidin-HRP. This avidin binding protein was present in different strains of subsp. *necrophorum* and subsp. *funduliforme* (Fig. 3.8).

3. Discussion

Bacterial attachment to host cells is crucial to the pathogenesis of infections in several bacterial species (2). The OMPs of many Gram-negative bacteria play a critical role in their attachment (14). Previous study showed that *F. necrophorum* does not have capsule or fimbriae (4). Our studies with *F. necrophorum* indicate that OMPs are involved in bacterial attachment to eukaryotic cells. The attachment of *F. necrophorum* to endothelial cells is mediated by OMPs is evidenced by the following observations:

1. Trypsinization of the outer membrane reduced the attachment.

2. Preincubation of the OMPs of subsp. *necrophorum* with EJG cells decreased binding of bacterium.

3. The polyclonal antibody raised against the OMPs of each subspecies when incubated with the bacterium, showed a decrease in the bacterial binding to EJG cells.

Outer membrane proteins that mediate attachment to the epithelial cells have been characterized in *Fusobacterium nucleatum,* a pathogen closely related to *F. necrophorum* that cause periodontal infections in humans (5). The OMPs of *F. nucleatum* include galactose binding adhesins, which mediate coaggregation with periodontopathogenic bacteria, as well as hemagglutination. FomA, a 39.5 kDa polypeptide, and a 30 kDa polypeptide have been suggested as other adhesins that are involved in interbacterial coggregation (Han et al., 2005). However, the role of the OMPs of *F. necrophorum* in mediating adhesions has not been described. Our previous study has shown that the OMP profile of *F. necrophorum* subsp.

necrophorum and subsp. *funduliforme* differ significantly with subsp. *necrophorum* a total of 19 protein bands out of which four bands of 38, 40, 60 and 74 kDa were more prominent than other bands while the OMPs of subsp. *funduliforme* showed a total of 20 proteins bands and of which, five were prominent (37.5, 58, 70, 140 and 150 kDa). The most prominent band was 40 and 37.5 kDa in subsp. *necrophorum* and subsp. *funduliforme,* respectively.

In liver abscesses of cattle, subsp. *necrophorum* is found more frequently (71% to 95% of liver abscesses) and occurs more often in pure culture (up to 75%) compared to subsp. *funduliforme* (6). The higher prevalence of subsp. *necrophorum* is because of increased virulence, generally attributed to higher leukotoxin production in subsp. *necrophorum* (18).

Fusobacterium necrophorum is a normal inhabitant of the rumen and the concentration of *F. necrophorum* in the rumen of cattle is influenced by the diet (8). Feeding rapidly-fermentable carbohydrates, such as cereal grains causes increased accumulation of volatile fatty acids and lactic acids in the rumen. Because *F. necrophorum* is a lactic acid-fermenting bacterium (20), grain feeding promotes their growth. Increased accumulation of acids and consequent decrease in ruminal pH (ruminal acidosis) causes irritation, erosion and ulceration of the ruminal wall. It is believed that compromised ruminal epithelium favors infection by *F. necrophorum* causing rumenitis. *F. necrophorum* is also a component of the epimural bacteria of the ruminal wall and in fact, the *F. necrophorum* in liver abscesses originates more often from the ruminal wall (10). *F. necrophorum* bound to the epithelium lining the rumen (17), causes abscesses in the ruminal wall and eventually reaches liver via portal circulation to cause hepatic abscesses (7, 12). Finally, the bacteria need to interact with the endothelial cells of the hepatic sinusoids in order to cross and establish infection in the liver. During these events in the rumen and the liver, the organisms must interact with the endothelial cells of the portal vein and capillaries of liver before

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it can establish in the liver parenchyma to cause infection. Hence, we used bovine endothelial cells to determine the role of OMPs in mediating attachment.

The attachment study showed that binding of subsp. *necrophorum* to endothelial cells was 6 to 8 times higher than subsp. *funduliforme.* The difference in the extent of attachment is not surprising considering the OMP profiles of the two subspecies differ significantly (1). The higher attachment may also contribute to increased occurrence of subsp. *necrophorum* in liver abscesses of cattle.

Preincubation of EJG cells with the outer membrane proteins of *F. necrophorum* decreased its binding to the cells indicating that the decrease could be due to saturation of potential receptors present on the EJG cell culture with OMPs incubated before addition of the bacteria. Furthermore, trypsin treatment of *F. necrophorum* also decreased the binding to EJG cells indicating the proteinaceous nature of the adhesins present on the outer membrane of *F. necrophorum*.

The inhibition in binding of *F. necrophorum* to EJG cells when the bacteria were preincubated with polyclonal antibody raised against their OMPs indicated a definite role of OMPs in mediating attachment to the EJG cells. Furthermore, the subspecies specific inhibition in attachment to EJG cells by these polyclonal antibody shows that OMPs composition of the two subspecies of *F. necrophorum* are different as seen in our previous studies.

The western blot analysis with rabbit sera raised against the OMP showed presence of four potential OMPs that bound with high affinity to the bovine endothelial cells. These four proteins of approximately 17, 24, 40 and 74 kDa could be responsible for the adherence of bacteria to the endothelial cells and in turn, help them in establishing the infection in the host.

Many bacteria show binding to extracellular matrix proteins in the host. Fibronectin is one such component which has been shown to bind with a variety of both Gram-positive and Gram-negative bacteria including the OMPs. Hence, we investigated if the OMPs of *F. necrophorum* also bind to fibronectin. The far-western analysis showed two OMP of subsp. *necrophorum* which is the primary etiologic agent in liver abscess of cattle, binds with high affinity with bovine fibronectin. In addition, we showed that the bovine endothelial cell line used in our study (EJG cells) produce fibronectin and hence, the attachment of *F. necrophorum* could also be contributed by fibronectin present in the cell culture.

Overall, this study showed that outer membrane proteins of *F. necrophorum* play an important role in its binding to bovine endothelial cells as trypsin treatment of bacterial led to decrease in binding to EJG cells, preincubation of EJG cells with OMPs also reduced the bacterial binding significantly and polyclonal antibody raised against the OMPs were able to inhibit bacterial binding to EJG cells when preincubated with *F. necrophorum*. The study also demonstrated that the binding to EJG cells in culture could also be contributed by binding to finbronectin which may serve as host receptor present in the extracellular matrix of the host.

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Figure Legends

Figure 3.1. *F. necrophorum* attached to bovine endothelial cells

Figure 3.2. Attachment of subsp. *necrophorum* and subsp. *funduliforme* to bovine endothelial cells.

Figure 3.3. Attachment of Subsp. *necrophorum* to EJG cells when subsp. *necrophorum* were pretreated with trypsin

Figure 3.4. Attachment of subsp. *necrophorum* when EJG cells were preincubated with outer membrane proteins of subsp. *necrophorum*

Figure 3.5. Attachment of subsp. *necrophorum* when preincubated with antibody raised against its own outer membrane proteins (OMPs) and with OMPs of subsp. *funduliforme* (A), Attachment of subsp. *funduliforme* when preincubated with antibody raised against its own OMP and with OMP of subsp. *necrophorum* (B).

Figure 3.6. Western blot analysis of potential adhesins of *F. necrophorum* subsp. *necrophorum*

Figure 3.7. Presence of fibronectin in EJG cell culture.

Figure 3. 8. A) Far-western analysis of OMPs of subsp. *necrophorum* showing two protein bands of 74 and 40 kDa bind with high affinity to bovine fibronectin (Lane 1). Incubation of OMPs only with avidin-HRP was used as control (Lane 2). B. Avidin binding protein present in the outer membrane protein of *F. necrophorum* subsp. *necrophorum* (1) and subsp. *funduliforme* (2), L: protein ladder.

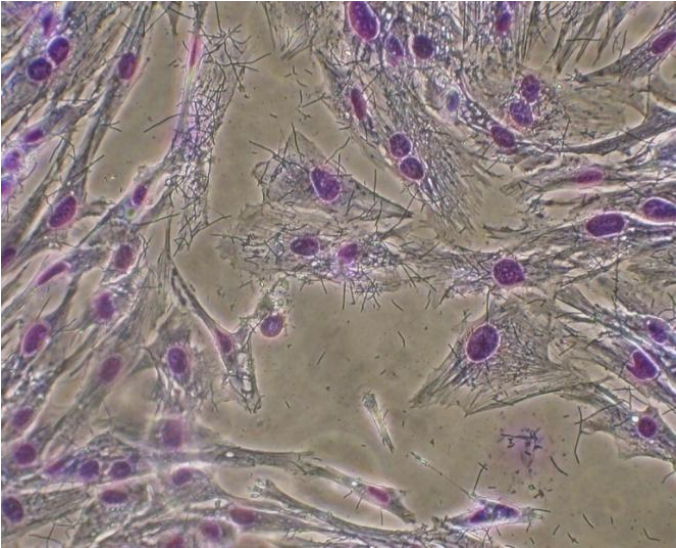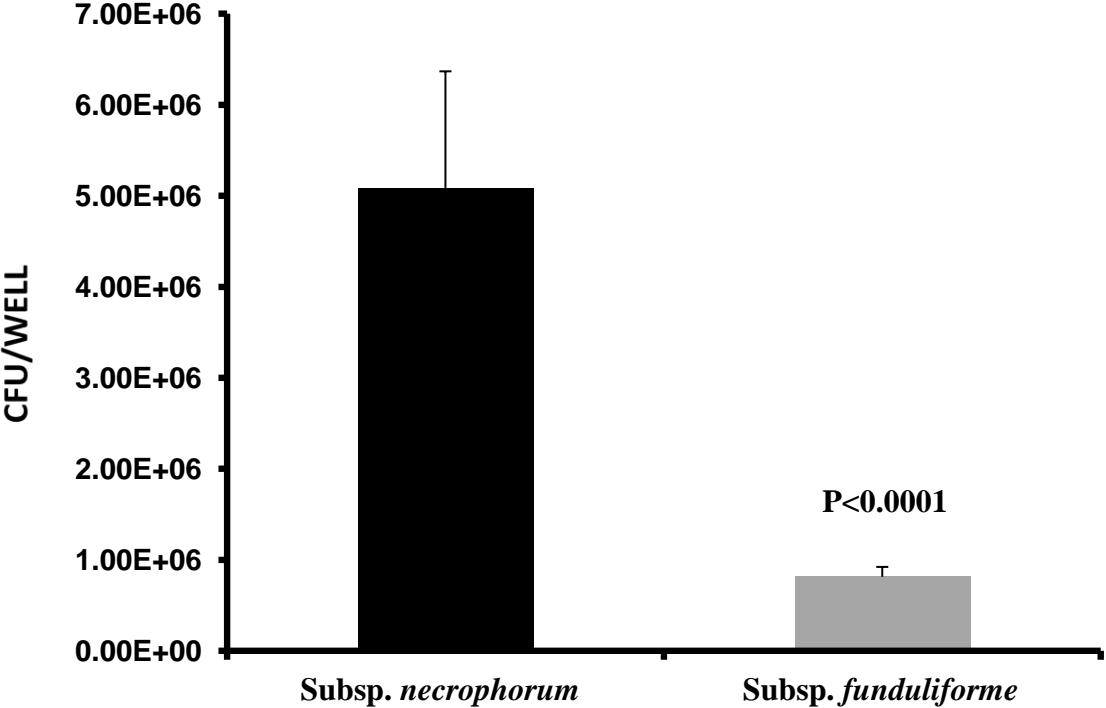**Figure 3.1.** *F. necrophorum* attached to bovine endothelial cells.

