CHARACTERIZATION OF A FUSOBACTERIUM NECROPHORUM SUBSPECIES NECROPHORUM OUTER MEMBRANE PROTEIN

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Abstract

Fusobacterium necrophorum is an anaerobic Gram-negative non spore forming rod shaped bacteria that is a normal inhabitant of the alimentary tract of humans and animals. Two subspecies of *F. necrophorum* have been recognized- subspecies *necrophorum* and subspecies *funduliforme*. Subspecies *necrophorum* is an opportunistic pathogen in animals causing diseases such as bovine hepatic abscesses and sheep foot rot while as subspecies *funduliforme* is linked with human oral and hepatic infections such as sore throats, Lemierre's syndrome and hepatic abscesses. The pathogenic mechanisms of *F. necrophorum* are complex and are not well understood or defined. Several virulence factors such as leukotoxin, haemolysin, haemagglutinin and adhesin have been described.

One of the most important factors in *F. necrophorum* bacterial pathogenesis is the adhesion of the bacteria to the host cell. The adhesion of the bacteria to the host cell helps it colonize the host tissue and this is followed by intracellular multiplication with dissemination to other tissues, which could ultimately lead to septicemia and death. Bacteria use adhesins which are proteins found in the outer membrane which help them bind with host receptors and this helps with the adhesion of the bacteria to the host cell. Not much is known about *F. necrophorum* adhesins. Here, we describe and characterize a novel adhesin.

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Dedication

This report is dedicated to my loving family Jaime and Surya, my parents and my brother who have always encouraged me and always believed in me even when I haven't believed in myself. Thank you for all your support!

Chapter 1 - Introduction

Fusobacterium necrophorum is a Gram-negative, anaerobic and rod shaped bacterium. It gets its genus name from the Latin word,"fusus", which means spindle, although it is not usually spindle shaped. *F. necrophorum* is a normal inhabitant of the ruminant gut flora of cattle, and is an opportunistic pathogen, and it is isolated from gastro-intestinal tracts, oral cavities and urogenital tracts from humans and animals.

F. necrophorum has been recognized as an animal pathogen since the late 1800s(Langworth, 1977). *F. necrophorum* is a much more common pathogen in animals than in humans and is an economically important bacterium to agriculture, as it causes liver abscesses in cattle. In feedlots, the average rate of prevalence is between 12-32% but in some cases, rates as high as 90-95% have been reported (Brink *et al.*, 1990; Nagaraja and Chengappa, 1998). Liver abscesses result in liver condemnation, reduced weight gain(up to 11%), decreased feed efficiency (up to 9%) and reduction in the dressing percentage of carcasses, costing approximately $66 per head in animals with severe abscesses (Brink *et al.*, 1990). Liver abscesses are the leading reason for liver condemnation and account for 50% of all condemnations. Livers are usually trimmed to get rid of abscesses, during which time, the abscesses may rupture, leading to the condemnation of the entire carcass and putting a huge economic burden on the meat packer.

Footrot is prevalent in sheep, goats and cattle, and to a certain extent, in horses (Bennett 2011). The hard keratin of the hoof is destroyed leading to lameness in the animal. It is thought to be caused by the synergistic interaction between two bacteria- *Dichelobacter nodosus* and *F. necrophorum*. Footrot is a contagious and debilitating disease (Zhou 2009) that causes losses in production and raises animal welfare issues. Recently, Zhou *et al*. have shown that *F.*

necrophorum is also involved in lameness in pigs, the presence of which was confirmed by testing the swabs from the lame hooves of six pigs for the presence of *F. necrophorum* leukotoxin gene by PCR (Zhou 2010). In pen trials, it has been shown that *F. necrophorum* is required for *D. nodosus* to establish a successful footrot infection (Roberts and Egerton 1969).

Evaluation of aerobic and anaerobic flora from liver abscesses has shown that *F. necrophorum* is the primary etiologic agent, with an isolation range ranging from 81-100%. In many cases, *F. necrophorum* is isolated as the sole pathogen, occasionally being isolated with a variety of both aerobic and anaerobic facultative bacteria (Scanlan and Hathcock 1983). *F. necrophorum* is also described as the cause of calf diphtheria (Loeffler), foot rot in cattle and sheep and hog cholera (Bang)

In humans, *F. necrophorum* accounts for <1% of bacteremia cases, with only a (few) hundred reported cases in the literature. However, among non-spore forming anaerobes, it is unique in having a very strong association with several clinically distinctive septicemic infections known as necrobacillosis, postanginal sepsis, or Lemierres syndrome (Riordan 2007). The human isolate of *F. necrophorum* subspecies *funduliforme* was first described by Jean Halle in 1898 as part of his Ph.D thesis on the bacteriology of the female genital tract.

A paper by Courmont and Cade (1900) is regarded as the first paper to describe Lemierre's Syndrome, which is postanginal septicemic infection with *F. necrophorum*. Lemierre's syndrome is also known as postanginal shock or human necrobacillosis and it usually occurs in children and young adults. The paper described a patient who complained of sore throat and cough. A few days later, there was onset of rigors which progressed to and overwhelming sepsis and caused a large abscess in the supraclavicular fossa. On autopsy, the lungs had multiple abscesses, which were thought to be septic emboli infarctions. Courmont and Cade were further

able to demonstrate the bacteria in the pus were anaerobic Gram-negative bacilli, which they then used to infect other animals. The colonies described were consistent with *F. necrophorum*. Lemierre's syndrome usually develops after a sore throat episode, usually caused by bacteria such as *Streptococcus*. The bacteria causes a peritonsillar abscess, inside of which, anaerobic bacteria such as *F. necrophorum* are able to flourish. The sore throat is accompanied by purulent exudate, high fever, cervical and submandibular lymphadenopathy, that rapidly leads to metastatic abscesses. *F. necrophorum* move from the abscesses into the neighboring tissues, including the jugular vein. Here the bacteria can result in the formation of a septic thrombosis. Once the thrombus enters the blood stream, it causes bacteremia and emboli are seeded throughout the rest of the body. If an emboli enters the pulmonary vasculature, it can block branches of the pulmonary artery bringing deoxygenated blood to the lungs from the heart. This can cause shortness of breath accompanied by chest pain and could lead to severe pneumonia. André Lemierre was the first to describe the disease in 1936 when he published a series of 20 cases of throat infection followed by anaerobic septicemia, in which 18 of the cases died.

Initially, there was much confusion about the causative organism, in part due to the many names given to *F. necrophorum*. André Lemierre first misidentified the organism as *B. fragilis* (Lemierre 1929), whereas some German authors referred to it as *Bacillus symbiophiles*, while some French scientists referred to it as *Bacillus funduliformis*. Such was the confusion that there were up to 52 names used to refer to *F. necrophorum.* The description of the associated cases and the properties of the organism lead people to believe that they were describing the same organism. Knorr (1923) introduced the genus *Fusobacterium* for pointed, non-spore forming , Gram-negative bacteria found in the mouth. Shinjo *et al.* (1991) proposed that *F. necrophorus* has two distinct subspecies, *F. necrophorum* subspecies *necrophorum* or biotype A and *F.*

necrophorum subspecies *funduliforme* or biotype B. this was based on differences at the biochemical, morphological and DNA homology differences between the two subspecies. His group also developed a one-step duplex PCR to detect and differentiate between the two subspecies. The separation into two subspecies is also based on the animal of origin of the bacteria. While *F. necrophorum* subspecies *necrophorum* was isolated from animals, *F. necrophorum* subspecies *funduliforme* was isolated form humans (Fig 1).

