CLOSTRIDIUM DIFFICILE IN HEALTHY FOOD ANIMALS AND DEVELOPMENT OF A PCR ASSAY FOR DETECTION IN ENRICHED FOOD AND FECAL SAMPLES

by

STEVEN ALTON LYON

(Under the Direction of Mark A. Harrison)

ABSTRACT

The overall goals of this research were to: 1) determine prevalence of *Clostridium difficile* from healthy beef cattle fecal samples by comparing a single versus a double alcohol shock method using selective (cycloserine-cefoxitin fructose agar [CCFA]) and non-selective (blood agar) media; 2) determine and compare toxigenic profiles of *C. difficile* isolated from feces of healthy swine and cattle and from the dairy cattle environment using PCR targeting *tcdA*, *tcdB*, and *cdtB* genes; and 3) develop a rapid, sensitive, and specific PCR assay to detect *C. difficile* in enriched food and fecal samples. Healthy beef cattle were noted as minor carriers of *C. difficile*. The overall prevalence of *C. difficile* was 6.3% (188/2,965 samples) regardless of method or media used. The single ethanol shock method was significantly better ($P < 0.0001$) at recovery compared to the double shock method for each media tested and across both agars. There were no significant differences between media within each method. Healthy food animals and the dairy environment were sources of toxigenic *C. difficile* strains. *C. difficile* isolates (n=478) from the feces of swine, cattle, and the dairy cattle environment were examined. Toxin genes *tcdA*, *tcdB*, and *cdtB* were identified in 67.4%, 75.7%, and 26.6% of the samples, respectively. Three hundred (62.8%) of 478 isolates were positive for both *tcdA* and *tcdB*. Of

those 300 isolates, 107 (35.7%) were also *cdtB* positive. Dairy (fecal and environmental) and swine isolates were significantly higher in *tcdA* and *tcdB* presence compared to beef isolates. Beef isolates were significantly higher in variant (*tcdA*-, *tcdB*+) strains. The PCR assay developed for *C. difficile* detection from enriched fecal (cattle, swine, broiler) and food (ground chuck, ground turkey, pork sausage) samples was rapid, specific, and sensitive. Detection was observed in ~ 32 h with as few as 20 *C. difficile* cells per 9 ml cycloserine-cefoxitin fructose broth with taurocholate (TCCFB) and at a level of 19 spores per 9 ml TCCFB without enrichment. The assay was specific to *C. difficile* only. This research provides a better understanding of the potential role that food animals play in *C. difficile*-associated disease.

INDEX WORDS: *Clostridium difficile*, alcohol shock, beef, swine, dairy, food, meat, *tcdA*, *tcdB*, *cdtB*, enrichment, PCR

CLOSTRIDIUM DIFFICILE IN HEALTHY FOOD ANIMALS AND DEVELOPMENT OF A PCR ASSAY FOR DETECTION IN ENRICHED FOOD AND FECAL SAMPLES

by

STEVEN ALTON LYON

B. S. A., The University of Georgia, 2004

M. S., The University of Georgia, 2006

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2009

CLOSTRIDUM DIFFICILE IN HEALTHY FOOD ANIMALS AND DEVELOPMENT OF A PCR ASSAY FOR DETECTION IN ENRICHED FOOD AND FECAL SAMPLES

by

STEVEN ALTON LYON

Major Professor:	Mark A. Harrison
Committee:	Paula J. Fedorka-Cray
	J. Stan Bailey
	Joseph F. Frank
	Mark E. Berrang

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
May 2009

DEDICATION

I would like to dedicate this dissertation to my beloved family and friends. It is with your love, encouragement, and support that have made me a better man. To my parents, Gene and Brenda Lyon, I thank you for teaching me about the importance of education and your patience with me as I've made my journey through college. Without you both, this goal of mine would not be possible. I would also like to thank my closest friends and wife, Kendra Bailey, who have meant so much to me over the past 9 years. I'll always cherish the times we spent together downtown Athens, tailgating, and watching the DAWGS win countless games and championships. It truly is 'great to be a Georgia Bulldog!'

ACKNOWLEDGEMENTS

Dr. Mark A. Harrison, I would like to thank you for your guidance and support throughout my tenure here at UGA in both undergraduate and graduate school. I have learned a lot from you as a professor and have enjoyed our conversations regarding SEC sports. Great appreciation is extended to Dr. Paula J. Fedorka-Cray for your support, suggestions, and guiding me through this journey. I thank you very much for taking me as your student and giving me the freedom at work to get the job done. I also want to thank you so very much for hiring me in 2002 to work for the USDA. That decision changed my life professionally as you paid for my education, and personally by me meeting my wife! Dr. J. Stan Bailey and Dr. Mark E. Berrang, thanks for being great bosses while I was employed at RRC and for serving on my committee. The help and the experience you both have given me have been very beneficial. Dr. Joseph F. Frank, thanks for being an excellent teacher to me and for serving on my committee. Last but not least thanks to Dr. Greg Siragusa, Dr. Sutawee Thitaram, Mrs. Johnna Garish, Ms. Meredith Broussard, and Mr. Steven Fini for all the technical help. You guys are the best!

TABLE OF CONTENTS

LIST OF TABLES

Page

CHAPTER 1

INTRODUCTION

Clostridium difficile is an important emerging pathogen of both humans and food animals. The strictly anaerobic bacterium is a gram-positive, spore-forming bacilli that induces disease in susceptible hosts via toxin production. *C. difficile*-associated disease (CDAD) can range from mild diarrhea (antibiotic-associated diarrhea) to fatal colitis (pseudomembranous colitis). CDAD is often associated with humans over the age of 65 with prior exposure to antimicrobials (Keel and Songer, 2006). In food animals, such as swine and dairy cattle, the disease is observed in neonates (Songer, 2004; Hammitt et al., 2008). CDAD develops via the fecal-oral route through the ingestion of inert *C. difficile* spores that contaminate the environment. Once antimicrobials reduce the normal anaerobic flora of the colon, *C. difficile* rapidly grows to dominate this habitat and produces toxins.

C. difficile toxins A (TcdA) and B (TcdB) are two virulence factors that initiate CDAD in susceptible hosts (Keel and Songer, 2006). TcdA is an enterotoxin and TcdB is a potent cytotoxin. Both toxins are classified as large clostridial toxins and induce damage to their host by inhibiting small GTP-binding proteins. This action leads to the disruption of actin filaments resulting in losses of cellular regulation, cell-to-cell contacts, and tight junctions. Host cellular damage is further induced by pro-inflammatory and neural responses. Some strains have recently been reported to produce an additional toxin, a protein binary toxin (CDT). The relation between CDAD and CDT is currently a mystery, but studies with cell culture assays have shown

that CDT induces changes in cellular morphology resulting in cellular death (Perelle et al., 1997).

The epidemiology of CDAD appears to be changing as mortality rates from 1999-2004 have increased as high as 30%, with 20,642 reported deaths (Redelings et al., 2007). Although CDAD has historically been considered a major nosocomial infection, cases have occurred outside the healthcare setting. Community-associated CDAD (CA-CDAD) cases and deaths have been reported in low risk individuals who reported no prior use of antibiotics or healthcare visits (Chernakl et al., 2005; Anonymous, 2008). Young food production animals have recently been reported to be susceptible hosts for CDAD, and highly virulent strains of *C. difficile* have been isolated from contaminated meat products. Furthermore, the emergence of hypervirulent strains, such as North American Pulsed Field Type 1 (NAP1), have proven to be difficult to treat due to increased antibiotic resistance and relapses of CDAD in susceptible patients (McDonald et al., 2005; Warny et al., 2005).

C. difficile is a major cause of enteritis in piglets and neonatal dairy calves. CDAD in these animals can lead to significant losses in production for the pork and dairy industries (Keel et al., 2007). The presence of TcdA and B in infected herds of swine can be as high as 67% of the litters and 35% of individual piglets (Waters et al., 1998; Yaeger et al., 2002). The prevalence in diarrheic dairy calves can reach 39.6% positive for TcdA and B (Porter et al., 2002). However, asymptomatic animals also act as carriers of *C. difficile*. Up to 74% of piglet feces (Yaeger, 2001; Yaeger et al., 2002) and 30.2% of dairy calf feces (Hammitt et al. 2008) have been noted to be TcdA and B positive. Healthy, on-farm swine, dairy cattle, and market poultry may also act as multiplying hosts for toxigenic *C. difficile*. Swine (Thitaram, 2008) and poultry (Simango and Mwakurudza, 2008) have been reported to be major carriers of *C. difficile*.

Furthermore, the dairy environment can be an important source of *C. difficile* spores (Thitaram, 2008). These spores could potentially spread to other animals or to humans. The prevalence of *C. difficile* from healthy beef cattle feces is unknown, as is the toxigenic capabilities of on-farm food animal isolates.

Retail meats are a source of *C. difficile* and may spread *C. difficile* among the community. Up to 29.6% of retail ground meats have been reported as positive for *C. difficile* (Rodriguez-Palacios et al., 2007; Songer, 2007). Contaminated products include ground meats such as beef, veal, pork, and turkey as well as sausages (chorizo, braunschweiger, pork sausage, and summer sausage). Prior contamination of the animal or the processing environment with *C. difficile* spores may lead to contamination of the final product. Given that each American annually consumes 67 pounds of ground beef (Davis and Lin, 2005); there is a distinct possibility of foods being vectors for CDAD. Thoroughly cooking contaminated meat at the recommended temperature (71°C) may not fully destroy *C. difficile* spores (Rodriguez-Palacios et al., 2007). Therefore, ready-to-eat (RTE) products may also pose a risk.

Evidence that *C. difficile* may be transmitted to humans via the food chain has focused on highly similar to indistinguishable types isolated from food animals, foods, and human clinical cases. Ribotype 078/Toxinotype V has been observed to be the most common *C. difficile* type among food animals (Keel et al., 2007; Jhung et al., 2008). This type was also routinely isolated from meats (Rodriguez-Palacios et al., 2007; Songer, 2007) and has been an emerging cause of human CA-CDAD cases (Goorhuis et al., 2008). Furthermore, CA-CDAD cases often involve CDT+ strains, and these strains have been noted to be highly prevalent in food animals (Keel, unpublished). In addition, NAP1 strains have been isolated from retail meat samples (Songer, 2007) indicating that meat sold to consumers has the potential to spread epidemic strains of *C.*

difficile among the community. The evidence of indistinguishable strains isolated from food animals, meats, and humans indicate the potential for *C. difficile* to be a zoonotic and foodborne disease.

Cultural isolation of *C. difficile* from feces and foods can be laborious, expensive, and time consuming, taking up to 5-15 days to produce results (Arroyo et al., 2005; Rodriguez-Palacios et al., 2006, 2007). Culture methods often include a selective procedure involving treating the sample with ethanol or heat to reduce background flora and vegetative cells. The sample is then transferred to an enrichment broth that selects for the germination of the surviving *C. difficile* spores. Isolates are obtained after growth on selective agar. Further characterization tests are required and results can be misinterpreted. As a result, diagnosing CDAD in animals and humans is typically performed by detecting TcdA and B in the feces by ELISA or by cell culture assays (Barbut et al., 2003). Other approaches to detect *C. difficile* have involved PCR targeting the 16S rRNA gene (Gumerlock et al., 1991; Kikuchi et al., 2002) or toxin genes (Kato et al., 1998; Stubbs et al., 2000). PCR based on 16S rDNA offers specific, sensitive, and rapid detection in pure cultures and fecal samples. PCR targeting toxin genes (*tcdA*, *tcdB*, *cdtB*) can be used to classify isolates based on their capability to produce toxins by distinguishing toxigenic strains from non-toxigenic strains.

Additional information is needed to determine if *C. difficile* is a foodborne disease. While there is evidence supporting that contaminated swine, dairy cattle, and meat may transmit the disease to humans, the prevalence of *C. difficile* in healthy, on-farm beef in the U.S. is not known. Also unclear are the toxigenic capabilities of *C. difficile* isolates recovered from healthy swine, dairy cattle, beef cattle, and the dairy environment. Information regarding healthy, on-farm animals may help researchers further understand the food animal's role, if any, with CA-

CDAD. In addition, development of a rapid, specific, and sensitive PCR detection method is needed by veterinarians, researchers, and food processors. Rapid detection of *C. difficile* in food animals would aid in timely diagnosis and treatment by veterinarians and the development and administration of intervention strategies. The rapid detection in food samples would aid processors in evaluating their food safety practices and allow for more testing of foods to determine their role, if any, in human CDAD transmission.

Objectives

The objectives of this dissertation were to:

a) Determine the prevalence of *C. difficile* in feces of healthy beef cows by comparing single and double alcohol shock methods.

b) Determine and compare the toxigenic profiles of *C. difficile* isolated from the feces of healthy swine and cattle using PCR targeting *tcdA*, *tcdB*, and *cdtB* genes.

c) Develop a rapid, specific, and sensitive PCR assay targeting the 16S rRNA gene to detect *C. difficile* in enriched food and fecal samples.

This dissertation is divided into 6 chapters. Chapter 2 is a review of the literature on *C. difficile*. The literature review is focused on the history and characteristics of *C. difficile*, culture methodologies, pathogenesis, CDAD, and prevalence of the pathogen in food animals and foods. In Chapter 3 the study comparing alcohol shocking methods to determine the prevalence of *C. difficile* from healthy beef feces is discussed. In Chapter 4 the study designed to identify toxin A, B, and CDT genes from *C. difficile* isolates recovered from healthy swine, bovine, and dairy environmental samples is presented. Chapter 5 presents the development of a 16S rDNA based PCR assay to detect *C. difficile* in enriched food and fecal samples. A summary and conclusion

of the research is discussed in Chapter 6. The Appendix provides the reader with additional data that was not discussed in Chapter 5. Specifically, it describes a study with methods and results on spore inoculation of meat and fecal samples for *C. difficile* detection by the enrichment PCR procedure.

References

Anonymous. 2008. Surveillance for Community-Associated *Clostridium difficile* --- Connecticut, 2006. MMWR. 57:340-343.

Arroyo, L., J. Rousseau, B. Willey, D. Low, H. Staempfli, A. McGreer, and J. Weese. 2005. Use of a selective enrichment broth to recover *Clostridium difficile* from stool swabs stored under different conditions. J. Clin. Microbiol. 43:5341-5343.

Barbut, F., M. Delmee, J. Brazier, J. Petit, I. Poxton, M. Rupnik, V. Lalande, C. Schneider, P. Mastrantonio, R. Alonso, E. Kuipjer, M. Tvede, and the ESCMID Study Group on *Clostridium difficile* (ESGCD). 2003. A European survey of diagnostic methods and testing protocols for *Clostridium difficile*. Clin. Microbiol. Infect. 9:989-996.

Chernakl, E., C. C. Johnson, A. Weltman, et al. 2005. Severe *Clostridium difficile*-associated disease in populations previously at low risk-four states. MMWR. 54:1201-1205.

Davis, C. and B. Lin. 2005. Factors affecting U.S. beef consumption. Found at www.ers.usda.gov/publications/ldp/Oct05/ldpm13502/ldpm13502.pdf. Accessed 2/12/2009.

Goorhuis, A., S. B. Debast, L. A. M. G. Van Leengoed, C. Harmanus, D. W. Notermans, A. A. Bergwerff, and E. J. Kuijper. 2008. *Clostridium difficile* PCR ribotype 078: an emerging strain in humans and pigs? J. Clin. Microbiol. 46:1157-1158.

Gumerlock, P., Y. Tang, F. Meyers, and J. Silva. 1991. Use of the polymerase chain reaction for the specific and direct detection of *Clostridium difficile* in human feces. Rev. Infect. Dis. 13:1053-1060.

Hammitt, M. C., D. M. Bueschel, M. K. Keel, R. D. Glock, P. Cuneo, D. W. DeYoung, C. Reggiardo, H. Trinh, and J. G. Songer. 2008. A possible role for *Clostridium difficile* in the etiology of calf enteritis. Vet. Microbiol. 127:343-352.

Jhung, M. A., A. D. Thompson, G. E. Killgore, W. E. Zukowski, G. Songer, M. Warny, S. Johnson, D. N. Gerding, L. C. McDonald, and B. M. Limbago. 2008. Toxinotype V *Clostridium difficile* in humans and food animals. Emerg. Infect. Dis. 14:1039-1045.

Kato, H., N. Kato, K. Watanabe, N. Iwai, H. Nakamura, T. Yamamoto, K. Suzuki, S. Kim, Y. Chong, and E. Wasito. 1998. Identification of toxin A-negative, toxin B-positive *Clostridium difficile* by PCR. J. Clin. Microbiol. 36(8):2178-2182.

Keel, M. K. and J. G. Songer. 2006. The comparative pathology of *Clostridium difficile*-associated disease. Vet. Pathol. 43:255-240.

Keel, M. K., J. S. Brazier, K. W. Post, S. Weese, and J. G. Songer. 2007. Prevalence of PCR ribotypes among *Clostridium difficile* isolates from pigs, calves, and other species. J. Clin. Microbiol. 45(6): 1963-1964.

Keel, M. K. 2008. Personal communication.

Kikuchi, E., Y. Miyamoto, S. Narushima, and K. Itoh. 2002. Design of species-specific primers to identify 13 species of *Clostridium* harbored in human intestinal tracts. Microbiol. Immunol. 46(5):353-358.

McDonald, L. C., G. E. Kilgore, A. Thompson, et al. 2005. An epidemic, toxin gene-variant strain of *Clostridium difficile*. N. Engl. J. Med. 353:2433-2441.

Perelle, S., M. Gilbert, P. Bourlioux, G. Corthier, and M. R. Popoff. 1997. Production of a complete binary toxin (actin-specific ADP-ribosyltransferase) by *Clostridium difficile* CD196. Infect. Immun. 65:1402-1407.

Porter, M. C., C. Reggiardo, D. M. Bueschel, M. K. Keel, and J. G. Songer. 2002. Association of *Clostridium difficile* with bovine neonatal diarrhea. In: Proc. 45th Ann. Mtg. Amer. Assoc. Vet. Lab. Diagn., St. Louis, Mo.

Redelings, M., F. Sorvillo, and L. Mascola. 2007. Increase in *Clostridium difficile*-related mortality rates, United States, 1999-2004. Emerg. Infect. Dis. 13:1417-1419.

Rodriguez-Palacios, A., H. Stampfli, T. Duffield, A. Peregrine, L. Trotz-Williams, L. Arroyo, J. Brazier, and S. Weese. 2006. *Clostridium difficile* PCR ribotypes in calves, Canada. Emerg. Infect. Dis. 12:1730-1736.

Rodriguez-Palacios, A., H. Staempfli, T. Duffield, and J. Weese. 2007. *Clostridium difficile* in retail ground meat. Canada. Emerg. Infect. Dis. 13:485-487.

Simango, C. and S. Mwakurudza. 2008. *Clostridium difficile* in broiler chickens sold at market places in Zimbabwe and their antimicrobial susceptibility. Int. J. Food Microbiol. 124:268-270.

Songer, J. G. 2004. The emergence of *Clostridium difficile* as a pathogen of food animals. Animal Health Res. Rev. 5(2):312-326.

Songer, J. 2007. Porcine CDAD. Presented to the National Pork Board Pork Safety Committee on July 30, 2007, Chicago, IL.

Stubbs, S., M. Rupnik, M. Gibert, J. Brazier, B. Duerden, and M. Popoff. 2000. Production of actin-specific ADP-ribosyltransferase (binary toxin) by strains of *Clostridium difficile*. FEMS Microbiol. Letters. 186:307-312.

Thitaram, S. N. 2008. Isolation and characterization of *Clostridium difficile* from porcine and bovine feces. Doctor of Philosophy Dissertation. University of Georgia, Department of Food Science and Technology, Athens, GA.

Warny, M., J. Pepin, A. Fang, et al. 2005. Toxin-production by an emerging strain of *Clostridium difficile* associated with outbreaks of severe disease in North America and Europe. Lancet. 336:1079-1084.

Waters, E. H., J. P. Orr, E. G. Clark, and C. M. Schaufele. 1998. Typhlocolitis caused by *Clostridium difficile* in suckling piglets. J. Vet. Diagn. Invest. 10:104-108.

Yaeger, M. 2001. *Clostridium difficile* and other emerging enteric diseases. Iowa Swine Disease Conference, November 9-10, 2001, Iowa State University, Ames, IA.

Yaeger, M., N. Funk, and L. Hoffman. 2002. A survey of agents associated with neonatal diarrhea in Iowa swine including *Clostridium difficile* and porcine reproductive and respiratory syndrome virus. J. Vet. Diagn. Invest. 14:281-287.

CHAPTER 2

LITERATURE REVIEW

***Clostridium difficile* Characteristics and History**

C. difficile can cause mild to fatal gastroenteritis in susceptible hosts usually post antimicrobial therapy. *C. difficile* is a member of the genus *Clostridium* which are anaerobic, Gram-positive, rod-shaped, spore-forming bacteria capable of producing toxins. Currently there are at least 100 identified strains of *C. difficile* (Durai, 2007). Clostridia species are ubiquitous in the environment and are commonly found in soil at depths void of oxygen and the intestinal tracts of animals. Several species are regarded as foodborne pathogens because of their propensity to be found in contaminated foods. Clostridia are also referred to as 'spoilers' when contaminating canned foods.

C. botulinum and *C. perfringens* both produce toxins capable of causing disease in humans and food animals (Setlow and Johnson, 2001). *C. nigrificans*, *C. bifermentans*, *C. butyricum*, *C. thermosaccharolyticum*, and *C. sporogenes* are involved in the spoilage of improperly thermally processed foods (Setlow and Johnson, 2001). While these clostridia are well established as problematic organisms in the food industry, *C. difficile* is important as a human nosocomial pathogen and agent of disease in neonatal food animals.

C. difficile was first isolated by Hall and O'Toole (1935) from the intestinal flora of newborn infants. They named it *Bacillus difficilus* due to its rod-shaped morphology and difficulty growing under the laboratory conditions available at that time. While *B. difficilus*

produces toxin, it was not considered an important pathogen since infants were asymptomatic carriers. Interestingly, the first described case of pseudomembranous colitis (PMC), a fatal disease now known to be caused by *C. difficile*, was described 42 years earlier (Finney, 1893). Finney observed that a patient had colitis with plaque-like, fibrinous membranes from their stomach to large bowel, and the bowel exhibited areas of hemorrhage and granular exudate. These affected areas or 'plaques' are now recognized as the definitive feature of PMC attributed to *C. difficile* infection (Price and Davies, 1977). During the late 1800's and early 1900's, PMC-like diseases were commonly reported. With the increased use of antibacterial agents in the mid-1900's, PMC cases began increasing again (Hummel et al., 1964). It was during this time that a link between PMC and antibiotic use was considered; however, it was not statistically significant and subsequently ignored (Pettet et al., 1954). As a result, PMC was often misdiagnosed and *Staphylococcus aureus* was implicated as the etiological agent (Pettet et al., 1954).

The etiology of *C. difficile* infection has changed over time. Once considered solely hospital-acquired in humans, *C. difficile* has become an agent of disease outside of the health care setting (Chernakl et al., 2005; Anonymous, 2008). *C. difficile*-associated disease (CDAD) has only recently been reported in food animals. Since 2000, there have been reports of human outbreaks in North America and Europe attributed to hypervirulent strains resulting in increased mortality due to hypertoxin secretion, increased antimicrobial resistance, and the ability to infect healthy (low-risk) people (Warny et al., 2005). As a result of both the food animal association and increase severity of disease, the epidemiological investigation of CDAD infections have increased worldwide.

Spores are an important aspect of the life cycle of clostridia. Microscopic examination of *C. difficile* reveals that vegetative cells harbor a subterminal spore termed an endospore. Once

the mother cell dies and lyses, due to harsh environmental conditions or nutrient starvation, this enclosed spore is released (free spore). Free spores are metabolically dormant but can remain viable in the environment for extremely long periods of time in the absence of nutrients. Once these spores are given the proper stimulus, they can return to a metabolically active state by germinating back into the vegetative form. The process of spore germination involves activation, germination, and outgrowth which can all be achieved within 1 h (Setlow and Johnson, 2001). Vegetative cells then undergo lag, log, and stationary growth phases before eventually dying.