Figure 3.2. Attachment of subsp. *necrophorum* **and subsp.** *funduliforme* **to bovine endothelial cells**

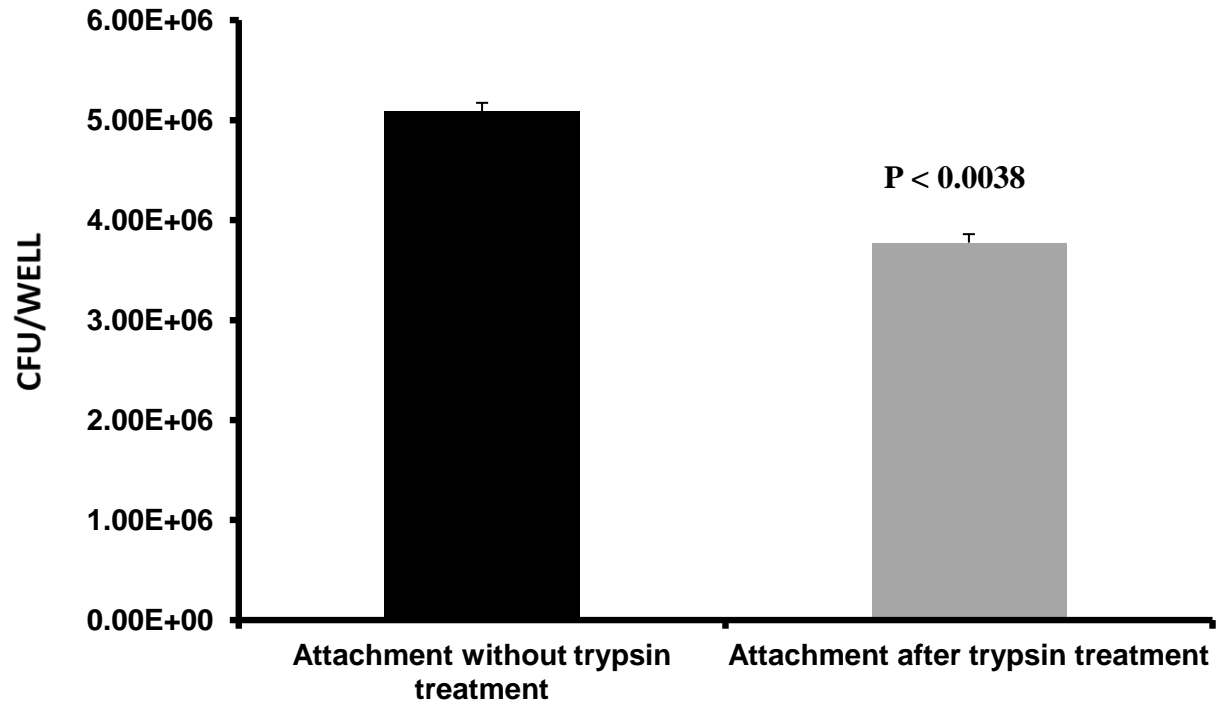**Figure 3.3. Effect on attachment to bovine endothelial cells when subsp. *necrophorum* were pretreated with trypsin.**

Figure 3.4. Attachment of subsp. *necrophorum* **to bovine endothelial cells when endothelial cells were preincubated with OMPs of subsp.** *necrophorum***.**
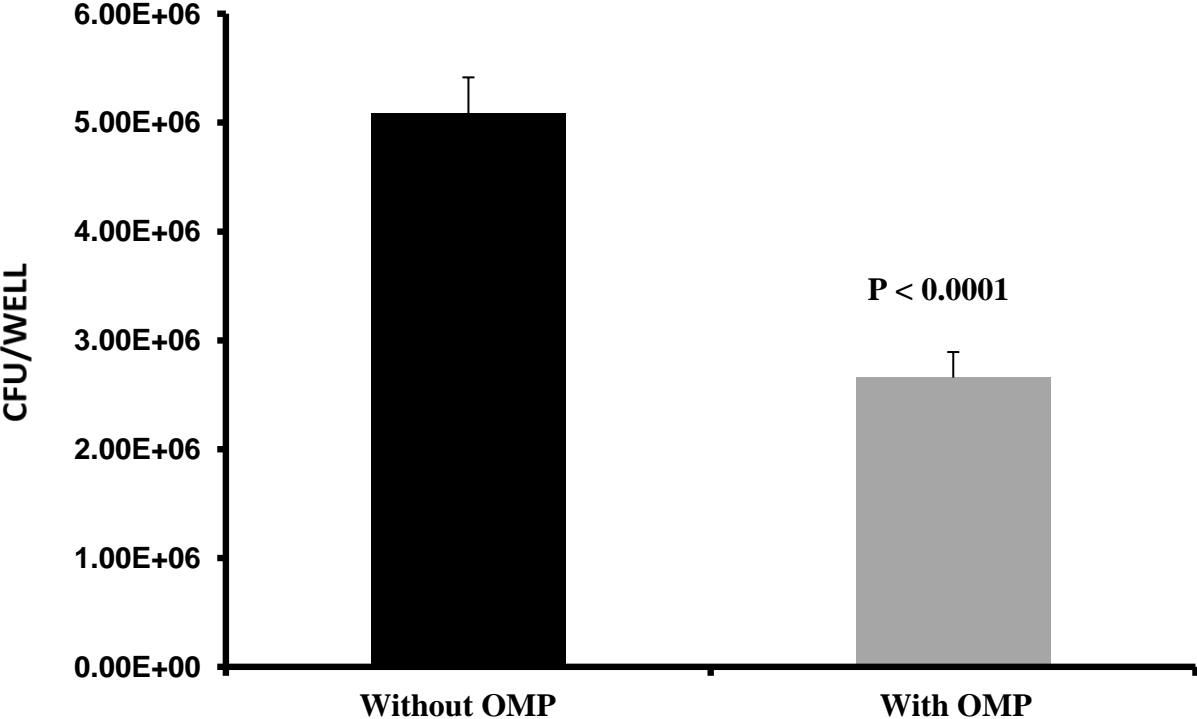
Figure 3.5. Effect of polyclonal antibody raised against OMPs of subsp. *necrophorum* **and subsp.** *funduliforme* **on their attachment to bovine endothelial cells.**
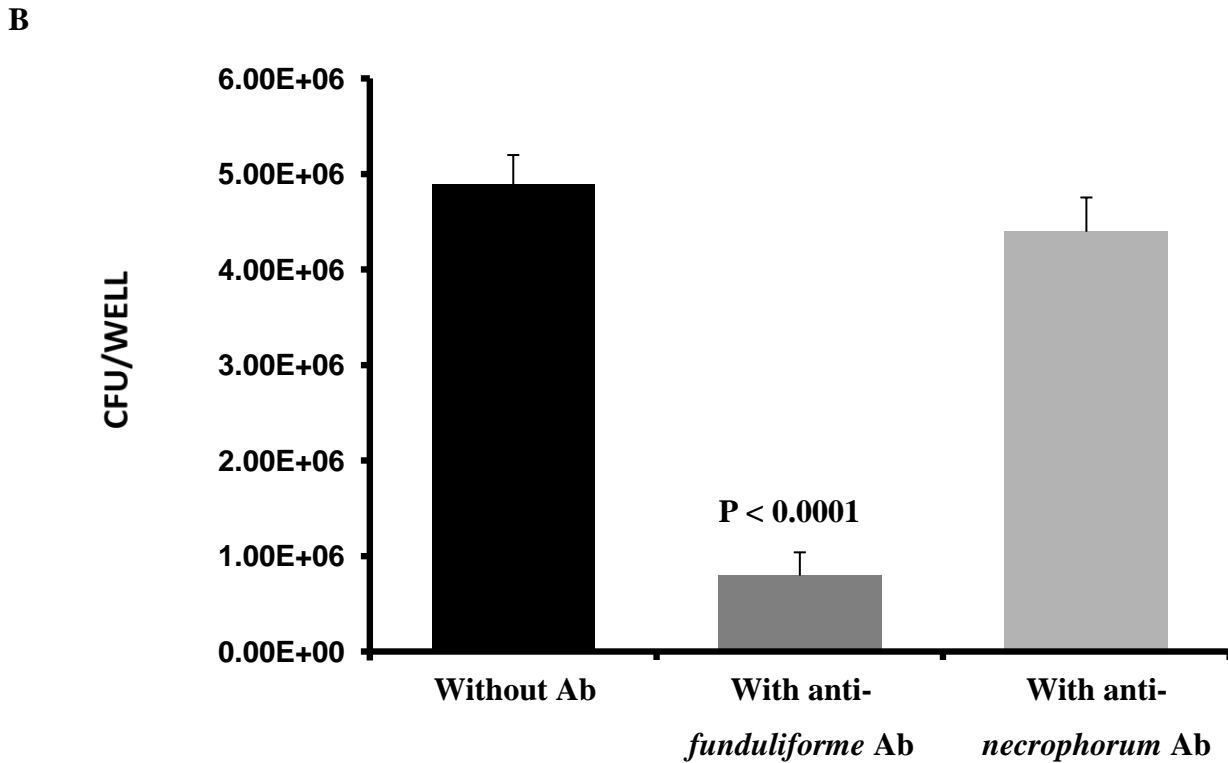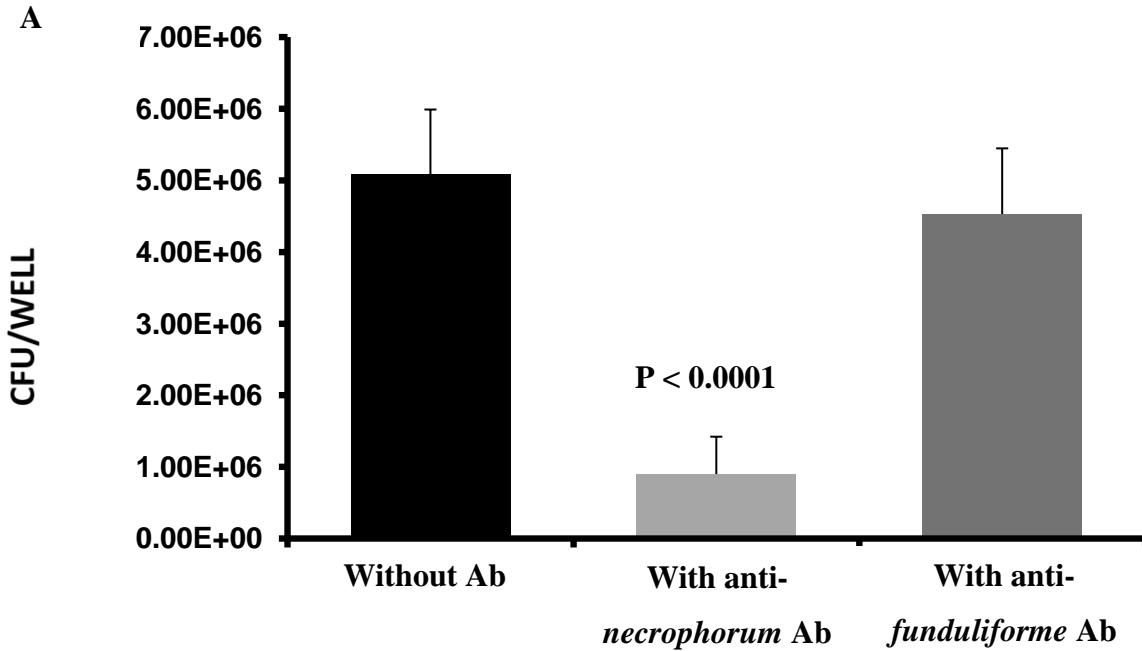
Figure 3.6. Western blot analysis of potential adhesins of subsp. *necrophorum*
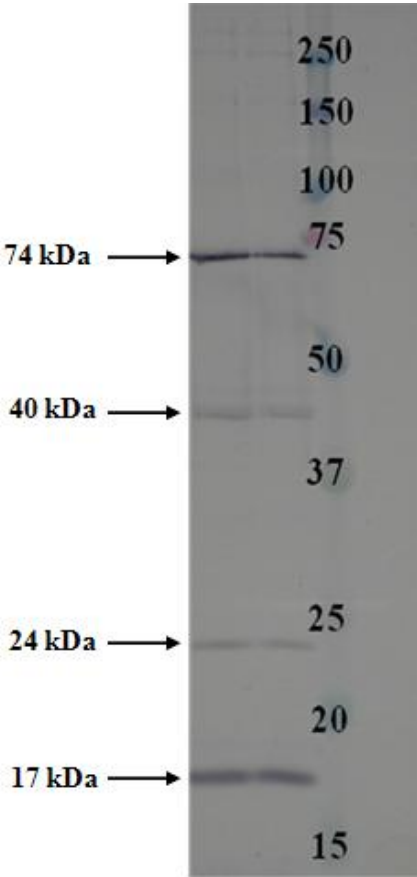
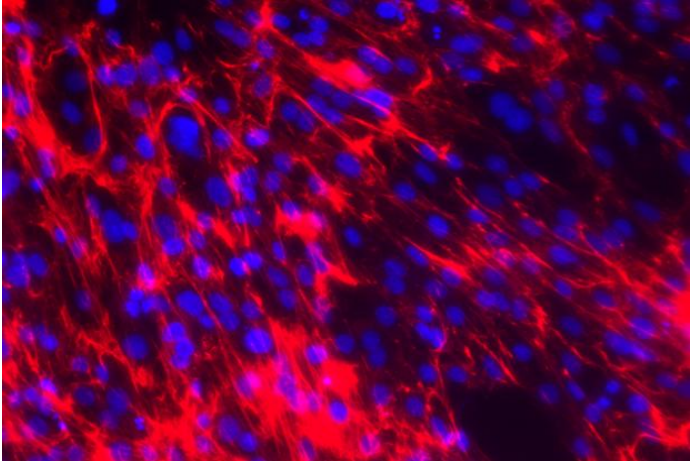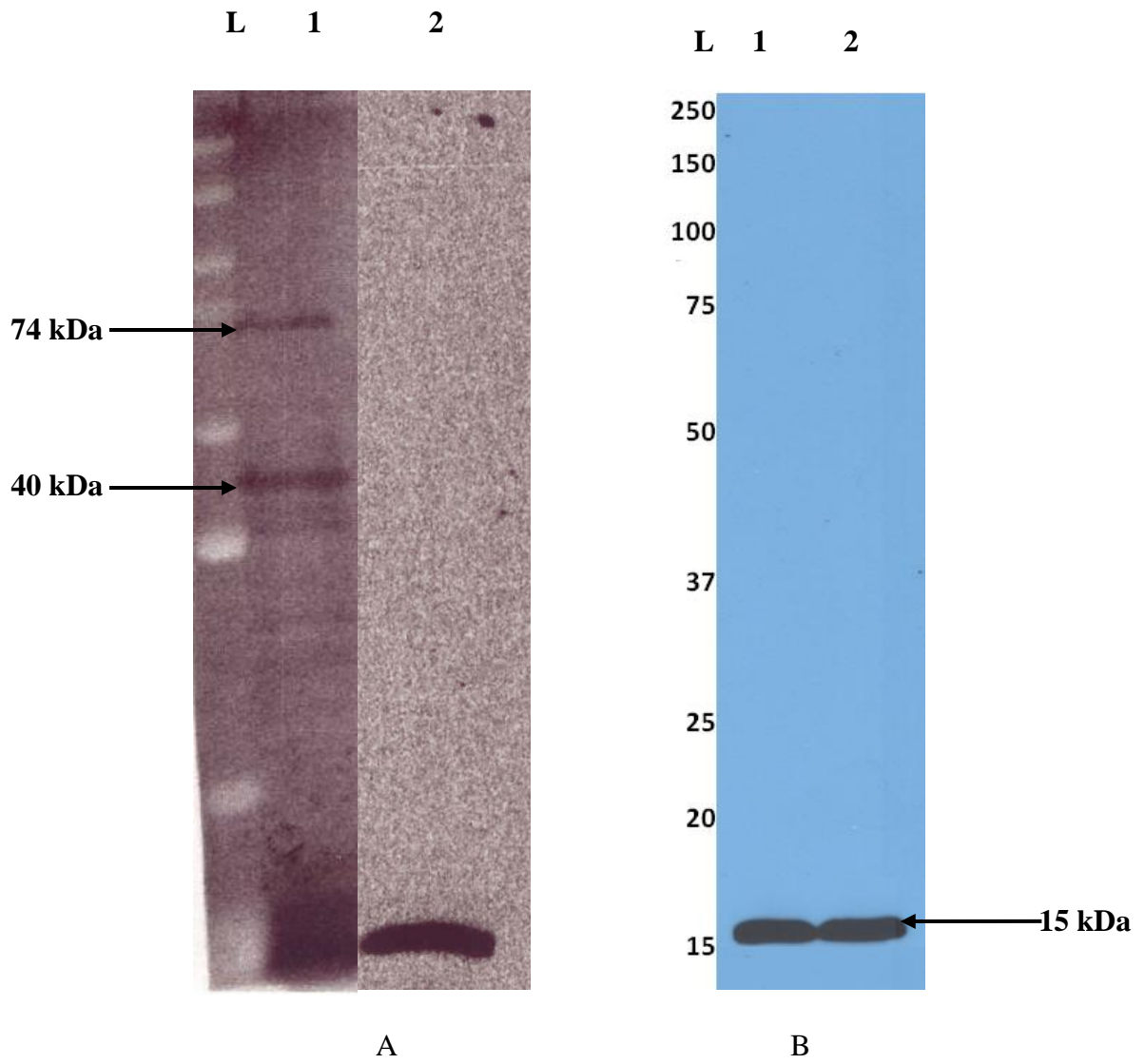**Figure 3.7. Presence of fibronectin in EJG cell culture.**