Pathogenic mechanisms of *F. necrophorum*

F. necrophorum is an opportunistic pathogen and it has many virulence factors which contribute to its pathogenesis. It is well established that subsp. *funduliforme* does not possess all the virulence factors of subsp. *necrophrum* and the toxins produced by subsp. *funduliforme* tend to be produced in smaller amounts than those of subsp. *necrophrum*. This might explain the difference in the severity of the infections and diseases between animals and humans caused by *F. necrophorum*. One of the striking aspects of *F. necrophorum* infections in humans is that it is most highly prevalent in a tight cluster based on age as it mainly affects people in the their late teens and early 20s. This is obvious not only in cases with Lemierre's syndrome but also in cases of tonsillitis and peritonsillar abscesses. The pathogenic mechanism depends on many bacterial and host factors such as bacterial strain, aspects of the host tonsil, presence or absence of immunity and synergistic factors.

Virulence factors of F. necrophorum

By definition, virulence factors refer to the properties (i.e., gene products) that enable a microorganism to establish itself on or within a host of a particular species and enhance its potential to cause disease. Virulence factors include bacterial toxins, cell surface proteins that

mediate bacterial attachment, cell surface carbohydrates and proteins that protect a bacterium, and hydrolytic enzymes that may contribute to the pathogenicity of the bacterium. Due to the economic importance of diseases it causes in animals, work has been done to study the virulence factors of *F. necrophorum*. In humans, not a lot of work has been done to study subsp. *funduliforme*, as the incidences of necrobacillosis and Lemierre's syndrome are very rare. Pham (look up) infected animals with human *F. necrophorum* isolates and was able to report that in animals, the more virulent human strains have an affinity for animal livers and lungs, while the less virulent strains seemed to affect joints, bones and muscles of the animals. *F. necrophorum* have a host of virulence factors that it uses to invade a host and establish a successful infection.

Leukotoxin

The best studied virulence factors of *F. necrophorum*, leukotoxin was first described by Roberts (1967). Since then, the toxins nature and action has been thoroughly characterized. Leukotoxin is a high molecular weight secreted protein-336kDa (Narayanan 2001), which is toxic to ruminant leukocytes. At very low concentrations, leukotoxin activates polymorphonuclear leukocytes (PMNs) and the leukocytes showed changes characteristic of apoptosis. At higher concentrations, leukotoxin induces it was shown that bovine mononuclear cells were induced to undergo apoptosis. At very high concentrations, leukotoxin causes necrotic cell death of bovine peripheral leukocytes. Non-leukotoxin producing strains of *F. necrophorum* are unable to induce foot rot in cattle following inoculation of the bacterium intradermally(Emery 1985). The sequence of the leukotoxin suggests that the leukotoxin is a novel protein not related to any other known bacterial leukotoxins or exotoxins.

Endotoxin

Lipopolysaccharide (LPS) is a very important virulence factor for Gram-negative bacteria, and purified LPS from *F. necrophorum* behaves like a classical endotoxin in animal models (Garcia 1975). Tan *et al*.,(1996) showed a difference between the LPS of *F. necrophorum* subsp *necrophorum* and subsp *funduliforme*, both in quantity and composition, with subsp *necrophorum* having higher LPS content than subsp *funduliforme*. Tan *et al*., also showed that *F. necrophorum* subsp *necrophorum* caused greater chicken embryo mortalities than that of subsp *funduliforme*.

Hemolysin

Divalent metal ions such as iron, magnesium, manganese, cobalt, copper, nickel and zinc, to name a few are often required for the survival of the bacteria for both subsp *necrophorum* and subsp *funduliforme*. These divalent metal ions in trace amounts are needed by the bacteria as cofactors for enzymes that catalyze biochemical reactions for various metabolic pathways required by the organism. Iron in particular is needed by the bacteria for almost all enzymatic and metabolic pathways. Since iron is essential in nature, vertebrates have developed elaborate mechanisms to sequester iron in body fluids (eg: transferring in blood and lactoferrin in external secretions help sequester free iron). These proteins create an environment depleted of iron at a level which is too low to support the growth of nearly all bacteria. Hemolytic activity is very important to *F. necrophorum* as the bacterium needs to acquire iron from the media to grow and multiply. Tan *et al*., was able to show that in liver abscesses contributed to *F. necrophorum* subsp *necrophorum*, there was hemolysin activity which appeared to contribute to abscess formation.

Hemagglutinin

Hemaglutinin refers to a substance that causes red blood cells to agglutinate. The process of agglutination is referred to as hemaglutination. Kanoe *et al*.,1989 has shown data that suggests that hemagglutinin is one of the components of the cell surfaces of *F. necrophorum* subsp *necrophorum* and is probably an outer membrane protein. Kanoe *et al*.,1989 also examined guinea pig mesenteric circulation in-vivo and demonstrated that both cell free hemagglutinin and *F. necrophorum* hemagglutinin could cause thrombus formation. The thrombi were first observed in the venules and later on in the arterioles. Immunofluorescence assays showed that the hemagglutinin bound to the thrombi in microcirculation and were able to deduce that thromobosis is one of the earliest steps in the pathogenesis of necrosis and is an important virulence factor for *F. necrophorum*.

Adhesins

Adhesion is one of the first steps that a bacterium has to do to for establishing a successful colonization. Bacteria produce adhesins, which are proteins that interact with host cell receptors and lead to the binding of the bacteria with the host cell. Tan *et al*., (1996) demonstrated the presence of fimbriae on the bacterial surfaces of both subspecies, which could be used to bind to the host cell receptor, although the host cell receptor has still not been identified.

Synergy

Since *F. necrophorum* are well known opportunistic pathogens, many studies have been carried out to see if they infect the host better in the presence of other bacteria which might make the condition more favorable for *F. necrophorum* to establish a successful infection. Smith *et al*., showed that *F. necrophorum* was able to produce fatal necrobacillosis in mice when injected in

doses greater than 10^6 organisms subcutaneously. When the mice were injected with sub-lethal doses in either diluted or undiluted broth cultures of other bacteria, especially *Escherischia. coli*, the infective dose to produce necrobacillosis in mice fell to less than 10 organisms of *F. necrophorum*. In the necrobacillosis lesions that developed from co-infection, *F. necrophorum* far outnumbered *E. coli*. They were also able to show that they achieved a similar synergistic effect when *F. necrophorum* was mixed with other bacteria such as *Pseudomonas aeruginosa, Bacteriodes fragilis* and *Fusobacterium nucleatum*. In the cases of alpha hemolytic *Streptococcus* and *B. fragilis*, *F. necrophorum* increased the persistence of alpha hemolytic *Streptococcus* and *B. fragilis* in-vivo and enabled the bacteria to multiply profusely showing that the synergistic effect was mutually beneficial.

TREATMENT AND MANAGEMENT/PREVENTION

F. necrophorum was a serious human disease in the age of pre-antibiotics. In the current day and age of antibiotics, *F. necrophorum* infections in humans are an uncommon problem and Lemierre's syndrome is rarely reported, with few cases reported, the disease is sometimes referred to as "the forgotten disease" (Weesner and Cisek, 1993). The current rate of incidence is 0.8 cases per million in the general population. In the U.K. 19 cases were reported in 1997 and 34 cases were reported in 1999. Prior to antibiotics, the mortality rate was 90%, now thanks to antibiotic therapy; mortality has dropped to 15%.

F. necrophorum, is generally very susceptible to beta-lactam antibiotics, metronidazole, clindamycin and third generation cephalosporins. Penicillin is often given as the first line of treatment and sometimes Clindamycin is given as monotherapy. There are many conflicting scientific studies as to which antibiotic to administer based on in-vitro studies. Many authors have a preference for metronidazole as it seems to have an excellent activity against all strains of

Fusobacterium species and they also cite excellent tissue penetration by the drug (Freeman *et al.*. 1997). There are some concerns about using metronidazole to treat humans (Rustia and Shubik, 1972), since metronidazole has been found to cause lung tumors and malignant lymphomas in mice. Despite this, metronidazole is widely used in Europe to treat *Fusobacterium* and other anaerobic bacterial infections.