Metabolic changes (morphological, physiological, and biochemical) in vegetative cells are noted after exposure to unfavorable growth conditions (Labbe and Shih, 1997). The sporulation process is an evolutionary adaptation response to the organism's immediate surroundings. The purpose is to preserve the organism's DNA with a series of structures capable of resisting environmental stress until more favorable conditions arise and the organism can grow again. In nature, the lack of a carbon source is the main factor which initiates sporulation. Other conditions that may result in sporulation include unfavorable intrinsic and extrinsic growth factors like temperature, pH, antimicrobial exposure, water activity, and oxygen presence. In vegetative cells, sporulation begins at the onset of the stationary phase with the detection of an unfit environment and the expression of sporulation (*spo*) genes. Sporulation can take from 3–10 h beginning with DNA replication for the new spore (endospore) and ending in the release of the mature spore (free spore) from the mother cell after autolysis. Once conditions are favorable for growth, germination back into the vegetative cell occurs in approximately 20 min.

It is a spore's ability to survive in harsh environments that makes them a problem for food processing and sanitation procedures. Unique small acid soluble proteins stabilize and

protect the DNA in spores from gamma irradiation, freezing, and drying. Heat resistance is due to dipicolonic acid and the low water activity of the spore's core. The sporecoat provides resistance to chemicals used in sanitation protocols. As a result, high concentrations of sanitizers are needed to overcome this resistance. Chlorine has been shown to be effective in reducing *C. difficile* spores in the health care setting by damaging the proteins of the sporecoat, not the DNA itself (Durai, 2007). Dry heat is more effective than wet heat at inactivating spores because it increases protein denaturation. The presence of all these protection mechanisms requires food processors achieve the proper D-value to ensure elimination of foodborne toxigenic and spoilage clostridial spores.

The use of sanitizers, alcohol rinses, UV radiation, and other antimicrobials in healthcare settings may not fully eliminate *C. difficile*. The capacity of *C. difficile* spores to resist killing by these procedures which dilute the spore load (Durai, 2007) is a major reason why it routinely contaminates the hospital environment resulting in transmission to patients. While other non-sporulating pathogens are destroyed, *C. difficile* spores are capable of survival. Sanitation procedures on-farm and in food processing plants may also not fully eliminate *C. difficile* spore contamination. The inability to eliminate spores in these environments increases the chances of food products or food animals becoming contaminated. Strict hand washing with soap and water and the use of chlorine based disinfectants seem to be most effective at reducing the spore load (Monaghan et al., 2008).

The complete genome of *C. difficile* strain 630, a virulent multi-drug resistant strain, was recently sequenced by Sebaihia et al. (2006). The authors noted a circular chromosome (4,290,252 bp) and plasmid (7,881 bp) DNA and genes involving antimicrobial resistance, toxin production, and sporulation were identified. The authors concluded the bacterium was suited for

growth and survival in the gut environment. *C. difficile* is well established as a pathogen of the lower gastrointestinal tract of mammals. The genome sequence of strain 630 helped shed more light on the encoded metabolic capabilities showing adaptation for niche colonization within the gut. Genes encoding pilus biosynthesis and specific binding to the collagen and fibronection of host cells were noted in addition to genes involving secretion of EPS and an S-layer which are important for non-specific host cell attachment. A large number of coding sequences are dedicated to metabolizing various carbohydrates suggesting that *C. difficile* may use a number of different sugars for carbon. The ability to tolerate bile acids was also reported and the authors believed *C. difficile* achieved this in a similar fashion to that of *Listeria monocytogenes*. A bacteriostatic metabolic byproduct of *C. difficile*, called *p*-cresol, gives the strict anaerobe a competitive advantage in the gut.

C. difficile strain 630 has a highly mobile genetic content, with 11% of the genome in the form of mobile genetic elements. This increases the likelihood that genetic exchange (via conjugation, transformation, or transduction) will occur. Seven putative conjugative transposons (CTn1–CTn7) were identified and another mobilizable transposon was found. Also discovered were 2 prophages with similar sequences and a skin element (prophage-like) inserted into the *sigK* gene involved in sporulation. These mobile elements were found to be responsible for *C. difficile* strain 630's acquisition of genes involved with virulence, host interaction, antimicrobial resistance, and surface structure products. Sebaihia et al. (2006) also concluded that the *C. difficile* genome was highly variable compared to other strains. As a result, certain strains of *C. difficile* can become more virulent and/or resistant to antimicrobials.

***Clostridium difficile* Culture, Isolation, and Diagnostic Methods**

The optimum growth temperature for *C. difficile* is 37°C with log phase occurring after 48–72 h of incubation. Compared to *C. perfringens*, *C. difficile* requires more reducing agents for growth, and it sporulates more readily (Songer and Uzal, 2005). *C. difficile* grows slower than aerobic bacteria due to the lower reduction potential of electron acceptors during anaerobic respiration, resulting in less ATP production. As an obligate (strict) anaerobe, vegetative *C. difficile* does not tolerate oxygen and will perish in its presence. Therefore, the use of anaerobic containers (jars, boxes, or bags) or chambers is required for laboratory culture. The gas composition used in anaerobic chambers is 5-10% CO_2, 5-10% H_2, and balanced N_2. In nature, the anaerobic regions of the large bowel of animals, including humans and food animals, is an ideal growth environment (Kelly and Lamont, 1998).

Under ideal growth conditions, *C. difficile* appears as non-motile rods when viewed as a wet mount under a microscope. Under harsh conditions (aerobic, 4°C, nutrient starvation), cell morphology changes and longer, thinner rods are observed. Purple rods (Gram-positive) are noted when performing a Gram-stain. The presence of free spores and endospores on a terminal end can be observed with or without spore staining.

Both selective enrichment broth and agar have been used to recover *C. difficile* from food and fecal samples (Arroyo et al., 2005; Rodriguez-Palacios et al., 2006, 2007). George et al. (1979) developed cycloserine-cefoxitin fructose agar (CCFA) and egg yolk medium that was selective for *C. difficile* and differentiated it between other anaerobes. CCFA currently contains lysed horse blood (for extra enrichment) instead of egg yolk because *C. difficile* is lecithinase and lipase negative (Marler et al., 1992). Arroyo et al. (2005) noted that CCFA was also effective at resuscitating *C. difficile* spores. Cycloserine-cefoxitin fructose broth supplemented

with 0.1% sodium taurocholate (TCCFB) is an enrichment broth used for *C. difficile* recovery. Taurocholate, a bile salt, induces spore germination (Wilson, 1983). Cycloserine and cefoxitin are antimicrobials that inhibit peptidoglycan synthesis. The innate resistance of *C. difficile* to these cephalosporins allows for their survival in the media while other susceptible anaerobes of the microflora are reduced. Typical post-incubation *C. difficile* colonies appear as swarming, flat, rough, are non-hemolytic, and 4–8 mm in diameter on CCFA (George et al., 1979).

Treating fecal and food samples through use of an ethanol or heat shock step has been shown to be an effective strategy at reducing unwanted background biota and induce *C. difficile* sporulation (Marler et al., 1992). Ethanol treatment of samples (mixed in a 1:1 vol/vol) was found to be more effective than shocking the sample with heat (70°C for 20 min). Clabots et al. (1989) found that human fecal samples treated with 100% ethanol prior to culture increased recovery rates of *C. difficile* in asymptomatic patients. A significant difference ($P < 0.005$) in sensitivity was reported when comparing fecal samples subjected to alcohol shock versus those that did not. Arroyo et al. (2005) used an alcohol shocking method post-enrichment to recover *C. difficile* from stool swabs. The authors observed that inoculating TCCFB and then alcohol shocking prior to plating significantly improved recovery compared to direct plating without the use of enrichment broth ($P < 0.001$). The authors described this selective technique as a sensitive method for the optimal recovery of spores, even after prolonged storage of the samples at room and refrigerated temperatures.

While a single alcohol (post-enrichment) shock has been successful for recovery (Rodriguez-Palacios et al., 2007), an additional shock performed prior to enrichment has been investigated. Using this double alcohol (pre-and post-enrichment) shock method, Rodriguez-Palacios et al. (2006) were successful in *C. difficile* recovery from symptomatic and

asymptomatic dairy calves. A comparison between a single alcohol and a double alcohol shock method was recently evaluated by Thitaram (2008) on the recovery of *C. difficile* from healthy swine and bovine fecal samples and the dairy environment. While the double shock procedure was significantly better at recovery from swine fecal ($P = 0.0004$) and dairy environmental ($P = 0.05$) samples, there was no significant difference using double shock on the dairy fecal samples. This suggests that the use of different shocking methodologies for isolation may be needed for different sample types in order to ensure optimal *C. difficile* recovery.

Current laboratory culture methods for *C. difficile* can be labor intensive, expensive, and time consuming, taking up to 15 days to produce results (Arroyo et al., 2005; Rodriguez-Palacios et al., 2006, 2007). Once a presumptive *C. difficile* colony is obtained, further testing is required for confirmation. Typical confirmation tests include the use of long-wave UV light to observe yellow/green fluorescence of the colonies (Knoop et al., 1993), presence of a horse dung odor, a typical Gram-stain appearance, and the detection of L-proline aminopeptidase activity (Rodriguez-Palacios et al., 2006). Gas-liquid chromatography may also be used to detect the presence of isocaproic acid or *p*-cresol (D'Ari and Barkes, 1985).

Isolates capable of producing TcdA and B (A+B+) are denoted as toxigenic while isolates incapable of producing TcdA and B (A-B-) are deemed non-toxigenic. Some strains are able to produce TcdB while not being able to produce TcdA (A-B+) due to mutations in the sequence of the TcdA gene (*tcdA*) (Kato et al., 1998). These strains are identified as variant strains and are still capable of causing disease. A third toxin, a binary cytolethal distending toxin (CDT), produced by some *C. difficile* strains may also be used in toxigenic classification. Detecting toxins A (TcdA) and B (TcdB) in feces is accomplished by use of enzymatic immunoassays, such as ELISA, or by use of cell culture (Barbut et al., 2003). In addition to commercial

availability, ELISA is rapid, sensitive, and specific. It can be used to detect TcdA and B in stool samples with a sensitivity of 71-94% and specificity of 92-98% (Pothoulakis and LaMont, 1993; Whittier et al., 1993).

Cellular cytotoxicity assays, which can take up to 2 days, are not as rapid as ELISA but offer better sensitivity (94-100%) and specificity (99%) (Pothoulakis and LaMont, 1993; Kelly and LaMont, 1998). Cultured HeLa, rhesus monkey kidney, and/or human fibroblast cells are exposed to fecal matter and TcdB induces a cytopathic effect. When *C. sordelli* antitoxins in the assay neutralize these effects, a diagnosis of CDAD is confirmed (Durai, 2007). Cytotoxin detection frequency increases as the severity of symptoms, duration, and pathology increases (Knoop et al., 1993).

Polymerase chain reaction (PCR) is a molecular alternative for the detection of bacterial genes. PCR assays for *C. difficile* are targeting either the toxin genes or 16S rRNA. Besides a rapid and specific detection (Matsuki et al., 2002), there are other advantages for using rRNA as the detection target. For example, each bacterial cell contains around 10,000 ribosomes compared to only 4 copies of chromosomal DNA (Watson et al., 1987). Therefore, primers targeting rRNA are more sensitive than primers targeting DNA. Another advantage of using 16S rRNA is that this gene has been highly conserved over time and contains many of the organism's housekeeping genes (Huysman and De Wachter, 1986). 16S rRNA is an excellent target for detection in complex ecosystems like that of feces and foods.

PCR assays with 16S rRNA targeting primers have been successful in differentiating *C. difficile* from other *Clostridium* spp. found in fecal samples. Gumerlock et al. (1991) developed an upstream primer targeting segment I of *C. difficile* 16S rRNA gene and a downstream primer targeting a highly conserved region. This assay amplified a 270 bp target and was used for the

direct and specific detection of *C. difficile* in human feces. Detection with as few as 10 *C. difficile* cells among 10^6 *E. coli* cells was reported. Also, the assay successfully discriminated against *C. sordelli* and *C. bifermentans*. Wilson et al. (1988) also developed species specific primers to distinguish between *C. difficile*, *C. bifermentans* and *C. sordelli*. These authors used 16S rRNA probes to accurately identify the clostridia even though the rRNAs of these three species were 97–98% similar in their sequences. Kikuchi et al. (2002) designed species-specific primers that were specific enough to distinguish between 13 known species of *Clostridium* that reside in the human intestines. The use of real-time PCR assays and a 16S rRNA target has also been successful in detecting and quantifying *C. difficile* (Rinttila et al., 2004, Tonooka et al., 2005; Fallani et al., 2006). 16S rRNA-based PCR methods have also been used to rapidly detect clostridia on raw poultry (Wang et al., 1994).

Toxin genes can also be identified using PCR as it is important to describe the types of strains isolated from human clinical and food animal isolates. *C. difficile* isolates may be classified based on their toxigenicity through toxin gene identification or toxin production. Kato et al. (1991) and Tang et al. (1994) used PCR to identify *C. difficile* isolates that are capable of producing TcdA. Kato et al. (1991) engineered an assay that differentiated between toxigenic and non-toxigenic isolates based on amplifications of the repeating and non-repeating regions of *tcdA*. Tang et al. (1994) were also able to create a rapid, specific, and sensitive PCR assay for detecting *tcdA*. Identification of the TcdB gene (*tcdB*) based on a non-repeating region of the gene has also been described by Kato et al. (1998). Detection of CDT can be performed by detecting *cdtA*, the enzymatic portion, or *cdtB*, the binding portion of the gene (Stubbs et al., 2000).

PCR amplification from fecal enrichment broth samples has been shown to be effective at detecting DNA from foodborne pathogens (Freschi et al., 2005; Levi and Towner, 2005) including *Clostridium* spp. (Dahlenborg et al., 2001; Tansuphasiri et al., 2005). Tansuphasiri et al. (2005) was able to detect phospholipase C (*plc*) and enterotoxin (*cpe*) genes from *C. perfringens* at a minimum of 10^4 cfu/ml enrichment broth. Dahlenborg et al. (2001) published a study describing a PCR enrichment method to detect *C. botulinum* types B, E, and F neurotoxin genes from slaughtered pig feces. The test was rapid (18 h) and sensitive (10 spores/g of fecal sample from type B, and 10^3 spores/g fecal sample from types E and F).

Detection of DNA from foodborne pathogens in enriched food samples has also been investigated (Fach et al., 1995, Lindqvist, 1997; Lantz et al., 1998). A common problem with PCR from enriched food and fecal samples (not an isolated colony) is the presence of inhibitors found in these complex matrices. PCR inhibitors may work by inactivating thermostable DNA polymerase, interfering with cell lysis, and degradation of nucleic acids (Wilson, 1997). *Taq* DNA polymerase may be the most important target site by inhibitors such as proteinases. Other inhibitors in foods and feces include bile salts (Lantz et al., 1997) and complex polysaccharides (Monteiro et al., 1997). Currently, no information is available on the detection of *C. difficile* from enriched fecal and food samples by use of PCR.

***Clostridium difficile* Pathogenesis**

CDAD in susceptible humans and animals is transmitted via the fecal-oral route. Contact with the contaminated environment, fomites, or carriers of *C. difficile* (animals and people) can lead to the ingestion of spores. In the stomach, spores are capable of survival due to their innate resistance to gastric acidity. Upon entering the small bowel, spores germinate into vegetative

cells when exposed to bile acids (Kelly and LaMont, 1998). *C. difficile* expresses peritrichous flagella to facilitate their movement and a polysaccharide capsule to evade phagocytosis. Growth continues in the colon and the pathogen attaches to colonic epithelium via fibronectin binding. Strains capable of causing CDAD usually produce TcdA and B (Songer and Anderson, 2006) which are secreted by attached cells just beginning the stationary growth phase (Dupuy and Sonenshein, 1998). TcdA and B are the two main virulence factors of *C. difficile* (Keel and Songer, 2006). These toxins are encoded by *tcdA* and *tcdB* located on a pathogenicity locus (PaLoc) found in chromosomal DNA (Cohen et al., 2000). TcdA is a potent enterotoxin with a large molecular weight of 308 kDa (Fiorentini and Thelestam, 1991). TcdB, a potent cytotoxin, is the second largest known bacterial toxin with a molecular weight of 270 kDa (Drudy et al., 2007).

TcdA and B work synergistically to cause CDAD in susceptible organisms by damaging the cytoskeleton of host cells. TcdA binds to a carbohydrate ligand followed by its internalization and activation within a host cell endosome (Wolfhagen et al., 1994) and inhibits small GTP-binding proteins Rho, Rac and Cdc42 in the cytoplasm of target cells by glycosylation of threonine (Drudy et al., 2007). As a result, actin filaments are disrupted leading to the loss of cellular function, tight junctions, and cell-to-cell contacts. Receptor sites for TcdB are unknown but it is hypothesized that it recognizes receptors on the basolateral side of enterocytes (Wolfhagen et al., 1994). With the loss of tight junctions from the action of TcdA, TcdB is thought to move between host cells towards the basement membrane and further damages cells (Lima et al, 1988) via glycosylation, neural stimulation, and induction of an inflammatory response. The inflammatory response from macrophages and monocytes results in further tissue damage, cellular necropsis, protein loss, and fluid exudation leading to diarrhea

(Canny et al., 2006). An additional component of TcdA is the disruption of mitochondria leading to ATP depletion and the generation of reactive oxygen intermediates (He et al., 2000). This results in an oxidative burst that may initiate the pro-inflammatory response (He et al., 2002). The neural reaction from TcdB exposure in the intestinal lumen causes mast cell degranulation (Keel and Songer, 2006). CDAD results when these multiple factors are initiated leading to host cell rounding and eventually apoptosis (Kim et al., 2007; Nottrott et al., 2007).

CDT is an actin-specific ADP-ribosyltransferase produced by some *C. difficile* strains that causes disruptions in the actin cytoskeleton but is unrelated to TcdA and B (Perelle et al., 1997). CDT is encoded by *cdtA* and *cdtB* which are located on the chromosome but not on the PaLoc. The role of CDT in CDAD is less certain than that of TcdA and B. However, cytopathic effects on cell culture is observed (Gulke et al., 2001) and suggests that CDT may be an additional virulence factor. This binary toxin is comprised of two units; a binding portion (CDTb) and an active enzymatic portion (CDTa). CDTa depolymerizes actin filaments leading to cytoskeletal disruption which changes the cell morphology leading to cellular death.

Clostridium difficile-associated Disease (CDAD)

CDAD is a disease of the distal intestinal tract (cecum and/or colon) typically occurring post-antimicrobial therapy (Keel and Songer, 2006) and ranges from mild diarrhea to fatal colitis. CDAD is an important disease which has proved difficult to control as case-fatality rates have been rising since 2005 (Zilberberg et al., 2008). A recent report (Redelings et al., 2007) showed that from 1999-2004, CDAD was reported as a cause of death for 20,642 people in the U. S. *C. difficile*-related deaths rose from 5.7 deaths per million population in 1999 to 23.7

deaths per million population in 2004. This report noted that CDAD caused more deaths than all other gastrointestinal diseases combined.

Two to 15% of adults harbor toxigenic *C. difficile* strains without showing CDAD signs or symptoms (Knoop et al., 1993). The carriage rate in infants may be as high as 50% (Bolton et al., 1984). However, CDAD is uncommon in infants perhaps due to the lack of toxin receptors (Borriello and Wilcox., 1998). Although CDAD is uncommon in infants, it is a major cause of enteritis in neonatal swine (Songer, 2006) and calves (Rodriguez-Palacios et al., 2006; Hammitt et al., 2008). The presence of toxin receptors and the absence of a developed microflora are thought to contribute to clinical illness.

Typical clinical signs and symptoms of CDAD include profuse watery (often non-bloody) diarrhea, abdominal pain/cramps, fever, leukocytosis, anorexia, nausea, and malaise (Kuijper et al., 2007). Patient feces may contain mucus, pus, and/or blood. CDAD is responsible for roughly 25% of all antibiotic-associated diarrheas (AAD) (Bartlett, 1992). The disease often advances to a more severe state represented by pseudomembranous colitis (PMC). PMC is confirmed via endoscopy where typical yellow patches of mucosal inflammation (pseudomembranes) on the intestinal epithelium are observed. Patients with PMC who encounter little to no diarrhea may develop toxic megacolon and a paralytic ileus. Toxic megacolon results in dilation of the colon whereas paralytic ileus results in the loss of colonic muscle tone. The colon may become perforated. PMC, ileus, and perforated colon are referred to as 'fulminant CDAD' and getting antibiotics to the infected site is difficult. Surgical removal of the colon may be performed to decrease the chance of death. Mortality rates for toxic megacolon can reach as high as 40% with reoccurrence of AAD in up to 40% of patients. In

addition, patients may develop systemic sepsis and die of septic shock and multi-organ failure (Kuijper et al., 2007).

CDAD outbreaks involving increased mortality and relapse rates have occurred in North America and Europe due to the emergence of a hypervirulent strain of *C. difficile* (McDonald et al., 2005; Warny et al., 2005). This strain is referred to as B1 (by restriction endonuclease analysis), North American pulsed-field type 1 (NAP1), PCR Ribotype 027, and Toxinotype III. *In vitro* studies of B1/NAP1/027 indicate that these strains hypersecrete both TcdA and B (Warny et al., 2005). Increased toxin production is hypothesized to occur due to an 18 bp deletion in the *tcdC* gene. B1/NAP1/027 strains also produce CDT. Besides increased ability to secrete toxins, B1/NAP1/027 strains show a high level of resistance to fluroquinolones (ciprofloxacin, gatifloxacin, and moxifloxacin). Resistance to this class of antimicrobials may be attributed to years of over prescribing. According to the CDC (2008), in 2005, 16 U.S. states were confirmed to have had CDAD cases involving B1/NAP1/027 strains. However, as of 2008, the number of states increased to 40. B1/NAP1/027 strains have caused CDAD epidemics worldwide and concerns are extending beyond the healthcare setting into the community.

Community acquired CDAD (CA-CDAD) cases are those occurring outside of the healthcare environment. The changing epidemiology of *C. difficile* strains, like B1/NAP1/027, is causing disease in healthy people with no previously known risk factors or healthcare exposure. CA-CDAD in 4 U.S. states was investigated in 2005. It was reported that there were 7.6 cases per 100,000 population (Chernakl et al., 2005) and severe CA-CDAD was confirmed in healthy people and peripartum women. From these patients, 24% reported no previous antimicrobial use 3 months prior to infection. Only 2 isolates were recovered and both were toxigenic (A+, B+, and CDT+), while 1 harbored the 18 bp *tcdC* deletion. In 2008, 456 cases of CDAD were

reported with 241 (53%) confirmed as community-acquired (Anonymous, 2008). Within this group, 25% of the patients had no underlying conditions and no inpatient healthcare exposures for 12 months prior to infection.

Known reservoirs of *C. difficile* within the community include household pets. Cats (30%) and dogs (21%) have been reported to be transient carriers of *C. difficile* (Borriello et al., 1983). Other sources include food animals and retail meat (Rodriguez-Palacios et al., 2007; Songer 2007). Considering CDAD is initiated by the ingestion of spores, increasing cases within the community may also be due to increased spore contamination within these environments. Animals and people may serve as vectors for *C. difficile* transmission.

Various host factors also play a significant role in CDAD. The most significant and common risk factor is the disruption of the colonic microflora by broad-spectrum antimicrobials. The most common antimicrobials that disrupt the natural microflora of the large bowel are cephalosporins, amoxicillin, fluoroquinolones, and clindamycin (Mohan et al., 2006). However, virtually any antimicrobial can result in an increased risk of acquiring CDAD (Keel and Songer, 2006). Indigenous microflora may employ various mechanisms to exclude *C. difficile* and other nonindigenous pathogens. These include bacteriocins, competition for nutrients and adhesion sites, production of volatile acids and H_2S, induction of an immune response, and stimulation of peristalsis.

