THE PENNSYLVANIA STATE UNIVERSITY
SCHREYER HONORS COLLEGE

DEPARTMENT OF VETERINARY AND BIOMEDICAL SCIENCES

FUSOBACTERIUM VARIUM INFECTION IN MICE AS A MODEL FOR THE STUDY OF VACCINE EFFICACY AND IMMUNOGENICITY

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ABSTRACT

Fusobacterium spp. are Gram-negative, obligate anaerobes, that have potentially both beneficial and pathological functions in humans and animals. *Fusobacterium necrophorum* has long been known as a causative agent of necrotic laryngitis, foot rot, and rumenitis-liver abscess complex in cattle, while *Fusobacterium varium* has been known to play a beneficial role as an integral constituent of the gut microflora. Recently, *F. varium* has become known as the most common pathogen to cause necrobacillosis in some white-tailed deer (*Odocoileus viginianus*) populations. Many deer farmers vaccinate their herds with a commercially available *Fusobacterium necrophorum* bacterin vaccine due to a lack of an acceptable alternative; however, many of these farms suffer from rampant cases of necrobacillosis. The objectives of this study were to investigate the effectiveness of four different vaccine preparations in preventing *F. varium* infection in mice. Additionally, the study explored the degree of cross-protection afforded by the vaccines prepared with *F. necrophorum*. Each vaccine *(F. varium* bacterin, *F. varium* toxoid, *F. necrophorum* bacterin, and *F. necrophorum* toxoid) was homogenized with an adjuvant and injected into mice on days 0 and 14. Mice were subsequently experimentally challenged with *F. varium* and *Trueperella (Arcanobacterium) pyogenes* on day 28. The *F. varium* toxoid vaccine induced the most protection in mice against *F. varium* infection, while the *F. necrophorum* toxoid vaccine did not induce any protective effect. This study provided information that suggests the current vaccine used by deer farmers may not be the most protective method against *F. varium* infection.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

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Chapter 1

Introduction

A Gram-negative, obligate anaerobe, *Fusobacterium varium* plays a beneficial role as an integral constituent of the gut microflora (Potrykus et al., 2008). Some recent studies, however, recovered *F. varium* from multiple tissues of farm-raised white-tailed deer with necrobacillosis (Brooks et. al., 2014). This severe septicemic infection results in purulent necrotic lesions that often spread to multiple organs of the body, ultimately causing death (Adler et al., 1990). Recently, *F. varium* was found to comprise a larger proportion of the clinical isolates recovered from gross pulmonary lesions in cases of respiratory tract infections in deer relative to any other *Fusobacterium* species (Brooks et al., 2014). With the exception of decubitus ulcers, *F. varium* isolates from human clinical specimens are somewhat rare, although it has recently been associated with ulcerative colitis as well (Legaria et al., 2005).

Necrobacillosis is of significant economic importance in the white-tailed deer-farming industry because of costs associated with decreases in total inventory. White-tailed deer in particular have been known to be valued at several thousand dollars each. Traditionally, these deer have been prized by a variety of groups such as hunters, outdoorsmen, and conservationists. As a result, the farming of white-tailed deer has become a growing industry in the United States (Hattel et al., 2004; Brooks et al., 2008). Recently, the state of Pennsylvania ranked 2nd and 3rd in the United States in numbers of commercial deer and elk farms and number of deer and elk sold, respectively (PDFA,

2006). Moreover, according to the United States Department of Agriculture, Pennsylvania displayed a 54% increase in farm numbers and a 33% increase in deer numbers between the years 2002 and 2007 (USDA, 2009). Therefore, it is essential to the cervid industry to determine the etiology, pathogenesis, and prevention of necrobacillosis, and subsequent disease conditions, on these farms.

As a result, due to a lack of an acceptable alternative, many deer farmers have responded by vaccinating their herds with the only commercially available *Fusobacterium* vaccine, which is a *F. necrophorum* bacterin. This opportunistic pathogen is a Gram-negative rod requiring anaerobic environmental conditions (Narayanan et al., 2003). *F. necrophorum* has long been known as a causative agent of necrotic laryngitis, foot rot, and rumentitis-liver abscess complex in cattle (Tan et al., 1996). Numerous studies have previously demonstrated the pathogenic effects of *F. necrophorum* in cattle and mice, while very few have been conducted on *F. varium*, as the organism has long been considered nonpathogenic (Mass, 1986).

