ISOLATION AND CHARACTERIZATION OF *CLOSTRIDIUM DIFFICILE* FROM PORCINE AND BOVINE FECES

by

SUTAWEE NARINT THITARAM

(Under the Direction of Joseph F. Frank)

ABSTRACT

Clostridium difficile is recognized as a nosocomial pathogen associated with antimicrobial drug-associated diarrhea and pseudomembranous colitis in humans. Community-acquired *C. difficile*-associated diarrhea has been increasingly reported. The presence of *C. difficile* in 345 swine fecal samples, 1,325 dairy cattle fecal samples, and 371 environmental samples from dairy cattle farms were examined. Isolates were tested for their susceptibility to 9 antimicrobials and characterized for their relatedness. In the process, two isolation techniques, single and double alcohol shock, were evaluated for the recovery of *C. difficile* and two typing methods, repetitive extragenic palindromic-polymerase chain reaction (rep-PCR) and pulse-field gel electrophoresis (PFGE), were compared. Fifty five (15.9%) swine fecal samples, 32 (2.4%) dairy cattle fecal samples and 32 (8.6%) dairy cattle environmental samples were positive for *C. difficile* by either single or double alcohol shock. Double alcohol shock was significantly better than single alcohol shock for the recovery of *C. difficile* in swine feces. There was no significant difference for the recovery of *C. difficile* between any combination of isolation methods and media observed in dairy cattle feces. While in environmental samples, double alcohol shock plating to cycloserine-cefoxitin fructose agar (CCFA) was found to be a method of choice for the recovery of *C. difficile*. All isolates were susceptible to amoxicillin-clavulanic acid, metronidazole, and vancomycin while a small number of isolates (1%) were susceptible to rifampicin. All isolates were resistant to levofloxacin. A total of 135 (71.8%), 30(16%), 25 (13.3%), and 10 (5.3%) isolates were resistant to clindamycin, ampicillin, erythromycin, and linezolid, respectively. Both typing methods demonstrated wide genetic variation among *C. difficile* strains. Rep-PCR findings were relatively comparable to those obtained by PFGE. A high percentage of indistinguishable isolates between human and animal was found. This suggests that there is potential for foodborne or interspecies transmission.

INDEX WORDS: *Clostridium*, *Clostridium difficile*, Isolation, Characterization, Pig, Swine, Porcine, Dairy, Bovine, Etest, Antimicrobial susceptibility testing, Rep-PCR, Pulse-field gel electrophoresis, PFGE

ISOLATION AND CHARACTERIZATION OF *CLOSTRIDIUM DIFFICILE* FROM PORCINE AND BOVINE FECES

by

SUTAWEE NARINT THITARAM

B.S., Khon Kaen University, Thailand, 1997

M.S., The University of Georgia, 2004

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

2008

ISOLATION AND CHARACTERIZATION OF *CLOSTRIDIUM DIFFICILE* FROM PORCINE AND BOVINE FECES

by

SUTAWEE NARINT THITARAM

Major Professor:	Joseph F. Frank
Committee:	Joseph Stan Bailey
	Mark E. Berrang
	Mark A. Harrison
	Robert L. Shewfelt

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
December 2008

To Bug Big, I miss you.

ACKNOWLEDGEMENTS

I would like to express my sincere appreciation to Dr. Joseph F. Frank, my major professor, for all his direction throughout my course work and research. I have learned a lot from you both as a professor and as a person. I would like to thank Dr. Robert L. Shewfelt, Dr. Mark A. Harrison, and Dr. Mark E. Berrang for being on my committee and for their advice during my research. I am grateful to Dr. J. Stan Bailey for his support throughout my course work and research, for hiring me to work for the USDA and also for being on my committee.

I would like to thank Dr. Paula J. Fedorka-Cray for her useful advice and kindly support. I am grateful to Dr. Gregory R. Siragusa and Dr. Nelson A. Cox for their guidance and helpful suggestions. I would like to thank Dr. Mark Wise, Bacterial Barcodes, Inc., for all his assistance. Special thanks to Johnna K. Garrish for her caring and technical advice, to Calvin L. Williams for his technical support, and to Cheryl Gresham for her work on PFGE. Appreciation is also extended to Bacterial Epidemiology and Antimicrobial Resistance Research Unit and Poultry Microbiological Safety Research Unit, Agricultural Research Service, USDA for providing all resources necessary to conduct this research.

I am grateful to Robert A. Johnson and Lisa Andrews at Pilgrim's Pride Corp., for their advice and support and to Dr. Niwat Sanoamuang, Khon Kaen University, for his believing in me and encouragement.

Lastly, I would like to thank my family, Pat and Kiwi Thitaram who are always there for me. I also would like to thank my parents, Nat and Em-on Narint.

Without these wonderful individuals, I would not be the person I am today.

TABLE OF CONTENTS

Page

ACKNOWLEDGEMENTS....v

LIST OF TABLES....viii

LIST OF FIGURES....x

CHAPTER

1 INTRODUCTION....1

Purpose of the Study....3

Experiments and Hypotheses....3

2 LITERATURE REVIEW....10

2.1 *Clostridium difficile*....10

2.2 Epidemiology and Typing of *Clostridium difficile*....21

2.3 Antimicrobial-Associated Risk Factors for CDAD....30

2.4 Antibiotic Use in Livestock Production and Its Impact....36

3 PREVALENCE AND COMPARISON OF CULTURAL AND ISOLATION METHODS FOR *CLOSTRIDIUM DIFFICILE* FROM PORCINE AND BOVINE FECES....74

4 AUTOMATED REPETITIVE EXTRAGENIC PALINDROMIC-POLYMERASE CHAIN REACTION (REP-PCR) AS A MOLECULAR TYPING METHOD FOR *CLOSTRIDIUM DIFFICILE*....88

5 ANTIMICROBIAL SUSCEPTIBILITY OF *CLOSTRIDIUM DIFFICILE* ISOLATED FROM PORCINE AND BOVINE FECES 125
6 CONCLUSIONS 150
APPENDICES 154
A MEDIA FORMULATIONS 154
B CHARACTERIZATION DATA 159
C ANTIMICROBIAL SUSCEPTIBILITY IN RELATION TO REP-PCR DATA 165
BIOGRAPHY 168

LIST OF TABLES

Page

Table 2.1: Agents implicated as causes of *Clostridium difficile* pseudomembranous colitis or diarrhea 33

Table 2.2: Selected reports on the use of antimicrobials in animals and associated public health implications 38

Table 3.1: Prevalence and comparison of isolation methods for recovery of *Clostridium difficile* 84