During antimicrobial therapy, anaerobic bacteria of the normal microflora are reduced. *C. difficile* is then able to recolonize the anaerobic regions of the colon sooner than repopulation of normal microflora can occur; resistance to antimicrobials may also facilitate colonization. *C. difficile* spores in the intestinal tract during or after therapy may play a role (Kelly and LaMont,

1993). With the lack of a competing microflora, *C. difficile* quickly establishes itself as the dominant bacterium. As a result, *C. difficile* toxins are secreted and CDAD may occur.

Another major risk factor for CDAD is the amount of time patients spend in hospitals where spore densities may be high. Antimicrobial use and sanitation within the hospital predisposes individuals to possible spore ingestion and colonization. Immunosuppressed individuals also are at risk due to the lack of antibodies (IgA) against *C. difficile* toxins (Durai, 2007). Other risk factors include age, diet, and the use of proton pump inhibitors. The use of proton pump inhibitors (PPI) has become an established risk factor for CA-CDAD (Dial et al., 2005) because ingested vegetative *C. difficile* cells may survive in the stomach due to an increased pH. Humans over the age of 65 are the most susceptible (Monaghan et al., 2008). Conversely, in food animals, neonates like piglets and calves are susceptible to CDAD (Rodriguez-Palacios et al., 2006; Songer and Anderson, 2006). Diets high in saturated fats, cholesterol, dietary casein, and corn starch caused increased CDAD in hamsters after antimicrobial treatment (Michelich et al., 1981; Blankenship-Paris et al., 1995).

Treatment of CDAD may or may not include antimicrobial therapy. If antimicrobials are to be used, the following are recommended, one-dose prophylaxis, avoid broad-spectrum antibiotics when possible, restrict intravenous antibiotics, use automatic stop dates, and employ an antibiotic pharmacist (Anonymous, 2007). The first step in curbing CDAD is to isolate the infected person, discontinue the inciting antibiotic, and switch to other therapies. Metronidazole, a nitrofuran that breaks down to a toxic nitrogenous compound among anaerobic respiration and breaks DNA nonspecifically, is the first drug of choice for treatment of CDAD (Durai, 2007). Metronidazole is reasonably priced but may not be 100% effective. The second drug of choice is vancomycin. Vancomycin (given orally) is a glycopeptide that is effective at killing Gram-

positive bacteria as it inhibits cell wall synthesis. It is more effective than metronidazole. However, the patient may be at an increased risk for vancomycin-resistant *Enterococcus* infection. Other effective drugs used include rifampicin, teicoplanin, and linezolid (Durai, 2007). While use of antimicrobials can resolve the disease, relapse due to reinfection occurs in 10-20% of patients (Pothoulakis and LaMont, 1993) and may be attributed to previous use of proven antimicrobials (Wilcox, 1996).

C. difficile can acquire multi-drug resistance. The complete genome sequencing of strain 630 showed resistances to various classes of antibiotics used in both human and animal therapy (Sebaihia et al., 2006). Several resistance genes (*ermB*, *tetM*, and *catD*) were located on conjugative transposons (Tn). Erythromycin resistance is due to *ermB* which encodes for methylation of the target (23S rRNA). It is located on Tn5398 which also encodes for macrolide, lincosamide, and streptogramin resistance (MLS-R). Tn5397 carries *tetM* which encodes for ribosomal protection proteins conferring tetracycline resistance. Tn4453 carries *catD* (chloramphenicol acetyltransferase) which confers resistance to chloramphenicol by chemically modifying the drug into an inactive form. It is possible for *C. difficile* to acquire resistance to rifampicin and metronidazole (Brazier et al., 2001) and there have been reports of *C. difficile* strains resistant to vancomycin (Pituch et al., 2005). Other coding sequences contribute to innate resistance to bacitracin, cephalosporins, and lantibiotics (Sebaihia et al., 2006).

Non-antimicrobial treatment is often employed to prevent relapses. The combination of probiotics and prebiotics has shown to be an effective alternative to antibiotic therapy. Probiotics are beneficial by several means; they prevent *C. difficile* colonization, adhesion, and invasion. Some may have antimicrobial activity (bacteriocins or acidic products), and they initiate an immune response (Doron and Gorbach, 2006). For example, *Lactobacillus* spp. is

antagonistic to *C. difficile* due to H_2O_2 and lactic acid production. Another example of an effective probiotic is *Saccharomyces boulardii* (McFarland et al., 1994). Prebiotics, or non-digestible oligosaccharides, serve as nutrients to stimulate the growth of probiotics. Normally pro- and prebiotics are orally administered. However, fecal bacteriotherapy is another method of inducing beneficial bacteria to prevent CDAD relapse. In this method, fecal enemas are prepared from healthy donors prior to administration (Tvede and Rask-Madsen, 1989). Other non-antimicrobial options include antibody and vaccines based treatments against TcdA and B (Monaghan et al., 2008).

Food Animals and Contaminated Foods

Infection with *C. difficile* affects production in the pork and cattle industries. Proper treatment in neonates, especially use of antibiotic therapy in a timely manner, can reduce production costs. Songer (2004), suggests that tiamulin, virginiamycin, and tylosin in sow feed may limit *C. difficile* colonization while tylosin given parenterally to piglets may be useful therapeutically.

Perhaps the most important uncontrolled cause of neonatal diarrhea in piglets is due to *C. difficile* (Songer and Anderson, 2006). CDAD has been reported as a major cause of neonatal piglet enteritis since 2000 and was first observed after accidental exposure of gnotobiotic pigs to *C. difficile* (Olson et al., 1994). Jones and Hunter (1983) were the first to observe natural CDAD in pigs. The disease was characterized by PMC and the presence of *C. difficile*.

CDAD develops in piglets at 1 to 7 days of age. Piglets can appear dyspneic and have distended abdomens, scrotal edema, and diarrhea (Waters et al., 1998). Mortality rates as high as

16% have been reported and outbreaks are often associated with respiratory distress due to the build up of ascites and severe mesocolonic edema (Waters et al., 1998; Kyne et al., 2000). Upon pathological examination, moderate to severe edema of the mesocolon and pasty-to-watery yellowish contents of the large intestines are observed (Songer and Uzal, 2005). Reports of sampling CDAD infected herds (> 1,500 piglets) indicate that approximately 67% of litters and 35% of individual pigs harbor toxigenic strains and are TcdA and B-positive (Waters et al., 1998; Yaeger et al., 2002). However, piglets without diarrhea may also harbor toxigenic strains (Yaeger 2001, 2002).

Evidence also points to *C. difficile* as a major cause of neonatal calf diarrhea (NCD) as dairy calves serve as multiplying hosts for this pathogen. Porter et al. (2002) concluded that TcdA and B were present in more than 25% of the calves, and of those, 40% were positive for both toxins. In 2006, Rodriguez-Palacios et al. were able to obtain isolates of *C. difficile* in stools from 31 (11.2%) of 278 dairy calves; 11 (7.6%) of 144 were from diarrheic calves while 20 (14.9%) of 134 were from asymptomatic calves. Toxins were also detected in the feces of 39.6% of the calves with diarrhea and 20.9% of the asymptomatic calves. In both cases, these differences were statistically significant ($P \leq 0.0009$).

Conversely, while Hammitt and coworkers (2008) found similarities in the rates of *C. difficile* isolation from asymptomatic calves (12.7% versus 14.9%) they observed a higher prevalence in diarrheic calves (25.3% versus 7.6%) and higher rates of toxin detection in asymptomatic calves (30.2% versus 20.9%). The authors' results for toxin detection in diarrheic calves were substantially lower (22.9% versus 39.6%). No reasons were given for the apparent discrepancies between these two studies.

Thitaram (2008) determined that market aged swine were carriers of *C. difficile*. The prevalence was reported to be 15.9% in healthy, on-farm porcine fecal samples versus 2.4% in dairy cattle feces. This prevalence is lower than that reported for the dairy calves (12.7% [Hammitt et al., 2008] and 14.9% 9 [Rodriguez-Palacios et al., 2006]). Reasons why *C. difficile* has been reported at higher levels in neonates may be due to its ability to colonize, proliferate, and produce toxins in animals with a less developed colonic microflora (Rodriguez-Palacios et al., 2006). Dairy farm environmental samples were also examined for *C. difficile* presence (Thitaram, 2008). She reported a prevalence of 8.6% and concluded that the environment can be a potential source of infection.

Avian species also carry *C. difficile.* Simango (2006) isolated *C. difficile* from free range chicken fecal samples in rural Zimbabwe and found 20 (17.4%) of 115 samples to be positive. Of these 20 isolates, 11 (55%) were positive for toxins A and B. Simango and Mwakurudza (2008) were able to recover *C. difficile* in 29 (29%) of 100 chicken fecal samples from a market place in urban Zimbabwe. These authors noted that 26 (89.7%) of these 29 isolates were positive for toxins A and B. Twenty-two soil samples near the birds were found to be positive for *C. difficile* and 95.5% (21/22) of these isolates were toxigenic (A+B+). The authors concluded that chickens were indeed a major reservoir of *C. difficile. C. difficile* has also been isolated from other avian species including ducks, geese, parakeets (Borriello et al., 1983) and ostrich chicks (Shivaprasad, 2003).

It is important to understand that food animals have the ability to shed toxigenic *C. difficile* vegetative cells and spores regardless of age or enteric disease status. In fact, it is quite common for asymptomatic animals to shed toxigenic strains into the environment (Songer,

2004). Once the environment is contaminated with spores, transmission to other hosts (human or animal) is likely.

Given that food animals are carriers of *C. difficile*, risks of acquiring CA-CDAD through the consumption and handling of contaminated foods has been studied. Results suggest that foods are a rich source of *C. difficile*. Rodriguez-Palacios et al. (2007) were the first to positively identify and isolate *C. difficile* spores from retail ground beef and veal samples intended for human consumption. In that study, *C. difficile* was obtained from 12 (20%) of 60 meat samples; 1 (14.3%) of 7 ground veal samples and 11 (20.8%) of 53 ground beef samples. Molecular typing was performed and 11 were classified as toxigenic. Of those, 8 (67%) were Toxinotype III (produced TcdA, B and CDT). Additionally, the authors reported that 3 (27%) of the 11 isolates were indistinguishable by PFGE from human isolates recovered in Ontario. Weese et al. (2005) found *C. difficile* in commercial turkey-based raw dog food and this isolate was also indistinguishable from human isolates.

Songer (2007) evaluated retail meats for the presence of *C. difficile*. Eighty-one ground meat samples were obtained from three grocery stores three times during monthly intervals. *C. difficile* was isolated from 9 (30%) of 30 ground beef samples, 1 (20%) of 5 summer sausage samples, 2 (33.3%) of 6 ground pork samples, 7 (38.8%) of 18 braunschweiger samples, 2 (25%) of 8 chorizo samples, 2 (18.2%) of 11 pork sausage samples, and 1 (11.1%) of 9 ground turkey samples. Overall, *C. difficile* was found in 24 (29.6%) of 81 meat samples. Isolates were characterized by PCR ribotyping, toxinotyping, toxin presence, and PFGE. Two isolates from braunschweiger were Ribotype 027, Toxinotype III, expressed toxin A, B and binary genes, and exhibited the PFGE pattern NAP1. One ground beef and one summer sausage isolate were also characterized as B1/NAP1/027.

C. difficile spores from feces may also contaminate food throughout slaughter and processing. Spores may also be located in skeletal muscle tissue which has been observed for other clostridial species (Vengust et al., 2003). Properly cooking raw meat destroys pathogens and vegetative cells but preformed *C. difficile* spores may survive. The heat resistance of *C. difficile* spores was recently investigated (Rodriguez-Palacios et al., 2007). Spores survived in ground beef cooked to recommended temperatures (71°C) and were still viable after holding at that temperature for 120 min. Further investigations are warranted to confirm that *C. difficile* contaminated meat, even post-cook, may serve as a vehicle for epidemic human CDAD infections.

Animals harbor pathogens which can be transmitted to humans via direct or indirect contact with animals, through contamination of the environment, or through food (Steinmuller et al., 2006). CA-CDAD may be included as a foodborne disease. Indistinguishable types of *C. difficile* isolates from animals, foods, and human clinical isolates have been reported. This includes PCR Ribotype 078/Toxinotype V (TcdA+, TcdB+, CDT+) which has been commonly found in swine and bovine samples.

Jhung et al. (2008) suggest that Toxinotype V may be a relatively common source of CA-CDAD. This toxigenic type has been reported to be increasing in human CDAD cases. While historically uncommon in humans, it is commonly found in animal CDAD cases. These strains have an 18 or 39 bp deletion in the *tcdC* gene sequence which allows them to hypersecrete their toxins in a fashion similar to B1/NAP1/027 strains. In a study comparing animal and human isolates, Jhung et al. (2008) noted that the food animal isolates (piglets and calves) shared a high degree of similarity with human isolates, and 2 isolates were indistinguishable. The authors suggested possible reasons for these types occurring in both humans and in food animals which

include: (1) exposure of humans and animals to a common *C. difficile* source; (2) direct contact and environmental contact; or (3) consumption of contaminated produce, water, or meat from infected animals.

Keel et al. (2007) performed a similar study and noted that PCR Ribotype 078 was most common among bovine (94%) and swine (83%) isolates. Only 4.4% of the human clinical isolates were this type. While PCR ribotype diversity is high in humans, it is low in food animals. Type 078 was predominant in both piglets and calves even though the swine were sampled in different U.S. regions (Southeast, West, and Midwest) and strict biosecurity practices were maintained. The authors were confident of zero interspecies contact. The only common factor noted was that both animals were in close contact with their human caretakers. The authors suggested that food animals may be exposed to various types of *C. difficile*; however, swine and cattle are more susceptible to colonization by Type 078.

While Keel et al. (2007) observed a low incidence of Type 078 in humans, Goorhuis et al. (2008) reported that Type 078/Toxinotype V was the third most prevalent type in certain Dutch hospitals, and that its prevalence was greater in Europe than in the U.S. The authors noted Type 078 has been the most frequently isolated *C. difficile* type among swine in the Netherlands. In addition, they reported Type 078/Toxinotype V was responsible for CA-CDAD in 54% of patients and that this type was a frequent cause of CA-CDAD in the Netherlands.

CA-CDAD often involves *C. difficile* strains that produce CDT in addition to TcdA and B. It has been reported that CDT+ strains are highly prevalent in piglets (83%) and calves (100%) (Keel, unpublished) but have been historically low in humans (1.6-10%) (Rupnik, 2007). However, CDT+ strains in human cases are increasing and are associated with CA-CDAD. Evidence indicates that foods and food animals are contaminated with *C. difficile* types capable

of causing disease and death in humans outside the healthcare setting. Types common among animals are becoming increasingly more prevalent in humans, suggesting that the emergence of CA-CDAD may be associated with consumption of contaminated meats or contact with carriers.

References

Anonymous. 2007. HPA Regional Microbiology Network: a good practice guide to control *Clostridium difficile*. Health Protection Agency.

Anonymous. 2008. Surveillance for Community-Associated *Clostridium difficile* --- Connecticut, 2006. MMWR. 57:340-343.

Arroyo, L., J. Rousseau, B. Willey, D. Low, H. Staempfli, A. McGreer, and J. Weese. 2005. Use of a selective enrichment broth to recover *Clostridium difficile* from stool swabs stored under different conditions. J. Clin. Microbiol. 43:5341-5343.

Barbut, F., M. Delmee, J. Brazier, J. Petit, I. Poxton, M. Rupnik, V. Lalande, C. Schneider, P. Mastrantonio, R. Alonso, E. Kuipjer, M. Tvede, and the ESCMID Study Group on *Clostridium difficile* (ESGCD). 2003. A European survey of diagnostic methods and testing protocols for *Clostridium difficile*. Clin. Microbiol. Infect. 9:989-996.

Bartlett, J. G. 1992. Antibiotic-associated diarrhea. Clin. Infect. Dis. 15:573-581.

Blankenship-Paris, T. L., B. J. Walton, Y. O. Hayes, and J. Chang. 1995. *Clostridium difficile* infection in hamsters fed an atherogenic diet. Vet. Pathol. 32:269-273.

Bolton, R. P., S. K. Tait, P. R. Dear, and M. S. Losowsky. 1984. Asymptomatic neonatal colonization by *Clostridium difficile*. Arch. Dis. Child. 59:466-472.

Borriello, S., P. Honour, T. Turner, and F. Barclay. 1983. Household pets as potential reservoir for *Clostridium difficile* infection. J. Clin. Pathol. 36:84-87.

Borriello, S. P. and M. H. Wilcox. 1998. *Clostridium difficile* infections of the gut: the unanswered questions. J. Antimicrob. Chemother. 41 (Suppl C):67-69.

Brazier, J. S., W. Fawley, J. Freeman, et al. 2001. Reduced susceptibility of *Clostridium difficile* to metronidazole. J. Antimicrob. Chemother. 48:741-742.

Canny, G., D. Drudy, P. Macmathuna, C. O'Farrelly, and A. W. Baird. 2006. Toxigenic *Clostridium difficile* induced inflammatory marker expression by human intestinal epithelial cells is asymmetrical. Life Sci. 79:920-925.

Chernakl, E., C. C. Johnson, A. Weltman, et al. 2005. Severe *Clostridium difficile*-associated disease in populations previously at low risk-four states. MMWR. 54:1201-1205.

Clabots, C., S. Gerding, M. Olson, L. Peterson, and D. Gerding. 1989. Detection of asymptomatic *Clostridium difficile* carriage by an alcohol shock procedure. J. Clin. Microbiol. 27:2386-2387.

Cohen, S., Y. Tang, and J. Silva Jr. 2000. Analysis of the pathogenicity locus in *Clostridium difficile* strains. J. Infect. Dis. 181:659-663.

Center for Disease Control. 2008. www.cdc,gov/ncidod/dhqp/ppt/State_Map_NAP1_10_2008.ppt. Accessed 2/13/09.

D'Ari, L., and H. A. Barkes. 1985. *p*-Cresol formation by cell-free extracts of *Clostridium difficile*. Arch. Microbiol. 143:311-312.

Dahlenborg, M., E. Borch, and P. Radstrom. 2001. Development of a combined selection and enrichment PCR procedure for *Clostridium botulinum* Types B, E, and F and its use to determine prevalence in fecal samples from slaughtered pigs. Appl. Environ. Microbiol. 67:4781-4788.

Dial, S., J. A. Delaney, A. N. Barkun, and S. Suissa. 2005. Use of gastric acid-suppressive agents and the risk of community-associated *Clostridium difficile*-associated disease. JAMA. 294:2989-2995.

Doron, S. and S. L. Gorbach. 2006. Probiotics: their role in the treatment and prevention of disease. Expert Rev. Anti. Infect. Ther. 4:261-275.

Drudy, D., S. Fanning, and L. Kyle. 2007. Toxin A-negative, toxin B-positive *Clostridium difficile*. Int. J. Infect. Dis. 11:5-10.

Dupuy, B. and A. L. Sonenshein. 1998. Regulated transcription of *Clostridium difficile* toxin genes. Mol. Microbiol. 27:107-120.

Durai, R. 2007. Epidemiology, pathogenesis, and management of *Clostridium difficile* infection. Dig. Dis. Sci. 52:2958-2962.

Fach, P., M. Gilbert, R. Griffais, J. P. Guillou, and M. R. Popoff. 1995. PCR and gene probe identification of botulinum neurotoxin A-, B-, E-, F-, and G- producing *Clostridium* spp. and evaluation in food samples. Appl. Environ. Microbiol. 61:389-392.

Fallani, M., L. Rigottier-Gois, M. Aguilera, C. Bridonneau, A. Collignon, C. Edwards, G. Corthier, and J. Dore. 2006. *Clostridium difficile* and *Clostridium perfringens* species detected in infant faecal microbiota using 16S rRNA targeted probes. J. Microbiol. Meth. 67:150-161.

Finney, J. M. T. 1893. Gastroenterostomy for cicatrizing ulcer of the pyloris. Bull. Johns Hopkins Hosp. 4:53-64.

Fiorentini, C. and M. Thelestam. 1991. *Clostridium difficile* toxin A and its effects on cells. Toxicon. 29:543-567.

Freschi, C., L. Carlvao, and C. de Oliveria. 2005. Comparison of DNA-extraction methods and selective enrichment broths on the detection of *Salmonella typhimurium* in swine feces by polymerase chain reaction (PCR). Braz. J. Microbiol. 36:363-367.

George, W. L., V. L. Sutter, D. Citron, and S. M. Finegold. 1979. Selective and differential medium for isolation of *Clostridium difficile*. J. Clin. Microbiol. 9:214-219.

Goorhuis, A., S. B. Debast, L. A. M. G. Van Leengoed, C. Harmanus, D. W. Notermans, A. A. Bergwerff, and E. J. Kuijper. 2008. *Clostridium difficile* PCR ribotype 078: an emerging strain in humans and pigs? J. Clin. Microbiol. 46:1157-1158.

Gulke, I., G. Pfeifer, J. Liese, M. Fritz, F. Hofmann, K. Atories, and H. Barth. 2001. Characterization of the enzymatic component of the ADP-ribosyltransferase toxin CDTa from *Clostridium difficile*. Infect. Immun. 69:6004-6011.

Gumerlock, P., Y. Tang, F. Meyers, and J. Silva. 1991. Use of the polymerase chain reaction for the specific and direct detection of *Clostridium difficile* in human feces. Rev. Infect. Dis. 13:1053-1060.

Hall, I. C. and E. O'Toole. 1935. Intestinal flora in newborn infants with a description of a new pathogenic anaerobe, *Bacillus difficillis*. Am. J. Dis. Child. 49:390-402.

Hammitt, M. C., D. M. Bueschel, M. K. Keel, R. D. Glock, P. Cuneo, D. W. DeYoung, C. Reggiardo, H. Trinh, and J. G. Songer. 2008. A possible role for *Clostridium difficile* in the etiology of calf enteritis. Vet. Microbiol. 127:343-352.

He, D., S. J. Hagen, C. Pothoulakis, M. Chen, N. D. Medina, M. Warny, and J. T. LaMont. 2000. *Clostridium difficile* toxin A causes early damage to mitochondria in culture cells. Gastroenterology. 119:139-150.

He, D., S. Sougioultzis, S. Hagen, J. Liu, S. Keates, A. C. Keates, C. Pothoulakis, and J. T. LaMont. 2002. *Clostridium difficile* toxin A triggers human colonocyte Il-8 release via mitochondrial oxygen radical generation. Gastroenterology. 122:1048-1057.

Hummel, R. P., W. A. Altemeier, and E. O. Hill. 1964. Iatrogenic staphylococcal enterocolits. Ann. Surg. 160:551-562.

Huysman, E. and R. De Wachter. 1986. Compilation of small ribosomal subunit RNA sequences. Nucleic Acid Res. 14 (Suppl):r73-r118.

Jhung, M. A., A. D. Thompson, G. E. Killgore, W. E. Zukowski, G. Songer, M. Warny, S. Johnson, D. N. Gerding, L. C. McDonald, and B. M. Limbago. 2008. Toxinotype V *Clostridium difficile* in humans and food animals. Emerg. Infect. Dis. 14:1039-1045.

Jones, M. A. and D. Hunter. 1983. Isolation of *Clostridium difficile* from pigs. Vet. Rec. 112:253.

Kato, N., C. Y. Ou, H. Kato, S. L. Bartley, V. K. Brown, V. R. Dowell, Jr., and K. Uneo. 1991. Identification of toxigenic *Clostridium difficile* by the polymerase chain reaction. J. Clin. Microbiol. 29:33-37.

Kato, H., N. Kato, K. Watanabe, N. Iwai, H. Nakamura, T. Yamamoto, K. Suzuki, S. Kim, Y. Chong, and E. Wasito. 1998. Identification of toxin A-negative, toxin B-positive *Clostridium difficile* by PCR. J. Clin. Microbiol. 36:2178-2182.

Keel, M. K. and J. G. Songer. 2006. The comparative pathology of *Clostridium difficile*-associated disease. Vet. Pathol. 43:255-240.

Keel, M. K., J. S. Brazier, K. W. Post, S. Weese, and J. G. Songer. 2007. Prevalence of PCR Ribotypes among *Clostridium difficile* isolates from pigs, calves, and other species. J. Clin. Microbiol. 45:1963-1964.

Keel, M. K. 2008. Personal communication.