As a consequence of this discrepancy, the efficacy of this commercial *F. necrophorum* vaccine for use in deer remains unclear and could potentially lead to severe economic turmoil for these farms. In order to investigate this problem, three initial pilot studies were conducted to refine key variables such as mouse strain, inoculation dose, and type of adjuvant. Additionally, the first pilot study revealed that *F. varium* alone could not establish infection in mice. Consequently, a second study determined that co-infection with *Trueperella (Arcanobacterium) pyogenes* was necessary in order to establish infection. *T. pyogenes* is a facultative anaerobe that has been suggested to

utilize oxygen and lower the redox potential to create an anaerobic environment (Tan et. al., 1996). Therefore, the consumption of excess oxygen by a facultative bacterium, such as *T. pyogenes,* seems to be a crucial synergistic property for the establishment of *F.varium*.

This study aims to measure the effectiveness of four different vaccine preparations in the prevention of *F. varium* infection in mice. Additionally, the study explores the degree of cross-protection afforded by the vaccines prepared with *F. necrophorum.*

Chapter 2

Materials and Methods

Culture

The *F. varium* isolate P11V used in this study was initially recovered from the respiratory tract of a white-tailed deer at the Animal Diagnostic Laboratory at The Pennsylvania State University. Identification was based upon the RapID ANA II system (Remel, Lenexa, KS, USA), 16S rDNA sequencing, and biochemical characteristics. The RapID ANA II panel is based on the detection of secreted bacterial enzymes and thus does not require live organisms (Burlage, 1985). It has been found to be an acceptable rapid test system for identifying many of the clinically significant anaerobic bacteria, such as *Fusobacterium* species (Celig, 1991). Cultures were maintained on sheep blood agar plates at 37°C in an anaerobic chamber. The environment of the chamber was mixed anaerobic gas with 5% carbon dioxide, 5% hydrogen, and 90% nitrogen. The *T. pyogenes* isolate used in this study was initially recovered from an abscess in a white-tailed deer at the Animal Diagnostic Laboratory at The Pennsylvania State University. Identification was based upon phenotypic characteristics and then confirmed by the Sensititre system (Trek Diagnostic System, Cleveland, OH, USA).

Vaccines

Ninety eight-week-old CF-1 female mice weighing 19-21 g were randomly divided into six groups of 15 mice each. Mice were allowed a one-week acclimation period prior to any experimental influence. Four of the groups received one of four

different vaccine preparations (*F. varium* bacterin, *F. varium* toxoid, *F. necrophorum* bacterin, *F. necrophorum* toxoid). The autogenous bacterin vaccines containing *F. varium* or *F. necrophorum* were prepared by growing the culture in a tube containing Brucella broth for 24 h at 37°C in an anaerobic chamber. Subsequently, the culture was killed with 0.3% formalin then placed on a shaker at 4°C for 24h; thus, leaving a suspension of dead cellular components for vaccination. The toxoid vaccines of *F. varium* or *F. necrophorum* were prepared in a similar fashion, including an additional step of centrifugation at 13,500 X g for 30 minutes at 4°C and sterile filtration of cellular components through a 0.22 μm membrane filter. Thus, the filtrate contained any inactivated toxins potentially secreted by the bacteria, but no bacterial structural components. All immunogens were emulsified with the Sigma Adjuvant System (Sigma Aldrich Co., St. Louis, MO, USA) according to the manufacturer's instructions. The two control groups received sterile Brucella broth combined with the Sigma Adjuvant System. Each mouse was injected subcutaneously on the back of the neck on days 0 and 14 with 0.2 ml of one of the above preparations. All procedures were conducted in compliance with IACUC protocols approved for this study.

Challenge with F. varium and T. pyogenes

Prior to injection into mice, *F. varium* and *T. pyogenes* were grown to an OD_{600} of approximately 0.8 and 1.4, respectively, in Hungate tubes containing Brain-Heart Infusion (BHI) broth. The culture of *F. varium* was diluted 2-fold with sterile Phosphate Buffered Saline (PBS). Similarly, the *T. pyogenes* culture was diluted 3-fold. The culture of *F. varium* and *T. pyogenes* had a bacterial concentration of 3.5×10^8 CFU/ml

and 5.15×10^{8} CFU/ml, respectively. The final CFU/ml concentrations were determined via spread plating. Dilutions of 10^{-6} through 10^{-8} were plated in duplicate onto sheep blood agar plates and incubated anaerobically at 37°C for 48 h. Colonies were counted and averaged on plates yielding 30-300 colonies. The 0.2 ml of inoculum of *F. varium* and *T. pyogenes* had a bacterial concentration of approximately 7×10^{7} CFU/mouse and 1.03×10^{8} CFU/mouse, respectively. Subsequently, mice in the four treatments and positive control groups were injected intraperitoneally with a 0.2 ml dose of each of these cultures on day 28. Mice in the negative control group received a 0.2 ml dose of sterile BHI broth. In order to record experimental end-points and mortalities, mice were observed twice daily for 4 days post-infection and once daily thereafter for a period of 10 additional days. Mice were euthanized upon reaching a surrogate endpoint defined by observations of labored respiration, loss of ability to ambulate, dehydration, or reduced body condition. Mice that survived for two weeks post-challenge were euthanized, necropsied, and observed for the presence of liver abscesses and peritonitis.