Figure 3.8. Far-western analysis of subsp. *necrophorum* OMPs with biotinylated fibronectin.

Chapter 4 - Identification of a high-affinity binding outer membrane proteins that binds *Fusobacterium necrophorum* subsp. *necrophorum* to bovine endothelial cells

Abstract

Abscesses formation in the liver of cattle is primarily associated with *Fusobacterium necrophorum,* a Gram-negative, obligate anaerobe. The other infections in cattle include foot rot, and necrotic laryngitis (calf-diphtheria). There are two subspecies; subsp. *necrophorum* and subsp. *funduliforme.* The two subspecies differ in morphological, biochemical and molecular characteristics. The abscess formation in liver is caused by subsp. *necrophorum* predominantly compared to subsp. *funduliforme* due to their higher virulence. Bacterial attachment to the host cell surface is critical to the pathogenesis in several Gram-negative bacterial species and outer membrane proteins (OMP) have been shown to play an important role in bacterial adhesion and establishment of infection. The information on the OMP of *F. necrophorum* that mediate attachment to host cells is lacking. In this study, we have identified an OMP which binds with high affinity to the bovine endothelial cells and is recognized by the sera from cattle with liver abscesses. The protein when sequenced showed similarity with other proteins found in other species of *Fusobacterium* such as *F. nucleatum*, *F. periodonticum* and *F. varium*. The PCR analysis showed that the gene for this adhesin protein was found to be present in different strains of subsp. *necrophorum*, subsp. *funduliforme* and *F. necrophorum* of human origin. The protein when preincubated with the endothelial cells, were able to prevent the attachment of subsp. *necrophorum* significantly. In addition, the polyclonal antibody produced against the adhesin protein was able to prevent the binding of subsp. *necrophorum* to bovine endothelial cells which

indicates that this protein could be a potential vaccine candidate for liver abscesses prevention in cattle.

1. Introduction

Fusobacterium necrophorum is a Gram-negative, anaerobic and rod-shaped to pleomorphic bacterium. It is frequently associated with a variety of necrotic infections of animals, and is a major pathogen of cattle (9). The organism is also a human pathogen, causes abscesses in internal organs and is also the etiological agent of acute pharyngitis, which in young adults leads to a syndrome called, Lemierre's syndrome (2). *F. necrophorum* is classified into subsp. *necrophorum* and subsp. *funduliforme*, and the two subspecies differ in morphological, biochemical and molecular characteristics (14, 15). Of the two subspecies, subsp. *necrophorum* is more frequently encountered in fusobacterial infections than subsp. *funduliforme* in animals. The subsp. *necrophorum* is more virulent for animals than subsp. *funduliforme* because of more potent or increased production of virulence factors, particularly the leukotoxin (9). The most common fusobacterial infections in cattle include hepatic abscesses, foot rot, and necrotic laryngitis (calf-diphtheria) (9, 17). Liver abscesses in feedlot cattle and foot rot in beef and dairy cattle are of significant economic importance to the cattle industry (3, 13).

Generally, Gram-negative bacterial cell attachment to the host eukaryotic cells is mediated by capsule, fimbrial proteins or outer membrane proteins (OMP) (1). Bacterial adhesion is a critical step in the establishment of infection and disease pathogenesis. *F. necrophorum* is not capsulated and fimbriae have not been demonstrated (4). We have shown that outer membrane proteins mediate attachment of *F. necrophorum* to bovine endothelial cells in our previous study. Earlier studies have implicated a putative outer membrane protein

hemagglutinin (agglutinates chicken erythrocytes) of 19 kDa, to mediate attachment of *F. necrophorum* to ruminal epithelial cells (5-7).

In this study, we show that a 40 kDa outer membrane protein (FnnA) of *F. necrophorum* subsp. *necrophorum* binds with very high affinity to bovine endothelial cells.

2. Materials and Methods

2.1. Bacterial strains and culturing

Fusobacterium necrophorum subsp. *necrophorum,* strains 8L1, A25, A21, A50, subsp. *funduliforme* strains B35, B47, B 17, B29, isolated from bovine liver abscesses (Tan *et al.*, 1992; Narayanan *et al.*, 1998) and four human clinical strains of *F. necrophorum* (RMA10682, RMA14786, RMA16505 and RMA16539; provided by Dr. Diane Citron, R. M. Alden Research Laboratory, Santa Monica, CA, USA) were used for this study. The organisms were cultured in prereduced, anaerobically sterilized Brain Heart Infusion (PRAS-BHI) broth (16). Bacterial cells for attachment assay were obtained by inoculating 0.1 ml of an overnight culture derived from a single colony into a 10 ml PRAS-BHI and grown to an OD_{600} of 0.6.

2.2. Extraction of outer membrane proteins

The outer membrane proteins from *F. necrophorum* subsp. *necrophorum* strain 8L1 were isolated according to the method described previously with slight modifications (12). Briefly, cultures were grown in 1 liter of PRAS-BHI broth for 12 to 14 h. The cells were pelleted by centrifugation, resuspended in 20 ml of cold 0.75M sucrose-10 mM Tris buffer at pH 7.8 with lysozyme (2 mg/ml of cell suspension) and incubated on ice for 20 min. Formation of spheroplasts was achieved by diluting the suspension with two volumes of cold 1.5 mM EDTA at constant rate of delivery. The spheroplasts were lysed by ultrasonication in an ice-water bath with a 3 mm microtip at 20W output pulse setting. The cell debris was removed by

centrifugation at 6,500 g for 15 min at 4 °C. The supernatant was then centrifuged at 386,984 g at 2-4°C for 2 h, and the supernatant was discarded. The pellet was resuspended 2 ml of 20 mg/ml Triton X-100 and 10 ml of cold 0.25 M sucrose, 3.3 mM Tris-1 mM EDTA, pH 7.8 (STE buffer) and incubated at room temperature for 45 min to dissolve the inner membrane and the volume was adjusted to that of the original sonicate suspension and the ultracentrifugation was repeated at 125,392 g for 2 h at 2-4 °C and the pellet was collected in cold STE buffer and stored at -80° C until use.

2.3. Culture of endothelial cells

Bovine adrenal gland capillary endothelial cell line (EJG cells CRL-8659, ATCC, Manassus, VA) was used for this study. Cells were grown in EMEM medium with 10 % fetal calf serum and 1 % antibiotic solution of streptomycin and penicillin (Invitrogen Inc., Carlsbad, CA)., The medium was changed every 3 to 4 days until the cells became monolayered. The cells were then trypsinized and subcultured to maintain the cell line for use in experiment.