Kelly, C. P. and J. T. LaMont. 1993. Treatment of *Clostridium difficile* diarrhea and colitis. Pages 199-212 in: Gastrointestinal Pharmacotherapy. M. M. Wolfe, ed. W. B. Saunders Co., Philadelphia, PA.

Kelly, C. P. and J. T. LaMont. 1998. *Clostridium difficile* infection. Ann. Rev. Med. 49:375-390.

Kikuchi, E., Y. Miyamoto, S. Narushima, and K. Itoh. 2002. Design of species-specific primers to identify 13 species of *Clostridium* harbored in human intestinal tracts. Microbiol. Immunol. 46:353-358.

Kim, H., S. H. Rhee, C. Pothoulakis, and J. T. Lamont. 2007. Inflammation and apoptosis in *Clostridium difficile* enteritis is mediated by PGE2 up-regulation of Fas Ligand. Gastroenterology. 133:875-886.

Knoop, F. C., M. Owens, and I. C. Crocker. 1993. *Clostridium difficile*: Clinical disease and diagnosis. Clin. Microbiol. Rev. 6:251-265.

Kuijper, E. J., J. T. van Dissel, and M. H. Wilcox. 2007. *Clostridium difficile*: Changing epidemiology and new treatment options. Current Opinions in Infect. Dis. 20:376-383.

Kyne, L., M. Warny, A. Qamar, and C. P. Kelly. 2000. Asymptomatic carriage of *Clostridium difficile* and serum levels of IgG antibody against toxin A. N. Eng. J. Med. 10:342-390.

Labbe R. G. and N-J. R. Shih. 1997. Physiology of sporulation of Clostridia. Pages 21-32 in: The Clostridia: Molecular Biology and Pathogenesis. J. I. Rood, B A. McClane, J. G. Songer, and R. W. Titball, eds. Academic Press, San Diego, CA.

Lantz, P. -G., M. Matsson, T. Wadstrom, and P. Radstrom. 1997. Removal of PCR inhibitors from human fecal samples through the use of an aqueous two-phase system for sample preparation prior to PCR. J. Microbiol. Methods. 28:159-167.

Lantz, P. -G., R. Knutsson, Y. Blixt, W. A. Al-Soud, E. Borch, and P. Radstrom. 1998. Detection of pathogenic *Yersinia enterocolitica* in enrichment media and pork by a multiplex PCR: a study of sample preparation and PCR-inhibitory components. Int. J. Food Microbiol. 45:93-105.

Levi, K. and K. Towner. 2005. Rapid detection of methicillin-resistant *Staphylococcus aureus* from screening enrichment broths from clinical samples. Eur. J. Clin. Micro. Infect. Dis. 24:423-427.

Lima, A., D. Lyerly, T. Wilkins, D. Innes, and R. Guerrant. 1988. Effects of *Clostridium difficile* toxins A and B in rabbit small and large intestine in vivo and on cultured cells in vitro. Infect. Immun. 56:582-588.

Lindqvist, R. 1997. Preparation of PCR samples from food by a rapid and simple centrifugation technique evaluated by detection of *Escherichia coli* O157:H7. Int. J. Food Microbiol. 37:73-82.

Marler, L. M., J. A. Siders, L. C. Wolters, Y. Pettigrew, B. L. Skitt, and S. D. Allen. 1992. Comparison of five cultural procedures for isolation of *Clostridium difficile* from stools. J. Clin. Microbiol. 30:514-516.

Matsuki, T., K. Watanabe, J. Fujimoto, Y. Miyamoto, T. Takada, K. Matsumoto, H. Oyaizu, and R. Tanaka. 2002. Development of 16S r RNA-gene targeted group-specific primers for the detection and identification of predominant bacteria in human feces. Appl. Environ. Microbiol. 68: 5445-5451.

McFarland, L. V., C. M. Surawicz, R. N. Greenberg, et al. 1994. A randomized placebo-controlled trial of *Saccharomyces boulardii* in combination with standard antibiotics for *Clostridium difficile* disease. JAMA. 271:1913-1918.

McDonald, L. C., G. E. Kilgore, A. Thompson, et al. 2005. An epidemic, toxin gene-variant strain of *Clostridium difficile*. N. Engl. J. Med. 353:2433-2441.

Michelich, V. J., O. Nunez-Montiel, G. S. Schuster, F. Thompson, and V. R. Dowell, Jr. 1981. Diet as a coadjuvant for development of antibiotic-associated diarrhea in hamsters (*Mesocricetus auratus*). Lab. Anim. Sci. 31:259-262.

Mohan, S. S., B. P. McDermott, S. Parchuri, and B. A. Cunha. 2006. Lack of value of repeat stool testing for *Clostridium difficile* toxin. Am. J. Med. 119:356.

Monaghan, T., T. Boswell, and Y. R. Mahida. 2008. Recent advances in *Clostridium difficile*-associated disease. Gut. 57:850-860.

Monteiro, L., D. Bonnemaison, A. Vekris, K. G. Petry, J. Bonnet, R. Vidal, J. Cabrita, and F. A. Megraud. 1997. Complex polysaccharides as PCR inhibitors in feces: *Helicobacter pylori* model. J. Clin. Microbiol. 35:995-998.

Nottrott, S., J. Schoentaube, H. Genth, I. Just, and R. Gerhard. 2007. *Clostridium difficile* toxinA-induced apoptosis is p53-independent but depends on glucosylation of Rho GTPases. Apoptosis. 12:1443-1453.

Olson, M. M., C. J. Shanholtzer, J. T. Lee, Jr., and D. N. Gerding. 1994. Ten years of prospective *Clostridium difficile*-associated disease surveillance and treatment at the Minneapolis VA Medical Center, 1982-1991. Infect. Control and Hospital Epidemiol. 15:371-381.

Perelle, S., M. Gilbert, P. Bourlioux, G. Corthier, and M. R. Popoff. 1997. Production of a complete binary toxin (actin-specific ADP-ribosyltransferase) by *Clostridium difficile* CD196. Infect. Immun. 65:1402-1407.

Pettet, J. D., A. H. Baggenstoss, W. H. Dearing, and E. S. Judd, Jr. 1954. Postoperative pseudomembranous enterocolitis. Surg. Gynecol. Obstet. 8:546-552.

Pituch, H., P. Obuch-Woszczatynski, D. Wultanska, F. Meisel-Mikolajczyk, and M. Luczak. 2005. A survey of metronidazole and vancomycin resistance in strains of *Clostridium difficile* isolated in Warsaw, Poland. Anaerobe. 11:197-199.

Pothoulakis, C. and J. T. LaMont. 1993. *Clostridium difficile* colitis and diarrhea. Gastroenterology Clinics of N. America. 22:623-637.

Porter, M. C., C. Reggiardo, D. M. Bueschel, M. K. Keel, and J. G. Songer. 2002. Association of *Clostridium difficile* with bovine neonatal diarrhea. In: Proc. 45th Ann. Mtg. Amer. Assoc. Vet. Lab. Diagn., St. Louis, Mo.

Price A. B. and D. R. Davies. 1977. *Pseudomonas* colitis. J. Clin. Pathol. 30:1-12.

Redelings, M., F. Sorvillo, and L. Mascola. 2007. Increase in *Clostridium difficile*-related mortality rates, United States, 1999-2004. Emerg. Infect. Dis. 13:1417-1419.

Rinttila, T., A. Kassinen, E. Malinen, L. Krogius, and A. Palva. 2004. Development of an extensive set of 16S rDNA-targeted primers for quantification of pathogenic and indigenous bacteria in faecal samples by real time PCR. J. Appl. Microbiol. 97:1166-1177.

Rodriguez-Palacios, A., H. Stampfli, T. Duffield, A. Peregrine, L. Trotz-Williams, L. Arroyo, J. Brazier, and S. Weese. 2006. *Clostridium difficile* PCR ribotypes in calves, Canada. Emerg. Infect. Dis. 12:1730-1736.

Rodriguez-Palacios, A., H. Staempfli, T. Duffield, and J. Weese. 2007. *Clostridium difficile* in retail ground meat. Canada. Emerg. Infect. Dis. 13:485-487.

Rupnik, M. 2007. Is *Clostridium difficile*-associated infection a potentially zoonotic and foodborne disease? Clin. Microbiol. Infect. 13:457-459.

Sebaihia, M., B. W. Wren, P. Mullany, et al. 2006. The multidrug-resistant human pathogen *Clostridium difficile* has a highly mobile, mosaic genome. Nature Genetics. 38:779-786.

Setlow, P. and E. A. Johnson. 2001. Spores and their significance. Pages 33-61. Food Microbiology Fundamentals and Frontiers, 2nd Ed. M. P. Doyle, L. R. Beuchat, and T. J. Montville, eds. ASM Press, Washington, D.C.

Shivaprasad, H. 2003. Hepatitis associated with *Clostridium difficile* in an ostrich chick. Avain Pathol. 35:57-62.

Simango, C. 2006. Prevalence of *Clostridium difficile* in the environment in a rural community in Zimbabwe. Transactions of the Royal Society of Tropical Med. and Hygiene. 100:1146-1150.

Simango, C. and S. Mwakurudza. 2008. *Clostridium difficile* in broiler chickens sold at market places in Zimbabwe and their antimicrobial susceptibility. Int. J. Food Microbiol. 124:268-270.

Songer, J. G. 2004. The emergence of *Clostridium difficile* as a pathogen of food animals. Animal Health Res. Rev. 5:312-326.

Songer, J. G. and M. A. Anderson. 2006. *Clostridium difficile*: an important pathogen of food animals. Anaerobe. 12:1-4.

Songer, J. 2007. Porcine CDAD. Presented to the National Pork Board Pork Safety Committee on July 30, 2007, Chicago, IL.

Songer, J. G. and M A. Anderson. 2006. *Clostridium difficile*: An important pathogen of food animals. Anaerobe. 12:1-4.

Songer, J. and F. Uzal. 2005. Clostridial enteric infections in pigs. J. Vet. Diagn. Invest. 17:528-536.

Steinmuller, N., L. Demma, J. B. Bender, M. Eidson, and F. J. Angulo. 2006. Outbreaks of enteric disease associated with animal contact: not just a foodborne problem anymore. Clin. Infect. Dis. 43:1596-1602.

Stubbs, S., M. Rupnik, M. Gibert, J. Brazier, B. Duerden, and M. Popoff. 2000. Production of actin-specific ADP-ribosyltransferase (binary toxin) by strains of *Clostridium difficile*. FEMS Microbiol. Letters. 186:307-312.

Tang, Y. J., P. H. Gumerlock, J. B. Weiss, and J. Silva Jr. 1994. Specific detection of *Clostridium difficile* toxin A gene sequences in clinical isolates. Mol. Cell. Probes. 8:463-467.

Tansuphasiri, U., C. Chanyasanha, and N. Cheaochantanakij. 2005. An enrichment broth culture-duplex PCR combination assay for the rapid detection of enterotoxigenic *Clostridium perfringens* in fecal specimens. Southeast Asian J. Trop. Med. Public Health. 36:1229-1238.

Thitaram, S. N. 2008. Isolation and characterization of *Clostridium difficile* from porcine and bovine feces. Doctor of Philosophy Dissertation. University of Georgia, Department of Food Science and Technology, Athens, GA.

Tonooka, T., S. Sakata, M. Kitahara, M. Hanai, S. Ishizeki, M. Takada, M. Sakamoto, and Y. Benno. 2005. Detection and quantification of 4 species of the genus *Clostridium* in infant feces. Microbiol. Immunol. 49:987-992.

Tvede, M. and J. Rask-Madsen. 1989. Bacteriotherapy for chronic relapsing *Clostridium difficile* diarrhoea in six patients. Lancet. 1:1156-1160.

Vengust, M., L. G. Arroyo, J. S. Weese, H. R. Baird, and J. D. Baird. 2003. Preliminary evidence for dormant clostridial spores in equine skeletal muscle. Equin. Vet. 35:514-516.

Wang, R., W. Cao, W. Franklin, W. Campbell, C. Cerniglia. 1994. A 16S rDNA-based PCR method for rapid and specific detection of *Clostridium perfringens* in food. Mol. Cell. Probes 8:131-138.

Warny, M., J. Pepin, A. Fang, et al. 2005. Toxin-production by an emerging strain of *Clostridium difficile* associated with outbreaks of severe disease in North America and Europe. Lancet. 336:1079-1084.

Waters, E. H., J. P. Orr, E. G. Clark, and C. M. Schaufele. 1998. Typhlocolitis caused by *Clostridium difficile* in suckling piglets. J. Vet. Diagn. Invest. 10:104-108.

Watson, J., N. Hopkins, J. Roberts, J. Steitz, and A. Weiner. 1987. Molecular biology of the gene, vol. 1. Benjamin/Cummings Publishing Co., New York.

Weese, J., J. Rousseau, and L. Arroyo. 2005. Bacteriological evaluation of commercial canine and feline raw diets. Can. Vet. J. 46:513-516.

Whittier, S., D. S. Shapiro, and W. F. Kelly. 1993. Evaluation of four commercially available enzyme immunoassays for laboratory diagnosis of *Clostridium difficile*-associated disease. J. Clin. Microbiol. 31:2861-2865.

Wilcox, M. H. 1996. Cleaning up *Clostridium difficile* infection. Lancet. 348:767-768.

Wilson, K. 1983. Efficacy of various bile salt preparations for stimulation of *Clostridium difficile* spore germination. J. Clin. Microbiol. 18:1017-1019.

Wilson, K., R. Blitchington, B. Hindenach, and R. Greene. 1988. Species-specific oligonucleotide probes for rRNA of *Clostridium difficile* and related species. J. Clin. Microbiol. 26:2484-2488.

Wilson, I. G. 1997. Inhibition and facilitation of nucleic acid amplification. Appl. Environ. Microbiol. 63:3741-3751.

Wolfhagen, J. H. M., R. Torensma, A C. Fluitt, and J. Verhoef. 1994. Toxins A and B of *Clostridium difficile*. FEMS Microbiol. Rev. 13:59-64.

Yaeger, M. 2001. *Clostridium difficile* and other emerging enteric diseases. Iowa Swine Disease Conference, November 9-10, 2001, Iowa State University, Ames, IA.

Yaeger, M., N. Funk, and L. Hoffman. 2002. A survey of agents associated with neonatal diarrhea in Iowa swine including *Clostridium difficile* and porcine reproductive and respiratory syndrome virus. J. Vet. Diagn. Invest. 14:281-287.

Zilberberg, M. D., A. F. Shorr, and M. H. Kollef. 2008. Increase in adult *Clostridium difficile*-related hospitalizations and case-fatality rates, United States, 2000-2005. Emerg. Infect. Dis. Jun; [Epub ahead of print].

CHAPTER 3

COMPARISON OF A SINGLE VERSUS A DOUBLE ALCOHOL SHOCK METHOD FOR THE ISOLATION OF *CLOSTRIDIUM DIFFICILE* FROM HEALTHY BEEF FECAL SAMPLES[1]

---

[1] S. A. Lyon, M. A. Harrison, P. J. Fedorka-Cray, J. S. Bailey, S. N. Thitaram, and G. R. Siragusa. To be submitted to Foodborne Disease and Pathogens

Abstract

Emerging evidence suggests that food animals may serve as reservoirs for *Clostridium difficile* and that contaminated meat may be a transmission vector for *C. difficile*-associated disease (CDAD). We investigated the prevalence of *C. difficile* in healthy, on-farm beef cattle feces by comparing two separate alcohol shock methods. One method consisted of a single alcohol shock administered post-enrichment in cycloserine-cefoxitin fructose broth supplemented with 0.1% sodium taurocholate (TCCFB). The second method involved a double alcohol shock performed pre-and-post enrichment in TCCFB. Samples were plated onto tryptic soy agar with 5% sheep blood (BA) and cycloserine-cefoxitin fructose agar supplemented with 7% lysed horse blood (CCFA) for isolation. The prevalence of *C. difficile* was compared using the Chi-square test for independence. *C. difficile* was isolated from 188 (6.3%) of 2,965 samples from either of the two methods regardless of plating agar. The single alcohol shock was significantly better for recovery compared to the double shock method. However, there were no significant differences between the plating agars within each treatment method. These results indicate that beef cattle are minor carriers of *C. difficile,* and a single alcohol shock method may be better at spore recovery in animals known to be minor carriers.

Keywords: *Clostridium difficile*, alcohol shock, *Clostridium difficile*-associated disease, beef cattle, food animals

Introduction

Clostridium difficile is an important nosocomial, Gram-positive, spore-forming, pathogenic bacterium responsible for intestinal diseases in humans (Warny et al., 2005) and other mammals including food production animals (Keel and Songer, 2006; Rodriquez-Palacios et al., 2006). *C. difficile*-associated disease (CDAD) can result in mild to severe diarrhea, colitis, and fatal diseases like pseudomembraneous colitis (PMC) and toxic megacolon, which can be brought about from antimicrobial treatment (Rupnik, 2007). Toxins A (enterotoxin) and B (cytotoxin) are the main virulence factors (Keel and Songer, 2006) resulting in CDAD. Highly virulent strains also produce a cytolethal distending toxin. *C. difficile* toxins destroy the colon epithelium leading to disease and/or death. Recently, human CDAD outbreaks have been increasing in incidence and severity due to the emergence of a hypervirulent strain designated NAP1 (pulsed-filed gel electrophoresis [PFGE] pattern), BI (restriction enzyme analysis [REA], toxinotype III (pathogenicity locus sequence by PCR), and PCR ribotype 027 (sequence between 16S and 23S rRNA) (McDonald et al., 2005). Hypervirulent strains like toxinotype III produce and secrete all three toxins (enterotoxin, cytotoxin, and cytolethal distending toxin) in higher concentrations compared to other types (toxinotypes 0 and V) (Jhung et al., 2008).

Research has established *C. difficile* as a causative agent of CDAD in neonatal pigs (Songer, 2004). Evidence also suggests it may be linked to neonatal calf diarrhea (NCD) (Rodriguez-Palacios et al., 2006; Hammitt et al., 2008), which is a typical illness observed in preweaning production calves that can result in death (Larson et al., 1998). Healthy food animals on the farm have also been found to be contaminated with *C. difficile* (Simango and Mwakurudza, 2008; Thitaram, 2008). Thitaram reported that 15.9% of fecal samples from healthy swine and 2.4% fecal samples from healthy dairy cattle were positive for *C. difficile*. In

that study, it was noted that 8.6% of dairy cattle environmental samples were also *C. difficile* positive. *C. difficile* positive feces in healthy animals prior to slaughter could lead to meat contamination. Retail ground meat, including ground beef, has tested positive for *C. difficile* including a NAP1 strain (Rodriguez-Palacios et al., 2007). Further evidence of transmission of *C. difficile* via food has focused on common PCR ribotypes and toxinotypes isolated from humans and food animals (Rodriguez-Palacios et al., 2006, Keel et al., 2007; Jhung et al., 2008). From this research, it has been noted that Ribotype 078/Toxinotype V is most common in neonatal pigs and calves but has increased in human cases post 2003, and its prevalence is rising (Rupnik et al., 2008). Cases of community-acquired CDAD (CA-CDAD) occurring outside the healthcare setting have been noted, and animals, including cattle, may be a source due to animal contact, environmental contamination or consumption of contaminated meat.

CDAD is diagnosed by detection of toxins A and B in feces by commercial enzymatic immunoassays or by examination of cytopathogenic effects in cell culture (Barbut et al., 2003). Bacterial culture of *C. difficile* is less common due to limited clinical value, lack of information regarding selective growth media, long incubation times (up to 10 d) and its strict anaerobic nature. Therefore, a method developed to optimize isolation could lead to further testing and characterization of *C. difficile* isolates. Culturing *C. difficile* requires selective enrichment broth and agar, often performed after shocking the sample with heat or alcohol to induce sporulation. Clabots et al., (1989) found that treating human fecal samples with ethanol prior to culturing increased *C. difficile* recovery in asymptomatic carriers. Arroyo et al., (2005) used a single alcohol shock method (post-enrichment) to recover *C. difficile* from stool swabs and concluded that the technique was a sensitive method for the optimal recovery of spores. Alcohol shocking of fecal samples acts as a selective agent for *C. difficile* by reducing competing flora and

vegetative *C. difficile* cells, thereby allowing for the selection of *C. difficile* spores. Surviving spores are later recovered as vegetative cells after being subjected to enrichment broth and agar plate incubation. Cycloserine-cefoxitin fructose broth supplemented with 0.1% sodium taurocholate (TCCFB) is an enrichment broth used for *C. difficile*. Taurocholate, a bile salt, has been shown to induce spore germination (Wilson, 1983). In stool samples, the use of enrichment broth has been shown to significantly improve *C. difficile* recovery compared with non-enriched samples (Buchanan, 1984). *C. difficile* cycloserine-cefoxitin fructose agar supplemented with 7% lysed horse blood (CCFA) may also be effective at spore recovery (Arroyo et al., 2005).

This study was designed to investigate the efficacy of alcohol shocking fecal samples from asymptomatic beef cattle on *C. difficile* recovery. The objectives of this study were: 1) to compare a single alcohol shock (post-enrichment) and double alcohol shock (pre-and-post enrichment) culture method on *C. difficile* recovery; 2) to compare plating medium using tryptic soy agar with 5% sheep blood (BA) and CCFA post enrichment in TCCFB; and 3) to investigate the prevalence of *C. difficile* isolated from healthy beef cattle fecal samples.

Materials and Methods

SAMPLES

A total of 2,965 fecal samples obtained from healthy (asymptomatic) beef cattle were processed between January 27th – April 15th and July 8th – August 19th, 2008. The samples processed were part of the National Animal Health and Monitoring System (NAHMS) surveillance program under the direction of the United States Department of Agriculture (USDA)-Agricultural Research Service, Russell Research Center located in Athens, GA. Fecal samples (approximately 200 g) from beef cattle around three different regions (West, Central,

and Southeast) of the United States were collected aseptically in whirl-pak bags™, stored at 4°C, and shipped to the laboratory within 24 hours.

C. DIFFICILE CULTURAL PROCEDURE

Fecal samples were processed immediately upon arrival at the lab. All media (broth and agar) were pre-reduced 24 hr prior to use in an anaerobic chamber (Sheldon Manufacturing, Bactron Anaerobic, Model BacII, Cornelius, OR) with a gas composition of 5% hydrogen, 5% CO_2 and 90% nitrogen. The procedure similar to that of Arroyo et al., (2005) was performed for the single alcohol shock (post enrichment). Briefly, a sterile cotton swab was used to place approximately 2 g of fecal sample into 9.0 ml of TCCFB (supplemented with *C. difficile* monolactam norfloxacin [C.D.M.N.] SR0173E, Oxoid, Columbia, MD) and incubated aerobically at 37°C for 7 d. Post incubation, tubes were vortexed and 3.0 ml samples were transferred to sterile 15 ml centrifuge tubes, mixed with 3.0 ml of 100% ethanol (Ultra Pure LLC, Darien, CT), and held at room temperature for 60 min. Samples were then centrifuged (Jouan, Model CR4-22, Manchester, VA) at 4,600 x g for 30 min and the supernatant fluid was discarded. The resulting pellet was struck for isolation onto BA (Remel, Lenexa, KS) and CCFA (*C. difficile* agar [Remel, Lenexa, KS, USA], supplemented with *C. difficile* SR0096 [Oxoid]), and then incubated in anaerobic jars for 3 d at 37°C. Suspect colonies typical of *C. difficile* (non-hemolytic, flat, rough, and swarming) were identified by distinctive horse dung odor, yellow/green fluorescence under long wave (365 nm) UV light, Gram-stain appearance (Gram-positive, spore-forming bacilli), and production of L-proline aminopeptidase (Pro Disc, Remel, Lenexa, KS, USA). To check for accuracy, all isolated colonies were tested and identified using PCR targeting 16S rRNA (Kikuchi et al., 2002).