Gross necropsy and histopathology

Following death or euthanasia, necropsies were performed on all mice with emphasis on the abdominal region and hepatic lesions. Representative samples of lung, heart, liver, spleen, and kidney from all mice were placed in 10% neutral buffered formalin. Tissues were processed routinely, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin.

Blood cultures

Approximately 1 ml of heart blood from mice was collected during necropsy in a 3 ml syringe. One drop was injected into tubes containing 5 ml of sterile BHI broth, while the remaining blood was collected into a Microtainer tube (Becton, Dickinson Co., Franklin Lakes, NJ, USA) for subsequent serum separation. BHI tubes with blood samples were incubated in an anaerobic chamber at 37° C for 48 h. These samples were then streaked on sheep blood, Laked Sheep Blood Kanamycin Vancomycin (LKV), and Phenylethyl Alcohol (PEA) agar plates (Remel, Lenexa, KS, USA). Plates were incubated in anaerobic chamber at 37° C for 48 h. Suspected *F. varium* and *T. pyogenes* colonies were identified using known morphology, Gram stain, and RapID ANA II and Sensititre system respectively according to manufacturer's instructions.

Liver cultures

At necropsy, approximately 1 g of liver from each mouse was collected into a Whirl-Pak® bag and homogenized by manually homogenizing the sample in 2 ml of PBS solution under aerobic conditions. Homogenate was then taken inside an anaerobic chamber and streaked on sheep blood, LKV, and PEA agar plates. Plates were incubated at 37 C for 48 h. Suspected *F. varium* and *T. pyogenes* colonies were identified using known morphology, gram stain and RapID ANA II and Sensititre system respectively.

Determination of antibody and cytokine titers

Blood for serum separation was collected from the facial vein of each mouse on days 0, 7, 14, 21, 28, and directly from the heart after death or euthanasia (day 42).

Antibody and cytokine titers for IgG total, IgG2a, IgG1, IgM, IFN-γ, IL-2, and IL-4 were assayed by Ready Set Go® ELISA kits (eBiosciences, Inc., San Diego, CA, USA).

Statistical analysis

Bacterial identification, mortalities, and liver abscess and peritonitis formation data were evaluated by contingency tables. Mortalities were further evaluated via a survivability curve. Standard statistical software (JMP, Cary, NC, USA) was used. For all analyses, a value of $P < 0.05$ was considered significant.