Table 4.1: General comparison of manual and automated rep-PCR 103

Table 4.2: Characteristics of *Clostridium difficile* strains from human 104

Table 4.3: Summary of overall grouping by rep-PCR and PFGE 105

Table 4.4: Distribution and frequency of *Clostridium difficile* isolates recovered from swine, dairy cattle, dairy cattle environment, human, and NAP type strains according to rep-PCR grouping 108

Table 4.5: Distribution and frequency of *Clostridium difficile* isolates recovered from swine, dairy cattle, dairy cattle environment, human, and NAP type strains according to PFGE grouping 111

Table 4.6: Discriminatory powers of different molecular methods for typing *Clostridium difficile* 113

Table 5.1: Antimicrobials used in the susceptibility testing and their properties 136

Table 5.2: Distribution of antimicrobials tested against 188 *Clostridium difficile* isolated from swine, dairy cattle and dairy cattle environment................................137

Table 5.3: Range of MIC values and resistance rates from 188 *Clostridium difficile* isolated from swine, dairy cattle and dairy cattle environment................................138

Table 5.4: Susceptibility profiles of 188 *Clostridium difficile* isolates grouped by sample type..139

Table 5.5: Antimicrobial resistance profile of 188 multiple resistant *Clostridium difficile* isolates..140

LIST OF FIGURES

Page

Figure 4.1: Fifty six distinct clusters of 220 *Clostridium difficile* isolates by rep-PCR 114

Figure 4.2: Virtual gel image of rep-PCR reproducibility and pattern stability of 12 *Clostridium difficile* isolates. A dendrogram and virtual gel images represent samples analyzed by 2 operators (2nd operator denoted by "-2" after sample ID) using 2 separate Diversilab system during multiple days 115

Figure 4.3: The two graphs show indistinguishable samples by rep-PCR that vary only in overall intensity. All peaks have proportionally different fluorescence units. Sample B has only a difference in the intensity of one peak (arrow) from the sample A.. 116

Figure 4.4: Scatter plot generated by the Diversilab software (version 3.3) represents the relationship of 220 *Clostridium difficile* isolates by rep-PCR.. 117

Figure 4.5: Sample graph overlay generated by Diversilab software (version 3.3) allows two graphs to be placed on the same scale which aids in interpretation. 118

CHAPTER 1

INTRODUCTION

Clostridium difficile is recognized as a nosocomial pathogen associated with antimicrobial drug-associated diarrhea and pseudomembranous colitis (PMC) in humans (George et al. 1977; George et al. 1978; Kelly et al. 1994) which is referred to as *C. difficile*-associated disease (CDAD) (George et al. 1977). The antimicrobial drugs most frequently associated with CDAD include clindamycin (Worsley 1998; Freeman and Wilcox 1999; Johnson et al. 1999b), cephalosporins (Zwiener et al. 1989; Gerding et al. 1995; Spencer 1998; Worsley 1998; Freeman and Wilcox 1999; Loo et al. 2005; Bartlett 2006), ampicillin, amoxicillin (Zwiener et al. 1989; Nelson et al. 1994; Gerding et al. 1995; Groschell 1996; Worsley 1998; Johnson et al. 1999a) and fluoroquinolones (McCusker et al. 2003; Loo et al. 2005; Pepin et al. 2005; Bartlett 2006; Brook 2008; Spigaglia et al. 2008) but almost all antibiotics can cause CDAD (Mylonakis et al. 2001).

The infection is generally believed to be acquired nosocomially (Simango 2006; Simango and Mwakurudza 2008). However, community-acquired CDAD is increasingly being recognized (Hirschhorn et al. 1994; Kyne et al. 1998; Kuijper et al 2006). As *C. difficile* diarrhea is thought to be a nosocomial problem, very few studies have been directed at the role of *C. difficile* as a potential cause of diarrhea in the environment outside hospitals. An Australian study showed that *C. difficile* was the most commonly isolated organism (5.5%) in outpatients with diarrhea presenting to general practices (Riley et al. 1991), which suggested exposure to the organism in the community. A second community-based study in Australia showed that *C. difficile* was the second most common pathogen (10.7%) in patients with diarrhea (Riley et al. 1995). Several reports indicated the presence of occasionally severe CDAD among healthy persons living in the community, including persons with no established risk factors for infection and had no recent exposure to antibiotics (MMWR 2005; MMWR 2008). Outbreaks of community-acquired CDAD have also been reported in Ireland (Kyne et al. 1998) and in the United States (Jhung et al. 2008; MMWR 2008).

The ability of *C. difficile* to form spores is thought to be a key feature in enabling the bacteria to persist in environment for long periods. Postulated risk factors for acquiring *C. difficile* in the community include contact with a contaminated health-care environment, contact with persons who are infected with and shedding *C. difficile*, and ingestion of contaminated food (MMWR 2008). Food animals are an important source of enteropathogenic microorganisms that can be spread to humans through consumption of foods of animal origin (Rupnik 2007; Simango and Mwakurudza 2008). *C. difficile* has been isolated from a variety of food animals such as calves (Rodriguez-Palacios et al. 2006; Keel et al. 2007), cattle (Simango 2006), chickens (Simango 2006; Simango and Mwakurudza 2008), and pigs (Post and Songer 2004; Keel et al. 2007; Pirs et al. 2008). It is not clear whether these food animals are a possible source of human *C. difficile* infection. However, *C. difficile* has been isolated from ground meat (20%) in the retail food system in Canada over a 10-month period in 2005 (Rodriguez-Palacios et al. 2007). Moreover, there is a high degree of similarity between *C. difficile* types present in humans and animals (Arroyo et al. 2005; Rodriguez-Palacios et al. 2006; Jhung et al. 2008) including two PCR ribotypes (017 and 027) associated with outbreaks of severe disease in humans in Canada (Rodriguez-Palacios et al. 2006), North America and Europe (Warny et al. 2005). The recent detection of *C. difficile* in food animals along with a high degree of similarity are cause for concern as an emerging foodborne pathogen.

Purpose of the Study

This study was designed to determine the prevalence of *C. difficile* in swine, dairy cattle and their environment, and to investigate the relationship of *C. difficile* isolated from food animal species and human clinical isolates. In the process, two isolation techniques and two typing methods for *C. difficile* were compared. In order to develop and better understand these two constrains, the following four main objectives were set:

1. To determine the prevalence of *C. difficile* in healthy swine and dairy cattle.
2. To compare single alcohol shock to double alcohol shock methods for the recovery of *C. difficile* from swine feces, dairy cattle feces, and dairy environment.
3. To evaluate automated rep-PCR as a molecular typing method for *C. difficile*
4. To determine the susceptibility of *C. difficile* isolated from swine feces, dairy cattle feces, and dairy environment to linezolid, amoxicillin-clavulanic acid, ampicillin, clindamycin, erythromycin, levofloxacin, metronidazole, rifampicin and vancomycin.