For the double alcohol shock method (pre-and-post enrichment) samples were processed similar to procedures reported by Rodriguez-Palacios et al., (2006). A sterile cotton swab was used to transfer approximately 2 g of fecal sample into a sterile 15 ml centrifuge tube. Six ml of 100% ethanol was added to each sample and vortexed. Samples were then rotated at room temperature for 60 min and centrifuged at 3,800 x g for 10 min. The supernatant fluid was decanted, and the resulting pellet was placed in 9.0 ml TCCFB for aerobic incubation for 7 d at 37°C. Post-incubation, 3.0 ml of vortexed sample were transferred to a sterile 15 ml centrifuge tube, mixed with an equal volume 100% ethanol (3.0 ml), and then held at room temperature for 60 min. Samples were centrifuged at 4,600 x g for 30 min. The supernatant fluid was discarded, and the sediment was struck to BA and CCFA for isolation followed by anaerobic incubation in jars for 3 d at 37°C. Suspected *C. difficile* colonies were identified as previously stated. All isolates were stored aerobically at ambient temperature in cooked meat broth.

STATISTICAL ANALYSIS

Chi-square tests for independence with Yates correction were performed based on the percentage of positive samples comparing the two shocking methods and types of plating agar used with GraphPad InStat version 3.05 statistical software (GraphPad Software, San Diego, CA, www.graphpad.com). P-values, odds ratios, and confidence intervals were obtained, and comparisons were deemed significantly different when $P < 0.05$.

Results and Discussion

C. difficile prevalence in feces of healthy beef cattle detected using two alcohol shocking methods are presented in Table 3.1. Of the 2,965 samples analyzed, 188 (6.3%) tested positive for *C. difficile* by at least one of the alcohol shocking methods regardless of plating agar. From

these 188 positive samples, 161 (5.43%) were detected using the single alcohol shock method while 48 (1.62%) were detected using the double alcohol shock method. Single alcohol shock was found to be a significantly better method ($P<0.0001$) at *C. difficile* recovery than the double shock method. Significant differences ($P<0.0001$) were observed for each media used (BA and CCFA) and across both agars when comparing alcohol shock methods. However, media had no significant effect within each method. The two sampling time periods of which samples were collected had no significance on *C. difficile* recovery (data not shown).

The 6.3% prevalence rate of *C. difficile* from healthy beef feces is slightly higher than that reported from dairy cattle feces (2.4%) and similar to that found in dairy environmental samples (8.6%) (Thitaram, 2008). The research performed by Thitaram noted that there were no significant differences in *C. difficile* recovery from dairy fecal samples when comparing single and double alcohol shocks. Rodriguez-Palacios et al., (2006) were successful in *C. difficile* recovery from sick and healthy dairy calves using a double alcohol shock method (pre- and post-enrichment). The authors detected *C. difficile* in 31 (11.2%) of 278 samples from calves: 11 (7.6%) of 144 samples from diarrheic calves and 20 (14.9%) of 134 samples from calves without diarrhea. The recovery rate in the current research using the double alcohol shock and healthy beef cattle was observed to be lower (1.62%). The higher incidence of *C. difficile* in younger animals may be due to an underdeveloped intestinal microflora leading to less competition for attachment sites. As a result, this may allow NCD or other CDAD to occur in younger animals. As the microflora continues to develop with age, competition by other anaerobes may make it difficult for *C. difficile* attachment and CDAD development. On-farm production cattle, like those tested in the current research, may be less likely to be contaminated with *C. difficile* and/or to be acute asymptomatic carriers.

C. difficile prevalence in healthy swine feces has been reported to be 15.9%; a double alcohol shock method was significantly better for recovery than a single shock method (Thitaram, 2008). It is uncertain why the prevalence of *C. difficile* in beef is lower than in swine, and/or why the single shock procedure was significantly more effective with beef than with swine. One explanation could be that *C. difficile* has a greater affinity for the swine colon compared to that of cattle. This might explain why swine are thought to be major reservoirs for *C. difficile* (Thitaram, 2008). Fitness difference among beef and swine samples may be another possibility. Alcohol shocking beef samples the second time may have further injured *C. difficile* cells recovering from the prior shock in a manner that reduced its growth while favoring surviving competitors. *C. difficile* strains isolated from swine samples may have been better adapted than the beef strains to survive a second shock while competitors did not. Another possible explanation could be differences in the spore coat protein composition between swine and beef strains which may affect the ability to resist ethanol. Variations in *C. difficile* prevalence between cattle and swine may also occur due to differences in diet rations of hormones, protein composition, and subtherapeutic and prophylactic antimicrobial use. An example with antimicrobial therapy differences is the use of levofloxacin in swine but not beef cattle. *C. difficile* isolated from swine and dairy were all found to be resistant to this quinolone (Thitaram, 2008), indicating levofloxacin may increase selection for *C. difficile* growth in swine but not beef cattle.

The *C. difficile* spore concentration in the environment may also be a factor in the discrepancy between prevalence in swine and cattle. The degree of spores in the environment could play a critical role in transmission and spread among animals. *C. difficile* spores can survive for prolonged periods of time in the environment even under stress from lack of

nutrients, unfavorable temperature, and exposure to antimicrobials. It has been reported that 8.6% of dairy cattle environmental samples were positive for *C. difficile* (Thitaram, 2008). Soil (37%) and water (6%) samples have tested positive for *C. difficile* (Simango, 2006). Therefore, an explanation as to why swine are major reservoirs of *C. difficile* compared to beef may be that swine have higher spore concentration in the environment. Sanitary conditions in the swine production environment may play a major role in the higher prevalence of *C. difficile.* Swine fecal samples, therefore, may have needed a second alcohol shock due to the level of contamination in the environment and to decrease competing microflora more so than beef fecal samples. Further research should investigate the numbers of *C. difficile* spores in the beef and swine environments. Thitaram (2008) found spore numbers higher in the dairy environment (8.6%) than in dairy fecal samples (2.4%). The same may be true for swine and beef. Any variations in diet, medication, and environment may affect the degree of stress, immunity, and microflora of these animals leading to differences in *C. difficile* presence.

Simango and Mwakurudza (2008) found 29% of live poultry to be positive for *C. difficile*. The authors used a single alcohol shock procedure similar to the method described in the current study. The high prevalence of *C. difficile* reported by these authors may be related to these birds being sold at an open urban market. Spore contamination, either with backyard production or at the market, may have been sufficient enough to cause contamination. Further research with organic and integrated broiler production should be investigated to determine if retail poultry is indeed a possible vector for transmission of *C. difficile* to humans.

Healthy beef fecal samples were observed to have low prevalence of *C. difficile*, indicating beef as a minor reservoir. However, beef may still be a means of transmitting CDAD along the food chain. The single shock procedure was capable of eliminating both the

background microflora and the *C. difficile* vegetative cells thoroughly enough to allow efficient recovery of spores without requiring a second shock procedure. Researchers and veterinarians in the cattle industry may benefit from this single shock method to increase recovery of *C. difficile* colonies. Further characterization and testing of animal *C. difficile* isolates is needed to investigate the pathogen's role, if any, in animal to human transmission. Obtaining large numbers of isolates from animal feces can be beneficial in determining more about its virulence, ecology, and antimicrobial resistance.

CDAD is a well documented important hospital-acquired infection and the increasing cases of CA-CDAD provides evidence that *C. difficile* could possibly be acquired from other sources. Moreover, the findings of toxigenic *C. difficile* strains in chickens (Simango, 2006), as well as indistinguishable types from human CDAD cases, and hypervirulent epidemic PCR ribotypes in retail meats (Rodriguez-Palacios et al., 2007; Songer, 2007), pigs (Songer, 2004), and calves (Rodriguez-Palacios et al., 2006) is further proof that the food chain may be a source of *C. difficile* transmission. Further investigation into toxin production, antimicrobial susceptibility, and characterization through genetic typing of *C. difficile* isolates from on-farm animals is currently being evaluated. Results obtained should help shed more light on the potential for food and food animals as vectors for CA-CDAD.

Table 3.1. The prevalence of *Clostridium difficile* from feces of healthy beef cattle using different isolation methods and media.

Isolation Method	**Sample % positive (# positive / total #)**
Single alcohol shock	
BA	4.89 (145/2,965)[a]
CCFA	5.30 (157/2,965)[a]
Total both agars	5.43 (161/2,965)[a]
Double alcohol shock	
BA	1.35 (40/2,965)[b]
CCFA	1.05 (31/2,965)[b]
Total both agars	1.62 (48/2,965)[b]

[*] Values with a different lower-case letter are significantly different ($P < 0.05$)

References

Arroyo, L., J. Rousseau, B. Willey, D. Low, H. Staempfli, A. McGreer, and J. Weese. 2005. Use of a selective enrichment broth to recover *C. difficile* from stool swabs stored under different conditions. J. Clin. Microbiol. 43:5341-5343.

Barbut, F., M. Delmee, J. Brazier, J. Petit, I. Poxton, M. Rupnik, V. Lalande, C. Schneider, P. Mastrantonio, R. Alonso, E. Kuipjer, M. Tvede, and the ESCMID Study Group on *Clostridium difficile* (ESGCD). 2003. A European survey of diagnostic methods and testing protocols for *Clostridium difficile*. Clin. Microbiol. Infect. 9:989-996.

Buchanan, A. 1984. Selective enrichment broth culture for detection of *Clostridium difficile* and associated cytotoxin. J. Clin. Microbiol. 20:74-76.

Clabots, C., S. Gerding, M. Olson, L. Peterson, and D. Gerding. 1989. Detection of asymptomatic *Clostridium difficile* carriage by an alcohol shock procedure. J. Clin. Microbiol. 27:2386-2387.

Hammitt, M., D. Bueschel, M. Keel, R. Glock, P. Cuneo, D. Young, C. Reggiardo, H. Trinh, and J. Songer. 2008. A possible role for *Clostridium difficile* in the etiology of calf enteritis. Vet. Microbiol. 127:348-352.

Jhung, M., A. Thompson, G. Killgore, W. E. Zukowski, G. Songer, M. Warny, S. Johnson, D. N. Gerding, L. C. McDonald, and B. Limbago. 2008. Toxinotype V *Clostridium diffiicle* in humans and food animals. Emerg. Infect. Dis. 14:1039-1045.

Keel, M. and J. Songer. 2006. The comparative pathology of *Clostridium difficile*-associated disease. Vet. Pathol. 43:225-240.

Keel, K., J. S. Brazier, K. W. Post, S. Weese, and J. G. Songer. 2007. Prevalence of PCR ribotypes among *Clostridium difficile* isolates from pigs, calves, and other species. J. Clin. Microbiol. 45:1963-1964.

Kikuchi, E., Y. Miyamoto, S. Narushima, and K. Itoh. 2002. Design of species-specific primers to identify 13 species of *Clostridium* harbored in human intestinal tracts. Microbiol. Immunol. 46:353-358.

Larson, R. L., V. L. Pierce, and R. F. Randle. 1998. Economic evaluation of neonatal health protection programs for cattle. J. Am. Vet. Med. Assoc. 213:810-816.

McDonald, L. C., G. E. Killgore, A. Thompson, R. C. Owens Jr., S. V. Kazakova, S. P. Sambol, et al. 2005. An epidemic, toxin gene-variant strain of *Clostridium difficile*. N. Eng. J. Med. 353:2433-2441.

Rodriguez-Palacios, A., H. Stampfli, T. Duffield, A. Peregrine, L. Trotz-Williams, L. Arroyo, J. Brazier, and S. Weese. 2006. *Clostridium difficile* PCR ribotypes in calves, Canada. Emerg. Infect. Dis. 12:1730-1736.

Rodriguez-Palacios, A., H. Staempfli, T. Duffield, and J. Weese. 2007. *Clostridium difficile* in retail ground meat. Canada. Emerg. Infect. Dis. 13:485-487.

Rupnik, M. 2007. Is *Clostridium difficile*-associated infection a potentially zoonotic and foodborne disease? Clin. Microbiol. Infect. 13:457-459.

Rupnik, M., A. Widmer, O. Zimmermann, C. Eckert, and F. Barbut. 2008. *Clostridium difficile* toxinotypeV, ribotypes 078, in animals and humans. J. Clin. Microbiol. 46(6):2146.

Simango, C. 2006. Prevalence of *Clostridium difficile* in the environment in a rural community in Zimbabwe. Transactions of the Royal Society of Tropical Med. and Hygiene. 100:1146-1150.

Simango, C. and S. Mwakurudza. 2008. *Clostridium difficile* in broiler chickens sold at market places in Zimbabwe and their antimicrobial susceptibility. Int. J. Food Microbiol. 124:268-270.

Songer, J. G. 2004. The emergence of *Clostridium difficile* as a pathogen of food animals. Animal Health Res. Rev. 5:312-326.

Songer, J. 2007. Porcine CDAD. Presented to the National Pork Board Pork Safety Committee on July 30, 2007 in Chicago, IL.

Thitaram, S. N. 2008. Isolation and characterization of *Clostridium difficile* from porcine and bovine feces. Doctor of Philosophy Dissertation. University of Georgia, Department of Food Science and Technology, Athens, GA.

Warny, M., J. Pepin, A. Fang, et al. 2005. Toxin-production by an emerging strain of *Clostridium difficile* associated with outbreaks of severe disease in North America and Europe. Lancet. 336:1079-1084.

Wilson, K. 1983. Efficacy of various bile salt preparations for stimulation of *Clostridium difficile* spore germination. J. Clin. Microbiol. 18:1017-1019.

CHAPTER 4

THE PRESENCE OF *CLOSTRIDIUM DIFFICILE* TOXIN GENES IN ISOLATES RECOVERED FROM HEALTHY BOVINE AND SWINE[1]

---

[1] S. A. Lyon, M. A. Harrison, P. J. Fedorka-Cray, J. S. Bailey, S. N. Thitaram, and G. R. Siragusa. To be submitted to Journal of Applied Microbiology

Abstract

Clostridium difficile infections represent an important human health problem in healthcare settings; however, community-acquired cases are emerging. Evidence suggests that food animals and contaminated meat could be possible transmission vectors for *C. difficile*-associated disease (CDAD). We used PCR to examine 478 *C. difficile* isolates from healthy, on-farm food animal feces (beef cattle [n=244], swine [n=130], and dairy cattle [n=43]) and from the dairy farm environment (n=61) for the presence of toxin genes (*tcdA*, *tcdB*, and *cdtB*). Overall, amplicons for toxin genes *tcdA*, *tcdB*, and *cdtB* were detected in 67.4% (322/478), 75.7% (362/478), and 26.6% (127/478), respectively. Three hundred (62.8%) of these isolates were capable of producing both toxins A and B, and 35.7% (107/300) of those showed positive bands for the binary toxin gene. Variant strains (A-B+) were identified in 12.5% (60/478) of the samples. The prevalence of isolates incapable of producing toxins A and B but positive for the *cdtB* gene was 1.9% (9/478). Dairy fecal (93%, 40/43), dairy environmental (85.2%, 52/61), and swine fecal (78.5%, 102/130) isolates were significantly greater in potentially toxigenic (A+B+) strains compared to beef fecal isolates. Overall, *C. difficile* from beef isolates were most likely to be non-toxigenic (A-B-) or of the variant types compared to the other samples. This research suggests that healthy swine, dairy cattle, and the dairy environment, may serve as sources to transmit virulent *C. difficile* strains to humans via the food supply chain.

Keywords: *Clostridium difficile*-associated disease, swine, cattle, *tcdA*, *tcdB*, CDT, food animals

Introduction

Clostridium difficile is an anaerobic, Gram-positive, sporeforming bacillus that causes more deaths in humans than all other gastrointestinal diseases combined (Redelings et al., 2007). *C. difficile*-associated disease (CDAD) symptoms can range from diarrhea, colitis, to septicemia (Kelly et al., 1994) and is brought about by chromosomal encoded toxins on a pathogenicity locus (PaLoc) (Hundsberger et al., 1997). Recently, concerns with CDAD have shifted to food production animals, because research indicates their ability to serve as reservoirs for *C. difficile* and possibly spread the pathogen within the community (Rodriquez-Palacios et al., 2006, Songer and Anderson, 2006, Simango and Mwakurudza, 2008; Thitaram, 2008). Community-acquired CDAD (CA-CDAD), or those cases occurring outside the health care setting, have increased recently. The chance for *C. difficile* transmission via direct contact with contaminated animals, their environment or consumption of their meat may be a possible explanation. Food animals, including neonatal swine and calves, are susceptible to CDAD (Songer et al., 2000; Rodriguez-Palacios et al., 2006) leading to significant losses in production for these industries. Healthy, food animals can be asymptomatic carriers with swine being a major reservoir (Thitaram, 2008) and cattle being minor reservoirs (Thitaram, 2008; Lyon, unpublished).

The documentation of toxigenic *C. difficile* strains in food production animals (Yaeger, 2001, Rodriguez-Palacios et al., 2006; Keel et al., 2007) and in foods (Rodriguez-Palacios et al., 2007; Songer, 2007) raises concerns that the foodchain may indeed serve as a vector for human CDAD outbreaks. A study investigating the ribotypes of *C. difficile* isolated from dairy calves reported that of the 8 types found, 7 have been identified in humans. Ribotypes 017 (*tcdA*-, *tcdB*+, *cdtB*-), 027 (*tcdA*+, *tcdB*+, *cdtB*+), and 078 (*tcdA*+, *tcdB*+, *cdtB*+) were the most common (Rodriguez-Palacios et al., 2006). Ribotypes 017 and 027 have been implicated in human

CDAD outbreaks (van den Berg et al., 2004; Warny et al., 2005) while type 078 has been the cause of recent human CA-CDAD cases (Goorhuis et al., 2008). Keel et al. (2007) found that neonatal swine (83%) and calves (94%) were most likely to be reservoirs of Ribotype 078. Furthermore, approximately 20 - 30% of retail ground meat has been shown to be contaminated with *C. difficile* including toxigenic Ribotypes 027, 078, 077, and 014 (Rodrigeuz-Palacios et al., 2007; Songer, 2007).

CDAD pathophysiology is initiated by colonization of the host's gastrointestinal tract by *C. difficile* followed by the production of its two main virulence factors, toxins A (TcdA) and B (TcdB) (Keel and Songer, 2006). These two toxins belong to the family of large clostridial cytotoxins and are the largest known bacterial toxins. TcdA is an enterotoxin that is 308 kDa in size and TcdB is a cytotoxin that is 270 kDa (Genth et al., 2008). The toxins are classified as glucosyltransferases because they glycosylate Rho proteins resulting in the loss of cellular regulation of the actin cytoskeleton (Aktories and Just, 2005). This action leads to loss of cellular contact, membrane ruffling, and cell rounding (Pothoulakis and Lamont, 2001). Apoptosis, or programmed cellular death, is also induced by TcdA and TcdB (Kim et al., 2007; Nottrott et al., 2007). Some *C. difficile* strains are capable of producing a third toxin, a binary protein toxin (CDT), which is an ADP-ribosyltransferase unrelated to TcdA and TcdB (Perelle et al., 1997). CDT is encoded by two genes; *cdtA* encodes for the enzymatic domain while *cdtB* encodes the binding domain. CDT is a cytolethal distending toxin that changes cell morphology when in cell culture, but its role in CDAD is currently unknown. CA-CDAD cases are often involved with CDT-producing strains (Terhes et al., 2004; Barbut et al., 2005). *C. difficile* binary toxin-positive strains from feces appear to be more prevalent in animals (ranging from 23

– 100%) compared to humans (1.6 – 10%) (Rupnik et al., 2003). Recently however, human cases involving these strains have increased (Rupnik, 2007).

Toxin production can be used to classify *C. difficile* strains. The majority of *C. difficile* produce both TcdA and TcdB (*tcdA*+, *tcdB*+) and are labeled as toxigenic, while others, classified as variant strains, produce TcdB but not TcdA (*tcdA*-, *tcdB*+). A deletion in the *tcdA* gene is responsible for the inability of these strains to produce TcdA. However, it has been shown that variant strains are still capable of causing CDAD (Barbut et al., 2002), including outbreaks (Alfa et al., 2000), indicating TcdB can still be an effective toxin independent of TcdA (Voth and Ballard, 2005). CDT production may also be used in classifying *C. difficile* strains based on toxicity.

C. difficile toxins are normally detected by using ELISA based analysis directly from feces. Detection of toxins from symptomatic humans and food animals is important for diagnosis and prompt antimicrobial therapy. However, in this study PCR was used as the means to identify toxin gene presence or absence in isolates. Currently, there is no data on the toxigenicity of *C. difficile* isolated from asymptomatic food animals. This study was designed to investigate the prevalence of *tcdA*, *tcdB*, and *cdtB* genes and the level of toxicity from *C. difficile* isolated from on-farm, healthy swine and bovine animals (dairy and beef cattle), and dairy cattle environmental samples. The objectives were: 1) to detect and assess the prevalence of *tcdA*, *tcdB*, and *cdtB* genes from these samples; 2) to compare the prevalence of these genes among the four sample types; and 3) to determine the number of strains that are capable of being toxigenic and compare them among the different sources.

Materials and Methods

SPECIMENS AND DNA EXTRACTION

A total of 478 *C. difficile* isolates recovered from culturing asymptomatic food animal fecal (beef n=244, swine n=130, dairy n=43) and dairy environmental samples (n=61) were tested for toxin gene presence by PCR. Dairy environmental isolates were recovered from alley ways, parlors, manure pit, holding pens, lagoons, water tanks, and feed areas. Isolates had previously been identified as *C. difficile* by PCR detection targeting the 16S rRNA gene (Kikuchi et al., 2002) and were stored aerobically at ambient temperature in sterile cooked meat broth until needed. The toxigenic strain *C. difficile* AE978 (*tcdA*+, *tcd*B+, *cdtB*+ and of PCR ribotype 027) obtained from University of Guelph (Canada) was used as the control for all PCR assays. Isolates were struck onto tryptic soy agar with 5% sheep blood (Remel, Lexana, KS) for isolation and incubated anaerobically for 3 d at 37°C. One to 2 isolated colonies were picked with a sterile needle, placed in 100µl molecular grade water (Mo-Bio, Carlsbad, CA), and then boiled for 10 min in order to lyse the cells. The extracted template DNA was stored at -20°C until needed for PCR analysis. Following PCR analysis, isolates were classified based on their capacity to produce toxins as follows: potentially toxigenic (*tcdA*+, *tcdB*+, *cdtB*-), potentially highly toxigenic (*tcdA*+, *tcdB*+, *cdtB*+), non-toxigenic (*tcdA*-, *tcdB*-, *cdtB*-), variant (*tcdA*-, *tcdB*+), and CDT+ only (*tcdA*-, *tcdB*-, *cdtB*+).

PCR ASSAYS FOR tcdA, tcdB, and cdtB DETECTION

All assays performed were a total of 25µl consisting of 12.5µl PCR Master Mix (Promega, catalog number M7502, Madison, WI), 5.5µl molecular grade water (Promega, Madison, WI), 5.0µl template DNA, 1.0µl forward primer, and 1.0µl reverse primer. PCR amplification reactions for all 5 assays were performed in a thermal cycler (MJ Research, Inc.

Model PT-2000, Waltham, MA) and times for each cycle (denaturation, annealing, and extension) were based on the authors' recommendations (Kato et al., 1993, 1998, Tang et al., 1994; Stubbs et al., 2000). Post-amplification, PCR products (10µl) were run through a gel electrophoresis chamber (120 volts) with 1 X TBE buffer stained with ethidium bromide. Visualization of amplicons was performed under UV light. All gels were run with the aforementioned positive control strain and a negative control that included all assay ingredients minus DNA.

Presence of *tcdA* was determined by using primers targeting the non-repeating and repeating sequences (Kato et al., 1993 and Kato et al., 1998). Non-repeating sequence of *tcdA* was amplified by using primers NK2 (5'-CCCAATAGAAGATTCAATATTAAGCTT-3') and NK3 (5'-GGAAGAAAAGAACTTCTGGCTCACTCAGGT-3') while the repeating sequence was amplified using primers NK9 (5'-CCACCAGCTGCAGCCATA-3') and NK11 (5'-TGATGCTAATAATGAATCTAAAATGGTAAC-3'). Amplicons for the non-repeating and repeating sequences were 252 bp and 1,200 bp, respectively. Isolates with positive amplicons for both sequences were classified as toxin A-positive (*tcdA*+) while those isolates showing no amplicons were classified as toxin A-negative (*tcdA*-). Isolates that were positive for either sequence but not the other were subjected to an additional assay (Tang et al., 1994). Those isolates that produced a 634 bp band using primers YT28 (5'-GCATGATAAGGCAACTTCAG-TGG-3') and YT29 (5'-GAGTAAGTTCCTCCTGCTCCATCAA-3') were then classified as *tcdA*+. Isolates with no bands were considered toxin A-negative (*tcdA*-).