2.4. Attachment of Fusobacterium necrophorum subsp. necrophorum to Karnovsky fixed bovine endothelial cells

The EJG cells were grown in 225 mm^2 flask (Corning Inc., Lowell, MA) to confluence. The cells were then trypsinized and seeded in a 6-well plate (Corning Inc.) with 3 ml of cell suspension containing a concentration of 1 x 10^{5}cells/ml and incubated in EMEM medium containing 10% FBS for 48 h to remove any effect of trypsinization on the eukaryotic surface proteins. The cells were then fixed with Karnovsky fixative (0.1 M cacodylate buffer, 2.5 % gluteraldehyde, 2 % paraformaldehyde) for 10 min. followed by washing with sterile PBS for 3 to 4 times. Overnight culture of strain 8L1, grown in PRAS-BHI to mid-log phase (grown to an OD$_{600}$ of 0.6) was used in the attachment assay. One ml of culture at mid-log phase was

inoculated into the wells containing the fixed cells and incubated at 37 °C for 1 h. After

incubation, the cells were washed vigorously with sterile PBS for 3 to 4 times and the bound

bacterial cells were observed under a light microscope.

2.5. Isolation of high-affinity binding adhesin

The EJG cells were plated in 6-well plates and incubated for 48 h. The cells were fixed

in Karnovsky fixative and then incubated with OMPs extracted from subsp. *necrophorum,* strain

8L1 (a total of 500 µl volume containing 400 µg of protein/well). After overnight incubation,

the solution containing unbound fraction was removed from the wells. The cells in the wells

were washed first with PBS twice, and then washed twice each with increasing strengths of

buffers; PBS containing 0.1% nonyl phenoxypolyethoxylethanol-40 (NP-40), modified radio-

immunoprecipitation assay (RIPA) buffer (25 mM Tris•HCl pH 7.6, 150 mM NaCl, 1% NP-40,

and 1% sodium deoxycholate). The bound OMP was then collected by washing with SDS

sample buffer (62.5 mM Tris-HCl pH 6.8, 25% glycerol and 10% SDS). Each of the washes was

concentrated to equal volume (10 times) and separated on a SDS-PAGE gel. The EJG cells

incubated without OMPs served as negative control.

2.6. N-terminal sequencing

The high affinity binding adhesin isolated in SDS-PAGE buffer was run on 4% stacking

and 10% separation gel after overnight polymerization to avoid and N-terminal blockage. The

protein was transferred on to a polyvinylidene fluoride (PVDF) membrane overnight by

electroblotting and stained with coomassie blue. The membrane was then destained for 15 min.

in 40% methanol/10% acetic acid followed by rinsing in 90% methanol/5% acetic acid followed

by rinsing in distilled water for 4 h with changing water several times. Finally, the membrane

was dried using Whatman No. 1 filter paper and stored. The protein present on dried PVDF

membrane was excised and submitted on dry ice to a sequencing facility in Iowa State University (Ames, IA). The N-terminal protein sequencing was carried out with 494 Procise Protein/peptide Sequencer/140C Analyzer (Perkin Elmer Inc., San Jose, CA) using Edman degradation method. The identified amino acid sequence was blasted in Pubmed database and investigated for similar proteins present in other bacterial species.

The search revealed that the binding protein was similar to FomA protein of *F. nucleatum* and *F. periodontium,* and a protein present in *F. vari*um.

2.7. Inhibition assay with FnnA

The outer membrane proteins of subsp. *necrophorum* strain 8L1 (400 µg) were incubated with Karnovsky fixed EJG cells grown in a 6-well plate. After overnight incubation, cells were washed with PBS twice to remove unbound proteins, followed by two washing of PBS + 0.1% NP-40 to leave only FnnA OMP bound to the fixed cells. Subsp. *necrophorum* strain 8L1 was grown to absorbance of 0.6 at 600 nm in PRAS-BHI medium and 1 ml of the culture was inoculated into each well containing Karnovsky fixed EJG cells. The bacteria were incubated with fixed cells for 1 h followed by washing with PBS vigorously for 3 to 4 times to remove unbound bacteria. The bound bacteria to the EJG cells were removed by quickly scraping the wells after adding 500 µl of trypsin and inactivating the trypsin with 1.5 ml of anaerobic EMEM medium containing 10% heat inactivated FBS. A 100 µl of the extracted medium containing the bacteria was serially diluted in PRAS-BHI and plated on blood agar, and incubated in an anaerobic glove box (Forma Scientific) to enumerate bacteria.

2.8.   Primer design and PCR conditions

The primers to amplify the gene for the adhesin protein was designed based on the DNA sequence of FomA protein present in *Fusobacterium nucleatum*. Forward primer was designed

starting from amino acid just after the 20 amino acid signal sequence of FomA of *F. nucleatum* (5'-GAA GTT ATG CCA GCA CCT ATG CCA GAA-3') and the reverse primer including the stop codon of the protein (5'-TTA GAA GCT AAC TTT CAT ACC AG-3').   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 Tm -5 ˚C for 30 s, and extension at 72 ˚C (1 min per kb of expected product) for 2 min and final extension at 72 °C for 4 min. Later, restriction sites were added to primers for cloning in to pET45b+ vector (5'-CGGGATCCAGAAGTTATGCCTGCACC-3' with *BamH*I site and reverse primer 5'-AACTGCAGTTA GAA GCT AAC TTT CATA -3'with pstI site).

2.9. DNA extraction and PCR analysis of different bovine strains of subsp. necrophorum, subsp. funduliforme and human strains of F. necrophorum for FnnA

The chromosomal DNA was prepared as per the procedure described by Narayanan *et al.*, 2001 (11). Briefly, each isolate were grown overnight in anaerobic BHI broth and the cells were pelleted by centrifugation at 6500 g. The pelleted cells were washed and resuspended in 100 mM Trish/HCl (pH 8.0), 10 mM EDTA. The cells were further treated with lysozyme (1mg/ml) and the resulting lysate was then further treated with 1% sarkosyl followed by RNase A (20 µg/ml) and pronase (50µg/ml) followed by incubation for 20 min. The sample was then sequentially extracted twice with phenol and choloroform. The DNA was precipitated with 0.1 vol. 3 M sodium acetate (pH 5.2) and equal volume of 2-propanol. Finally, DNA was resuspended in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA and stored at 4 °C until used.

The PCR analysis was carried out using four strains of subsp. *necrophorum* (A25, A21, A50 and 8L1), four strains of subsp. *funduliforme* (B17, B29, B47, and B35) and four clinical

strains of *F. necrophorum* (RMA10682, RMA14786, RMA16505 and RMA16539) to determine

if the gene for FnnA is present.

2.10. Analysis for presence of FnnA protein in different subsp. *funduliforme* and human strains of F. necrophorum

The outer membrane proteins of different strains of subsp. *funduliforme* and human

isolates of *F. necrophorum* were extracted as mentioned before and run on SDS-PAGE gel.

2.11. Cloning, sequencing and expression of FnnA from subsp. *necrophorum*

pET45 b+ vectors (EMD Bioscience) was used to clone and express the *fnnA* gene from

subsp. *necrophorum* strain 8L1 DNA as mentioned before. The amplified product was cloned in

pET45b+ vector in-frame. The pET45b+ vector has N-terminal histidine tag for protein

purification using Ni^+ column. The cloned vector was transformed into *E. coli* BL21(DE3) cells

and the right clones were verified using colony PCR. Positive clones were subjected for protein

expression using IPTG by inducing the cells for 24 h after inoculating the overnight grown

clones in fresh LB medium. The DNA sequence of the insert from clone expressing the protein

was determined by DNA sequencing. The amino acid sequence of the cloned DNA was deduced

from the DNA sequence analysis and further analyzed for its similarity with homologous

proteins present in the database.

2.12. Western blot analysis of native and recombinant FnnA with bovine antiserum against F. necrophorum subsp. necrophorum strain 8L1

The natively purified FnnA and the *E. coli* BL21(DE3) cells expressing the recombinant

FnnA protein was subjected for SDS-PAGE analysis and blotted on nitrocellulose membrane.

The blotted proteins were detected using serum from steers that were experimentally induced

with liver abscesses by intraportal inoculation with subsp. *necrophorum* strain 8L1 in another

study as primary antibody and goat anti-bovine IgG antibody conjugated with alkaline phosphatase as secondary antibody. The color was developed using BCIP/NBT substrate (Sigma-Aldrich Co**.).**

2.13. Purification of recombinant adhesin protein FnnA

The proteins containing histidine tag at its N-terminal will be purified using PrepEaseTM His-Tagged Protein Purification Kit (USB Inc., Cleveland, OH) under denaturing conditions as per the manufacturer's instructions. Briefly, the *E. coli* culture was grown to an absorbance (600 nm) of 0.4 and induced with 1 mM IPTG to produce the cloned protein. The culture was harvested after 6 h of induction by pelleting. The cells were then resuspended in LEW buffer (400 mM NaH_2PO_4, 2.4 M NaCl, pH 8.0) and lysozyme was added to a final concentration of 1 mg/ml and incubated for 30 min on ice. The cells were then sonicated, followed by centrifugation to remove supernatant. The pellet was then resuspended in 1 X LEW buffer, followed by centrifugation to collect the pellet. The pellet was again solubilized in 1 X LEW denaturing buffer (LEW buffer + 8M urea) and was applied to Ni-TED column equilibrated with 1 X LEW denaturing buffer. The column was then washed with 1 x LEW denaturing buffer and the protein was eluted by using 1 X Elution denaturing buffer (50 mM NaH_2PO_4, 300mM NaCl, 250 mM imidazole, pH 8.0., 8.0 M urea).

2.14. Production of polyclonal antisera against native and recombinant FnnA adhesin
   protein

Polyclonal antisera against the recombinant FnnA adhesin protein were produced in rabbit. The protein was expressed, purified and sent to Cocalicobiologicals Inc. (Reamstown, PA) for the antibody production. The 40 kDa native protein from outer membrane of *F. necrophorum* was cut off from the SDS-PAGE gel after running the final fraction of proteins

obtained during isolation of FnnA. The protein was sent in the gel for polyclonal antibody production directly.

2.15. Inhibition assay with polyclonal antibody raised against the native and cloned FnnA protein

The cultured EJG cells were seeded in a 6-well plate (Corning Inc., Lowell, MA) by adding 3 ml of cells at a concentration of 1×10^5 cells/ml and incubated in EMEM medium for 48 h to remove any effects of trypsinization on membrane proteins. After 48 h, the cells were washed with sterile PBS for 3 or 4 times. The cells were then incubated with subsp. *necrophorum* or subsp. *funduliforme* either pretreated with or without 1:100 dilution of polyclonal antiserum for 1 h at multiplicity of infection of 100:1 and incubated for 1 h. Cells were washed 4 times with sterile PBS and the EJG cells along with attached bacteria were scraped using 500 µl of trypsin-versen (Lonza Walkersville Inc., Walkersvile, MD), and neutralizing it quickly with 1.5 ml of anaerobic EMEM containing 10% fetal bovine serum The collected cells were serially diluted and spread-plated on blood agar plate. The colonies were counted after incubating the plates anaerobically for two days.

3. Results

3.1. Attachment of F. necrophorum subsp. necrophorum to Karnovsky fixed cells

The cells of *Fusobacterium* subsp. *necrophorum* were able to bind to the EJG cells fixed with Karnovsky's fixative. The binding affinity to the fixed cells was similar to the binding to unfixed EJG cells.

3.2. Isolation and identification of high affinity binding OMP FnnA

Washing of Karnovsky's fixed EJG cells incubated overnight with OMPs of *Fusobacterium* subsp. *necrophorum* with PBS or detergents with increasing stringency, no

protein was detected in either washed fraction. However, the final wash with SDS-PAGE buffer containing 10 % SDS, which could detach the tightly bound OMP revealed a band of 40 kDa protein (Fig. 4.1). The N-terminal amino acid sequence of the 40 kDA protein was K-E-V-M-P-A-P-M-P-E-D/E-E. The Blast-P sequence analysis revealed that the protein has 96% homology to a 40 kDa OMP of *F. nucleatum* and *F. periodonticum* (FomA) and *F. varium.* Sequence analysis also revealed that the proteins in other fusobacteria have a 20-aa signal peptide that is cleaved upon its expression into the periplasm.