Experiments and Hypotheses

Experiment 1: Three hundred forty-five swine fecal samples, 1,325 dairy cattle fecal samples and 371 dairy cattle environmental samples from healthy food animals were tested for the presence of *C. difficile*. Two different methods (single and double alcohol shock) and 2 different plating media (tryptic soy agar supplemented with 5% sheep blood; BA and cycloserine-cefoxitin fructose agar; CCFA) were employed to isolate *C. difficile.* First, double alcohol shock method may increase the recovery rate of *C. difficile* by further inhibiting the growth of other organisms and inducing more spore germination. The simplest hypothesis to test here is whether or not double alcohol shock increases the recovery rate of *C. difficile*.

$H\text{-}1_O$: Double alcohol shock method does not increase the recovery rate of *C. difficile*

$H\text{-}1_A$: Double alcohol shock method increases the recovery rate of *C. difficile*

Secondly, CCFA was expected to inhibit more background colonies than that of BA (non-selective media) because of the addition of selective agents (cycloserine, cefoxitin, and sodium taurocholate). The simplest hypothesis to test here is whether CCFA has more or less background colonies than that of BA.

$H\text{-}2_O$: CCFA has more background colonies than BA

$H\text{-}2_A$: CCFA has less background colonies than BA

Experiment 2: the applicability of rep-PCR in typing *C. difficile* was evaluated by using a commercial kit that supplied a set of primers complementary to interspersed non-coding repetitive sequences. The results obtained by rep-PCR were compared to those obtained by the reference method, pulsed-field gel electrophoresis (PFGE). Ninety-four swine isolates, 94 dairy cattle isolates from experiment 1, and 33 human clinical isolates were typed using both techniques.

Experiment 3: *Clostridium difficile* strains isolated from experiment 1 (94 swine and 94 dairy) were tested for susceptibility to linezolid, amoxicillin-clavulanic acid, ampicillin, clindamycin, erythromycin, levofloxacin, metronidazole, rifampicin and vancomycin. Antimicrobials were selected based on their importance in both human and veterinary medicine.

Literature Cited

Arroyo, L.G., Rousseau, J., Willey, B.M., Low, D.E., Staempfli, H., McGeer, A., and Weese J.S. (2005) Use of a selective enrichment broth to recover *Clostridium difficile* from stool swabs stored under different conditions. *J Clin Microbiol* **43**(10), 5341-5343.

Bartlett, J.G. (2006) The new epidemic of *Clostridium difficile*-associated enteric disease. *Ann Intern Med* **145**, 758-764.

Brook, I. (2008) Pseudomembranous colitis. In *Anaerobic Infections Diagnosis and Management* ed. Brook, I. pp. 325-330. New York: Informa Healthcare USA, Inc.

Freeman, J. and Wilcox, M.H. (1999) Antibiotics and *Clostridium difficile*. *Microbes Infect* **1**, 377-384.

George, R.H., Symonds, J.M., Dimock, F., Brown, J.D., Arabi, Y., Shinagawa, N., Keighley, M.R., Alexander-Williams, J., and Burdorn, D.W. (1978) Identification of *Clostridium difficile* as a cause of Pseudomembranous colitis. *BMJ* **1**, 695.

George, W.L., Sutter, V.L., and Finegold, S.M. (1977) Antimicrobial agent-induced diarrhea-a bacterial disease. *J Infect Dis* **136**, 822-828.

Gerding, D.N., Johnson, S., Peterson, L.R., Mulligan, M.E., and Silva, J., Jr. (1995) *Clostridium difficile*-associated diarrhea and colitis. *Infect Control Hosp Epidemiol* **16**, 459-477.

Groschell, D.H. (1996) *Clostridium difficile* infection. *Crit Rev Clin Lab Sci* **33**, 203-245.

Hirschhorn, L.R., Trnka, Y., Onderdonk, A., Lee, M.L., and Platt, R. (1994) Epidemiology of community-acquired *Clostridium difficile*-diarrhea. *J Infect Dis* **169**, 127-133.

Jhung, M.A., Thompson, A.D., Killgore, G.E., Zukowski, W.E., Songer, G., Warny, M., Johnson, S., Gerding, D.N., McDonald, C., and Limbago, B.M. (2008) Toxinotype V *Clostridium difficile* in humans and food animals. *Emerg Infect Dis* **14**(7), 1039-1045.

Johnson, S., Samore, M.H., and Farrow, K.A. (1999a) Epidemics of diarrhea caused by a clindamycin-resistant strain of *Clostridium difficile* in four hospitals. *N Engl J Med* **341**, 1645-1651.

Johnson, S., Samore, M.H., Farrow, K.A., Killgore, G.E., Tenover, F.C., Jyras, D., Rood, J. I., DeGirolami, P., Baltch, A.L., Rafferty, M.E., Pear, S.M., and Gerding, D.N. (1999b) Epidemics of diarrhea caused by a clindamycin-resistant strain of *Clostridium difficile* in four hospitals. *N Engl J Med* **341**, 1645-1651.

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

Kelly, C.P., Pothoulakis, C., and LaMont, T.J. (1994) *Clostridium difficile* colitis. *N Engl J Med* **330**, 257-262.

Kuijper, E.J., Coignard, B., and Tull, P. (2006) Emergence of *Clostridium difficile*-associated disease in North America and Europe. *Clin Microbiol Infect* **12**(Suppl.6), 2-18.

Kyne, L., Merry, C., O’Connell, B., Keane, C., and O’Neill, D. (1998) Community-acquired *Clostridium difficile* infection. *J Infect* **36**, 287-288.

Loo, V.G., Poirier, L., Miller, M.A., Oughton, M., Libman, M.D., Michaud, S., Bourgault, A.M., Nguyen, T., Frenette, C., Kelly, M., Vibien, A., Brassard, P., Fenn, S., Dewar, K., Hudson, T.J., Horn, R., Rene, P., Monczak, Y., and Dascal, A. (2005) A predominantly clonal multi-institutional outbreak of *Clostridium difficile*-associated diarrhea with high morbidity and mortality. *N Eng J Med* **353**, 2442-2449.

McCusker, M.E., Harris, A.D., Perencevich, E., and Roghmann, M.-C. (2003) Fluoroquinolone use and *Clostridium difficile*-associated diarrhea. *Emerg Infect Dis* **9**(6), 730-733.