Like many bacteria, *Fusobacterium* species seem to be gaining resistance to antibiotics. Simon, (1977) tested 25 isolates of *Fusobacterium* against 37 antimicrobials for antibiotic susceptibility using the disc diffusion method and reported that *Fusobacterium* was susceptible to 15 antimicrobials and resistant to 12 where as for the remaining 10 antimicrobials, some isolates were completely resistant where as others showed partial resistance. Moreno *et al*., (1989) reported that in a study of 11 patients with Lemierre's syndrome two patients showed resistance to ampicillin and treatment with cefoxitin or metronidazole was resumed to cure the infection. Brazier (2006) evaluated 100 human isolates of *F. necrophorum* submitted to the U.K. ARL and found a 15% resistance to erythromycin, with a 2% resistance to penicillin and 1% resistant to tetracycline. In animals, Sheldon *et al*., looked at minimum inhibitory concentrations (MICs) of candidate antibiotics for the principal bacteria associated with uterine infections. The bacteria were isolated from Holstein-Friesian cows which had clinical metritis and/or endometritis. They found that *F. necrophorum* had the highest MIC50 values for enrofloxacin and oxytetracycline but had values of 0.06 microgram/ml or below for all cephalosporins. Nagaraja *et al*., (1998) evaluated 37 isolates of *F. necrophorum* from liver abscesses in cattle and showed that subsp *necrophorum* and subsp *funduliforme* were both susceptible to chlortetracycline, oxytetracycline, tilmicosin, tylosin and virginiamycin, while they were somewhat less susceptible to bacitracin, lasalocid and monensin. While the main line for

combating *F. necrophorum* infections is by the administration of antibiotics, this has lead to the bacterium gaining resistance to many antibiotics and antimicrobials

Saginala *et al*., (1996) did a study using serum antileukotoxin antibody response and protection in steers which were vaccinated with a crude *F. necrophorum* leukotoxoid against a subsequent experimental challenge with *F. necrophorum*. The steers were injected on days 0 and 21subcutaneously with a concentrated culture supernatant of *F. necrophorum* containing leukotoxoid and were challenged intraportally with *F. necrophorum* culture on day 42. The steers were euthanized on day 63 and their livers were examined for assessment of protection. They concluded that antileukotoxin antibody titers provided some degree of protection against the induced liver abscesses (8 out of 25 vaccinated steers developed abscesses while as in the control group, 3 out of 5 control steers developed abscesses). In another study Saginala *et al*., (1996) determined the efficacy of leukotoxin –based *F. necrophorum* vaccines in providing protection against experimentally induced liver abscesses in steers. They were able to find that the culture supernatant vaccine was more effective than whole-cell culture or semipurified leukotoxin in protecting the steers against liver abscesses and partial purification of leukotoxin appeared to reduce its protective immunity.

Emerging antimicrobial resistance and an increasing concern in public health and meat consumers over the use of antibiotics for growth promotion in feed animals have led several European countries to ban the use of antibiotics as growth promoters. The concerns make the development of an effective vaccine an important priority. The leukotoxoid vaccine provided immunity to the cattle against *F. necrophorum* infection but it was not 100% effective, providing around 43% protection to the animals. Unfortunately, the leukotoxoid vaccine is a crude vaccine and contains bacterial components, such as lipopolysaccharide which causes adverse reaction in

cattle and other cellular derivatives or debris which causes injection site abscesses and transient illness followed by reduced feed intake and weight gain in cattle (Checkley *et al.*, 2005). As a result, this vaccine was taken off the market. Another commercially available vaccine Fusogard® (Novartis) is a bacterin (suspension of killed or attenuated bacteria). This vaccine demonstrated about a 31.5% reduction in liver abscesses. The protection provided by either vaccine is not better than the current method of abscess treatment in cattle (tylosin supplementation in cattle diet).

Considering the current mindset in public health and animal welfare and in the big picture against the use of antibiotics for growth promotion and in some cases, for treatment of animal diseases, the development of a vaccine which has high efficacy and minimal side effects is warranted. Identification of crucial outer membrane proteins required by *F. necrophorum* to adhere to the host surface to start colonization would make an ideal vaccine target if antibodies are raised in animals and humans against these proteins. These critical immunodominant outer membrane protein/proteins, once identified could be used to generate recombinant proteins. Recombinant protein vaccines generally stimulate superior immunity since the host immune system has evolved to recognize these antigens and the purified proteins do not usually cause adverse reactions in animals and humans.

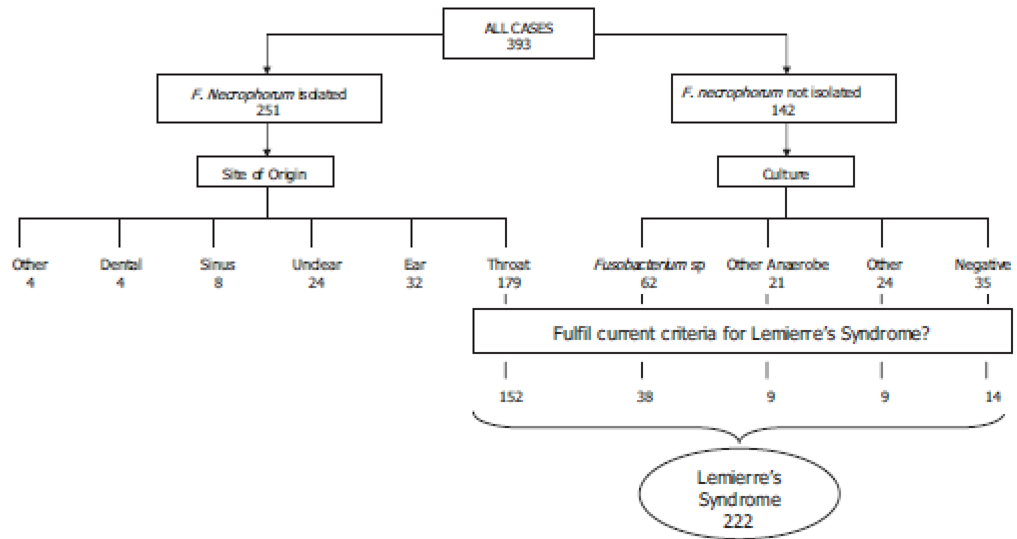


Figure 1.1:The difference between F. necrophorum subspecies necrophorum and F. necrophorum subsecies funduliforme, from a summary of cases. Courtesy of Riordian

Importance of outer membrane proteins in Gram-negative bacteria for adhesion and infection

One of the most important and critical events in bacterial pathogenesis is the adhesion of the pathogen to host cells. Many Gram-negative bacteria produce outer membrane proteins which help the bacteria bind to receptors on the host cell surface and initiate colonization of the host. It is this first initial attachment of the pathogen to host cells which help the bacteria in a successful colonization of the host. The outer membrane proteins of many bacteria such as the OmpA of *E. coli* (Wang Y. 2002), and FomA (Liu. *et al*.2013), protein of *F. nucleatum* are well characterized and have been shown to be important for establishing successful host colonizations. Greater understanding of *F. necrophorum* OMP can provide valuable insights into disease pathogenesis as well as provide potential targets for vaccine development.