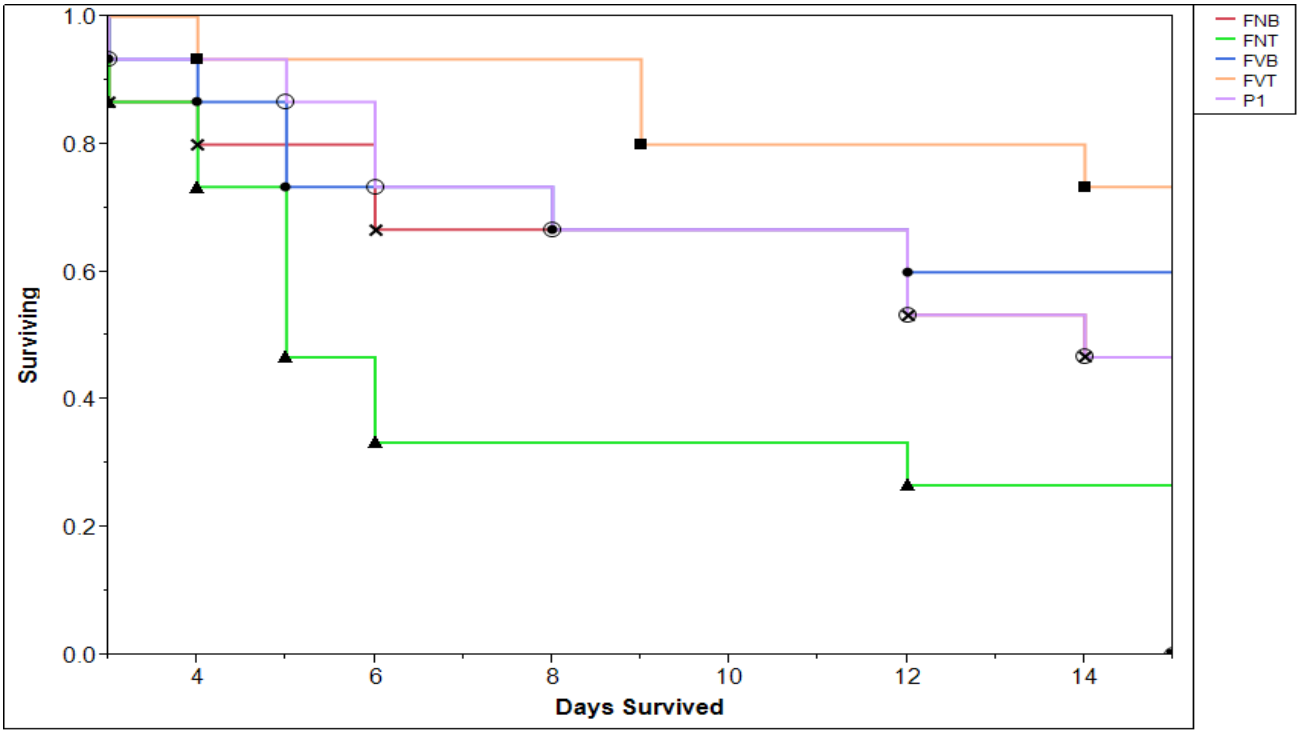
Chapter 3

Results

Survivability

Following the challenge with *F. varium* and *T. pyogenes*, mice in all groups, except the negative control, exhibited mortalities during the two-week period post-challenge. The survivability curve shown below depicts the percent of the total mice surviving on a particular day throughout the two-week period post-infection (Figure 1 and Table 1)

Figure 1: Days survived by treatment groups following intraperitoneal challenge with *F.varium* and *T. pyogenes*



The scale on the y-axis signifies the % of the surviving mice in each group divided by 100. The abbreviations in the legend are described as follows: FNB: *F. necrophorum* bacterin, FNT: *F. necrophorum* toxoid, FVB: *F. varium* bacterin, FVT: *F. varium* toxoid, P1: positive control

Hepatic and peritoneal gross pathology

Of the 90 mice studied, 34 died prior to the two-week period post-challenge. Four out of 15 mice (27%) vaccinated with the *F. varium* bacterin developed either liver abscesses, peritonitis, or both. Two of the 15 mice (13%) that received the *F. varium* toxoid vaccine had either liver abscesses or peritonitis. Five out of 15 mice (33%) vaccinated with the *F. necrophorum* bacterin developed liver abscesses, peritonitis, or both. Seven out of 15 mice (47%) that received the *F. necrophorum* toxoid vaccine had either liver abscesses or peritonitis; one mouse exhibited both conditions. Seven out of 15 mice (47%) in the positive control group developed liver abscesses, peritonitis, or both. None of the mice in the negative-control group died nor developed gross lesions (Table 1).

Table 1. Mortality, liver abscess formation, and peritonitis presence of mice vaccinated with different preparations after experimental challenge with *Fusobacterium varium*

Vaccine Preparations	Number of dead mice	Number of mice with liver abscess (%)	Number of mice with peritonitis (%)	Number of mice with both liver abscess and peritonitis (%)
***F. varium* bacterin**	6/15 (40)[a,b]	3/15 (20)[a]	3/15 (20)[a]	2/15 (13.3)
***F. varium* toxoid**	3/15 (20)[a]	1/15 (6.7)[a]	1/15 (6.7)[a]	0/15 (0)
***F. necrophorum* bacterin**	7/15 (47)[a,b]	2/15 (13.3)[a]	5/15 (33.3)[a]	2/15 (13.3)
***F. necrophorum* toxoid**	11/15 (73)[b]	4/15 (26.7)[a]	3/15 (20)[a]	1/15 (6.7)
Positive Control	7/15 (47)[a,b]	7/15 (46.7)[a]	3/15 (20)[a]	3/15 (20)
Negative Control	0/15 (0)[c]	0/15 (0)[c]	0/15 (0)[c]	0/15 (0)

Within a column, means that do not have the same superscript letters (a-c) differ (P<0.05).

Histopathology

Mice from the group that received the *F. varium* toxoid vaccine displayed the least number of microscopic lesions. From this group, two mice displayed moderate to severe, multifocal, suppurative hepatic lesions. The positive control group, mice that were injected with sterile media as vaccination, displayed the highest number of microscopic lesions. One of the eight mice presented necrotizing suppurative hepatitis, with a large number of neutrophils at the periphery of the portal veins. Four of the eight mice exhibited moderate, multifocal, suppurative hepatitis with bacterial presence at the site of inflammation. Finally, three of the eight mice showed mild, multifocal, suppurative hepatitis. Additionally, three mice in the positive control group displayed mild, multifocal, splenic necrosis.