MMWR (2005) Severe *Clostridium difficile*-associated disease in populations previously at low risk-four states, 2005. The MMWR report, December 2, 2005/54; 1201-1205. http://www.cdc.gov/mmwr/preview/mmwrhtml/mm5447a1.htm

MMWR (2008) Surveillance for community-associated *Clostridium difficile*-Connecticut, 2006. The MMWR report, April 4, 2008/57(13); 340-343. http://www.cdc.gov/mmwr/preview/mmwrhtml/mm5713a3.htm

Mylonakis, E., Ryan, E.T., and Calderwood, S.B. (2001) *Clostridium difficile*-associated diarrhea: a review. *Arch Intern Med* **161**, 525-533.

Nelson, D.E., Auerbach, S.B., Baltch, A.L. Desjarardin, E., Beck-Sague, C., Rheal, C., Smith, R.P., and Jarvis, W.R. (1994) Epidemic *Clostridium difficile*-associated diarrhea: role of second- and third-generation cephalosporins. *Infect Control Hospit Epidemiol* **15**, 88-94.

Pepin, J., Saheb, N., Coulombe, M.A., Alary, M.E., Corriveau, M.P., Authier, S., Lebranc, M., Rivard, G., Bettez, M., Primeau, V., Nguyen, M., Jacob, C.E., and Lanthier, L. (2005) Emergence of fluoroquinolones as the predominant risk factor for *Clostridium difficile*-associated diarrhea: a cohort study during an epidemic in Quebec. *Clin Infect Dis* **41**, 1254-1260.

Pirs, T., Ocepek, M., and Rupnik, M. (2008) Isolation of *Clostridium difficile* from food animals in Slovenia. *J Med Microbiol* **57**, 790-792.

Post, K.W. and Songer, J.G. (2004) Antimicrobial susceptibility of *Clostridium difficile* isolated from neonatal pigs with enteritis. *Anaerobe* **10**, 47-50.

Riley, T.V., Wetheral, F., Bowman, J., Mogyorosy, J., and Golledge, C.L. (1991) Diarrhoeal disease due to *Clostridium difficile* in general practice. *Pathology* **23**, 346-349.

Riley, T.V., Cooper, M., Bell, B., and Golledge, C.L. (1995) Community-acquired *Clostridium difficile*-associated diarrhoea. *Clin Infect Dis* **20**(Suppl.2), S263-S265.

Rodriguez-Palacios, A., Stampfli, H.R., Duffield, T., Peregrine, A.S., Trotz-Williams, L.A., Arroyo, L.G., Brazier, J.S., and Weese, J.S. (2006) *Clostridium difficile* PCR ribotypes in calves, Canada. *Emerg Infect Dis* **12**(11), 1730-1736.

Rodriguez-Palacios, A., Stampfli, H.R., Duffield, T., and Weese, J.S. (2007) *Clostridium difficile* in retail ground meat, Canada. *Emerg Infect Dis* **13**(3), 485-487.

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

Simango, C. (2006) Prevalence of *Clostridium difficile* in the environment in a rural community in Zimbabwe. *Trans R Soc Trop Med Hyg* **100**, 1146-1150.

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

Spencer, R.C. (1998) The role of antimicrobial agents in the aetiology of *Clostridium difficile*-associated disease. *J Antimicrob Chemother* **41**(Suppl. C), 21-27.

Spigaglia, P., Barbanti, F., Mastrantonio, P., Brazier, J.S., Barbut, F., Delmee, M., Kuijper, E., and Poxton, R. (2008) Fluoroquinolone resistance in *Clostridium difficile* isolates from a prospective study of *C. difficile* infections in Europe. *J Med Microb* **57**, 784-789.

Warny, M., Pepin, J., Fang, A., Killgore, G., Thompson, A., Brazier, J., Frost, E., McDonald, L.C. (2005) Toxin production by an emerging strain of *Clostridium difficile* associated with outbreaks of severe disease in North America and Europe. *Lancet* **366**, 1079-1084.

Worsley, M.A. (1998) Infection control and prevention of *Clostridium difficile* infection. *J Antimicrob Chemother* **41** (Suppl.C), 59-66.

Zwiener, R.J., Belknap, W.M., and Quan, R. (1989) Severe pseudomembranous enterocolitis in a child: case report and literature review. *Pediatr Infect Dis J* **8**, 876-882.

CHAPTER 2

LITERATURE REVIEW

2.1 *Clostridium difficile*

C. difficile was first isolated from feces and meconium of asymptomatic newborn infants, and was originally named *Bacillus difficilis* because of its morphology and difficulties encountered in cultivating it (Hall and O'Toole 1935). During the past 40 years, *C. difficile* received attention in the medical literature as a nosocomial pathogen that may be associated with antibiotic-associated diarrhea in humans (George et al. 1978; Larson et al. 1978). Later, the organism was renamed *Clostridium difficile* (Kuijper and Surawicz 2008), and reports of *C. difficile* in the literature were mainly limited to chance findings in studies directed at other areas, such as McBee's report of *C. difficile* in a seal (McBee 1960) and Stevenson's finding in the gut contents of a desert locust (Stevenson 1966). Smith and King in 1962 looked specifically for reports of *C. difficile* in human infections and reported eight cases of extra-intestinal infections in which they concluded it was not playing a pathogenic role (Smith and King 1962). It was not until the late 1970s that cumulative research pointed the finger of suspicion at *C. difficile* as an important cause of nosocomial pathogens of humans (Brazier 1998). This discovery was a result of the efforts of researchers investigating a significant increase in a previously very rare condition, antibiotic-associated pseudomembranous colitis (PMC), a disease which at that time was being linked to the use of specific antibiotics, especially clindamycin and the related lindamycin (Poxton 2006). The discovery resulted from the findings that antisera to *C. sordellii* neutralized toxic activity found in fecal filtrates from patients with antibiotic-associated colitis.

However, *C. sordellii* could not be isolated from the patients. *C. difficile*, on the other hand, had been isolated previously from many patients, but had been ignored since it was “non-pathogenic”. Further investigation showed that toxic activity in culture filtrates from *C. difficile* was neutralized by *C. sordellii* antisera. Thus the fortuitous cross-neutralizing activity of *C. sordellii* antisera led to the discovery of *C. difficile* as the cause of antibiotic-associated colitis (Moncrief and Wilkins 2000). At present, *C. difficile* is recognized as a nosocomial pathogen associated with antibiotic-associated diarrhea and pseudomembranous colitis in humans (Bartlett 1992; Kelly et al. 1994; Voth and Ballard 2005; Kuijpers and Surawicz 2008). They are capable of causing severe gastrointestinal disease in individuals undergoing antibiotic therapy (Delaney et al. 2007), and are responsible for both sporadic cases and epidemic outbreaks (Johnson et al. 1990). Most cases are nosocomially acquired, but community-acquired *Clostridium difficile*-associated disease (CDAD) is being increasingly recognized (Hirschhorn et al. 1994; Kyne et al. 1998; Kuijper et al 2006a).