Toxin B gene presence was detected using primers NK104 (5'GTGTAGCAATGAA-AGTCCAAGTTTACGC-3') and NK105 (5'-CACTTAGCTCTTTGATTGCTGCACCT-3') derived from the non-repeating sequence of *tcdB* (Kato et al., 1998). Samples with a 204 bp

PCR product were classified as toxin B-positive (*tcdB*+) and those with no amplicon were toxin B-negative (*tcdB*-). The detection of the binary toxin gene was performed with primers targeting the binding portion of the gene (*cdtB*) (Stubbs et al., 2000). Primers cdtBpos (5'-CTTAATGC-AAGTAAATACTGAG-3') and cdtBrev (5'-AACGGATCTCTTGCTTCAGTC-3') were used and isolates with a 510 bp amplicon were considered binary toxin positive (*cdtB*+).

STATISTICAL ANALYSIS

A Fischer's exact test was used to analyze proportions of toxin gene presence and toxicity classifications among the swine and bovine isolates using GraphPad Instat version 3.05 statistical software (GraphPad Software, San Diego, CA, www.graphpad.com). Two-sided *P*-values, odds ratios, and 95% confidence intervals were obtained, and statistical significance was based on $P < 0.05$.

Results and Discussion

C. difficile toxin genes *tcdA* and *tcdB* were identified in significantly greater numbers ($P < 0.0001$) among isolates from dairy samples (environmental and fecal) and swine feces than in beef fecal samples (Table 4.1). Dairy fecal isolates were 95.3% (41/43) positive for *tcdA* and 93.0% (40/43) positive for *tcdB* while dairy environmental isolates were 86.9% (53/61) positive for *tcdA* and 88.5% (54/61) positive for *tcdB*. Isolates from swine feces were similar with 84.6% (110/130) positive for *tcdA* and 87.7% (114/130) positive for *tcdB*. Only 48.4% (118/244) of beef fecal isolates were positive for *tcdA* and 63.1% (154/244) were identified to contain *tcdB*. The presence of *cdtB* was significantly greater (*P* values ≤ 0.0421) in dairy environmental isolates (49.2%) than in dairy fecal (27.9%), swine fecal (32.3%), and beef fecal isolates (17.6%). *C. difficile* from dairy feces were not significantly different in *cdtB* presence compared

to swine and beef fecal isolates. However, beef isolates were observed to be significantly lower in *cdtB* gene presence compared to that of swine isolates ($P = 0.0018$).

Results for the toxigenic classifications are in Table 4.2. Potentially toxigenic *C. difficile* strains (*tcdA*+, *tcdB*+, *cdtB*-) were highest amongst the swine and dairy isolates and lowest in beef isolates. Among the 43 dairy fecal samples, 65.1% (28) were classified potentially toxigenic. This value was significantly greater ($P = 0.0179$) than the dairy environment samples (41% positive or 25/61 samples). Our findings in healthy dairy cattle samples were considerably greater than that previously reported for dairy calves (Rodriguez-Palacios et al., 2006) in which 8 (25.8%) of 31 *C. difficile* isolates were toxigenic. For swine isolates, it was found that 46.9% (61/130) isolates were potentially toxigenic which was not significantly different from dairy fecal isolates. Beef isolates exhibited significantly less potentially toxigenic strains compared to swine ($P = 0.007$) and dairy fecal isolates ($P < 0.0001$).

Potentially highly toxigenic *C. difficile* strains (*tcdA*+, *tcdB*+, *cdtB*+) were observed from every sample type tested. There were no significant differences in the prevalence of the potentially highly toxigenic type among dairy environmental (44.3%), dairy fecal (27.9%), and swine fecal (31.5%) isolates. However, beef fecal isolates (11.1%) were significantly lower in potentially highly toxigenic strains compared to the other sample types (P values ≤ 0.005). The prevalence of these strains reported in dairy fecal (27.9%) samples was similar to that reported by Rodriguez-Palacios et al., (2006) who noted that 11 (35.5%) of 31 *C. difficile* isolates from dairy calf feces were of this toxin classification.

Recently, the presence of *C. difficile* toxins A and B has been investigated from food animal and environmental isolates. However, those reports have involved neonates (piglets and dairy calves) and urban marketplace poultry. In the current study, a total of 417 isolates from

porcine and bovine feces showed that 248 (59.5%) were positive for the *tcdA* and *tcdB* (potentially toxigenic or highly toxigenic classifications). Previous research from dairy calves was noted to be 61.3% and 100% positive for these strains (Rodriguez-Palacios et al., 2006; Hammitt et al., 2008). Those results are similar to those reported in the current study which noted that 93% (40/43) of the isolates of market-ready dairy cattle are of these types. The presence of toxins A and B as detected by ELISA in neonatal swine has been previously reported to be 35% in piglets with enteritis (Songer, 2004) and 74% in non-diarrhetic piglets (Yaeger, 2001 and Yaeger et al., 2002). The percentage of *tcdA* and *tcdB* genes from healthy swine fecal *C. difficile* isolates in the current research was 78.5% (102/130). Based on earlier and current data it appears the age of swine does not affect the prevalence of these strains. This same trend is evident for dairy cattle. This suggests that asymptomatic dairy cattle and swine are just as likely to be carriers of potentially highly toxigenic *C. difficile* as are symptomatic piglets and calves. However, asymptomatic animals serving as multiplying hosts heading to slaughter may pose a greater human health risk due to their lack of CDAD signs and symptoms. This scenario could increase the spread of potentially highly toxigenic *C. difficile* spores through the processing plant environment leading to the contamination of meat and meat products.

To our knowledge this is the only research performed on beef cattle isolates (neonatal or adult) to determine the prevalence of *C. difficile* toxin genes. Healthy beef cattle isolates were less likely to be of the A+B+ type (43.4%) compared to swine and dairy cattle. However, those beef cattle contaminated with *C. difficile* on-farm should still be considered a possible source of CA-CDAD.

Simango and Mwakurudza (2008) used enzymatic assays to detect toxins A and B from poultry and soil isolates from a rural Zimbabwe marketplace. Twenty-six (89.7%) of 29 poultry

isolates were of strains producing TcdA and B. This may imply that poultry, swine, and dairy cattle are more likely to be carriers of these *C. difficile* types compared to beef cattle. Environmental isolates obtained from soil samples taken randomly around the marketplace were also contaminated with toxigenic isolates (95.5%). This value is comparable to the prevalence seen in the dairy cattle environmental isolates of the current study (85.2%). Thus, the environment surrounding food animal seems like a reasonable arena for *C. difficile* transmission to other animals, people, and food which could result in CA-CDAD outbreaks.

C. difficile strains producing toxin B but not A (variant) are uncommon in humans although they have been implicated in human CDAD cases (van den Berg et al., 2004). The majority of variant strains were found from the beef isolates. Of the 244 beef isolates examined, 18.9% (46) were of this type. This prevalence was significantly greater than (P values ≤ 0.02) those found in swine feces (9.2%), dairy environment (3.3%), and dairy feces (0%). Variant strains were significantly higher in swine fecal isolates compared to dairy fecal isolates ($P = 0.0393$) but not dairy environmental samples. The absence of variant strains from dairy cattle isolates in this study is in agreement with work previously performed by Porter et al., (2002). However, Rodriguez-Palacios et al., (2006), found that 9 (29%) of 31 dairy calf isolates were of this type. The differences between these prevalences could be due to geographical and environmental conditions.

As stated previously, CDT+ *C. difficile* strains have been implicated in human CA-CDAD cases. Neonatal food animals have been shown to be major carriers of these strains (piglets, up to 83% positive and calves, up to 100% positive) (Keel, unpublished data). The prevalence of these strains among healthy food animals in the current research was observed to be lower among the dairy environment (47.5%), swine fecal (32%), dairy fecal (27.9%), and beef

fecal (18%) samples (data not listed in tables). Dairy environmental and swine fecal isolates were significantly higher ($P < 0.0001$ and $P < 0.0028$, respectively) in the prevalence of these strains than beef fecal isolates. Our findings vary from those by Keel, however, Rodriguez-Palacios et. al., (2006), previously reported similar findings of positive samples to ours from dairy calves (45.2%). Isolates capable of producing only CDT (*tcdA*-, *tcdB*-, *cdtB*+) were found in 3.2% of dairy environmental, 2.4% of beef fecal, 0.8% swine fecal, and 0% of dairy fecal samples. There were no significant differences among the sample types for CDT+ only strains. *C. difficile* isolates of these types have been previously reported to be low. Rodriguez-Palacios et al., (2006) found CDT+ only strains in just 2 (6.5%) of 31 dairy calves. Our data further supports evidence that food animals are not a major source of CDT+ only strains.

Non-toxigenic strains (*tcdA*-, *tcdB*-, *cdtB*-) were significantly more prevalent ($P < 0.0001$) among beef fecal isolates (30.3%) and were the second most common type found from beef fecal samples. This suggests that the beef intestinal tract may serve as a better environment for these non-virulent types compared to dairy and swine intestinal tracts. It is unclear why, but differing diets (protein levels, hormones, and growth promoters) and grow out environments may also be possible reasons for these differences. The prevalence of non-toxigenic strains in the dairy environment, swine fecal, and dairy fecal samples was 6.6%, 5.4%, and 4.7%, respectively. The observed prevalence (4.7%) of non-toxigenic strains in asymptomatic dairy fecal is similar to that reported by Rodriguez-Palacios et al., (2006) for symptomatic dairy calves (3.2%). Twenty-two (4.6%) of the total isolates tested were of a A+B- type.

A grand total of 478 *C. difficile* isolates from healthy food animals and the dairy cattle environment were tested by PCR for the presence of toxin genes *tcdA*, *tcdB*, and *cdtB*. Three hundred (62.8%) were found to be positive for both *tcdA* and *tcdB* genes and of those, 35.7%

(107/300) were positive for *cdtB*. Among the animals compared, *C. difficile* originating form swine and dairy cattle were the most capable of producing toxins related to CDAD and beef cattle the least capable. These data, along with that of Thitaram (2008) and Lyon (unpublished) on the prevalence of *C. difficile* in these food animals, suggests that healthy, on-farm beef cattle are not as likely to be a major concern among food animals transmitting CDAD as are swine and dairy cattle. The reason why beef harbors less *C. difficile* and less toxigenic strains compared to swine and dairy cattle needs to be further investigated. *C. difficile* strains containing all three toxin genes were observed in all four sample types implying that production food animals and their environment may serve as reservoirs for highly virulent strains. Asymptomatic healthy food animals, especially swine and dairy cattle, may be more likely to harbor potentially toxigenic *C. difficile* strains than symptomatic neonates. The food animal environment also appears to be a major source of *C. difficile* spore contamination. Thus, there is a risk for transmitting *C. difficile* spores throughout the slaughter plant. Contaminated processing equipment and workers can serve as transmission vectors to spread the spores to meat products. In addition, variant (A-B+) strains were observed in 12.5% (60/478) of all the samples (significantly greatest in beef isolates) and CDT+ only strains were also found (1.9%) indicating that healthy asymptomatic food animals can harbor various types of toxigenic *C. difficile* that have been responsible for human CDAD outbreaks in health care settings and amongst the community.

Table 4.1. Prevalence[1] and comparison[2] of toxin genes in *Clostridium difficile* isolated from healthy animal fecal samples and dairy environment[3]

	Toxin gene		
Sample	***tcdA***	***tcdB***	***cdtB***
Dairy environment	86.9 (53/61)[a]	88.5 (54/61)[a]	49.2 (30/61)[a]
Dairy fecal	95.3 (41/43)[a]	93.0 (40/43)[a]	27.9 (12/43)[b,c]
Swine fecal	84.6 (110/130)[a]	87.7 (114/130)[a]	32.3 (42/130)[b]
Beef fecal	48.4 (118/244)[b]	63.1 (154/244)[b]	17.6 (43/244)[c]

[1] Values represent percentage of samples testing positive for toxin gene; followed by number positive and total numbers tested in parenthesis.

[2] Values within each column with a different lower-case letter are significantly different ($P<0.05$)

[3] Dairy cattle environmental isolates included samples from alley ways, parlors, manure pits, holding pens, lagoons, water tanks, and feed area.

Table 4.2. Prevalence[1] and comparison[2] of toxigenic *Clostridium difficile* strains isolated from healthy animal fecal samples and dairy environment[3]

	Toxigenic Classification					
Sample	**Toxigenic[4]**	**Highly Toxigenic[5]**	**Non-toxigenic[6]**	**Variant[7]**	**CDT+ only[8]**	**A+B-[9]**
Dairy environment	41.0 (25/61)^{b,c}	44.3 (27/61)^{a}	6.6 (4/61)^{b}	3.3 (2/61)^{b,c}	3.2 (2/61)^{a}	1.6 (1/61)^{a}
Dairy fecal	65.1 (28/43)^{a}	27.9 (12/43)^{a}	4.7 (2/43)^{b}	0 (0/43)^{c}	0 (0/43)^{a}	2.3 (1/43)^{a}
Swine fecal	46.9 (61/130)^{a,b}	31.5 (41/130)^{a}	5.4 (7/130)^{b}	9.2 (12/130)^{b}	0.8 (1/130)^{a}	6.2 (8/130)^{a}
Beef fecal	32.4 (79/244)^{c}	11.1 (27/244)^{b}	30.3 (74/244)^{a}	18.9 (46/244)^{a}	2.4 (6/244)^{a}	4.9 (12/244)^{a}

[1] Values represent percentage of samples testing positive for toxin classification; followed by number positive and total numbers tested in parenthesis.
[2] Values within each column with a different lower-case letter are significantly different (P<0.05)
[3] Dairy cattle environmental isolates included samples from alley ways, parlors, manure pits, holding pens, lagoons, water tanks, and feed area.
[4] *tcdA* +, *tcdB* +, *cdtB*-
[5] *tcdA* +, *tcdB* +, *cdtB* +
[6] *tcdA* -, *tcdB* -, *cdtB*-
[7] *tcdA* -, *tcdB* +
[8] *tcdA* -, *tcdB* -, *cdtB* +
[9] *tcdA* +, *tcdB*-

References

Aktories, K. and I. Just. 2005. Clostridial Rho-inhibiting protein toxins. Current Topics in Microbiol. and Immun. 291:113-145.

Alfa, M. J., A. Kabani, D. Lyerly, S. Moncrief, L. M. Neville, A. Al-Barak, G. K. H. Harding, B. Dyck, K. Olekson, and J. M. Embil. 2000. Characterization of a toxin A-negative, toxin B-positive strain of *Clostridium difficile* response for a nosocomial outbreak of *Clostridium difficile*-associated diarrhea. J. Clin. Microbiol. 38:2706-2714.

Barbut, F., V. Lalande, B. Burghoffer, H. V. Thien, E. Grimprel, and J. C. Petit. 2002. Prevalence and genetic characterization of toxin A variant strains of *Clostridium difficile* among adults and children with diarrhea in France. J. Clin. Microbiol. 40:2079-2083.

Barbut, F., D. Decre, V. Lelande *et al*. 2005. Clinical features of *Clostridium difficile*-associated diarrhea due to binary toxin (actin-specific ADP-ribosyltransferase)-producing strains. J. Med. Microbiol. 54:181-185.

Genth, H., S. Dreger, J. Huelsenbeck, and I. Just. 2008. *Clostridium difficile* toxins: More than mere inhibitors of Rho proteins. Int. J. Biochem. Cell Biol. 40:592-597.

Goorhuis, A., S. Debast, L. Van Leengoed, C. Harmanus, D. Notermans, A. Bergwerff, and E. Kuijper. 2008. *Clostridium difficile* PCR ribotype 078: an emerging strain in humans and pigs? J. Clin. Microbiol. 46:1157-1158.

Hammitt, M. C., D. M. Bueschel, M. K. Keel, R. D. Glock, P. Cuneo, D. W. DeYoung, C. Reggiardo, H. Trinh, and J. G. Songer. 2008. A possible role for *Clostridium difficile* in the etiology of calf enteritis. Vet. Microbiol. 127:343-352.

Hundsberger, T., V. Braun, M. Wiedmann, P. Leukel, M. Sauerborn, and C. von Eichel-Streiber. 1997. Transcription analysis of the genes tcdA-E of the pathogenicity locus of *Clostridium difficile*. Eur. J. Biochem. 244:735-742.

Kato, N., C. Y. Ou, H. Kato, S. L. Bartley, C. C. Luo, G. E. Killgore, and K. Ueno. 1993. Detection of toxigenic *Clostridium difficile* in stool specimens by the polymerase chain reaction. J. Infect. Dis. 167:455-458.

Kato, H., N. Kato, K. Watanabe, N. Iwai, H. Nakamura, T. Yamamoto, K. Suzuki, S. Kim, Y. Chong, and E. Wasito. 1998. Identification of toxin A-negative, toxin B-positive *Clostridium difficile* by PCR. J. Clin. Microbiol. 36:2178-2182.

Keel, M. K. and J. G. Songer. 2006. The comparative pathology of *Clostridium difficile*-associated disease. Vet. Pathol. 43:255-240.

Keel, K., J. S. Brazier, K. W. Post, S. Weese, and J. G. Songer. 2007. Prevalence of PCR Ribotypes among *Clostridium difficile* isolates from pigs, calves, and other species. J. Clin. Microbiol. 45:1963-1964.

Keel, M. K. 2008. Personal communication.

Kelly, C. P., C. Pothoulakis, and T. J. Lamont. 1994. *Clostridium difficile* colitis. N. Eng. J. Med. 330:257-262.

Kikuchi, E., Y. Miyamoto, S. Narushima, and K. Itoh. 2002. Design of species-specific primers to identify 13 species of *Clostridium* harbored in human intestinal tracts. Microbiol. Immunol. 46:353-358.

Kim, H., S. H. Rhee, C. Pothoulakis, and J. T. Lamont. 2007. Inflammation and apoptosis in *Clostridium difficile* enteritis is mediated by PGE2 up-regulation of Fas Ligand. Gastroenterology. 133:875-886.

Lyon, S. A. 2008. Unpublished data.

Nottrott, S., J. Schoentaube, H. Genth, I. Just, and R. Gerhard. 2007. *Clostridium difficile* toxinA-induced apoptosis is p53-independent but depends on glucosylation of Rho GTPases. Apoptosis. 12:1443-1453.

Perelle, S., M. Gilbert, P. Bourlioux, G. Courthier, and M. R. Popoff. 1997. Production of a complete binary toxin (actin-specific ADP-ribosyltransferase) by *Clostridium difficile* CD196. Infect. Immun. 65:1402-1407.

Porter, M. C., C. Reggiardo, R. D. B. M. K. Glock, M. K. Keel, and J. G. Songer. 2002. Association of *Clostridium difficile* with bovine neonatal diarrhea [abstract]. In: Abstracts of the Proceedings of the American Association of Veterinary Laboratory Diagnosticians. 45th annual meeting. October 19-21, 2002, Saint Louis, MO.

Pothoulakis, C. and J. T. Lamont. 2001. Microbes and microbial toxins: paradigms for microbial-mucosal interactions II. The integrated response of the intestine to *Clostridium difficile* toxins. Am. J. Physiol. Gastrointest. Liver Physiol. 280:G178-G183.

Redelings, M., F. Sorvillo, and L. Mascola. 2007. Increase in *Clostridium difficile*-related mortality rates, United States, 1999-2004. Emerg. Infect. Dis. 13:1417-1419.

Rodriguez-Palacios, A., H. Stampfli, T. Duffield, A. Peregrine, L. Trotz-Williams, L. Arroyo, J. Brazier, and S. Weese. 2006. *Clostridium difficile* PCR ribotypes in calves, Canada. Emerg. Infect. Dis. 12:1730-1736.

Rodriguez-Palacios, A., H. Staempfli, T. Duffield, and J. Weese. 2007. *Clostridium difficile* in retail ground meat. Canada. Emerg. Infect. Dis. 13:485-487.

Rupnik, M., M. Grabnar, and B. Geric. 2003. Binary toxin producing *Clostridium difficile* strains. Anaerobe. 9:289-294.

Rupnik, M. 2007. Is *Clostridium difficile*-associated infection a potentially zoonotic and foodborne disease? Clin. Microbiol. Infect. 13:457-459.

Simango, C. and S. Mwakurudza. 2008. *Clostridium difficile* in broiler chickens sold at market places in Zimbabwe and their antimicrobial susceptibility. Int. J. Food Microbiol. 124:268-270.

Songer, J., K. Post, D. Larson, B. Helen Jost, and R. Glock. 2000. Enteric infection of neonatal swine with *Clostridium difficile*. Swine Health Produc. 8:185-189.

Songer, J. G. 2004. The emergence of *Clostridium difficile* as a pathogen of food animals. Animal Health Res. Rev. 5:312-326.

Songer, J. G. and M. A. Anderson. 2006. *Clostridium difficile*: an important pathogen of food animals. Anaerobe. 12:1-4.

Songer, J. 2007. Porcine CDAD. Presented to the National Pork Board Pork Safety Committee on July 30, 2007, Chicago, IL.

Stubbs, S., M. Rupnik, M. Gibert, J. Brazier, B. Duerden, and M. Popoff. 2000. Production of actin-specific ADP-ribosyltransferase (binary toxin) by strains of *Clostridium difficile*. FEMS Microbiol. Letters. 186:307-312.

Tang, Y. J., P. H. Gumerlock, J. B. Weiss, and J. Silva Jr. 1994. Specific detection of *Clostridium difficile* toxin A gene sequences in clinical isolates. Mol. Cell. Probes. 8:463-467.

Terhes, G., E. Urban, J. Soki, K. A. Hamid, and E. Nagy. 2004. Community-acquired *Clostridium difficile* diarrhea caused binary toxin, toxin A, and toxin B gene positive isolates in Hungary. J. Clin. Microbiol. 42:4316-4318.

Thitaram, S. N. 2008. Isolation and characterization of *Clostridium difficile* from porcine and bovine feces. Doctor of Philosophy Dissertation. University of Georgia, Department of Food Science and Technology, Athens, GA.

van den Berg, R. J., E. C. Claas, D. H. Oyib, C. H. Klaassen, L. Dijkshoorn, J. S. Brazier et al. 2004. Characterization of toxin A-negative, toxin B-positive *Clostridium difficile* isolates from outbreaks in different countries by amplified fragment length polymorphism and PCR ribotyping. J. Clin. Microbiol. 42:1035-1041.

Voth, D. E., and J. Ballard. 2005. *Clostridium difficile* toxins: Mechanism of action and role in disease. Clin. Microbiol. Rev. 18:247-263.

Warny, M., J. Pepin, A. Fang, et al. 2005. Toxin-production by an emerging strain of *Clostridium difficile* associated with outbreaks of severe disease in North America and Europe. Lancet. 336:1079-1084.

Yaeger, M. 2001. *Clostridium difficile* and other emerging enteric diseases. Iowa Swine Disease Conference, November 9-10, 2001, Iowa State University, Ames, IA.

Yaeger, M., N. Funk, and L. Hoffman. 2002. A survey of agents associated with neonatal diarrhea in Iowa swine including *Clostridium difficile* and porcine reproductive and respiratory syndrome virus. J. Vet. Diagn. Invest. 14:281-287.