References

1) **Langworth BF.** 1977. *Fusobacterium necrophorum*: its characteristics and role as an animal pathogen. Bacteriol Rev. 41(2):373-90.
2) **Brink DR, Lowry SR, Stock RA, Parrott JC.** 1990. Severity of liver abscesses and efficiency of feed utilization of feedlot cattle. J Anim Sci. 68(5):1201-7.
3) **Nagaraja TG, Chengappa MM.** 1998. Liver abscesses in feedlot cattle: a review. J Anim Sci. 76(1):287-98.
4) **Bennett GN, Hickford JG.** 2011Ovine footrot: new approaches to an old disease. Vet Microbiol. 148(1):1-7. doi: 10.1016/j.vetmic.2010.09.003. Epub 2010 Sep 15.
5) **Zhou H, Bennett G, Hickford JG.** 2009. Variation in *Fusobacterium necrophorum* strains present on the hooves of footrot infected sheep, goats and cattle. Vet Microbiol. 135(3-4):363-7. doi: 10.1016/j.vetmic.2008.09.084. Epub 2008 Oct 9.
6) **Zhou H, Dobbinson S, Hickford JG.** 2010. *Fusobacterium necrophorum* variants present on the hooves of lame pigs. Vet Microbiol. 141(3-4):390. doi: 10.1016/j.vetmic.2009.09.014. Epub 2009 Sep 11.
7) **Roberts DS, Egerton JR.** 1969. The aetiology and pathogenesis of ovine foot-rot. II. The pathogenic association of *Fusiformis nodosus* and *F. necrophorus*. J Comp Pathol. 79(2):217-27.
8) **Scanlan CM, Hathcock TL.** 1983. Bovine rumenitis - liver abscess complex: a bacteriological review. Cornell Vet. 73(3):288-97.
9) **Riordan T.** 2007. Human infection with Fusobacterium necrophorum (Necrobacillosis), with a focus on Lemierre's syndrome. Clin Microbiol Rev. 20(4):622-59.
10) **Courmont P, Cade A.** 1900. Sur une septico-pyohémie de l'homme simulant la peste et causée par un strepto-bacille anaérobie. Arch. de Méd. Exp. 12:393-418.

11) **Lemierre A, Guy A, Rudolf M.** 1929. Septicémies à *Bacillus fragilis*. Presse Méd. 37:1669.

12) **Lehmann KB, Knorr M.** 1923. Ueber die fusospirilläre symbiose, die Gattung Fusobacterium (K. B. Lehmann) und Spirillum sputigenum. II Mitteilung. Knorr, M. Die Gattung Fusobacterium. Zentbl. Bakteriol. Parasitenkd. Infekt. Hyg. Abt. 1 Orig. 89:4-22.

13) **Shinjo T, Fujisawa T, Mitsuoka T.** 1991. Proposal of two subspecies of *Fusobacterium necrophorum* (Flügge) Moore and Holdeman: *Fusobacterium necrophorum* subsp. *necrophorum* subsp. nov., nom. rev. (ex Flügge 1886), and *Fusobacterium necrophorum* subsp. *funduliforme* subsp. nov., nom. rev. (ex Hallé 1898). 1991. Int. J. Syst. Bacteriol. 41:395-397.

14) **Roberts DS.** 1967. The pathogenic synergy of *Fusiformis necrophorus* and *Corynebacterium pyogenes*. I. Influence of the leucocidal exotoxin of F. necrophorus. Br J Exp Pathol. 48(6): 665–673.

15) **Narayanan SK, Nagaraja TG, Chengappa MM, Stewart GC.** 2001. Cloning, sequencing, and expression of the leukotoxin gene from *Fusobacterium necrophorum*. Infect Immun. 69(9):5447-55.

16) **Emery DL, Vaughan JA, Clark BL, Dufty JH, Stewart DJ.** 1985. Cultural characteristics and virulence of strains of *Fusobacterium necrophorum* isolated from the feet of cattle and sheep. Aust. Vet. J. 62:43-46.

17) **Garcia MM, Charlton KM, McKay KA.** 1975. Characterization of endotoxin from *Fusobacterium necrophorum*. Infect. Immun. 11:371-379.

18) **Tan ZL, Nagaraja TG, Chengappa MM.** 1996. *Fusobacterium necrophorum* infections: virulence factors, pathogenic mechanism and control measures. Vet Res Commun. 20(2):113-43.

19) **Kanoe MM, Yamanaka, Inoue M.** 1989. Effects of *Fusobacterium necrophorum* on the mesenteric microcirculation of guinea pigs. Med. Microbiol. Immunol. 178:99-104.

20) **Freeman CD, Klutman NE, Lamp KC.** 1997. Metronidazole a therapeutic review and update. Drugs 54:679-708.

21) **Rustia M, Shubik P.** 1972. Induction of lung tumors and malignant lymphomas in mice by metronidazole. J. Natl. Cancer Inst. 48:721-726.

22) **Simon PC.** 1977. Susceptibility of *Fusobacterium necrophorum* to antimicrobials. Part I: as determined by the disc method. Can J Comp Med. 41(2):166-8.

23) **Moreno S, Altozano JG, Pinilla B, López JC. de Quirós B, Ortega A, Bouza E.** 1989. Lemierre's disease: postanginal bacteremia and pulmonary involvement caused by *Fusobacterium necrophorum*. Rev. Infect. Dis. 11:319-324.

24) **Brazier JS.** 2006. Human infections with *Fusobacterium necrophorum*. Anaerobe. 12:165-172.

25) **Lechtenberg KF, Nagaraja TG, Chengappa MM.** 1998. Antimicrobial susceptibility of *Fusobacterium necrophorum* isolated from bovine hepatic abscesses. Am J Vet Res. 59(1):44-7.

26) **Saginala S, Nagaraja TG, Tan ZL, Lechtenberg KF, Chengappa MM, Kemp KE, Hine PM.** 1996. Serum neutralizing antibody response and protection against experimentally induced liver abscesses in steers vaccinated with *Fusobacterium necrophorum*. Am. J. Vet. Res. 57:483-488.

27) **Saginala S, Nagaraja TG, Tan ZL, Lechtenberg KF, Chengappa MM, Hine PM.** 1996. The serum neutralizing antibody response in cattle to *Fusobacterium necrophorum* leukotoxoid and possible protection against experimentally induced hepatic abscesses. Vet Res Commun. 20(6):493-504.

28) **Checkley SL, Janzen ED, [...], . McKinnon JJ.** 2005. Efficacy of vaccination against *Fusobacterium necrophorum* infection for control of liver abscesses and footrot in feedlot cattle in western Canada. Can Vet J. 48(11): 1002-1007.

29) **Wang Y.** 2002. The function of OmpA in *Escherichia coli*. Biochem Biophys Res Commun. 292(2):396-431.

30) **Liu PF, Huang IF, Shu CW, Huang CM.** 2013. Halitosis vaccines targeting FomA, a biofilm-bridging protein of *Fusobacteria nucleatum*. Curr Mol Med. 13(8):1358-67.

31) **Loeffler F**. Mitt Gesundh Amte 1884;2:489

32) **Weesner CL, Cisek JE**. 1993. Lemierre syndrome: the forgotten disease. Ann Emerg Med. 22(2):256-8.