Incidence of CDAD has been increasing since 2003 (Pepin et al. 2004; Loo et al. 2005; Kuijper et al. 2006a) and increasingly involves a more severe course, higher mortality, increased risk of relapse, more complications and difficult in control (Kuijper et al. 2006a). These changes in the incidence and severity of CDAD may be associated with the emergence of a more virulent strain of *C. difficile* (Kuijper et al. 2006b). This increased virulence is presumably associated with higher levels of toxin production by fluoroquinolone-resistant strains belonging to polymerase chain reaction (PCR) ribotype 027, North America pulsed-field gel electrophoresis (NAP) type 1, restriction endonuclease analysis (REA) type BI and toxinotype III (McDonald et al. 2005; Warny et al. 2005; Kuijper et al. 2006b). They produce 16 times more toxin A, 23 times more toxin B than other strains (McDonald et al. 2005; Warny et al. 2005), produce an extra toxin known as binary toxin (Blossom and McDonald 2007), and posses an 18-base pair deletion gene (Loo et al. 2005; McDonald et al. 2005). Recent outbreaks of hypervirulent strains of *C. difficile* have been reported in the United States (Joseph et al. 2005; Loo et al. 2005; Smith 2005; McDonald et al. 2005; Kuijper et al. 2006a), Canada (Rodriguez-Palacios et al. 2006; Hubert et al. 2007; Rodriguez-Palacios et al. 2007), and Europe (Joseph et al. 2005; Smith 2005; Kuijper et al. 2006a; Kuijper et al. 2006b; Goorhuis et al. 2007).

Characteristics of *C. difficile*. *C. difficile* is an obligately anaerobic Gram-positive, spore-forming bacillus, 3-5 µm in length. On blood based media, colonies are 3-5 mm in diameter with an irregular, lobate or rhizoidal edge, grey, opaque and non-haemolytic (Brazier and Borriello 2000). They ferment glucose, levulose, mannitol, salicin, and xylose, but not lactose, sucrose, galactose, glycerol, inulin, or raffinose (Willis 1986). They produce two major toxins; toxin A, primarily an enterotoxin, and toxin B, a cytotoxin (Banno et al. 1981; Taylor et al. 1981; Sullivan et al. 1982; Banno et al. 1984). The organism causes gastrointestinal infections in mammals (Songer 1996) and humans that range in severity from asymptomatic colonization to severe diarrhea, PMC, toxic megacolon, colonic perforation, and death (McFarland and Stamm 1986; Lyerly et al. 1988; Gerding 1989; Brook 2008).

Pathogenesis of *C. difficile*. *C. difficile* is spread via the fecal-oral route (Poutane and Simor 2004; Sunenshine and McDonald 2006). The organism is ingested as hardy spores, which can survive for long periods in the environment and can traverse the acidic stomach (Poutane and Simor 2004). In the small intestine, spores germinate into the vegetative form. In the large intestine, CDAD can arise if the normal flora has been disrupted by antibiotic therapy. *C. difficile* reproduces in the intestinal crypts, releasing toxin A and B, causing severe inflammation (Bongaerts and Lyerly 1994; Borriello 1998; Poxton et al. 2001). Mucous and cellular debris are expelled, leading to the formation of pseudomembrane. Toxin A attracts neutrophils and monocytes, and toxin B degrades the colonic epithelial cells, both leading to colitis, watery diarrhea and pseudomembrane formation (Poutane and Simor 2004).

For *C. difficile* to establish and proliferate in the colonic mucosa, the normal flora of the colon must be disrupted (as with antibiotics) and *C. difficile* must be ingested. To cause the disease, *C. difficile* must colonize, grow and form toxins (Poutanen and Simor 2004). Although these events need not necessarily occur in that order (Barbut and Petit 2001), once all three occur, one can develop CDAD (Sunenshine and McDonald 2006).

CDAD ranges from mild diarrhea to fulminant, pseudomembranous colitis, sepsis, multi-organ failure, and death. Mild cases may present with slight fever, loose stools, and abdominal cramps (Tedesco et al. 1979; Finegold 1986; George 1980). Diagnosis is commonly delayed (Blossom and McDonald 2007). Wanahita et al. 2003 reported that 50% of patients with unexplained leucocytosis had CDAD and the leucocytosis often preceded signs of colitis. *C. difficile* colitis can start with non-specific signs of edema and erythema on endoscopy. Inflammation usually predominates in the colon. The disease can progress to severe colitis with typical adherent pseudomembranes. These pseudomembranes can coalesce to obscure the mucosa. Severe CDAD is associated with fever, leucocytosis, hypoalbuminemia, and high serum concentrations of C-reactive protein. Such patients need aggressive therapy because severe colitis can result in toxic megacolon, colon perforation, and progressive multi-organ failure (Kuipers and Surawicz 2008). Mortality rates in patients with CDAD range from 3-30% (Redelings et al. 2007; Kuipers and Surawicz 2008) which is attributable to comorbidity in elderly patients (Kuipers and Surawicz 2008). It is unclear why some patients develop disease and others do not; however, toxin production is essential for disease to occur (Sunenshine and McDonald 2006).

Sporulation and Survival in Human Host. Vegetative cells of *C. difficile* are very sensitive to oxygen (Sorg and Sonenshein 2008), but the spores are not and are resistant to most commonly used disinfectants (Fekety et al. 1981; Struelens et al. 1991; Rutala et al. 1993). They persist in the environment and are difficult to eradicate (Kuijper and Surawicz 2008). To survive outside the anaerobic environment of the large bowel of the host, *C. difficile* has to be in the spore form (Sorg and Senenshein 2008). Since toxins are produced by cells, not spores, the spores presumably germinate in the gastrointestinal tract, grow out as vegetative cells, and produce toxin. Any *C. difficile* that are excreted by the host have to be in the spore form to survive for long periods (Jump et al. 2007). The morphological changes during sporulation are very similar in *Clostridium* and *Bacillus subtilis*. In brief, sporulation is initiated under conditions of nutrient limitation and leads to formation of asymmetrically placed division septum that divides the cell into two unequal compartments, each of which contains one copy of the chromosome. The larger, mother cell compartment then engulfs the forespore and helps the forespore mature (Hilbert and Piggot 2004). The addition of a peptidoglycan cortex and several layers of coat proteins precede release into the environment by lysis of the mother cell (Henriques and Moran 2007). Once released from the mother cell, the spore is metabolically dormant but highly resistant to many types of environments (Fekety et al. 1981; Struelens et al. 1991; Rutala et al. 1993; Kuijper and Surawicz 2008). When conditions become suitable for growth, the spores germinate and grow out as vegetative cells. In *B. subtilis*, germination can be induced by L-alanine or by a mixture of asparagine, glucose, fructose, and potassium ions. Receptors involved in sensing these environmental cues are GerA, GerB, and GerK (Moir et al.