CHAPTER 5

DEVELOPMENT OF A 16S rDNA BASED PCR ASSAY TO DETECT *CLOSTRIDIUM DIFFICILE* IN ENRICHED FOOD AND FECAL SAMPLES[1]

---

[1] S. A. Lyon, M. A. Harrison, P. J. Fedorka-Cray, J. S. Bailey, and G. R. Siragusa. To be submitted to Foodborne Disease and Pathogens

Abstract

Clostridium difficile is an emerging pathogen of food animals and an established agent of disease in humans. Retail meat has been identified as a source of the pathogen. In this study, a rapid, specific, and sensitive PCR assay method targeting the 16S rRNA gene was developed for the detection of *C. difficile* in food enriched broth (ground chuck, ground pork sausage, ground turkey) and fecal enriched broth (swine, dairy cattle, beef cattle, broiler). Based on examination of 484 *C. difficile* isolates, 15 strains of other *Clostridium* spp., and 11 other bacteria associated with food and feces, it was concluded that the assay detected *C. difficile* specifically. Sensitivity tests were conducted with feces and food enriched media (cycloserine-cefoxitin fructose broth supplemented with 0.1% sodium taurocholate [TCCFB]) post 24 h incubation at 37°C. Samples were inoculated with various concentrations of *C. difficile* strain 630 (ATCC BAA 1382) prior to DNA extraction and PCR. The detection limits post-enrichment were established to be as few as 20 *C. difficile* organisms per 9 ml TCCFB for swine feces, dairy cattle feces, broiler feces, ground turkey, and ground chuck samples. Forty *C. difficile* organisms per 9 ml TCCFB was the detection limit for beef feces and pork sausage samples. The addition of bovine serum albumin (BSA) dramatically increased detection, especially in ground turkey and broiler fecal samples. The procedure produced results in ~32 h which includes the 24 h selective enrichment step. This method may be used as an alternative to laborious and lengthy (5-10 days) standard cultural procedures to diagnose symptomatic food animals for CDAD and detect *C. difficile* in contaminated meat products quickly and accurately.

Keywords: *Clostridium difficile*, 16S rRNA, PCR, fecal enriched broth, food

Introduction

Clostridium difficile is a ubiquitous anaerobic, spore-forming bacterium that causes disease in humans and food animals. *C. difficile*-associated disease (CDAD) is associated primarily with the production of an enterotoxin (TcdA) and a cytotoxin (TcdB) (Keel and Songer, 2006). These toxins are glucosyltransferases that work synergistically to alter host cell regulation by affecting the actin filaments (Aktories and Just, 2005). As a result, loss of cellular contact, membrane ruffling, and cell rounding occur (Pothoulakis and LaMont, 2001). Cellular damage is additionally brought about by an inflammatory response and apoptosis, or programmed cellular death, which are also induced by TcdA and TcdB (Kim et al., 2007; Nottrott et al., 2007). Some *C. difficile* strains are capable of producing a third toxin, a binary protein toxin (CDT), which is an ADP-ribosyltransferase (Perelle et al., 1997). CDT is a cytolethal distending toxin that changes cell morphology when in cell culture. However, its role in CDAD is currently unknown. CDAD, which is often associated with prior exposure to antimicrobial therapy, can result in mild diarrhea to severe colitis in susceptible hosts (Kelly et al., 1994). Mortality rates increased 35% each year from 1999-2004 (Redelings et al., 2007) and the emergence of hypervirulent and antibiotic resistant epidemic strains such as North American Pulsed-Field Gel Electrophoresis Type 1 (NAP1) have caused outbreaks in North America and Europe (Warny et al., 2005).

Community-associated CDAD (CA-CDAD) cases, or those occurring outside the healthcare setting, have emerged and evidence suggests that food animals may be a potential source for these infections. Urban market poultry (29%), healthy swine (15.9%), and dairy calves (14.9%) have been noted to be important sources of toxigenic *C. difficile* (Rodriguez-Palacios et al., 2006, Simango and Mwakurudza, 2008; Thitaram, 2008). CA-CDAD cases are

often associated with CDT+ strains which are highly prevalent in food animals (Keel et al., 2007, Goorhuis et al., 2008; Jhung et al., 2008). Furthermore, *C. difficile* strains that are of Toxinotype V/Ribotype 078 (TcdA+, TcdB+, CDT+) are the most common types among food animals (Keel et al., 2007, Goorhuis et al., 2008; Jhung et al., 2008). Once considered rare in human disease, this same type has recently emerged as a common cause of CA-CDAD (Goorhuis et al., 2008; Jhung et al., 2008).

C. difficile has been reported to contaminate 20-41% of retail ground meats and toxigenic strains such as NAP1 and Ribotypes 077, 014, and 078 have been isolated from various ground meat products (Rodriguez-Palacios et al., 2007; Songer, 2007). It has also been noted that a minority of the *C. difficile* strains isolated from these meats were indistinguishable from human clinical isolates. Food animals serve as transmission vectors for zoonotic diseases, including foodborne disease, due to direct or indirect contact, environmental contamination, and/or consumption of their contaminated meat (Steinmuller et al., 2006). Therefore, it is believed that food animals may also transmit CDAD to humans via these same routes (Jhung et al., 2008).

C. difficile is an important agent of enteritis in neonatal piglets (Songer and Anderson, 2006) and dairy calves (Porter et al., 2002, Rodriguez-Palacios et al., 2006; Hammitt et al., 2008) which can lead to significant losses in production (Keel et al., 2007). Proper treatment, especially antibiotic therapy, in a timely manner by animal producers may save money. Previous investigation into the diagnosis of CDAD has been based on detection of TcdA and TcdB from feces by immunoassays such as ELISA (Songer, 2004, Rodriguez-Palacios et al., 2006; Simango and Mwakurudza, 2008) or by fibroblastic culture assay in which cytopathic effects are observed. ELISA assays may result in false-positives and are only available for fecal specimens and not foods. Cell culture assays are difficult and costly to perform. Laboratory isolation by

cultural techniques for *C. difficile* are labor intensive, expensive, and time consuming, from 5-15 days to achieve results (Arroyo et al., 2005, Rodriguez-Palacios et al., 2006, 2007; Simango and Mwaurudza, 2008). Afterwards, further characterization tests are required to confirm a *C. difficile* positive sample. Therefore a rapid, sensitive, and specific test for *C. difficile* presence in foods and feces is needed for veterinary diagnosis and treatment, to provide researchers with a new tool to investigate food as a potential vector for CDAD, and for the accurate analysis of processing and sanitation programs for food processors.

In recent years, a number of PCR assays targeting the 16S rRNA gene to detect *C. difficile* have been developed (Gumerlock et al., 1991, Wang et al., 1994, Kikuchi et al., 2002, Rinttila et al., 2004; Tonooka et al., 2005). Advantages of targeting the 16S rRNA gene are: 1) there are 10,000 copies of 16S rRNA molecules compared to only a single copy for most other genes (Watson et al., 1987); 2) 16S rRNA gene has been highly conserved over time (Huysman and De Wachter, 1986); and 3) 16S rRNA is an excellent target in complex ecosystems like feces and meat (Matsuki et al., 2002). However, a PCR assay involving an enrichment step prior to DNA extraction has not yet been established for fecal and food samples to rapidly detect *C. difficile*. PCR assays from enriched samples have been employed to detect other foodborne pathogens in food and pig feces (Lindqvist, 1997, Lantz et al., 1998; Dahlenborg et al., 2001). These assays offer rapid (up to 36 hours), sensitive (as few as 5 cfu/g food), and specific results. The objective of this study was to develop a rapid, sensitive, and specific PCR assay targeting the 16S rRNA gene to detect *C. difficile* from enriched food and fecal samples.

Materials and Methods

BACTERIAL STRAINS

Four hundred and eighty-four strains of *C. difficile*, 15 strains of other *Clostridium* spp., and 11 other non-*Clostridium* strains (6 gram-positive and 5 gram-negative) were used in this study (Tables 5.1, 5.2, and 5.3, respectively). All *Clostridium and Bacillus* spp. were struck from cooked meat broth (Oxoid, Columbia, MD) (stored aerobically at 25°C) onto tryptic soy agar with 5% sheep blood (BA) (Remel, Lexana, KS) and incubated anaerobically at 37°C for 3 d. All other strains were grown aerobically on BA at 37°C for 24 h with the exception of *Campylobacter* spp.. *C. jejuni* and *C. coli* were grown on BA for 48 h under microaerophilic conditions (10% CO_2, 5% O_2, and balanced N_2). DNA was extracted from these pure cultures by picking 1 to 2 isolated colonies with a sterile needle and boiling them in 100 µl molecular grade water for 10 min. The extracted DNA was then stored at -20°C until needed for PCR.

SAMPLE PREPARATION

The PCR assay was evaluated for sensitivity using inoculated feces (swine, broiler, dairy cattle, beef cattle) and meat (ground chuck, ground pork sausage, ground turkey) samples. Swine, dairy, and beef cattle feces were originally obtained from the National Animal Health Monitoring System (NAHMS) and were from healthy animals with no signs of diarrhea. Samples (approximately 200 g) were collected aseptically in whirl-pak™ bags and sent on ice to the laboratory within 24 h. For the broiler fecal samples, viscera were collected from the evisceration line of a local poultry slaughter processing plant and placed in a sterile stomacher bag. The intestines were packed in ice and immediately sent to the laboratory within 1 h. Cecum and gut contents were removed aseptically into whirl-pak™ bags. Food samples (approximately 460 g) were purchased from a local grocery store and taken to the laboratory

within 1 h. Food and fecal samples were initially cultured for *C. difficile* presence by employing alcohol shock methods previously described by Thitaram (2008). Food and fecal samples used in this study were negative for *C. difficile*. These samples were stored at -20°C. Before use, the samples were thawed at 4°C for 16 h.

All media was pre-reduced in an anaerobic chamber (Bactron Anaerobic, Model BacII, Sheldon Manufacturing, Cornelius, OR) with a gas composition of 5% hydrogen, 5% CO_2 and 90% nitrogen for 24 h prior to use. Each sample type was prepared by mixing 25 g of sample with 225 ml 0.9% sterile saline in a sterile stomacher bag and homogenized for 2 min in a stomacher. For each sample type, two replications were performed. One ml of sample homogenate was transferred to 9 ml peptone buffered saline (PBS) dilution blank and vortexed. Large debris was avoided when withdrawing the samples. From this solution, 1 ml each was placed into 9 sterile 9 ml cycloserine-cefoxitin fructose broth supplemented with 0.1% sodium taurocholate (TCCFB) (supplemented with *C. difficile* monolactam norfloxacin [C.D.M.N.] SR0173E, Oxoid, Columbia, MD) tubes. Eight of the 9 tubes represented a different concentration of *C. difficile* inoculum (10^8 cells to 10^1 cells) and one tube was used as an uninoculated negative control. A positive control was prepared using *C. difficile* ATCC BAA 1382 (strain 630). Preparation involved inoculating a 9 ml TCCFB tube with a 10 µl loop full of fresh 3 d old culture from a *C. difficile* cycloserine-cefoxitin fructose agar supplemented with 7% lysed horse blood (CCFA) (*C. difficile* agar [Remel, Lenexa, KS], supplemented with *C. difficile* SR0096 [Oxoid]) plate. All tubes were incubated aerobically at 37°C for 24 h.

Twenty-four hours later, serial dilutions were performed from the positive control tube using PBS blanks. One ml aliquots were placed into each of the 8 TCCFB tubes so that each tube received a specific concentration (10^8 cells to 10^1 cells). Standard plate counts from the

PBS dilution blanks were performed to determine the initial and inoculating concentrations by plating onto CCFA and incubating them at 37°C for 3 d. The negative control TCCFB tube received no *C. difficile* inoculation (only the sample). All TCCFB tubes (8 samples with different concentrations and positive and negative controls) were then vortexed thoroughly and centrifuged (Jouan, Model CR4-22, Manchester, VA) at 3,800x g for 10 m. The supernatant fluid was decanted and the resulting pellet was saved for DNA extraction using the Mo Bio UltraClean™ Fecal DNA Kit (Mo Bio Laboratories, Inc., Carlsbad, CA). Template DNA was stored at -20°C until needed for PCR.

PCR ASSAY FOR *CLOSTRIDIUM DIFFICILE* DETECTION

The total volume of the PCR assay developed was 25 µl. It consisted of 12.5 µl PCR Master Mix containing *Taq* DNA polymerase, $MgCl_2$, and dATP, dGTP, dCTP, dTTP (Promega, catalog number M7502, Madison, WI), 4.5 µl molecular grade water (Promega, Madison, WI), 1.0 µl of bovine serum albumin (BSA) (Idaho Technology Inc., Salt Lake City, UT), 5.0 µl template DNA, and 1.0 µl of each primer. The 16S rRNA gene detection was accomplished using primers ClDIF-F (5'-CCT GAA TAT CAA AGG TGA GCC A-3') and ClDIF-R (5'-CTA CAA TCC GAA CTG AGA GTA-3'). These sequences were developed by Kikuchi et al. (2002) who designed them to distinguish *C. difficile* from 13 other *Clostridium* spp.

PCR amplification was carried out with a thermal cycler (MJ Research, Inc. Model PT-2000, Waltham, MA) consisting of 1 cycle for 2 min at 94°C, followed by 35 cycles for 30 sec at 94°C, 30 sec at 60°C, and 2 min at 72°C, and one cycle for 2 min at 72°C. Post-amplification, PCR products (10 µl) were separated by using a gel electrophoresis chamber (120 V) with 1X TBE buffer stained with ethidium bromide. Visualization of bands was performed under UV

light. All gels were tested with the aforementioned positive control strain and a negative control. A positive detection was noted when an amplicon of 1,085 bp was observed.

Results and Discussion

For the specificity test, the PCR assay fully distinguished all 484 *C. difficile* strains from all the other bacteria tested. Only *C. difficile* strains showed a positive detection with the 1,085 bp amplicon while the other strains were not amplified. These results indicate that the assay is specific to *C. difficile* and that there is no cross-reaction with the other bacteria such as *E. coli*, *Salmonella* Typhimurium, other clostridia, *Campylobacter*, *Enterococcus*, *Pseudomonas*, *Bacillus*, *Staphylococcus*, and *Listeria*, that might be expected in food and fecal samples.

In the PCR sensitivity test with varying concentrations of *C. difficile* inoculated into fecal/food enriched media (TCCFB), from 20 to 10^8 cells were detectable after 24 h incubation. There were no differences between replications for each sample type. In some instances, cell numbers below 10^4 were observed to have decreased intensities of bands (PCR products). PCR assay detection results for the food enriched samples are shown in Table 5.4. Post 24 h enrichment, the assay was sensitive enough to detect 20 *C. difficile* organisms with the ground chuck and ground turkey samples and 40 *C. difficile* organisms with pork sausage samples. Band intensities remained high to medium with the ground chuck and pork sausages samples, but were faint with the ground turkey with the 2.0×10^2 and 2.0×10^1 cfu inoculated samples.

The rapid (32 h) and low cell number detection observed in this current study is similar to that of research regarding foodborne pathogen detection in enriched meat samples by PCR. Wang et al. (1994) were able to detect 2 cells of *C. perfringens* on inoculated poultry after a 17 h enrichment step using a 16S rDNA based PCR assay. Another PCR enrichment broth assay

targeting the 16S rRNA gene detected 400 cfu/ml enrichment of *Y. enterocolitica* in minced pork (Lantz et al., 1998). PCR assays targeting toxin genes have also been successful in enriched food samples. Fach et al. (1995) used an assay to detect *C. botulinum* after a 18 h enrichment step. Targeting the BoNT genes, the authors detected 10 cfu/g *C. botulinum* types A and E in inoculated meat samples. Lindqvist (1997) spiked minced beef samples with *E. coli* O157:H7. After only 8 h enrichment, the PCR assay targeting *eae* and verocytotoxin genes (*VT1* and *VT2*) was able to detect 5 cfu/g of the pathogen. The use of toxin genes (*tcdA*, *tcdB*, *cdtB*) as a target in a multiplex PCR enrichment assay for the detection of toxigenic *C. difficile* from feces and foods should be evaluated.

PCR detection results of inoculated fecal enrichment broth samples are presented in Table 5.5. The sensitivities of this assay with the fecal samples were equal to the food samples. At 24 h post-enrichment, the assay was sensitive enough to detect 20 *C. difficile* organisms in the swine, dairy, and broiler fecal enriched samples, and 40 *C. difficile* organisms in the spiked beef fecal enrichment samples. PCR product amplicons (band) intensities declined to low levels under 10^4 cfu with the swine and dairy fecal enrichment samples. Only 10^1 cfu samples produced a light intensity band with broiler fecal enrichment samples and the bands from the beef fecal enrichment samples remained at a high level of intensity through all inoculum concentrations. The use of a PCR enrichment procedure has been shown to be effective to detect *C. botulinum* in pig feces. Dahlenborg et al. (2001) successfully detected *C. botulinum* types B (non-proteolytic) at 10 spores/g feces and 3.0×10^3 spores/g feces with *C. botulinum* types E and F after 18 h enrichment.

The rapid, sensitive, and specific PCR assay developed in the current study could be useful to veterinarians, researchers, and food processors to detect *C. difficile* in feces and food

enriched TCCFB broth. The total time needed for positive detection is ~32 h (24 h enrichment in TCCFB, 4 h DNA extraction step, and 3 h PCR process). This time frame is substantially shorter than cultural procedures that could require 10 d to obtain results. Quicker diagnosis by veterinarians could lead to prompt therapy and/or isolation of *C. difficile* infected animals which in turn would reduce horizontal transmission among herds/flocks. The assay may be important for food processors concerned with the safety and processing quality of their products. Rapid detection of *C. difficile* with this PCR assay could also save time and labor compared to the laboratory cultural method. Furthermore, the ability for a PCR assay to detect as few as 20 *C. difficile* organisms, as was the case in this study, represents a 100-fold increase in sensitivity compared to culture (Gumerlock et al., 1991). The shorter time to obtain results coupled with increased sensitivity could be of value to regulatory decision makers, and scientists responsible for ensuring the safety of meat products.

Both food and feces are contaminated by bacteria and other microorganisms. As a result, high concentrations of non-target DNA are present in these samples. Using previously frozen (-20°C) samples for this PCR procedure allowed for some of this non-target microflora to be reduced. Evidence has shown that storage at -20°C does not affect recovery with regard to an enrichment PCR assay with *C. botulinum* (Lindqvist, 1997) nor with *C. difficile* culture involving alcohol shocking and TCCFB enrichment (Thitaram, 2008). Freezing the samples prior to enrichment may act as a selective process for *C. difficile* as the spores that survived freezing are later recovered as vegetative cells in the TCCFB broth. The enrichment step, prior to DNA extraction, is needed for the *C. difficile* vegetative cells to grow and multiply to PCR detectable levels. Since there are 10,000 copies of 16S rRNA molecules in living cells (Watson et al., 1987), the enrichment step allows for a high concentration of the target. High concentrations of

the primers' target may also help in reducing PCR inhibitors that are frequently found in food and feces (Wang et al., 1994).

PCR inhibitors have been identified in fecal (Lantz et al., 1997) and food samples (Powell et al., 1994). Inhibitors of PCR reactions may act by: 1) interfering with cell lysis; 2) degradation or capture of nucleic acids; or 3) inactivation of thermostable DNA polymerase (Wilson, 1997). Inhibitory components found in foods include bile salts, complex carbohydrates, proteinases, and non-target DNA (Al-Soud and Radstrom, 2000). Inhibitors in food include proteinases (Powell et al., 1994) and non-target DNA. To overcome such inhibitors, an amplification facilitator may be used in the assay. Bovine serum albumen (BSA) has been shown to increase amplification with *Taq* DNA polymerase. Al-Soud and Radstrom (2000) reported positive detection of *L. monocytogenes* in 4% fecal samples with BSA in the PCR assay compared to detection in 0.4% fecal and 0.2% meat samples with an assay excluding BSA. The authors noted that BSA had no synergistic or additive effects. Powell et al., (1994) used BSA to overcome proteinase inhibitors in milk. BSA may act by removing the effects of protease inhibitors by stabilizing enzymes during DNA digestion (Al-Soud and Radstrom, 2000) and may prevent adhesion of DNA polymerase to reaction tubes.

In the current study, PCR inhibition was initially observed. To overcome the inhibition, the sample concentration was diluted to 0.1% wt/vol (3 x 1:10 serial dilutions). This sample concentration was sufficient to allow detection of *C. difficile* in all fecal/food enriched samples tested without the use of BSA. However, the sensitivity without BSA was considerably less (data not shown). The biggest effect was with poultry samples (ground turkey and broiler fecal enriched broth). 10^6 and 10^5 *C. difficile* organisms were required for detection in bovine fecal and ground turkey enriched media respectively, without BSA in the assay. When BSA was

added to the assay, detection sensitivity increased to 20 *C. difficile* organisms for both types of samples. The addition of BSA also improved detection in the swine, dairy, and beef fecal enriched samples. In those samples, 10^4 *C. difficile* organisms were required for detection without BSA; the assay that included BSA was able to amplify the target and allow detection of 20 *C. difficile* organisms for swine and dairy fecal enriched samples and 40 *C. difficile* organisms in beef fecal enriched samples. The presence of BSA increased the intensity of bands for the 10^2 and 10^1 *C. difficile* cells inoculated into ground chuck enriched samples. However, BSA had no effect on *C. difficile* detection in pork sausage enriched samples.

While rapid (~32 h), sensitive (20 *C. difficile* organisms), and specific results were obtained with vegetative cell inoculated into fecal/food enriched broth samples, further tests need to be conducted. Recovery of cells and spores from direct inoculation of food and fecal samples needs to be determined. *C. difficile* spores would be more likely to contaminate retail meats than would vegetative cells. Also, spores would be prevalent in fecal samples. Therefore, performing a controlled low concentration spore inoculation study would be appropriate. Heat shocking (70°C for 10 min) samples prior to enrichment in TCCFB would reduce background flora and initiate spore germination. Samples taken at different time intervals from TCCFB broth could be evaluated for *C. difficile* presence using this assay. In addition, the assay could be used to target *tcdA*, *tcdB*, and *cdtB* genes to detect toxigenic strains in these samples. Furthermore, additional replications and inoculation with various levels of background biota would be useful in determining the efficiency of this assay.

In conclusion, the goal of this study was to develop a PCR assay targeting 16S rRNA gene sequences to detect *C. difficile* in food and fecal enriched samples. The assay is capable of distinguishing *C. difficile* from other anaerobic and foodborne bacteria commonly associated

with these samples. The assay is sensitive, as evidenced by detection of 20-40 *C. difficile* organisms with inoculated samples. Compared to cultural isolation procedures, this assay is rapid. Although further validation is needed, the assay shows promise for use by veterinarians to quickly and accurately diagnose and treat food animals with CDAD. Furthermore, this assay could be employed by researchers and food processors to detect *C. difficile* in meat products, as well as other foods, which would lead to more information about the possibility of foods as potential sources for human CDAD. Data could be used by government regulatory agencies to develop a *C. difficile* risk assessment and to establish regulatory standards in RTE foods.

Table 5.1. List of *Clostridium difficile* strains[a], sources[b], and the PCR assay results[c] for specificity test

***Clostridium difficile* strain**	**Source**	**PCR result**
630	ATCC BAA 1382	+
VPI 10463	ATCC A3255	+
9689	ATCC 9689	+
NAP1	CDC	+
NAP8	CDC	+
AE978	U. of Guelph	+
WT	NAHMS swine	+
WT	NAHMS beef	+
WT	NAHMS dairy	+
WT	NAHMS dairy environment	+

[a] NAP, North American Gel Electrophoresis type; WT, Wild Type strains (total n = 478, swine n = 130, beef n = 244, dairy n = 43, and dairy environment n = 61) were isolated from fresh fecal samples and confirmed by biochemical tests and PCR.
[b] ATCC, American Type Culture Collection; CDC, Centers for Disease Control; U. of Guelph, University of Guelph, Canada; NAHMS, National Animal Health Monitoring System.
[c] +, Positive detection with 1,085 bp amplicon.

Table 5.2. List of other clostridia strains, sources[a], and the PCR results[b] for specificity test

***Clostridium* strain**	**Source**	**PCR result**
Clostridium spiroforme	ATCC 29900	-
Clostridium limosum	ATCC 25620	-
Clostridium bifermentans	ATCC 638	-
Clostridium paraputrificum	ATCC 25780	-
Clostridium innocum	ATCC 14501	-
Clostridium tetani	ATCC 19406	-
Clostridium perfringens	ATCC 13124	-
Clostridium perfringens	ATCC 3624	-
Clostridium sporogenes	ATCC 3584	-
Clostridium sordelli	ATCC 9714	-
Clostridium beijerckeii	ATCC 8260	-
Clostridium noyvi	ATCC 19402	-
Clostridium histolyticum	ATCC 19401	-
Clostridium septicum	ATCC 12464	-
Clostridium coccoides	ATCC 29236	-

[a] ATCC, American Type Culture Collection.
[b] -, Negative detection with no amplification.