3.3. Inhibition assay

When *F. necrophorum* **s**ubsp. *necrophorum* were incubated with EJG which were previously saturated with the 40 kDa protein, there was a significant decrease in the binding of the bacterium compared to control where no OMP was preincubated (Fig. 4.2).

3.4. Presence of FnnA OMP in different subspecies of F. necrophorum

Polymerase chain reaction (PCR) analysis showed that the FnnA gene is present in both the subspecies of *F. necrophorum* of bovine and human origins examined (Fig. 4.3a and 4.3b). In addition, the size of amplified PCR product in different strains of subsp. *funduliforme* was less than that of subsp. *necrophorum*.

3.5. Analysis for presence of FnnA protein in different subsp. funduliforme and human strains of F. necrophorum

To further evaluate if 40 kDa protein can be visualized in subsp. *funduliforme* and human strains of *F. necrophorum* as in subsp. *necrophorum*. The outer membrane proteins of subsp. *funduliforme* and human isolates of *F. necrophorum* were run on SDS-PAGE gel. The 40 kDa protein was not present in any strain except RMA16505 of human isolate (Fig. 4.4).

3.6. Cloning, sequencing and expression of fnnA from subsp. necrophorum

Cloning of *fnnA* gene using primers design based on the N-terminal sequence identified during protein sequencing and FomA protein of *Fusobacterium nucleatum* in pET45b+ vector resulted in successful expression of the protein of 363 amino acids. The protein had identical N and C-terminal amino acid sequences to FomA of *F. nucleatum* extending beyond the amino acids that are formed due to the forward and reverse primers.

3.7. Western blot analysis of native and recombinant FnnA

The native identified adhesin protein and the recombinant adhesin proteins found to be immunodominant when detected with sera from steers challenged with subsp. *necrophorum* in another study indicating that this protein antigen is being recognized by the immune system of cattle easily (fig. 4.5).

3.8. Inhibition assay with polyclonal antibody raised against cloned FnnA protein

When *F. necrophorum* **s**ubsp. *necrophorum* were preincubated with the antibody raised against the 40kDa adhesin, FnnA. There was a significant decrease in the binding of bacterium to the EJG cells (Fig. 4.6). However, the inhibition with polyclonal antibody raised against natively purified 40 kDa protein from *subsp. necrophorum* showed less inhibition compared to the polyclonal antibody raised against the recombinant 40 kDa protein which is due to the less amount of native protein injected in the rabbit to produce the polyclonal antibody.

4. Discussion

OMP of many Gram-negative bacteria have been shown to play an important role in their establishment and causing infection in the host. Previous studies conducted in our lab showed that the polyclonal antibody raised against the OMPs when preincubated with *F. necrophorum* were able to prevent their binding to bovine endothelial cells significantly. The objective of the

current study was to identify specific OMP that can play an important role binding of *F. necrophorum* susbp. *necrophorum* to the bovine endothelial cells. In our previous study, we have shown that *F. necrophorum* binds to bovine endothelial cells. However, the protocol designed for isolation of OMP with high affinity for the endothelial cells involved fixing of the cells with Karnovsky fixative; we first studied if subsp. *necrophorum* binds to the Karnovsky fixed EJG cells. Our study showed that subsp. *necrophorum* binds to the Karnovsky fixed EJG cells. To identify an OMP that binds with high affinity to EJG cells, the total OMP was incubated to the fixed EJG cells and the high affinity binding OMP was isolated after serially washing the cells with detergents of increasing stringency and finally the tightly bound OMP was extracted with SDS-PAGE buffer which contains 10% SDS. No protein could be detected in any fraction except in the highly stringent SDS-PAGE buffer that contains 10 % SDS which showed that the isolated protein was tightly bound to the EJG cells. N-terminal protein sequencing of the identified adhesin showed that this protein is present in other closely related *Fusobacterium* such as *F. nucleatum* and *F. periodonticum* and is termed as FomA. FomA is a porin protein of *F. nucleatum* has been shown to play important role by acting as adhesin which help other bacteria to coaggregate along with *F. nucleatum* and form bacterial biofilm during oral infections in humans (8). Recently, a vaccine prepared using FomA of *F. nucleatum* showed to decrease the oral infections in mice model (8). In addition, the FomA protein of *F. nucleatum* is also found to bind with statherin protein of human saliva which play a significant role in plaque formation and later development of *F. nucleatum* associated peiodontitis (10). These studies indicate a potential role of this group of protein as adhesins and hence, the protein identified in *F. necrophorum* subsp. *necrophorum* might be responsible for binding to host eukaryotic cells in establishing the infection in cattle.

The identified adhesion protein when preincubated with the EJG cells, was able to reduce the binding of subsp. *necrophorum* to the cells which indicate that the protein blocked the binding sites of endothelial cells to be utilized by the subsp. *necrophorum* which in turn decreased the binding. The western blot analysis of the identified adhesion protein using sera from steers infected with subsp. *necrophorum* and showed liver abscesses, showed that this protein is immunodominant and hence is being easily recognized by the cattle immune system.

The PCR analysis with different strains of subsp. *necrophorum*, subsp. *funduliforme* and human strains of *F. necrophorum* showed that the gene for the protein was present universally. However, the SDS-PAGE analysis of the OMP of subsp. *funduliforme* and human strains of *F. necrophorum* did not show any dominant band in the 40 kDa range as was seen with OMPs of subsp. *necrophorum*. The absence of the protein could either be due to less expression of the protein or mutation in the gene encoding this protein in subsp. *funduliforme*. The sequence analysis of subsp. *funduliforme* showed gene mutation in the sequence which makes the protein out of frame leading to no expression of this protein.

As subsp. *necrophorum* is more virulent and is prevalent in liver abscesses formation in cattle, it is possible that this protein which is expressed dominantly in the OMP of subsp. *necrophorum*, helps the bacterium to better attach to the host cells surface and help them to transport better from ruminal wall to the liver and establishing the infection.

The polyclonal antibody raised against the natively purified and recombinant adhesin in rabbit when incubated at 1:100 dilution with subsp. *necrophorum,* showed significant decrease in the bacterial binding indicating that the antibody was blocking the adhesin present on the surface of subsp. *necrophorum*.

The recognition of identified adhesin with sera of steers challenged with subsp. *necrophorum*, ability of the adhesin as well as the antibody raised against it to reduce binding of subsp. *necrophorum* to EJG cells shows that it can be used as a potential candidate for vaccine preparation against the liver abscess formation in cattle.

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Figure Legends

Figure 4.1. Isolation of FnnA. OMP from subsp. *necrophorum* were incubated overnight with fixed EJG cells and unbound protein were removed (lane 2) and washed with PBS (lane 3 as treated cells and lane 4 as control cells) and detergents with increasing stringency, no protein was detected in washing with PBS + 0.1% NP-40 (lane 5 as treated cells and 6 as control cells), modified RIPA (lane 7 as treated cells and 8 as control cells). The final wash with SDS-PAGE buffer containing 10 % SDS revealed a band of (lane 10 as treated cells and lane 11 as control cells) 40 kDa protein.

Figure 4.2. Inhibition assay with FnnA. The EJG cells were saturated with 40 kDa OMP and attachment study was carried out with subsp. *necrophorum*. The bacteria were incubated with fixed cells and the unbound bacteria were washed off using PBS. The bound bacteria to the EJG cells were removed from the wells and were serially diluted in PRAS-BHI and plated on blood agar to determine the concentration of bound bacteria.

Figure 4.3a. Presence of 40 kDa adhesin gene in different strains of subsp. *necrophorum* (1:A25, 2: A21, 3: A50, 4: 8L1) and subsp. *funduliforme* (6: B17, 7:B29, 8:B47, 9: B35). Lane 5: 1kb DNA ladder.

Figure 4.3b. Presence of 40 kDa adhesin gene in human *F. necrophorum* strains. 1: 1kb DNA ladder, 2-5: human strains, 6: subsp. *necrophorum* strain A25 7: subsp. *necrophorum* strain 8L1 8: subsp. *funduliforme*, 9: negative control.

Figure 4.4. Analysis of OMPs of different strains of subsp. *funduliforme* of bovine and human origin showed absence of 40 kDa protein except in strain RMA16505. The protein was present in all different strains of subsp. *necrophorum.*

Figure 4.5. Western blot analysis of native identified FnnA adhesin protein (lane1), recombinantly expressed FnnA adhesin protein in *E. coli* lysate transformed with pET45b+ vector (lane 2), E. coli control lysate (lane 3) and purified recombinant FnnA adhesin (lane 4) with sera from steer challenged with subsp. *necrophorum.*

Figure 4.6. Inhibition assay with polyclonal antibody raised against the adhesin protein. Subsp. *necrophorum* when preincubated with the antibody (Third bleed), showed a significant decrease in bacterial binding to bovine endothelial cells compared to subsp. *necrophorum* not treated with any antibody (control) or when preincubated with pre-bleed sera obtained from rabbit before injecting the antigen for antibody production (Pre-bleed). A) Inhibition with antibody raised against the native 40 kDa protein. B) Inhibition with antibody raised against recombinant 40 kDa protein.

Figure 4.7. 2-D model of the 40 kDa proteins identified in study. A) FomA of *F. nucleatum*, B) protein cloned in pET45b+ vector. The model is created using PRED-TMBB software based on hidden Markov model method.

Figure 4.1 Isolation of FnnA.
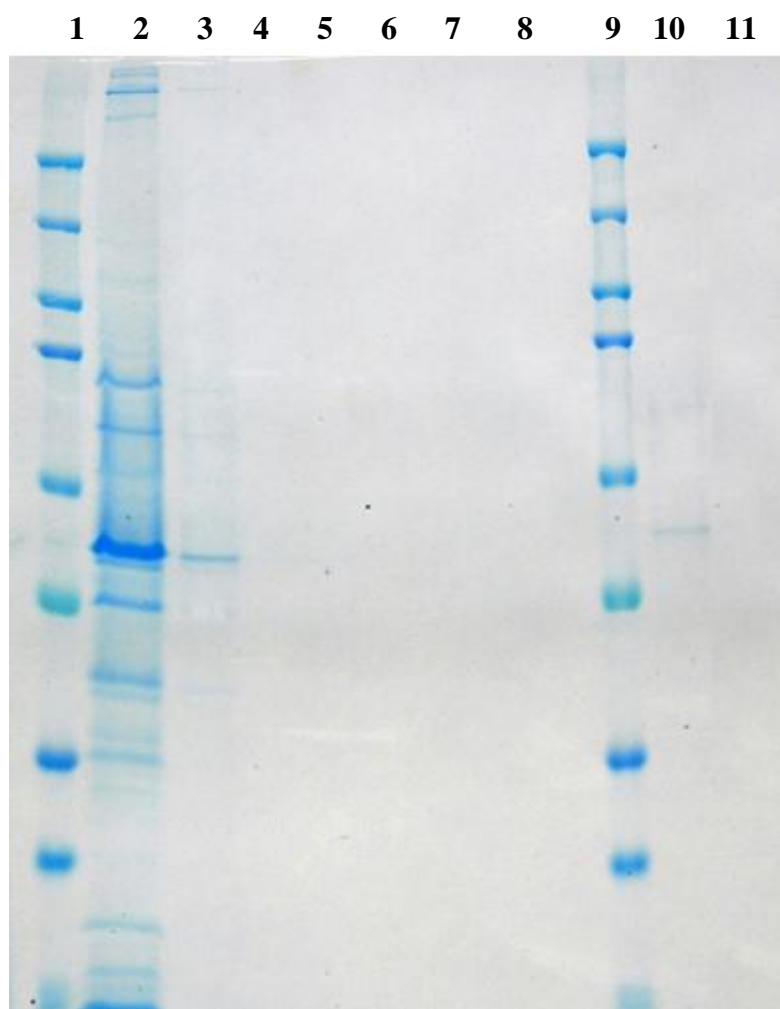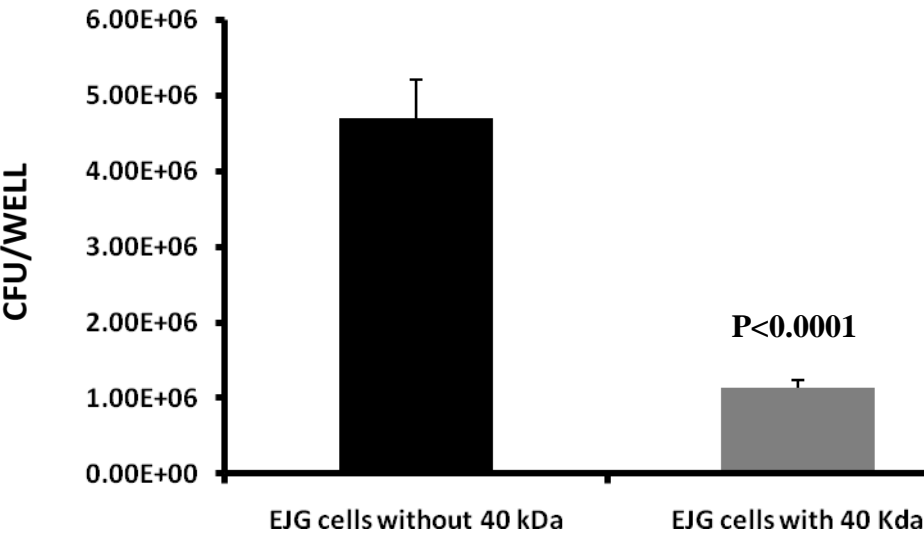
Figure 4.2. Inhibition assay with FnnA