1979; Irie et al. 1996). After the germinant is sensed, a large depot of calcium dipicolinate (Ca^{2+} -DPA) is released, the core hydrates, the cortex is degraded, and metabolism begins (Setlow 2003). Homologs of GerA, GerB, and GerK exist in several *Bacillus* species as well as in many *Clostridium* species but are absent in *C. difficile*, suggesting that *C. difficile* responds to different kinds of environmental cues (Paredes et al. 2005; Sebaihia et al. 2006). Previous studies showed that taurocholate, a bile salt, enhances *C. difficile* spores germination recovered from environment and stool (Wilson et al. 1982; Bliss et al. 1997; Weese et al. 2000).

Bile is produced by the liver and stored in the gall bladder. To aid in digestion, the gall bladder secretes bile into the duodenum, where it helps to absorb fat and cholesterol (Curtis and Barnes 1994). The primary bile produced by the liver consists mainly of cholate and chenodeoxycholate conjugated with either taurine or glycine (Ridlon et al. 2006). During passage through the distal ileum, bile is actively reabsorbed and recycles to the liver (Curtis and Barnes 1994). However, 400-800 mg of bile passes daily from the ileum into the cecum, where it becomes a substrate for biotransforming reactions by the normal bacterial flora (Vlahcevic et al. 1996; Thomas et al. 2001). Cholate derivatives and the amino acid glycine act as cogerminants and are sufficient to induce germination of *C. difficile* spores (Sorg and Sonenshein 2008). Deoxycholate, a metabolite of cholate produced by the normal microflora also induces the germination but inhibits the growth of vegetative cells (Sorg and Sonenshein 2008).

When a normal healthy host ingests *C. difficile* spores, the spores survive the passage through the stomach and pass through the duodenum and into the jejunum, where the concentrations of primary bile salts (cholate derivatives) and nutrients are high (Rao et al. 2006; Ridlon et al. 2006). *C. difficile* spores germinate in response to cholate derivatives and glycine. The germinated spores then pass through the ileum and finally into the anaerobic environment of the cecum. Here, the normal microflora metabolizes the cholate derivatives that escape enterohepatic circulation to deoxycholate, a predominant bile salt in the feces of healthy humans (Carey and Watson 1955; Makita and Wells 1963; Thomas et al. 2001). The deoxycholate produced prevents vegetative growth of *C. difficile* and the host remains uncolonized. Spores that did not germinate during passage through the upper part of the digestive system may germinate in response to deoxycholate but are then unable to grow further. Upon antibiotic treatment, the normal microflora is perturbed and the species that are capable of 7α-dehydroxylation of primary bile salts are significantly reduced (Samuel et al. 1973). This reduction would lead to an increase in the concentration of primary bile salts (cholate derivatives) and a decrease in the concentration of secondary bile salts (deoxycholate) in the cecum (Samuel et al. 1973). This decrease in secondary bile acids may provide an environment in which *C. difficile* can grow and colonize.

The presence of primary bile acids in the colon may contribute to the extent of *C. difficile* colonization by providing a germinant for spores that remain within the colon. Therefore, the important protective role normal microflora plays may be that of metabolizing cholate derivatives to deoxycholate, an inhibitor of *C. difficile* growth. The first-line of therapy for CDAD is treatment with vancomycin or metronidazole (Kelly et al. 1994; McFarland et al. 2002; Zar et al. 2007). It is not surprising that patients suffer relapses after completing treatment regiment with these drugs, since the microflora needs time to repopulate the colon and restore the normal balance of primary and secondary bile acids.

Role of Toxins in Disease. The major virulence factors of *C. difficile* are enterotoxin A and cytotoxin B which are the largest bacterial toxins known (molecular masses of 308 and 207 kDa, respectively) (Banno et al. 1984; Aktories et al. 1997). The corresponding genes *tcd*A (toxin A) and *tcd*B (toxin B) are located on a large pathogenicity island in the bacterial chromosome (Voth and Ballard 2005). Both toxin A and B have been recognized to be monoglucosyltransferase, which modify low molecular mass GTP-binding proteins of the Rho subfamily by using UDP-glucose as a co-substrate. Modification of RhoA appears to be the underlying mechanism of the cytotoxic effects of the toxins (Aktories et al. 1997). Inactivation of Rho by C3-induced ADP-ribosylation was shown to induce alterations of the intestinal tight junctions and to increase the permeability of enterocyte monolayer (Nusrat et al. 1995). Similar alterations of the functions of enterocytes were observed after treatment with *C. difficile* toxins (Moore et al. 1990), suggesting that the pathogenic effects induced by these toxins, diarrhea and intestinal epithelial injury, are based on their ability to inactivate Rho subfamily proteins by glucosylation (Aktories et al. 1997). The presence of the toxins can be demonstrated in the feces of 95% of patients with PMC and 30% of patients with antibiotic-associated colitis (George 1984; Anand and Glatt 1993; Kelly et al. 1994; Bartlett 1996; Borriello and Dodson 1996; Jones et al. 1997; Spencer 1998). Toxin A and toxin B act synergistically (Keel and Songer 2006). Toxin A creates widespread damage to the mucosa, permitting toxin B to affect epithelial cells (Kamaras and Murrell 2001; Lima et al 1988). Toxin A is thought to play more critical role than toxin B (Borriello 1998) because it is associated with extensive tissue damage and fluid accumulation (Bongaerts and Lyerly 1994). Toxin B plays a role only after the gastrointestinal wall has been damaged by toxin A (Poxton et al 2001). Both toxins disrupt the cytoskeleton by acting on regulatory proteins involved in actin polymerization (Lyerly et al. 1988). It once was thought that toxigenic strains produced both toxins whereas non-toxigenic strains did not produce either (Alonso et al. 2005). However, the isolation of toxigenic strains that produced either toxin A or toxin B have been reported (Torres 1991; Lyerly et al. 1992; Cohen et al. 1997; Toyokawa et al. 2003; Kuijpers and Surawicz 2008), and it was soon demonstrated that this phenomenon was not as rare among clinical isolates as previously thought (Depitre et al. 1993; Kato et al. 1998; Rupnik et al. 2003). The presence of an additional toxin in *C. difficile* has also been detected. This actin-specific ADP-ribosyltransferase toxin has been designated binary toxin (CDT) due to its two independent proteins, *cdt*A, the catalytic component, and *cdt*B, the binding component. *C. difficile* binary toxin is related genetically, immunologically and functionally to the group of clostridial binary toxins, which includes the well known iota toxin of *Clostridium perfringens* (Popoff et al. 1988). It is unclear how the production of the binary toxin by strains of *C. difficile* can affect its virulence (Voth and Ballard 2005). It has been suggested that, although strains that produce binary toxin only have a relatively low virulence, the toxin could act synergistically in strains that produce both toxin A and toxin B (Geric et al. 2003; Stubbs et al. 2000). Interestingly, strains that do not produce toxin A and toxin B appear to produce binary toxin (Florin and Thelestam 1991). Toxins A and B cause increased vascular permeability by opening tight junctions between cells, and both cause hemorrhage (Borriello 1998; Poxton et al. 2001). They both also induce the production of tumour necrosis factor-alpha, cytokines, proinflammatory interleukins, and the activation of the enteric nervous system, which contribute to the associated inflammatory response, polymorphonucleocyte (PMN) chemotaxis and fluid secretion (Johnson et al. 1999a; Poxton et al. 2001). Patchy epithelial necrosis is common, and the neutrophil exudation into the lumen often produces a “volcano lesion”. Epithelial necrosis becomes diffuse, with ulceration and pseudomembrane formation occurs (Price and Davies 1977).