Table 5.3. List of other bacterial strains, sources[a], and the PCR results[b] for specificity test

Bacterial strains	Source	PCR result
Bacillus subtilis	ATCC 6633	-
Bacillus fragilis	ATCC 25285	-
Bacillus megaterium	ATCC 9885	-
Staphylococcus aureus	ATCC 29213	-
Enterococcus faecalis	ATCC 29212	-
Listeria monocytogenes	ATCC 19114	-
Escherichia coli	ATCC 25922	-
Campylobacter jejuni	ATCC 33560	-
Campylobacter coli	ATCC 33559	-
Pseudomonas aeruginosa	ATCC 27853	-
Salmonella Typhimurium	ATCC 14028	-

[a] ATCC, American Type Culture Collection.
[b] -, Negative detection with no amplification.

Table 5.4. PCR results[a] for food enriched samples[b] containing indicated number of cells of *Clostridium difficile* in 9 ml TCCFB tube[c]

Sample	10^8	10^7	10^6	10^5	10^4	10^3	10^2	10^1	- C[d]
Ground chuck	+	+	+	+	+	+	+	+	-
Pork sausage	+	+	+	+	+	+	+	+	-
Ground turkey	+	+	+	+	+	+	±	±	-

[a] Symbols: +, high intensity band; ±, low intensity band; -, no band.
[b] Sample concentration of 0.1% wt/vol (3 X 1:10 serial dilutions of original sample).
[c] Samples incubated 24 h prior to inoculation from a 2.0 x 10^8 cfu/ml fresh culture solution.
[d] – C, negative control, contained sample but had zero cells.

Table 5.5. PCR results[a] for fecal enriched samples[b] containing indicated number of cells of *Clostridium difficile* in 9 ml TCCFB tube[c]

Sample	10^8	10^7	10^6	10^5	10^4	10^3	10^2	10^1	- C[d]
Swine	+	+	+	+	+	±	±	±	-
Dairy	+	+	+	+	+	±	±	±	-
Beef	+	+	+	+	+	+	+	+	-
Broiler	+	+	+	+	+	+	+	±	-

[a] Symbols: +, high intensity band; ±, low intensity band; -, no band.
[b] Sample concentration of 0.1% wt/vol (3 X 1:10 serial dilutions of original sample).
[c] Samples incubated 24 h prior to inoculation from a 2.0 x 10^8 cfu/ml fresh culture solution.
[d] – C, negative control, contained sample but had zero cells.

References

Aktories, K. and I. Just. 2005. Clostridial Rho-inhibiting protein toxins. Current Topics in Microbiol. and Immun. 291:113-145.

Arroyo, L., J. Rousseau, B. Willey, D. Low, H. Staempfli, A. McGreer, and J. Weese. 2005. Use of a selective enrichment broth to recover *C. difficile* from stool swabs stored under different conditions. J. Clin. Microbiol. 43:5341-5343.

Al-Soud, W. A. and P. Radstrom. 2000. Effects of amplification facilitators on diagnostic PCR in the presence of blood, feces, and meat. J. Clin. Microbiol. 38:4463-4470.

Dahlenborg, M., E. Borch, and P. Radstrom. 2001. Development of a combined selection and enrichment PCR procedure for *Clostridium botulinum* Types B, E, and F and its use to determine prevalence in fecal samples from slaughtered pigs. Appl. Environ. Microbiol. 67:4781-4788.

Fach, P., M. Gilbert, R. Griffais, J. P. Guillou, and M. R. Popoff. 1995. PCR and gene probe identification of botulinum neurotoxin A-, B-, E-, F-, and G- producing *Clostridium* spp. and evaluation in food samples. Appl. Environ. Microbiol. 61:389-392.

Goorhuis, A., S. B. Debast, L. A. M. G. Van Leengoed, C. Harmanus, D. W. Notermans, A. A. Bergwerff, and E. J. Kuijper. 2008. *Clostridium difficile* PCR ribotype 078: an emerging strain in humans and pigs? J. Clin. Microbiol. 46:1157-1158.

Gumerlock, P., Y. Tang, F. Meyers, and J. Silva. 1991. Use of the polymerase chain reaction for the specific and direct detection of *Clostridium difficile* in human feces. Rev. Infect. Dis. 13:1053-1060.

Hammitt, M. C., D. M. Bueschel, M. K. Keel, R. D. Glock, P. Cuneo, D. W. DeYoung, C. Reggiardo, H. Trinh, and J. G. Songer. 2008. A possible role for *Clostridium difficile* in the etiology of calf enteritis. Vet. Microbiol. 127:343-352.

Huysman, E. and R. De Wachter. 1986. Compilation of small ribosomal subunit RNA sequences. Nucleic Acid Res. 14 (Suppl):r73-r118.

Jhung, M. A., A. D. Thompson, G. E. Killgore, W. E. Zukowski, G. Songer, M. Warny, S. Johnson, D. N. Gerding, L. C. McDonald, and B. M. Limbago. 2008. Toxinotype V *Clostridium difficile* in humans and food animals. Emerg. Infect. Dis. 14:1039-1045.

Keel, M. K. and J. G. Songer. 2006. The comparative pathology of *Clostridium difficile*-associated disease. Vet. Pathol. 43:255-240.

Keel, K., J. S. Brazier, K. W. Post, S. Weese, and J. G. Songer. 2007. Prevalence of PCR ribotypes among *Clostridium difficile* isolates from pigs, calves, and other species. J. Clin. Microbiol. 45:1963-1964.

Kelly, C. P., C. Pothoulakis, and T. J. Lamont. 1994. *Clostridium difficile* colitis. N. Eng. J. Med. 330:257-262.

Kikuchi, E., Y. Miyamoto, S. Narushima, and K. Itoh. 2002. Design of species-specific primers to identify 13 species of *Clostridium* harbored in human intestinal tracts. Microbiol. Immunol. 46:353-358.

Kim, H., S. H. Rhee, C. Pothoulakis, and J. T. Lamont. 2007. Inflammation and apoptosis in *Clostridium difficile* enteritis is mediated by PGE2 up-regulation of Fas Ligand. Gastroenterology. 133:875-886.

Lantz, P. -G., M. Matsson, T. Wadstrom, and P. Radstrom. 1997. Removal of PCR inhibitors from human fecal samples through the use of an aqueous two-phase system for sample preparation prior to PCR. J. Microbiol. Methods. 28:159-167.

Lantz, P. -G., R. Knutsson, Y. Blixt, W. A. Al-Soud, E. Borch, and P. Radstrom. 1998. Detection of pathogenic *Yersinia enterocolitica* in enrichment media and pork by a multiplex PCR: a study of sample preparation and PCR-inhibitory components. Int. J. Food Microbiol. 45:93-105.

Lindqvist, R. 1997. Preparation of PCR samples from food by a rapid and simple centrifugation technique evaluated by detection of *Escherichia coli* O157:H7. Int. J. Food Microbiol. 37:73-82.

Matsuki, T., K. Watanabe, J. Fujimoto, Y. Miyamoto, T. Takada, K. Matsumoto, H. Oyaizu, and R. Tanaka. 2002. Development of 16S r RNA-gene targeted group-specific primers for the detection and identification of predominant bacteria in human feces. Appl. Environ. Microbiol. 68: 5445-5451.

Nottrott, S., J. Schoentaube, H. Genth, I. Just, and R. Gerhard. 2007. *Clostridium difficile* toxinA-induced apoptosis is p53-independent but depends on glucosylation of Rho GTPases. Apoptosis. 12:1443-1453.

Perelle, S., M. Gilbert, P. Bourlioux, G. Corthier, and M. R. Popoff. 1997. Production of a complete binary toxin (actin-specific ADP-ribosyltransferase) by *Clostridium difficile* CD196. Infect. Immun. 65:1402-1407.

Porter, M. C., C. Reggiardo, D. M. Bueschel, M. K. Keel, and J. G. Songer. 2002. Association of *Clostridium difficile* with bovine neonatal diarrhea. In: Proc. 45th Ann. Mtg. Amer. Assoc. Vet. Lab. Diagn., St. Louis, Mo.

Pothoulakis, C. and J. T. Lamont. 2001. Microbes and microbial toxins: paradigms for microbial-mucosal interactions II. The integrated response of the intestine to *Clostridium difficile* toxins. Am. J. Physiol. Gastrointest. Liver Physiol. 280:G178-G183.

Powell, H. A., C. M. Gooding, S. D. Garrett, B. M. Lund, and R. A. McKee. 1994. Proteinase inhibition of the detection of *Listeria monocytogenes* in milk using the polymerase chain reaction. Lett. Appl. Microbiol. 18:59-61.

Redelings, M., F. Sorvillo, and L. Mascola. 2007. Increase in *Clostridium difficile*-related mortality rates, United States, 1999-2004. Emerg. Infect. Dis. 13:1417-1419.

Rinttila, T., A. Kassinen, E. Malinen, L. Krogius, and A. Palva. 2004. Development of an extensive set of 16S rDNA-targeted primers for quantification of pathogenic and indigenous bacteria in faecal samples by real time PCR. J. Appl. Microbiol. 97:1166-1177.

Rodriguez-Palacios, A., H. Stampfli, T. Duffield, A. Peregrine, L. Trotz-Williams, L. Arroyo, J. Brazier, and S. Weese. 2006. *Clostridium difficile* PCR ribotypes in calves, Canada. Emerg. Infect. Dis. 12:1730-1736.

Rodriguez-Palacios, A., H. Staempfli, T. Duffield, and J. Weese. 2007. *Clostridium difficile* in retail ground meat. Canada. Emerg. Infect. Dis. 13:485-487.

Simango, C. and S. Mwakurudza. 2008. *Clostridium difficile* in broiler chickens sold at market places in Zimbabwe and their antimicrobial susceptibility. Int. J. Food Microbiol. 124:268-270.

Songer, J. G. 2004. The emergence of *Clostridium difficile* as a pathogen of food animals. Animal Health Res. Rev. 5:312-326.

Songer, J. G. and M. A. Anderson. 2006. *Clostridium difficile*: an important pathogen of food animals. Anaerobe. 12:1-4.

Songer, J. 2007. Porcine CDAD. Presented to the National Pork Board Pork Safety Committee on July 30, 2007, Chicago, IL.

Steinmuller, N., L. Demma, J. B. Bender, M. Eidson, and F. J. Angulo. 2006. Outbreaks of enteric disease associated with animal contact: not just a foodborne problem anymore. Clin. Infect. Dis. 43:1596-1602.

Thitaram, S. N. 2008. Isolation and characterization of *Clostridium difficile* from porcine and bovine feces. Doctor of Philosophy Dissertation. University of Georgia, Department of Food Science and Technology, Athens, GA.

Tonooka, T., S. Sakata, M. Kitahara, M. Hanai, S. Ishizeki, M. Takada, M. Sakamoto, and Y. Benno. 2005. Detection and quantification of 4 species of the genus *Clostridium* in infant feces. Microbiol. Immunol. 49:987-992.

Wang, R., W. Cao, W. Franklin, W. Campbell, C. Cerniglia. 1994. A 16S rDNA-based PCR method for rapid and specific detection of *Clostridium perfringens* in food. Mol. Cell. Probes 8:131-138.

Warny, M., J. Pepin, A. Fang, et al. 2005. Toxin-production by an emerging strain of *Clostridium difficile* associated with outbreaks of severe disease in North America and Europe. Lancet. 336:1079-1084.

Watson, J., N. Hopkins, J. Roberts, J. Steitz, and A. Weiner. 1987. Molecular biology of the gene, vol. 1. Benjamin/Cummings Publishing Co., New York.

Wilson, I. G. 1997. Inhibition and facilitation of nucleic acid amplification. Appl. Environ. Microbiol. 63:3741-3751.

CHAPTER 6

SUMMARY AND CONCLUSION

This research was the first to describe the prevalence of toxigenic *C. difficile* in healthy, on-farm swine and cattle. The data collected in this dissertation may help food scientists and veterinarians better understand the potential role of food animals and foods in CA-CDAD. While much of the focus regarding CDAD in animals has been on symptomatic neonates, this research proves that healthy, on-farm swine and cattle are also carriers of potentially pathogenic strains of *C. difficile* that could be transmitted to humans. In addition, with the discovery of NAP1 strains in meat; CA-CDAD deaths in low risk individuals, and indistinguishable types found among food animals, humans, and meat; researchers, physicians, and food scientists need to be cognizant that *C. difficile* may be a foodborne biological hazard. In order to answer the question of whether CDAD may be a zoonotic and foodborne disease that impacts human health, it is apparent that researchers need to characterize isolates originating from food animals, people, and foods.

This research describes an improved method to recover *C. difficile* isolates from the feces of beef cattle. Applying an alcohol shock post-enrichment step was found to be significantly more effective for recovery than applying an additional shock prior to enrichment (double alcohol shock). This information is important for beef cattle veterinarians because this method is significantly more sensitive than what which found optimal for recovering *C. difficile* from swine feces and from dairy environmental samples using the double shock procedure. Depending on

the type of sample and host, optimum culture methods for *C. difficile* vary. The single shock method described in this study allows for the optimal recovery of *C. difficile* isolates from healthy beef feces. These recovered isolates can be further characterized to determine beef cattle's role, if any, in regards to *C. difficile* transmission to humans via the food chain.

The results of this research indicate that healthy, on-farm swine, cattle, and the dairy farm environment are reservoirs for potentially toxigenic strains of *C. difficile*. Asymptomatic animals with *C. difficile* going to market have the potential to contaminate the slaughter plant. Spore contamination in the environment can lead to contamination of equipment, finished product, packaging, and workers. Given the resistant nature of *C. difficile* spores, contamination may be difficult to control, and thus become a major, long term problem within the facility. *C. difficile* contaminated raw meat may also bring the pathogen into cook plants. Spores surviving the thermal process may result in contamination of RTE foods. As a result, *C. difficile* tainted foods may then be shipped from the plant. These contaminated foods (raw or RTE) would then be available for consumption by susceptible humans. *C. difficile* contaminated food shipped to and consumed within healthcare facilities may be an additional source for nosocomial CDAD infection.

The 16S rDNA based PCR assay described in this research is the first to be reported for *C. difficile* detection in food and fecal enriched medium. *C. difficile* was successfully detected in a variety of fecal and meat enriched sample types using this assay. The assay could be used by veterinarians, researchers, and food processors with both meat and fecal samples while eliminating lengthy and laborious culture methods. For veterinarians, the assay could be used in place of or in conjunction with ELISA and cell culture to directly detect the agent of CDAD. The assay would be easier and cheaper than the cell culture procedure and would not result in

false positives as can be the case with ELISA. Researchers could utilize this single assay's rapid results, specificity, and sensitivity to detect *C. difficile* in a variety of fecal and food enriched media specimens. Surveying a variety of many different types of food and fecal samples within the environment would be important to assess which additional foods and animals are possible vectors for CDAD transmission. Food processors could use the assay to evaluate processing and sanitation procedures within their particular processing procedures.

The data collected from this research is important because it further supports the theory that CDAD is potentially a zoonotic and foodborne disease. Healthy, on-farm food animals act as multiplying hosts for potentially toxigenic *C. difficile* strains that could possibly transmit CDAD to humans by contact or environmental and food contamination. Therefore, these animals may be involved with human acquisition of CDAD within the community. Furthermore, the enrichment PCR assay developed from this research provides scientists with a powerful tool to detect *C. difficile* from food and fecal enriched samples. Culture methods vary widely based on the sample source and no established method is currently available for foods. This single assay could be used for many other sample types besides materials evaluated in this study. For example, other sample types to be evaluated to determine their ability to transmit *C. difficile* to humans include, seafood, frozen foods, RTE foods, organically and commercially grown fruits and vegetables, juices, and animal feed. Furthermore, the assay provides government regulatory agencies (Food Safety and Inspection Service and Food and Drug Administration) a method to determine *C. difficile* risk assessment of the aforementioned foods. The assay would be an important tool in establishing and providing for regulatory standards for food processors.

Further research is needed to determine the role of food and food animals with regards to CDAD and what is the direct impact on human health. While Zimbabwe urban market poultry

was noted to be a major carrier of toxigenic *C. difficile*, evaluation of integrated poultry in the U.S. needs to be investigated. RTE foods need to be evaluated for *C. difficile* contamination given the thermal resistant nature of their spores. Dairy products should also be evaluated due to dairy cattle and the dairy farm environment being established sources of *C. difficile*. Genetic typing comparisons between healthy swine and cattle need to be studied. Applying the toxin genes (*tcdA*, *tcdB*, and *cdtB*) as targets for a PCR enrichment assay should be developed. The research discussed in this dissertation is a critical step in determining *C. difficile* as a possible agent of foodborne disease.

APPENDIX

DETECTION OF *CLOSTRIDIUM DIFFICILE* SPORES FROM FOOD AND FECAL SAMPLES BY A 16S rDNA BASED PCR ASSAY

Materials and Methods

SPORE PREPARATION

Clostridium difficile spores from strain 630 (ATCC BAA 1382) were produced in Duncan Strong sporulation media. A primary culture was made by placing a 10 µl loop full of fresh colonies grown on tryptic soy agar with 5% sheep blood (Remel, Lenexa, KS) into 10 ml freshly degassed fluid thioglycolate broth and incubating anaerobically at 37°C for 18 h. A second 10 ml thioglycolate tube was inoculated with 1 ml of the primary culture and incubated at 37°C for 4 h under anaerobic conditions. Post-incubation, 2.5 ml from the secondary culture tube was inoculated into 250 ml (1%) fresh Duncan Strong sporulation medium. Duncan Strong medium was incubated anaerobically for 4 d at 37°C to induce nutrient starvation. Afterwards, the spore solution was centrifuged (Jouan, Model CR4-22, Manchester, VA) at 1,300 x g for 15 min and the pellet was washed with 90 ml sterile double deionized water (ddH_2O). This solution was further concentrated by centrifugation at 4,600 x g for 20 min. The resulting pellet was washed again with 15 ml ddH_2O and centrifuged for 20 min at 4,600 x g. The concentrated spores were finally suspended in 3 ml ddH_2O and stored aerobically at 4°C. Wet mounts of the suspension viewed under a microscope revealed both free spores and terminal endospores. The enumeration of spores from the spore stock was performed weekly via the standard plate count

method involving microdilutions in peptone buffered saline (PBS) plated onto *C. difficile* cycloserine-cefoxitin fructose agar supplemented with 7% lysed horse blood (CCFA). The concentration was exactly 1.9 x 10^5 spores per ml for each count.

LAB PROCEDURE

Fecal (beef cattle, dairy cattle, swine) and meat (ground chuck, pork sausage, ground turkey) samples were inoculated with spores of *C. difficile* strain 630 for this study. Fecal samples (approximately 200 g) were obtained from healthy animals (non-diarrheic) and collected aseptically in whirl-pak™ bags. Samples were sent on ice to the laboratory within 24 h. Meat samples (mean weight of 455 g) were purchased from a local supermarket and taken to the lab within 1 h. All samples were stored at -20°C and were thawed at 4°C for 16 h prior to spore inoculation and homogenization.

All media was pre-reduced in an anaerobic chamber (Bactron Anaerobic, Model BacII, Sheldon Manufacturing, Cornelius, OR) with a gas composition of 5% hydrogen, 5% CO_2 and balanced nitrogen for 24 h prior to use. Each sample type was prepared by mixing approximately 1 g of sample and 100 µl of spore stock (19,000 spores) with 9 ml 0.9% sterile saline in a sterile stomacher bag and vortexed at maximum speed for 2 min. For each sample type, 3 replications were performed. One ml of sample homogenate was heat shocked at 70°C for 10 min to induce spore germination and to reduce background microflora. After heating, this volume was transferred to a 9 ml PBS dilution blank and vortexed. From this solution, 1 ml (19 spores) was placed into 6 sterile 9 ml cycloserine-cefoxitin fructose broth supplemented with 0.1% sodium taurocholate (TCCFB) (supplemented with *C. difficile* monolactam norfloxacin [C.D.M.N.] SR0173E, Oxoid, Columbia, MD) tubes. Three TCCFB tubes represented 0 h and the other 3 tubes represented 24 h. A positive control was prepared using *C. difficile* ATCC

BAA 1382 (strain 630). Preparation involved inoculating two 9 ml TCCFB tubes (0 h and 24 h) with a 10 µl loop full of fresh 3 d old culture from a CCFA (*C. difficile* agar [Remel, Lenexa, KS], supplemented with *C. difficile* SR0096 [Oxoid]) plate. Two negative controls were used (0 h and 24 h) by making a sample homogenate as previously described except that no spores were added. TCCFB tubes to be processed after 24 h enrichment were incubated anaerobically at 37°C. TCCFB tubes to be processed immediately (0 h) were vortexed thoroughly and centrifuged at 3,800 x g for 10 m. The supernatant was decanted and the resulting sediment was saved for DNA extraction using the Mo Bio UltraClean™ Fecal DNA Kit (Mo Bio Laboratories, Inc., Carlsbad, CA, USA). Samples to be processed after 24 h enrichment were processed in the same manner. Template DNA was stored at -20°C until needed for PCR.

PCR PROCEDURE FOR *CLOSTRIDIUM DIFFICILE* DETECTION

The total volume of the PCR assay was 25 µl. It consisted of 12.5 µl PCR Master Mix containing *Taq* DNA polymerase, $MgCl_2$, and dATP, dGTP, dCTP, dTTP (Promega, catalog number M7502, Madison, WI), 4.5 µl molecular grade water (Promega, Madison, WI), 1.0 µl of bovine serum albumin (BSA) (Idaho Technology Inc., Salt Lake City, UT), 5.0 µl template DNA, and 1.0 µl of each primer. The 16S rRNA gene detection was accomplished using primers ClDIF-F (5'-CCT GAA TAT CAA AGG TGA GCC A-3') and ClDIF-R (5'-CTA CAA TCC GAA CTG AGA GTA-3').

PCR amplification was carried out with a thermal cycler (MJ Research, Inc. Model PT-2000, Waltham, MA) consisting of 1 cycle for 2 min at 94°C, followed by 35 cycles for 30 sec at 94°C, 30 sec at 60°C, 2 min at 72°C, and one cycle for 2 min at 72°C. Post-amplification, 10 µl of the PCR products were run through a gel electrophoresis chamber (120 V) with 1X TBE buffer stained with ethidium bromide. Visualization of bands was performed under UV light.

All gels were tested with the aforementioned positive control strain and a negative control. A positive detection was noted when an amplicon of 1,085 bp was observed.

Results

Overall, *C. difficile* spores were detected in all sample types immediately following spore inoculation (0 h) and post 24 h enrichment in TCCFB (Table A.1). Band intensities were observed to be medium in the 0 h samples and high in the 24 h enriched samples. The assay was able to detect 19 *C. difficile* spores per 9 ml TCCFB enriched sample or 19,000 *C. difficile* spores per gram of meat/feces. The developed assay could be used to detect *C. difficile* spores in food and feces without enrichment giving results within 8 h.

Table A.1. PCR results[a] for *Clostridium difficile* spore detection for 3 replications from meat and fecal samples[b]

	Enrichment time							
	0 hours				24 hours			
Sample	**- C[c]**	**Rep 1**	**Rep 2**	**Rep 3**	**- C**	**Rep1**	**Rep 2**	**Rep 3**
Meat								
Ground chuck	-	+	+	+	-	+	+	+
Pork sausage	-	+	+	+	-	+	+	+
Ground turkey	-	+	+	+	-	+	+	+
Feces								
Beef	-	+	+	+	-	+	+	+
Dairy	-	+	+	+	-	+	+	+
Swine	-	+	+	+	-	+	+	+

[a] Symbols: +, high intensity band; -, no band.
[b] Sample concentration of 0.1% (3 X 1:10 serial dilutions of original sample) and spore concentration of 19 spores per 9 ml TCCFB tube.
[c] – C, negative control, contained sample but had zero spores.