Table 2: Histopathological analysis of liver and spleen samples

Vaccine Preparation	Number of mice with suppurative hepatitis (%)	Number of mice with suppurative splenitis (%)	Number of mice with gross liver abscess (%)
***F. varium* toxoid**	2/15 (13.3)	0/15 (0)	1/15 (6.7)
Positive Control	8/15 (53.3)	3/15 (20)	7/15 (47)

Identification of F. varium and T. pyogenes in liver tissue and heart blood

F. varium and *T. pyogenes* were both isolated from homogenized liver tissue and heart blood from 33 out of 34 mice (97%) that died during the two-week period post-challenge. Of the 56 mice that survived this period, both *F. varium* and *T. pyogenes* were isolated from homogenized liver tissue from four mice (7%) and heart blood from one

mouse (1.8%). Either *F. varium* or *T. pyogenes* was isolated from homogenized liver tissue from three mice (5%) and heart blood from one mouse (1.8%) (Table 2).

Table 3. Isolates of *F. varium* or *T. pyogenes* recovered from homogenized liver and heart blood in all mice post-mortem

Vaccine Preparations	No. of liver samples		No. of heart blood samples	
	F. varium	***T. pyogenes***	***F. varium***	***T. pyogenes***
***F. varium* bacterin**	$6/15^{a,b}$	$6/15^{a,b}$	$6/15^{a,b}$	$6/15^{a,b}$
***F. varium* toxoid**	$3/15^{a}$	$3/15^{a}$	$3/15^{a}$	$3/15^{a}$
***F. necrophorum* bacterin**	$7/15^{a,b}$	$8/15^{a,b}$	$7/15^{a,b}$	$8/15^{a,b}$
***F. necrophorum* toxoid**	$11/15^{b}$	$11/15^{b}$	$11/15^{b}$	$11/15^{b}$
Positive Control	$9/15^{a,b}$	$7/15^{a,b}$	$7/15^{a,b}$	$7/15^{a,b}$
Negative Control	$0/15^{c}$	$0/15^{c}$	$0/15^{c}$	$0/15^{c}$

Within a column, means that do not have the same superscript letters (a-c) differ (P<0.05).

Antibody and cytokine response in mice

No significant data was generated via Ready Set Go® ELISA analysis of antibody and cytokine titers. Multiple attempts were made to analyze the serum samples using a variety of conditions and dilutions; however, background variability was excessively large to allow for interpretation of the ELISA data.

Chapter 4

Discussion

Multiple responses including mortality, presence of liver abscesses and peritonitis, histopathology, antibody and cytokine titers and bacterial identification in liver and heart blood were considered to evaluate the effectiveness of various vaccine preparations in providing protection against experimental co-infection with *F. varium* and *T. pyogenes*. The *F. varium* toxoid vaccine was an effective immunogen as evidenced by low mortalities (20%) and showed the least number of hepatic lesions and abdominal inflammation. Furthermore, bacterial presence in the livers and heart blood of mice from this group was the lowest relative to the other treatment groups.

The *F. necrophorum* toxoid vaccine was a poor immunogen resulting in 11 mortalities in this group after challenge with *F. varium* and *T. pyogenes*. Moreover, the number of deaths from this treatment group was significantly greater than even the positive control. These results suggest that some secreted factor had a negative influence on mice vaccinated with the *F. necrophorum* toxoid vaccine. It is possible that *F. necrophorum* leukotoxin, an endotoxin known to be secreted by *F. necrophorum*, caused the mice to succumb to endotoxic shock. As a result, the mice may have become more susceptible to infection in comparison to mice that received no vaccination.

In contrast, there is currently no evidence of the leukotoxin gene or leukotoxin production in *F. varium*; therefore, the protective affects elicited by the *F. varium* toxoid vaccine may stem from alternate factors (Brooks et. al., 2014). For example, previous studies have identified butyric acid as a potential virulence factor in *F. varium* (Ohkusa et. al., 2003). Butyric acid has been identified to cause colonic lesions and host inflammatory responses by inducing apoptosis (Okayasu, 2012). Ultimately, this investigation suggests the presence of a secreted virulence factor, other than leukotoxin, within the *F. varium* toxoid vaccine.

Further studies are necessary in order to focus experiments on vaccines prepared with *F. varium*. This study provided information that suggests the current vaccine used by deer farmers may not be the most protective method against *F. varium* infection.

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