Development of Culturing and Isolation Methods for *C. difficile*. Isolation of *C. difficile* from fecal specimens has been facilitated by the development of a selective and differential medium. George and his colleges first developed a selective and differential medium for *C. difficile*, a medium that contains cycloserine, cefoxitin, fructose, and egg yolk (cycloserine –cefoxitin fructose agar; CCFA). They tested CCFA and other media that have been reported to be selective for clostridia (cycloserine fructose agar; CFA consist of egg yolk, fructose base and cycloserine), clostridial agar, reinforced clostridial agar with cresol, and egg yolk-neomycin agar. Among media tested, they concluded that CCFA was the most sensitive, selective for the recovery of *C. difficile*, and provided a rapid method for the screening of fecal samples from patients with CDAD (George et al. 1979). Borriello and Hohour (1981) introduced the use of absolute alcohol as a selective procedure (alcohol shock) for the detection and isolation of *C. difficile* in feces. In addition, their results indicated that this method can be successfully performed on the bench with a minimum of anaerobic microbiology equipment. Subsequent, researchers have focused on developing isolation techniques that incorporate the enrichment step with alcohol shock. For example, Riley and his colleges (1987) compared two enrichment methods for their ability to recover *C. difficile* from feces. One method used selective enrichment broth (brain heart infusion broth containing cycloserine and cefoxitin) followed by detection with a latex particle agglutination reagent. The other method used non-selective enrichment broth following treatment of the specimen with absolute alcohol shock. All samples were plated onto CCFA. Enrichment culture was significantly more successful at detecting *C. difficile* than direct plating. Alcohol shock enrichment was twice as effective as direct culture, while selective enrichment broth was three times more effective. A work done by Clabots et al. (1989) also demonstrated that alcohol shock is significantly more sensitive than that of direct plating to CCFA. Enrichment may greatly facilitate the recovery of *C. difficile* studies of the environmental distribution of *C. difficile* where similarly low numbers of organisms may be present in specimens. The ratio of vegetative cells to spores is of some importance in determining the success of the enrichment method. Thus in situations where spores are present, an alcohol shock procedure should be as successful as selective enrichment. The CCFA devised by George et al. (1979) and the modification of it where egg yolk is replaced by horse blood (Oxoid CCFA), have been widely and successfully used for the isolation of *C. difficile* from fecal samples (George et al. 1979; Enevold et al.1980; Holst et al. 1981; Nash et al. 1982). The addition of bile salts, such as sodium cholate or taurocholate, to a medium is believed to enhance recovery by inducing the germination of spores from environmental samples or feces after alcohol shock (Brazier 1998). Wilson et al. (1982) and Buggy et al. (1985) have reported that the incorporation of sodium taurocholate in CCFA (in place of egg yolk) enhances the recovery of *C. difficile* spores from solid media. O'Farrell et al. (1984) incorporated 0.1% sodium taurocholate into cycloserine cefoxitin fructose broth (CCFT broth; same ingredients as CCFA but no agar added) as a selective enrichment broth for the isolation of *C. difficile* from vaginal and fecal specimens. They found that CCFT broth enrichment significantly increased the isolation rates from vaginal swabs compared to direct plating onto CCFA. This suggested that low numbers of *C. difficile* may be present in specimens. In contrast to the results obtained with vaginal swabs, the isolation rate in fecal samples obtained using CCFT enrichment broth was not significantly different to that obtained using direct plating CCFA. This may be explained by the relatively high counts of *C. difficile* and/or other organisms present in fecal samples.

***Clostridium difficile* Carriage in Food Animals.** *C. difficile* also appears to be an important cause of enteric disease in food animals such as pigs (Songer et al. 2000, Songer 2004, Songer and Anderson 2006) and dairy calves (Songer 2004; Songer and Anderson 2006; Keel et al. 2007). Food animals are a source of human enteropathogenic microorganisms which can be spread to humans through consumption of foods of animal origin (Steinmuller et al. 2006). *C.*

difficile has been isolated from food animals such as poultry (Al Saif and Brazier 1996; Simango 2006; Simango and Mwakurudza 2008), calves (Rodriguez-Palacios et al. 2006), cattle (Simango 2006; Pirs et al. 2008), and pigs (Waters et al. 1998; Songer et al. 2000; Simango 2006; Songer and Anderson 2006; Keel et al. 2007; Pirs et al. 2008). A study by Rodriguez-Palacios and colleagues in 2007 reported that *C. difficile* has been isolated from 12 (20%) of 60 retail ground meat purchased over a 10-month period in Canada. Eleven isolates were toxigenic, and 8 were classified as toxinotype III. Moreover, molecular typing of *C. difficile* isolates from calves has shown similarities in PCR ribotypes to human isolates including two PCR ribotypes associated with outbreaks of severe disease in Canada (Rodriguez-Palacios et al. 2006), North America, and Europe (Warny et al. 2005).