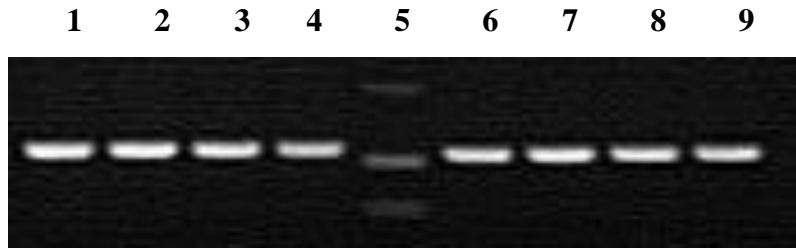**Figure 4.3. PCR analysis for *fnnA* gene in *F. necrophorum***
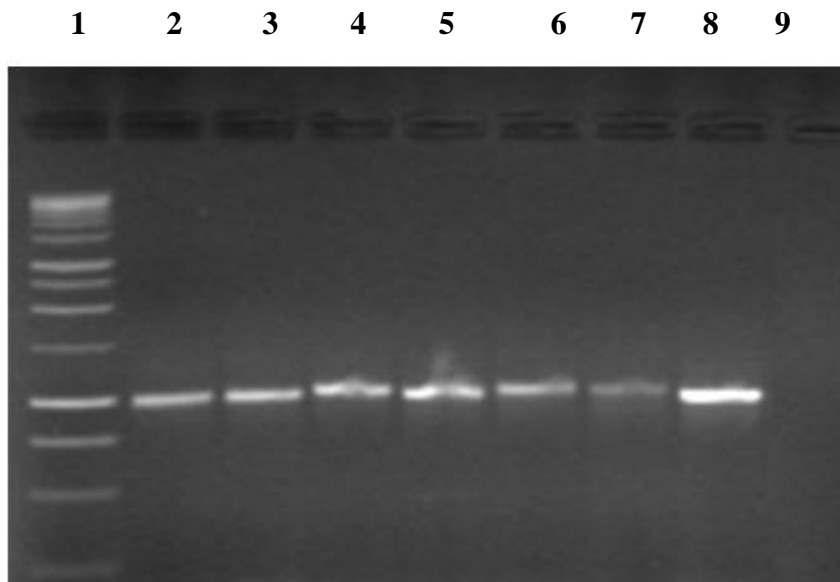
Fig. 4.3a.

Fig. 4.3b.

Figure 4.4. SDS-PAGE analysis of to investigate presence of 40 kDa FnnA protein in *F. necrophorum* subsp. *funduliforme* and subsp. *necrophorum*.
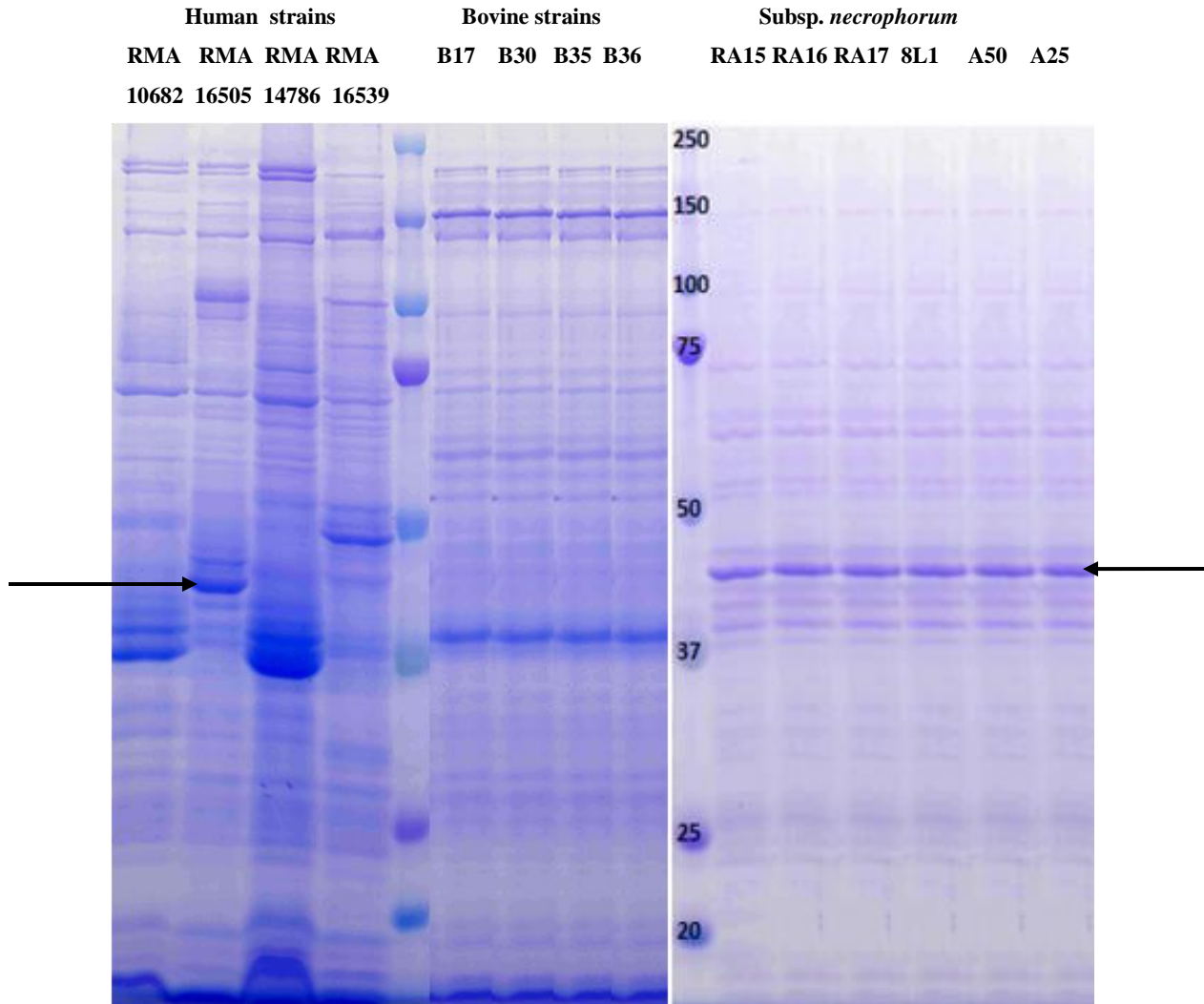
Figure 4.5. Western blot analysis 40 kDa native adhesin and recombinant adhesin.
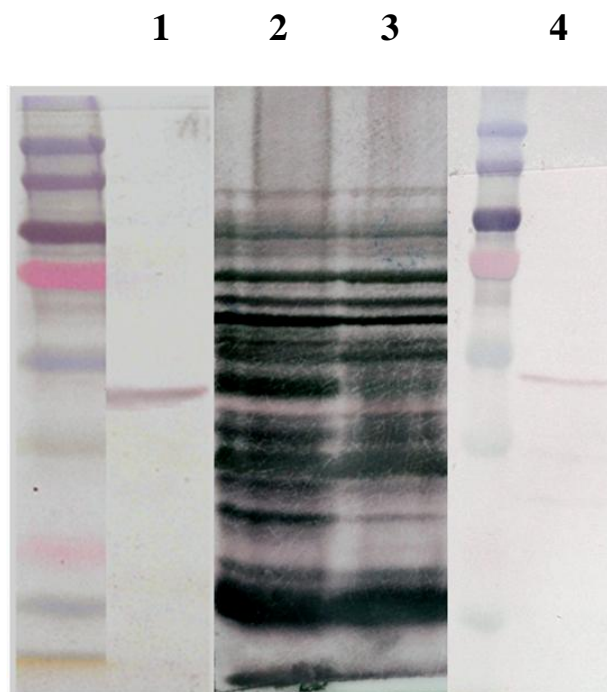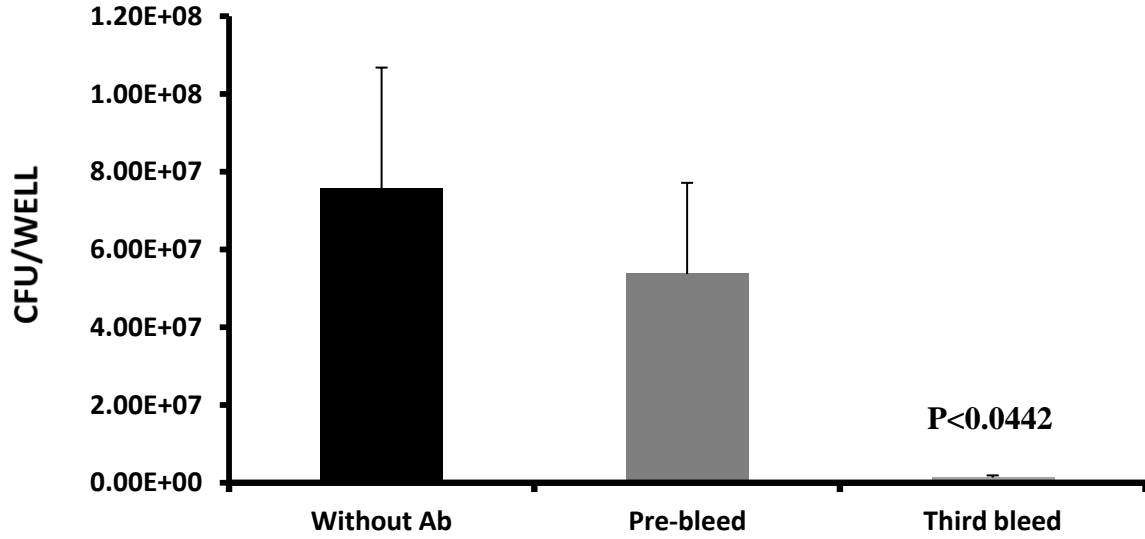
Figure 4.6. Inhibition assay with polyclonal antibody raised against the 40 kDa FnnA adhesin.
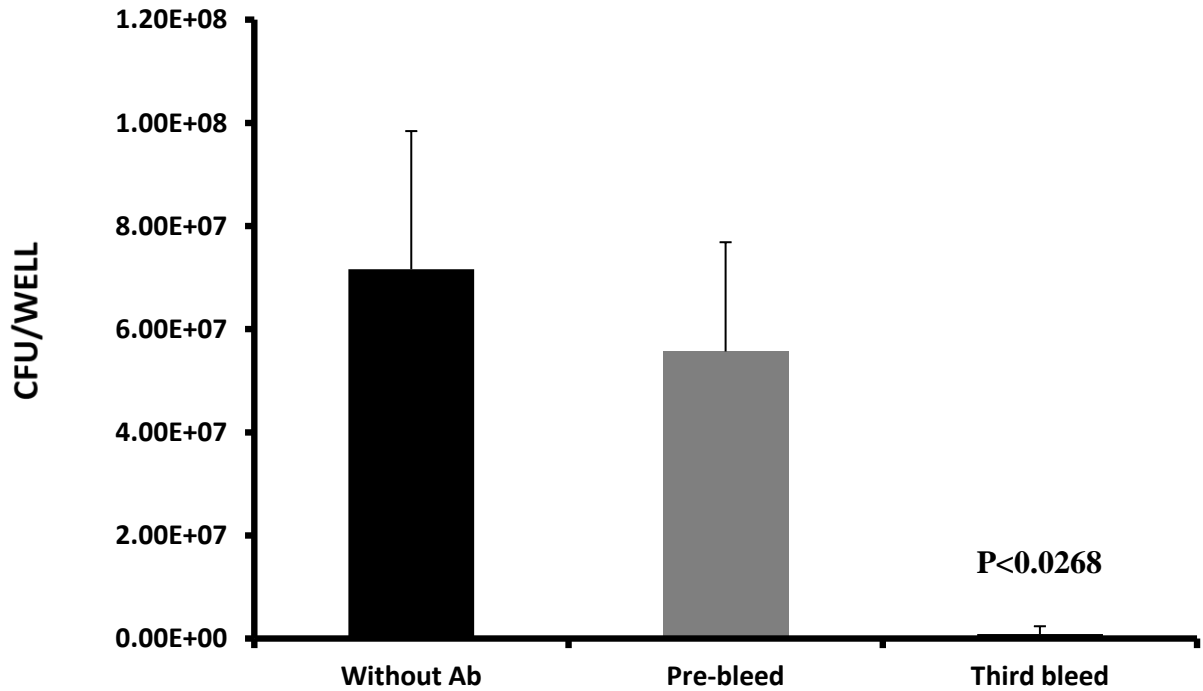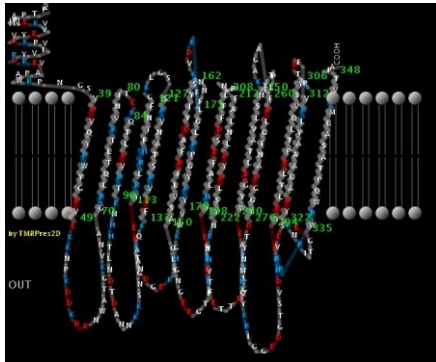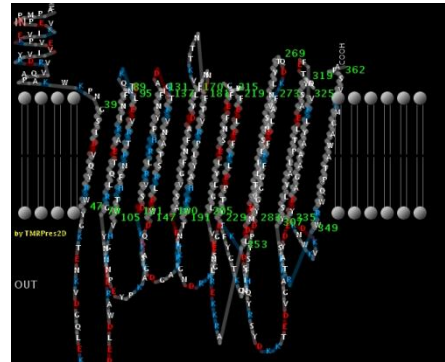
A)