Chapter 2 - Abstract

Liver abscesses are of major economic importance to the cattle industry. Liver abscesses are mainly associated with the presence of *Fusobacterium necrophorum*, a non-spore forming and Gram-negative anaerobe. There are two main subspecies of this bacterium, which differ molecularly, morphologically, biochemically and in virulence. Previous studies have shown that the outer membrane proteins of *F. necrophorum* subsp. *necrophorum* are important for its successful binding to immobilized bovine adrenal gland capillary endothelial (EJG) cells. An outer membrane protein of *F. necrophorum* subsp. *necrophorum* with the highest binding capacity to EJG cells was characterized. The gene was cloned into pFLAG-CTS vector and subsequently expressed on the surface of *E. coli* BL21 DE3 cells. When *E. coli* carrying the recombinant plasmid was induced using IPTG, it had significantly enhanced binding to immobilized EJG cells compared to both the uninduced control and the *E. coli* carrying control vector only. When fixed EJG cells were incubated with purified native outer membrane proteins, *E. coli* carrying the induced recombinant plasmid, showed lowered levels of binding, comparable to the uninduced control and the *E. coli* carrying control vector only. This gain of function by recombinant *E. coli* confirms the ability of this protein to act as an adhesin to help *F. necrophorum* subsp. *necrophorum* bind to host cells. Functional characterization of this novel adhesin further expands our limited understanding of the pathogenesis of this poorly studied but economically significant and highly pathogenic bacterium.

Introduction

Fusobacterium necrophorum is a Gram-negative, rod shaped and obligate anaerobic bacterium which is frequently associated with necrotic infections in animals and humans (1). *F. necrophorum* is classified into two subspecies (11)- subsp *necrophorum* which is associated with liver abscess and foot rot in animals, while subsp *funduliforme* is associated with necrobacillosis and Lemierre's syndrome in humans. In feedlot cattle, subsp *necrophorum* is a major cause of infections including hepatic abscesses, necrotic laryngitis, resulting from aggressive grain-feeding programs (2). Foot rot and lameness in dairy and beef cattle and hepatic abscesses in

feedlot cattle are of major economic concerns in the cattle industry (2, 3). In feedlots, liver abscesses range from 12-32%, depending on various management and dietary factors (2). Liver abscesses and subsequently liver condemnations are of major economic concern to beef producers and packers, since the liver accounts for roughly 2% of the weight of the carcass.

In-vitro studies have shown that *F. necrophorum* uses outer membrane proteins (OMPs) to mediate adhesion to bovine adrenal gland capillary endothelial (EJG) cells (4). Four OMPs of 17, 24, 43 and 74kDa demonstrated an important role in adhesion of bacteria to fixed endothelial cells, with the 43kDa protein (FunA) showing the highest affinity for EJG cells. In these studies, we cloned the 43kDa protein gene (*funA*) into pFLAG-CTS expression vector and consequently cloned it into *E. coli* BL21 DE3 cells to characterize the protein. We were able to show that this protein expressed in *E. coli* increases binding of *E. coli* to EJG cells, when compared to the uninduced or control vector expressed in *E. coli*. We conclude that this 43kDa OMP serves as an adhesin for endothelial cells for bovine strains of *F. necrophorum*. Neutralization of *F. necrophorum* adherence is an attractive strategy to prevent *Fusobacterium* colonization of the liver.

MATERIALS AND METHODS

Bacterial strains

Fusobacterium necrophorum subsp *necrophorum* strain 8L1 was used for cloning the 43kDa gene in this study. 8L1 was grown in pre-reduced, anaerobically sterilized Brain Heart Infusion (PRAS-BHI) broth (6). *E. coli* TOP10 (Invitrogen) was used for cloning and sequencing the gene. *E. coli* BL21DE3 was used for the expression of the recombinant adhesin proteins.

Isolation of outer membrane proteins

Outer membrane proteins from *F. necrophorum* subsp. *necrophorum* strain 8L1 were isolated using standard procedure, as described by Osborn and Munson (5). 8L1 was grown overnight in 1L of PRAS-BHI. Bacterial cells were harvested by centrifugation at 1,200 RPM for 15 minutes at 43C. The pelleted cells were resuspended in 10mL cold 0.75M sucrose-10mM tris,

pH 7.8. Lysozyme was added at a concentration of 2mg/mL and the cells were incubated on ice for 20 minutes. The suspension was diluted with 20 ml of cold 1.5mM EDTA delivered over 15 minutes. Following the addition of EDTA, the mixture was sonicated in an ice water bath, using a 3mm microtip at 20W output pulse settings. The sonication was repeated 10 times and was done in 10 second intervals with a minute pause between each sonication. The sonicated liquid was then centrifuged at 1,200g for 15 minutes at 43C. The supernatant from the above step was centrifuged at 65,000 RPM for 2 hours at 43C. The membrane pellet was resuspended in a small volume of cold 0.25M sucrose and 20mg/mL Triton X 100 (Sigma-Aldrich, St. Louis, MO), and incubated for 45 minutes at room temperature to dissolve the inner membrane. The suspension was centrifuged at 37,000 RPM for 2 hours. The pellet obtained consisted of the OMP fraction and was resuspended in cold STE buffer and stored at -80 until use.

Cloning and expression of the 43KDa OMP

The *funA* gene was amplified from *F. necrophorum* subsp. *necrophorum* strain 8L1 and subsequently cloned, as a *HindIII/SalI* fragment into the expression vector pFLAG-CTS (Sigma-Aldrich, St. Louis, MO) (7). The sequence of the forward and reverse primers was 5' CAT AAGCTT CT GAA ATT ATG CCT GGA CC 3' and 5' TAA GTCGAC GAA GCT AAC TTT CAT ACC A 3' respectively, the underlined sequences being the restriction enzyme sites. The PCR conditions were as follows: 1 cycle 96˚C for 3 min followed by 35 cycles of denaturation at 96˚C for 15 s, annealing at 50˚C for 30 s, and extension at 72˚C for 1 min and a final extension at 72°C for 5 min using Ex taq polymerase (Takara Inc., Mountain View, CA). The fragment was cloned to include the FLAG sequence at the N-terminal of the protein. The plasmid was named pSM2013 and was transformed into *E. coli* BL21DE3(8) cells. *E. coli* BL21 DE3 cells carrying pSM 2013 were now labelled as SM 2013. For expression, SM 2013 or the pFLAG control vector were grown in LB broth supplemented with ampicillin at a final concentration of 100μg/ml to an OD_{600} of approximately 0.7 at 37^0C. IPTG was added to a final concentration of 0.5M and protein was expressed for 2 hours at 37^0C.

Expression and Localization of the expressed protein on the cells surface

The purified native OMP preparation containing FunA and recombinantly expressed FunA protein from *E. coli* BL21 (DE3) were subjected for SDS-PAGE analysis and blotted on nitrocellulose membrane (Whatman Optitran, Dassel, Germany). Mouse antisera recognizing FunA protein was produced and was utilized in Western blots to determine if the protein was expressed in SM 2013 clone.

EJG attachment assay

Bovine adrenal gland capillary endothelial cells (EJG) were acquired from ATCC (American Type Culture Collection, Manassas, VA), catalog # CRL-8659. Cells were grown and maintained in Eagle's minimum essential medium, supplemented with 10% fetal bovine serum and 1% antibiotics per 1 liter of media. The cell media was changed every 3 days until the cells formed a monolayer. The cells were then treated with trypsin and were seeded into a 6 well plate at 120,000 cells/well and let stand alone for 48 hours to negate the effects of trypsin.

Forty eight hours post seeding, the EJG cells were fixed with modified Karnovsky's solution(0.1 M cacodylate buffer, 2.5% glutaraldehyde, 2% paraformaldehyde, adjusted to pH 7.4)(10) for 30 minutes at 43C, and then rinsed three times with double distilled water. Each well was then incubated with either the induced or un-induced SM 2013 clone or *E. coli* BL21DE3 cells containing the pFLAG-CTS-BAP control vector for 2 hours at 37^0C. After two hours, the wells were washed three times using double distilled water. One ml of LB broth was then added to each well, and the cells were scraped off the bottom using a cell scraper. The LB broth containing the bacteria and the EJG cells were serially diluted and plated on LB agar plates containing ampicillin at a final concentration of 100μg/ml and incubated overnight at 37^0C. Attached bacteria were enumerated by counting colonies.