B)

Figure 4.7. 2-D model of 40 kDa FnnA adhesin in comparison to FomA of *F. nucleatum*.

A

B

Appendix A - Characterization of *Fusobacterium necrophorum* isolated from llama and alpaca

Abstract

Fusobacterium necrophorum, a Gram negative and rod-shaped anaerobe, is an opportunistic animal and human pathogen that causes a variety of infections termed necrobacillosis. There are two subspecies of *F. necrophorum*, subsp. *necrophorum* and subsp. *funduliforme,* which differ morphologically, biochemically and genetically. In cattle, *F. necrophorum* is a major pathogen and causes liver abscesses, foot rot and calf diphtheria. The objective of this study was to characterize the *F. necrophorum* isolates obtained from necrotic infections of llama and alpaca. We characterized seven isolates from alpaca and two isolates from llama. The PCR analysis showed that all isolates belonged to subsp. *necrophorum* and showed the presence of putative hemagglutinin gene. Western blot analysis with anti-leukotoxin antibodies raised in rabbit showed the presence of leukotoxin protein in the culture supernatant of all isolates. Furthermore, flow cytometry of the culture supernatants demonstrated cytotoxicity to bovine and alpaca polymorphonuclear leukocytes. *F. necrophorum* isolates from llama and alpaca appear to be similar to bovine isolates and leukotoxin may be a major virulence factor.

1. Introduction

 Fusobacterium necrophorum is a Gram-negative, anaerobic and rod-shaped pathogen, which causes a variety of infections in animals and humans, often called necrobacillosis. Two subspecies of *F. necrophorum* exist (15). The subsp. *funduliforme* is less pathogenic than the subsp. *necrophorum* in animals; however, clinical isolates from human infections belong to subsp. *funduliforme* (16). *Fusobacterium necrophorum* is a major pathogen of cattle, in which it causes hepatic, interdigital and laryngeal necrobacillosis (19). In wild animals, necrobacillosis has been reported in white-tailed deer (*Odocoileus virginianus),* mule deer (*Odocoileus heminous),* pronghorn antelopes (*Antilocapra americana*), and blue duikers (*Cephalophus monticola fusicolor*), generally affecting the oral cavity (3, 4, 14, 20). In camelids, *F. necrophorum* infections cause fever, anorexia and depression; the other clinical signs depending on the location of the lesion (5). Necrobacillosis may occur on the lips, tongue, pharynx, interdigital spaces, foot pad, larynx, mandible or maxillary bones (5). In alpaca, it has been reported to cause osteomyelitis of mandibles and many disseminated necrotic lesions (5). Lesions in the mouth and pharynx may extend to cause mandibular and alveolar osteomyelitis. Occasionally, the infection may be aspirated to the lung causing severe necrotizing pneumonia while infections in the interdigital space and foot pad lead to lameness (5).

 The subsp. *necrophorum* is more virulent and more frequently encountered in necrotic infections of animals (6, 9). Strains belonging to subsp. *necrophorum* produce high levels of virulence factors, such as leukotoxin, hemolysin and hemagglutinin (10). Of these factors, leukotoxin is considered to be the major virulence determinant (16). Leukotoxin is a 336 kDa protein with no significant homology to any known proteins (12), and induces apoptosis and necrosis of bovine peripheral polymorphonuclear leukocytes in a dose-dependent manner (11).

All pathogenic strains of *F. necrophorum* (of both subspecies) possess leukotoxin gene (*lktA*) (9, 12, 17). However, a study conducted in the UK (7) suggested that leukotoxin is not an universal virulence factor in *F. necrophorum*. The biochemical and biologic characteristics of *Fusobacterium* strains isolated from camelids have not been described. We obtained *Fusobacterium* isolates from a variety of necrotic infections in llama and alpaca to speciate, identify subspecies status of the isolates, and determine whether the strains have leukotoxic activities.

2. Materials and Methods

2.1. Bacterial strains and culture

Two strains isolated from llama (69521 and 61557) and seven strains isolated from alpaca (11034, PSU, 61715, 68690, 68699, 73556 and 118610) were used in this study. *Fusobacterium necrophorum* subsp. *necrophorum,* strain A25 and subsp. *funduliforme,* strain B35, previously isolated from liver abscesses of cattle served as control (6), The organisms were cultured in brain heart infusion broth (Becton-Dickinson, Franklin Lakes, NJ) that was pre-reduced with the addition of 0.05% cysteine hydrochloride and anaerobically sterilized (PRAS-BHI; (18).

2.2. Biochemical characteristics

The isolates were tested biochemically using RapID-ANA II kit (Remel, Lenexa, Ks). Isolates, grown overnight in PRAS-BHI, were used to prepare bacterial lawn testing with RapID-ANA II test panel.

2.3. Isolation of chromosomal DNA

The chromosomal DNA was prepared as per the procedure described by Narayanan *et al.,* (2001) Briefly, each isolate was grown overnight in anaerobic BHI broth and the cells were

pelleted by centrifugation at 6,500 g. The pelleted cells were washed and resuspended in 100

mM Tris-HCl (pH 8.0), 10 mM EDTA. The cells were further treated with lysozyme (1 mg/ml)

and the resulting lysate was then treated with 1% sarkosyl, RNase A (20 μg/ml), and pronase (50

μg/ml) followed by incubation for 20 min at 55 °C. The sample was then sequentially extracted

twice with phenol and chloroform. The DNA was precipitated with 0.1 vol. of 3 M sodium

acetate (pH 5.2) and equal volume of 2-propanol. Prepared DNA was resuspended in 10 mM

Tris-HCl (pH 8.0), 1 mM EDTA and stored at 4 °C until used.

2.4. PCR assays

The genus confirmations of isolates, based on 16S rRNA sequence, were done by the

procedure previously (8). The species confirmation was based on the hemagglutination gene and

the promoter for the leukotoxin gene (1, 21). The primer sequences and their targets are shown

in table 1. The PCR conditions were optimized keeping the annealing temperature as T_m-5 °C

for each primer set. PCR amplifications were carried out under the following conditions using

ExTaq (Takara Corp., Madison, WI, USA) was as follows: initially 94 °C for 3 min; 35 cycles of

denaturation at 94 °C for 1 min, Tm-5 °C for 30 s and 72 °C for 1–3 min (1 min/kb of expected

product size); and a final extension at 72 °C for 4 min.

2.5. Preparation of bovine and alpaca polymorphonuclear cells

Polymorphonuclear leukocytes (PMNs) from peripheral blood from cattle and alpaca

were prepared as described previously (17). Briefly, 50 ml of blood was collected in heparinized

tube from healthy cattle and alpaca by venipuncture of the jugular vein. The blood was

transported on ice, centrifuged at 700 g for 20 min at 4 °C and the buffy coat was discarded. The

RBCs were lysed for 2-3 min using 10 ml of sterile distilled water and incomplete RPMI

medium was added to the cells quickly. The cells were centrifuged again at 700 g and the

supernatant was discarded. This process was repeated to assure that there were no RBCs remaining in the cell suspension. The PMN cells were resuspended in RPMI medium containing 5% fetal calf serum (13). The numbers of viable cells were counted using 0.4% trypan blue dye exclusion assay in a hemocytometer (11).

2.6. Preparation of culture supernatants

The bovine, llama and alpaca isolates of *F. necrophorum* were grown in PRAS-BHI to an absorbance of 0.6-0.7 at 600 nm and pelleted by centrifugation at 6,500 g for 20 min at 4 °C. The supernatant was collected, filtered through a 0.22 μm filter (Millipore) and concentrated 60-fold with a 100 kDa molecular mass cut-off filters (Millipore). The aliquots of supernatants were store at -80 °C until used for western blot.

2.7. Western blot

The concentrated supernatants from all the strains were separated on SDS-PAGE in 4% stacking and 8% resolving gel under denaturing conditions. The proteins were then transferred to nitrocellulose membrane (Thermofisher Sci., Carlsbad, MA) using transblot apparatus (Biorad Inc. Hercules, CA). The membranes were then blocked with 0.2% skimmed milk for 3 h. The leukotoxin proteins were then detected using rabbit polyclonal antiserum raised against strain A25 affinity purified leukotoxin (11) at 1:1,000 dilutions as primary antibody followed by incubation with goat anti-rabbit IgG conjugated with alkaline phosphatase. The immunoreactive proteins were detected by colorimetric reaction using 5-bromo-4-chloro-3-indolylphosphate/nitro blue tetrazolium (BCIP/NBT) solution as substrate (BioRad Inc.).

2.8. Cytotoxicity assay

The cytotoxic effects of different llama and alpaca strains were determined on alpaca leukocytes by a cell viability assay using propidium iodide (11). The culture supernatants were

collected and prepared as before. The culture supernatant of each isolate was also autoclaved to compare the cytotoxicity with normal supernatant. The viable PMNs (1 x$10^{6)}$ cells were treated with culture supernatants for 45 min at 37 °C and 5% CO2, washed twice with PBS, resuspended in PBS and stained with 10 ml of propidium iodide (50 mg/ml stock) in dark for 5 min. Autoclaved supernatant from each strain with PMNs and PMNs suspended in complete RPMI 1640 and not treated with supernatant served as controls. 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).