OMP inhibition assay

EJG cells were grown and maintained as previously described, and were seeded into 6 well plates at 120,000 cells/well and were let to stand alone for 48 hours to negate the effect of trypsin. The cells were fixed with modified Karnovsky's solution for 30 minutes, following three rinses with double distilled water. The fixed cells were incubated with 250μl of preparations containing OMPs of *F. necrophorum* subsp. *necorphorum* strain 8L1 for 2 hours at 43C. The

unbound OMPs were washed off three times using double distilled water. The OMP coated cells were then incubated with either the induced or un-induced SM 2013 clone or *E. coli* BL21DE3 cells containing the pFLAG-CTS control vector for 2 hours at 37^0C. The attached bacteria were enumerated using the procedure previously described.

Proteinase K assay

For surface localization studies, we carried out surface proteolysis using proteinase K treatment(9). SM 2013 clone was induced for 2 hours at 37^0C. The induced intact bacteria were then split up into 4 aliquots and subject to 0, 25, 50 or 100 µg/ml of proteinase K for 2 hours. 6 well plates containing EJG cells at 120,000 cells/ well were then incubated with the treated bacteria for 2 hours. The unattached bacteria were washed off using sterile water and the attached bacteria were serially diluted and enumerated as described before.

Statistical analysis

All assays were done in triplicate and were repeated a minimum of three times. Graph Pad Prism (La Jolla, CA) version 5.1 statistical software was used for the analysis of data. The data was analyzed using repeated measures ANOVA followed by a Tukey posttest. A *P*value of <0.05 was considered significant.

Results

Expression and Localization of the expressed protein on the cells surface

The mouse serum recognized the native and recombinant protein according to its predicted mass of 43kDa. This band was not present in *E. coli* BL21 DE3 cells carrying the control plasmid.

EJG attachment assay

When SM2013 was incubated with fixed EJG cells, there was a significant increase in the binding of bacterial cells when compared to the EJG cells that were incubated with either uninduced SM2013 or the induced or uninduced pFLAG control vector in *E. coli* BL21DE3 cells.

OMP inhibition assay

When SM2013 was incubated with fixed EJG cells that were saturated with the OMP preparation, there was a significant ($P < 0.0001$) decrease in the binding of the bacterial cells compared to the control EJG cells that were not saturated with the OMP prep.

Proteinase K assay

The Western blots showed that the proteins were cleaved by proteinase K in a dose dependent manner. The protein was also shown to be expressed on the surface of *E. coli* as the intact bacteria were incubated with proteinase K.

Discussion

Outer membrane proteins (OMPs) are a very important feature of Gram-negativel bacteria as they are the first proteins to interact with the host cell surface. These proteins are often key for the bacteria in its pathogenesis. OMP are diverse in nature and play multiple roles; they may act as adhesins, porins, or receptors for various host molecules, to name a few. OMP of many Gram-negative bacteria have been shown to play an important role in adhesion to host cells and subsequently leading to successful colonization and infection of the host (12).

In the case of Fusobacterium species, OMPs have been shown to be critical for adhesion and infection in *F. nucleatum*(13). It has been shown that OMPs of *F. necrophorum* subsp. *necrophorum* are important in mediating attachment of bacterial cells to endothelial cells (4). Previous studies have shown that pre-incubating *F. necrophorum* with trypsin reduced the ability of the bacteria to bind with fixed EJG cells, showing that bacterial attachment is most likely mediated through outer membrane proteins. We were able to successfully clone and express the 43kDa OMP in the pFLAG-CTS vector. This vector carries the leader region of the OmpA of *E.coli*, which allows expression of the recombinant protein in the periplasm of *E.coli*. The recombinant protein was sequenced and was compared against OMPs of *F. necrophorum* strain 8L1 and was successfully identified as the right size band through Western blots. SM 2013 was subject to proteolytic degradation in intact bacteria, showing that the OmpA leader sequence was successful in sending the recombinant protein to the outer membrane of *E. coli*.

When SM2013 was induced, it showed a significantly higher binding to fixed EJG cells, when compared against the non-induced SM2013 or the induced or non induced control vector expressed in *E.coli* BL21DE3 cells. Preincubating fixed EJG cells with OMPs of *F.*

necorphorum strain 8L1 reduced the binding efficiency of SM2013 to the EJG cells, indicating that some of the OMPs were binding to the same cell receptor.

References

1) **Tan ZL, Nagaraja TG, Chengappa MM.** 1996. *Fusobacterium necrophorum* infections: virulence factors, pathogenic mechanism and control measures. Vet Res Commun. **20**:113-43.
2) **Nagaraja TG, Chengappa M M.** 1998. Liver abscesses in feedlot cattle: a review. J. Anim. Sci. **76:**287-98.
3) **Nagaraja TG, Lechtenberg KF.** 2007. Liver abscesses in feedlot cattle. Vet Clin North Am Food Anim Pract. **23**:351-69.
4) **Kumar A, Gart E, Nagaraja TG, Narayanan S.** 2013. Adhesion of Fusobacterium necrophorum to bovine endothelial cells is mediated by outer membrane proteins. Vet Microbiol. **162**:813-8.
5) **Osborn MJ, Munson R.** 1974. Separation of the inner (cytoplasmic) and outer membranes of Gram-negative bacteria. Methods Enzymol. **31**:642-53.
6) **Tan ZL, Nagaraja TG, Chengappa MM.** 1992. Factors affecting leukotoxin activity of *Fusobacterium necrophorum*. Vet. Microbiol. **32:**15-28.
7) **Asvadi P, Fletcher A, Raison RL.** 2002. Expression and functional analysis of recombinant scFv and diabody fragments with specificity for human RhD. J. Mol. Recognit. **15:** 321–30.
8) **Fass R, van de Walle M, Shiloach A, Joslyn A, Kaufman J, Shiloach J.** 1991. Use of high density cultures of Escherichia coli for high level production of recombinant Pseudomonas aeruginosa exotoxin A. Appl Microbiol Biotechnol. **36:** 65-9.
9) **Pinne M, Matsunaga J, Haake DA.** 2012. Leptospiral Outer Membrane Protein Microarray, a Novel Approach to Identification of Host Ligand-Binding Proteins. J. Bacteriol. **194:** 6074-87.
10) **Babinski MA, Costa WS, Sampaio FJ, Cardoso LE.** 2007. Structural organization of fibrous connective tissue in the periacinar region of the transitional zone from normal human prostates as revealed by scanning electron microscopy. BJU Int. **100:** 943-4.

11) **Shinjo T, Fujisawa T, Mitsuoka T.** 1991. Proposal of Two Subspecies of *Fusobacterium necrophorum* (Flügge) Moore and Holdeman: *Fusobacterium necrophorum* subsp. *necrophorum* subsp. nov., nom. rev. (ex Flügge 1886), and *Fusobacterium necrophorum* subsp. *funduliforme* subsp. nov., nom. rev. (ex HallÉ 1898). IJSEM. **41:** 395-7.
12) **Bavington C, Page C.** 2005. Stopping bacterial adhesion: a novel approach to treating infections. Respiration. **72:** 335-44
13) **Nakagaki H, Sekine S, Terao Y, Toe M, Tanaka M, Ito H, Kawabata S, Shizukuishi S, aro Fujihashi K, Kataoka K.** 2010. *Fusobacterium nucleatum* Envelope Protein FomA Is Immunogenic and Binds to the Salivary Statherin-Derived Peptide. Infect Immun. **78:** 1185-92.

Figure legends

Figure 1A. Western blot analysis of recombinant FunA adhesin protein expressed in *E. coli*. Protein ladder (lane A), SM 2013 un-induced (lane B), SM 2013 induced for 2 hours (lane C) and protein ladder (lane D). Figure 1B. Coomassie blue staining of the OMP extract. Protein ladder (lane A), SM 2013 un-induced (lane B), SM 2013 induced for 2 hours (lane C) and protein ladder (lane D).

Figure 2. EJG attachment assay. The bacteria were incubated with fixed EJG cells and the unbound bacteria were washed off using sterile water. The bound bacteria to the EJG cells were removed from the wells and were serially diluted in LB broth and plated on LB agar plates containing a final concentration of Ampicillin at 50µg/ml to enumerate surface bound bacteria.SM 2013 Induced (lane A), SM 2013 Uninduced (lane B), pFLAG-CTS-BAP Induced (lane C), pFLAG-CTS-BAP Uninduced (lane D).

Figure 3. OMP inhibition assay. Fixed EJG were coated with OMPs from *F. necrophorum* subsp. *necrophorum* starin 8l1. Unbound OMPs were washed off and bacteria were incubated with the pretreated EJG cells. SM 2013 Induced (lane A), SM 2013 Uninduced (lane B), pFLAG-CTS-BAP Induced (lane C), pFLAG-CTS-BAP Uninduced (lane D).

Figure 4. Proteinase K assay. Intact induced bacteria were incubated with increasing concentrations of proteinase K and were then whole cell lysates were subject to Western blot. Protein ladder (lane A)

Figure 5. Proteinase K assay. Intact induced bacteria were incubated with increasing concentrations of proteinase K and were then incubated on fixed EJG cells. SM2013 Uninduced (lane A), SM2013 Induced (lane B), SM2013 Induced and incubated with 25µg/ml proteinase K (lane C), SM2013 Induced and incubated with 50µg/ml proteinase K (lane D), SM2013 Induced and incubated with 100µg/ml proteinase K (lane E). A- significantly different from all other groups.