3. Results

3.1. Species and subspecies confirmation

All the isolates when tested with RapID-ANA II kit, were positive for production of alkaline phosphatase and indole

PCR analysis using genus specific primers Fuso 1 and 2 showed that all the isolates belong to the genus *Fusobacterium* (Fig. A.1,1A). A PCR assay using primers specific for hemagglutinin gene (HAEM) amplified a 315 bp product from all the llama and alpaca isolates (Fig. A.1, 1B). The PCR analysis with Fund primer gave negative result with all the isolates which generally amplifies a 317 bp product in susbp. *funduliforme* (Fig. A.1. 1C). Hence, all the isolates from llama and alpaca belonged to subsp. *necrophorum*. We further tested the presence of leukotoxin gene in these isolates using PCR targeting the promoter region of leukotoxin (*lkt* promoter. All the strains and control subsp. *necrophorum* strain A25 amplified a 571 bp product (Fig. A.1, 1D).

3.3. Leukotoxin and leukotoxicity

The western blot analysis of the concentrated culture supernatant with rabbit polyclonal antiserum raised against the affinity purified leukotoxin from subsp. *necrophorum* showed banding pattern similar to subsp. *necrophorum* with 250, 150, 130 and 110 kDa bands in the western blot analysis.

In addition, two very high intensity bands were also present in strain PSU. A 250 kDa band was absent from strain 68690 and 68699 and other bands were of lesser intensities. Strain 61715 and strain 73556 showed two additional bands at 30 kDa range which have been previously identified to be breakdown products of leukotoxin (16). The difference in the banding pattern in different strains may arise from proteolytic enzymes or other unidentified factors present in the different strains (Fig. A.2).

3.4. Leukotoxin activity

The flow cytometry analysis showed that the culture supernatants from llama and alpaca isolates were toxic to PMNs of alpaca and bovine origin. There was a considerable variation in the susceptibilities of alpaca and bovine PMNs to leukotoxin of *F. necrophorum* strains. Subsp. *necrophorum* strain A25 and subsp. *funduliforme* strain B35 were more toxic to bovine PMNs compared to alpaca PMNs. All the strains isolated from llama and alpaca were more toxic to alpaca PMNs compared to bovine PMNs except strain 73556, 118610 and 69521 which were more toxic to bovine PMNs than alpaca PMNs (Fig. A.3). There was no difference in the toxicity of PMNs treated with the autoclaved supernatant or PMNs suspended in RPMI-1640 as control (data not shown).

4. Discussion

To our knowledge, this is the first study to characterize *Fusobacterium* isolates from camelids. Biochemical and molecular characterizations revealed that the *Fusobacterium* isolates from alpaca and llama belonged to the species *necrophorum*. The biochemical and culture characteristics PCR study for hemagglutinin, promoter region of leukotoxin showed that the isolates were of subsp. *necrophorum*. This supports the previous observations that necrotic infections caused by *F. necrophorum* in animals are mostly caused by subsp. *necrophorum* (2). In addition, leukotoxin, which is considered a major virulence factor responsible for the necrotic infections caused by *F. necrophorum,* were produced by these isolates as shown by western blot analysis of the culture supernatants. The several bands that appeared in the analysis supports the previous observation that leukotoxin from *F. necrophorum* is highly unstable (16). Furthermore, the cell viability assay using alpaca and bovine PMNs showed that the supernatant was toxic to PMNs of alpaca and bovine origin. Most of the strains of llama and alpaca isolates were more toxic to the alpaca PMNs compared to bovine PMNs. The autoclaved supernatant did not show cytotoxicity, suggesting the toxic activity is a protein and not due to LPS.

Furthermore, most of the isolates (except tongue abscess and oral swab isolate) obtained from llama and alpaca were associated with infections involving the bone. The isolates do have alkaline phosphatase activity which might help the bacteria in bone resorption creating a deep wound and thus, making a more anaerobic environment for the bacterial growth. However, the association of *F. necrophorum* in camelids with necrotic infections in bone needs further study and explanation.

In a recent study reported that leukotoxin is not a universal virulence factor of *F. necrophorum* responsible for necrotic infections in animals and man (7). However, in our study,

all the isolates from llama and alpaca were shown to have a functional leukotoxin which was toxic to the PMNs. Hence, in camelids, the study shows that leukotoxin plays a major role in the establishment of necrotic infections.

In conclusion, we found that the *Fusobacterium* isolates obtained from the necrotic infections in alpaca and llama belongs to *F. necrophorum* subsp. *necrophorum* and that the leukotoxin were produced in these isolates which could be major virulence factor.

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Figure legends:

Figure A.1. PCR analysis of different strains of *F. necrophorum* isolates from llama and alpaca using genus specific primers (1A), specific primer for subsp. *necrophorum* of hemagglutinin gene (1B), specific primer for subsp. *funduliforme* (1C) and primers for leukotoxin promoter region of *F. necrophorum* (1D) (M: 100bp DNA ladder, 1 - 2: llama isolates, 3-9: alpaca isolates, 10-11: bovine isolates (10:subsp. *necrophorum*, 11:subsp. *funduliforme*).

Figure A.2. Western blot analysis of culture supernatants of *F. necrophorum* isolates from llama and alpaca. M: 100bp DNA ladder, 1:A25 (Positive control), 2: 11034, 3: PSU, 4: 61557, 5:61715, 6:69521, 7:68690, 8:68699, 9: 73556, 10:118610.

Figure A.3. Cytotoxicity assay with the culture supernatants of subsp. *necrophorum* strain A25, subsp. *funduliforme* strain B35 along with different isolates of *F. necrophorum* from alpaca and llama using polymorphonuclear cells of alpaca (A.3A) and bovine (A.3B) origins.

Tables

Table A.1. Primers for PCR of different gene targets.

Primer	Targets	Product size (base pairs)
Haem forward and Haem reverse	Genus-specific 16S ribosomal DNA for *Fusobacterium*	610
Fund forward and Fund reverse	Putative Hemagglutinin gene specific for subsp. *necrophorum*	311
Fund forward and Fund reverse	Promoter region of leukotoxin gene (specific for subsp. *funduliforme)*	337
5'lktpxmxh and 3'lktpxmxh	Promoter region of leukotoxin gene in subsp. *necrophorum* and subsp. *funduliforme*	571in subsp. *necrophorum* and 449 in subsp. *funduliforme* (not efficiently)

Table A.2. PCR analysis of different isolates of *F. necrophorum* from llama and alpaca.

Isolate	Animal	Source	Haem	Fund	5'lktp-XmXh	Fuso 1 & 2
B35	Bovine (KSU)	Liver abscess	-	+	-	+
A25	Bovine (KSU)	Liver abscess	+	-	+	+
69521	Llama (KSU)	Sequestrum of hind limb	+	-	+	+
61557	Llama (KSU)	Sequestrum of hind limb	+	-	+	+
68699	Alpaca (KSU)	Tooth abscess, osteomyelitis of mandible	+	-	+	+
61715	Alpaca (KSU)	Necrotic infection in maxillary bone	+	-	+	+
68690	Alpaca (KSU)	Sequestrum of forelimb	+	-	+	+
11034	Alpaca (KSU)	Necrotic mandible	+	-	+	+
73556	Alpaca (Cornell Univ. NY)	Oral swab	+	-	+	+
PSU	Alpaca (Pennsylvania)	Tongue abscess	+	-	+	+
118610	Alpaca (Cornell Univ. NY)	Bone	+	-	+	+

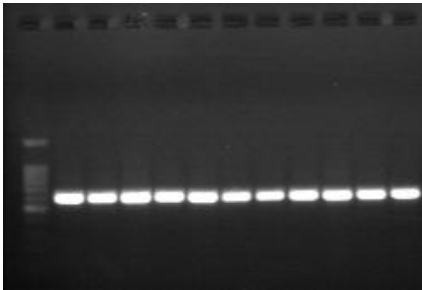**Figure A.1. PCR analysis of different isolates of *F. necrophorum* from llama and alpaca.**

Fig. 1A. PCR assay with Fuso 1and 2 primer
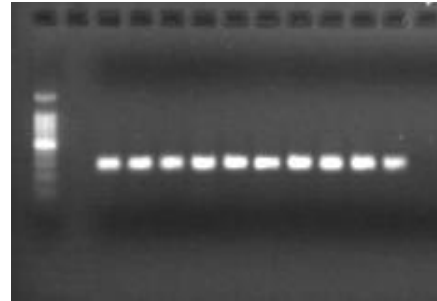
Fig. 1B. PCR assay with Haem primer
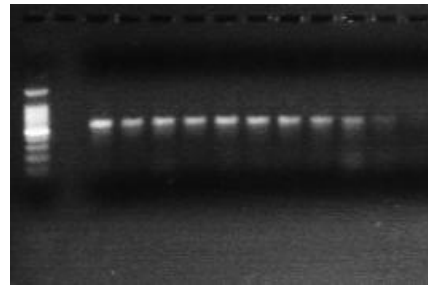

Fig. 1C. PCR assay with Fund primer

Fig. 1D. PCR assay with 5'lktpXmXh primer

Figure A.2. Western blot analysis of culture supernatants of *F. necrophorum* isolates from llama and alpaca.
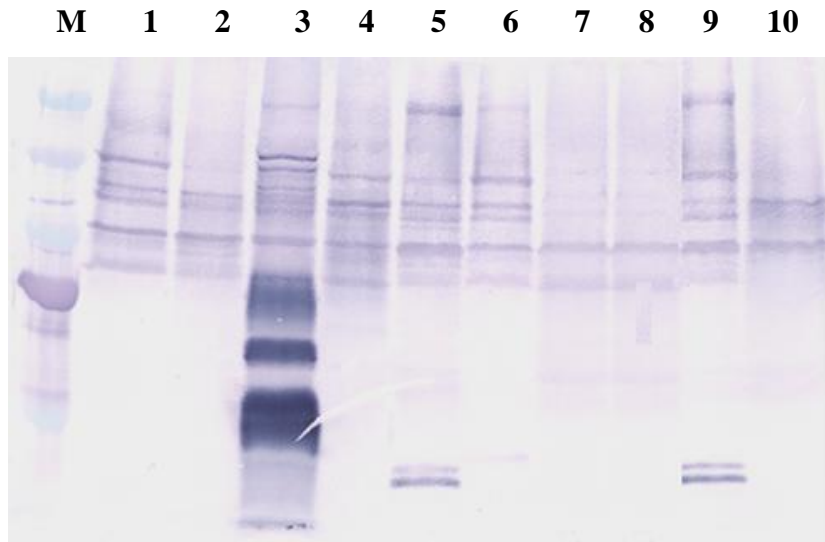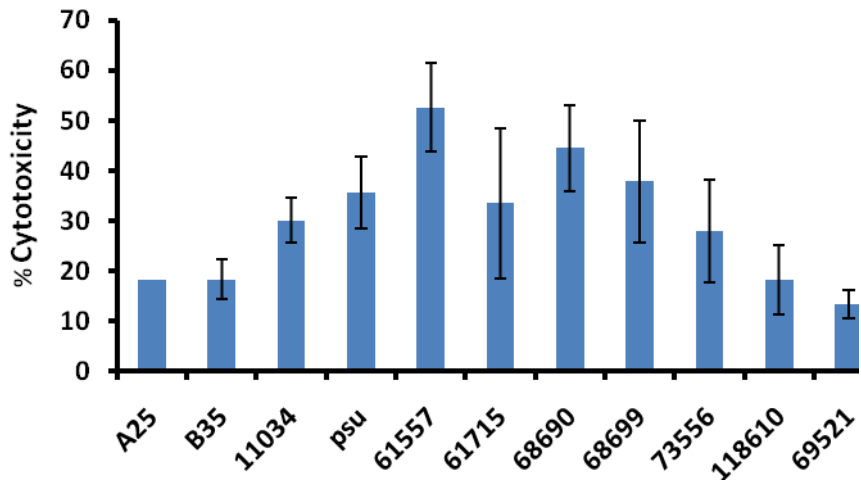
Figure A.3. Cytotoxicity assay.

A)

B)