Figure 1A

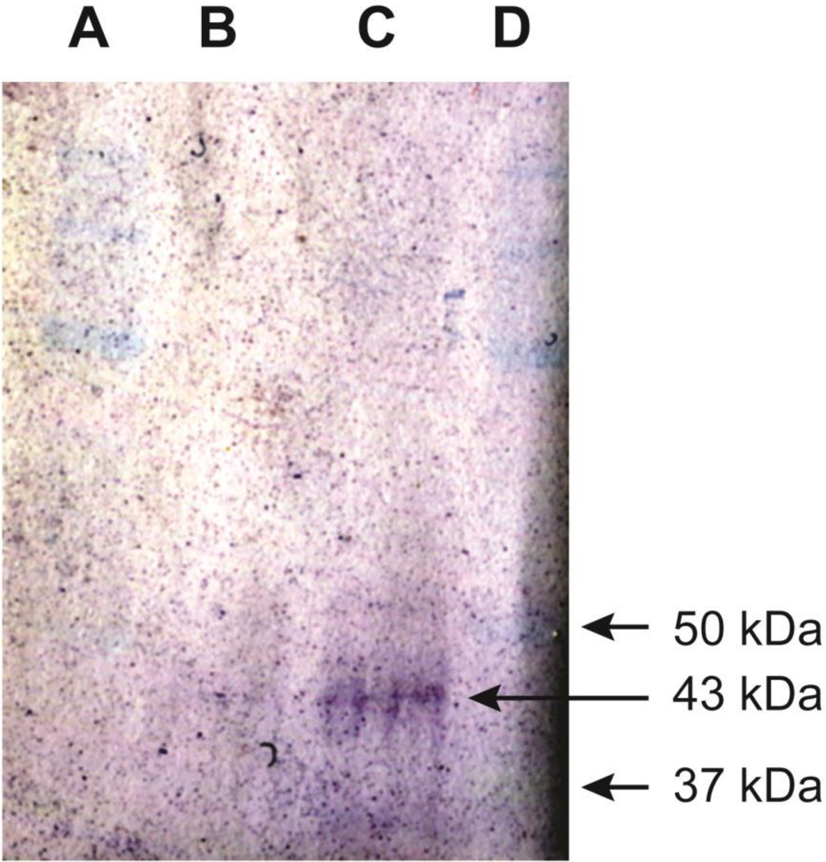


Figure 1B

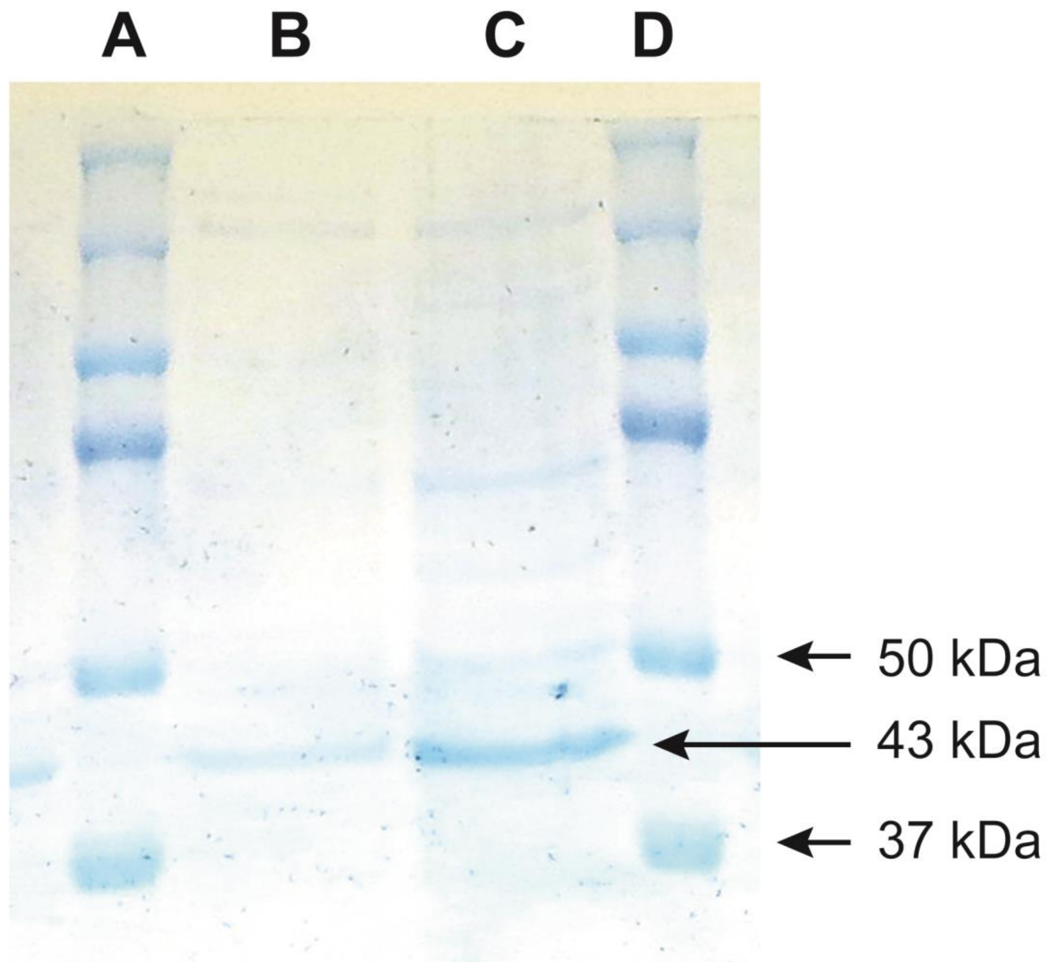


Figure 2

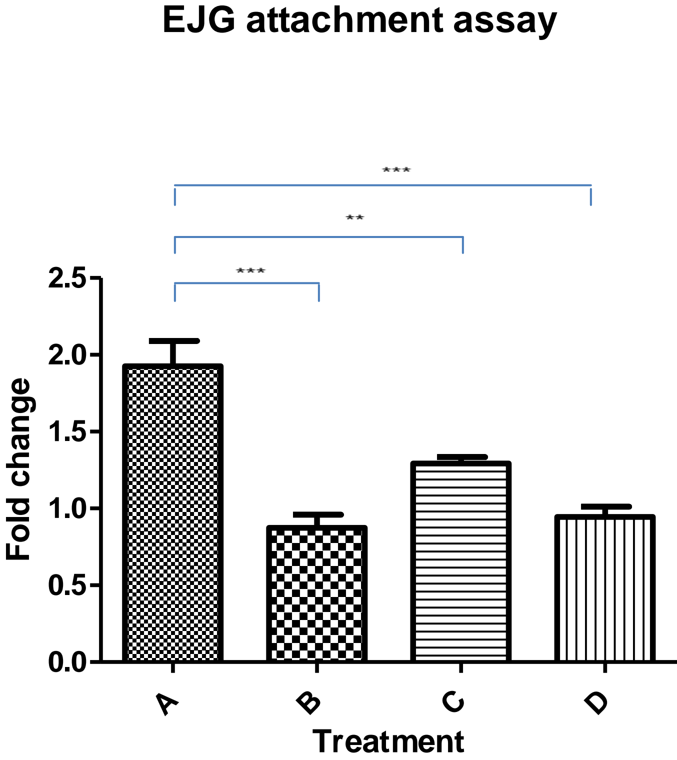


Figure 3.

EJG cells coated with *Fusobacterium necrophorum* subsp. *necrophorum* OMPs

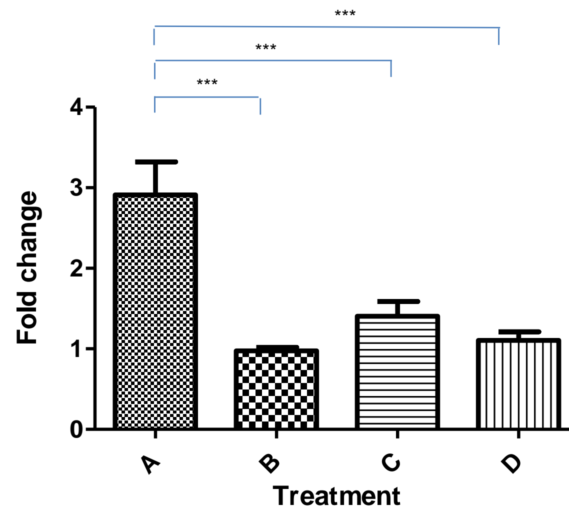


Figure 4

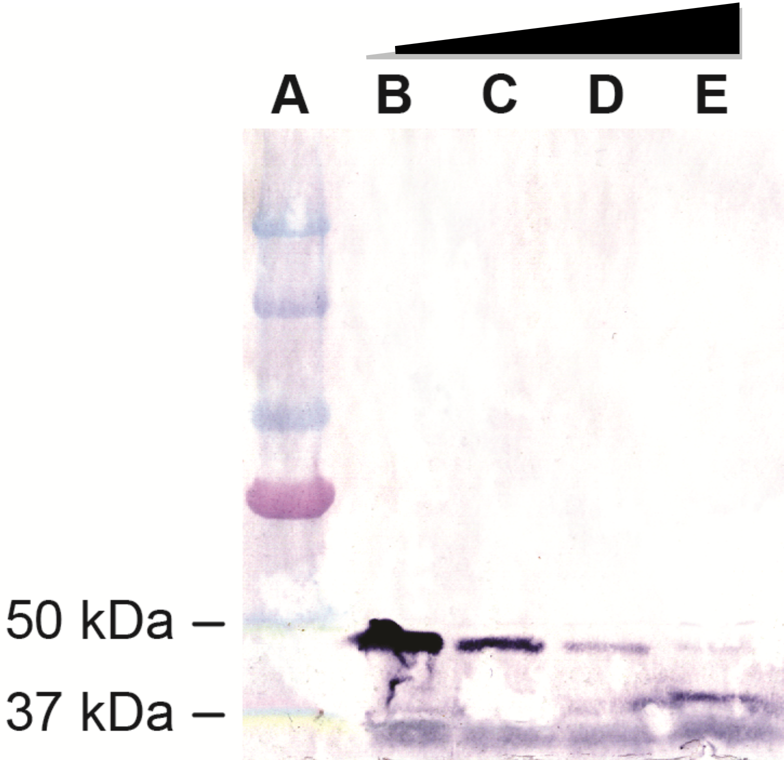
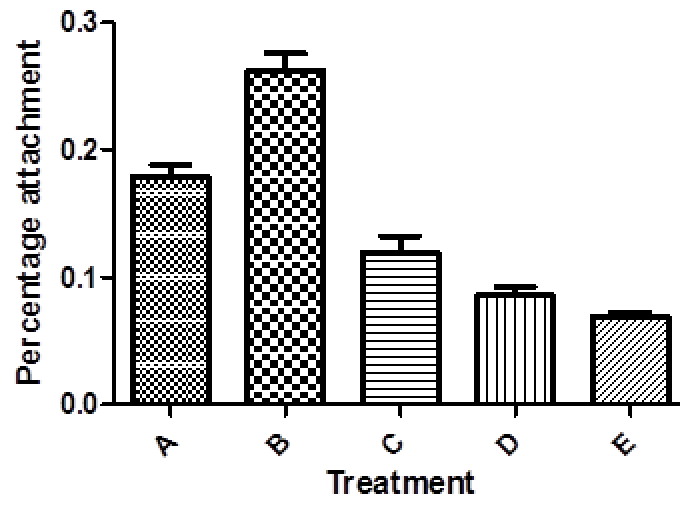


Figure 5



Chapter 3-Summary of my study

F. necrophorum subsp. *necrophorum* has been an economically important bacterium to the cattle industry ever since feed lots have been used to speed up the production of beef to meet the demand of the public for higher amounts of protein to supplement their diets. One way to counteract this bacterium would be to reduce the amount of animal protein consumed by people and replace it with more sustainable protein such as plant proteins. This would reduce the need for animal feedlots, which in turn would decrease the number of cases of *F. necrophorum* subsp. *necrophorum* infections in cattle. Another attractive strategy would be to not feed these animals with highly fermentable grain in feedlots, but give them what they would consume regularly, which would be cattle fodder. Since there is a huge opposition to switch from animal protein to plant protein and since feedlots are in the business of making copious amounts of money, regardless of the cost to the animal on the feedlot or the human who will consume the meat, these animals are fattened using every which method, many of them which could be considered as borderline illegal and unethical. A third strategy would be to produce a vaccine which could successfully prevent the binding of *F. necrophorum* subsp. *necrophorum* to the bovine liver and therefore prevent abscesses from forming.

The in-vitro experiments carried out suggest that this 43kDa OMP is a protein which binds with high affinity with fixed bovine EJG cells. These results suggest that this could be a very important pathogenic mechanism that this bacterium may use to establish initial attachment with EJG cells. Once initial attachment is established, the bacterium could use its vast array of virulence factors to colonize a host.

This protein could lead to a successful vaccine, but at the heart of the matter is that, "Is this the right way to treat the disease?" If it is mainly a management issue, should one not take a

step back and correct what one is doing wrong, instead of try and produce a stop gap method to try and treat this disease? If we do successfully prevent *F. necrophorum* subsp. *necrophorum* from establishing itself and causing an infection, what guarantees are there that a more potent and pathogenic bacteria will not take its place? These are questions which will probably be answered in time and I hope that when these questions are answered, that it is not too late to undo all the harm we are doing to these animals, to humans and to the planet.