2.2 Epidemiology and Typing of *Clostridium difficile*

Strain typing is an integral part of epidemiological investigations of nosocomial infections. Methods to distinguish bacterial strains have improved due mainly to the introduction of molecular technology. Although not all molecular techniques are equally effective for typing all organisms, pulsed-field gel electrophoresis (PFGE) is the technique currently favored for most nosocomial pathogens (Tenover et al. 1997). Bacterial typing is based on the phenotypic or genotypic analysis of multiple isolates within a particular species to identify characteristics that may subdivide the strains into smaller groupings (Power 1996). Such analyses have several uses: to investigate outbreaks which may in turn influence or focus epidemiological investigations; to examine sequential isolates relapse; to establish whether certain strains are associated with specific clinical syndromes; and in a wider context, to increase our understanding of the epidemiology of infection.

The development and application of new methods that allow the typing of different strains of *C. difficile* have been crucial for defining the epidemiology of CDAD. It is important to note that many epidemiologic studies can be conducted only if there are organisms available for typing. Even though culture of *C. difficile* is somewhat difficult and often not available in many laboratories, obtaining cultures is essential for most epidemiologic investigations. Typing methods can be used to identity different strains and thus allow investigations of endemic disease as well as outbreaks. Modes of transmission, as well as efficacy of interventions, can be examined. Among typing methods employing phenotypic characteristics of *C. difficile*, susceptibility testing was exploited early on and provided some of the first evidence for nosocomial spread of the organism in England (Burdon 1982). However, antibiograms have limited usefulness, as the MICs of most antimicrobials for *C. difficile* fall within a narrow range (Tabaqchali 1990). Several electrophoretic methods based on cellular and surface protein patterns have been developed into very useful investigative tools; these include polyacrylamide gel electrophoresis combined with radiolabeling (Tabaqchali et al. 1984) or with immunoblotting (Mulligan et al. 1988; Kato et al. 1993). Rapid serotyping system using slide agglutination also has been used successfully and has been refined by the removal of cross-reacting flagella antigens (Delmee et al. 1990). A system based on susceptibilities of *C. difficile* to bacteriocins and bacteriophages has been developed and exploited for epidemiological studies; but some strains are not typeable and the method is not widely available (Dei 1989; Sell et al. 1983).

All typing systems can be characterized in terms of typeability, reproducibility, discriminatory power, ease of performance, and ease of interpretation (Hunter and Gaston 1988; Arbeit 1995). Typeability refers to the ability of a technique to assign an unambiguous result (type) to each isolate (Hunter 1990). Although non-typeable isolates are more common with phenotypic methods, they have been recognized with most methods. For example, with PFGE, a technique that is almost uniformly applicable to bacteria (Tenover et al. 1995), some strains of *C. difficile* remain nontypeable because the chromosomal DNA is degraded, presumably by endogenous nucleases, before it can be cleaved properly by the restriction endonucleases used in the PFGE protocol (Kato et al. 1994). A reproducible method is one that yields the same results upon repeat testing of a bacterial strain (Hunter 1990). In the context of an epidemiological study, this means that the same strain recovered from epidemiologically linked patients will give the identical typing (or nearly identical) result. Poor reproducibility may reflect technical variation in the method or biologic variation occurring during in vivo or in vitro passage of the organisms to be examined. Over time (a few weeks or years, depending on the species), the typing patterns produced by DNA-based methods, such as PFGE and Arbitrarily Primed-PCR (AP-PCR), will show some minor, natural variation (Maslow et al. 1993; van Belkum and Meis 1994). The discriminatory power of a technique refers to its ability to differentiate among epidemiologically unrelated strains, ideally assigning each to a different type (Hunter 1990). Traditional phenotypic methods, such as antimicrobial susceptibility testing, serotyping, and biotyping, frequently show lower discriminatory power than molecular methods (Swaminathan and Matar 1993; Arbeit 1995)

Antimicrobial Susceptibility Testing. Antimicrobial susceptibility testing has relatively poor discriminatory power, because antimicrobial resistance is under tremendous selective pressure in healthcare institutions (Tenover and McGowan 1996) and often is associated with mobile genetic elements (for example, transposons and plasmids) (Davies 1994). Changes in antibiograms also may reflect spontaneous point mutations, such as seen with fluoroquinolones (Nakamura et al. 1989). Thus, isolates that are epidemiologically related and otherwise genetically indistinguishable may manifest different antimicrobial susceptibilities due to acquisition of new genetic material over time (Locksley et al. 1982) or the loss of plasmids (Mickelsen et al. 1985). Conversely, unrelated isolates may have indistinguishable resistance profiles, which may represent acquisition of the same plasmid by multiple species (Tenover et al. 1997).

Antibiotic resistance among many pathogenic anaerobic bacteria has increased significantly over the past three decades, paralleling similar trends among aerobic pathogens (Letournel-Glomaud et al. 2003; Hecht 2006). Human strains of *C. difficile* have a wide range of susceptibility to erythromycin and tetracycline (Delmee and Avesani 1988). Nearly all human isolates of *C. difficile* are susceptible to ampicillin, penicillin G, metronidazole, carbapenesm, and vancomycin and 90% are susceptible to tetracycline and erythromycin; minimum inhibitory concentration (MIC) for clindamycin and fluoroquinolones are variable and most are resistant to cephalosporins (Dzink and Bartett 1980; Chow 1985). The first-lines of antibiotics to treat CDAD are metronidazole and vancomycin (Kelly et al. 1994; McFarland et al. 2002; Zar et al. 2007). Awareness of these disturbing findings is limited among both clinicians and microbiologists due to a number of confounding factors, including lack of isolation, standardization of susceptibility testing methods, and lack of correlation of clinical outcome with resistance. Monitoring the development of antimicrobial resistance in bacteria isolated from animals as well as humans is necessary to ensure the proper use and prolonged lifespan of current antimicrobials (Tollefson et al. 1998; Tollefson et al. 1999; Fedorka-Cray et al. 2002; Fedorka-Cray et al. 2005) and may allow us to provide optimal therapy in difficult cases (Poilane et al. 1999). The reference methods for susceptibility testing of anaerobic bacteria are the agar dilution method and broth microdilution as recommended by the Clinical and Laboratory Standard Institute (CLSI; formerly NCCLS) (CLSI 2007).

In 1991, the Etest was introduced (AB Biodisk, Sweden) as the first new method for anaerobe susceptibility testing since commercial broth microdilution (Hecht 2002). The Etest employs plastic strips coated with antimicrobials that produce a concentration gradient as they diffuse into agar on a plate that has been streaked with the test organism. The face-up side of the strip has a continuous minimum inhibitory concentration (MIC) interpretative scale; the MIC is read from the point where the zone of inhibition intersects with the strip (Rosenblatt and Gustafson 1995). The Etest was reported as an alternative user friendly, agar gradient method with a high degree of correlation to agar dilution for testing anaerobic bacteria (Citron et al. 1991), including *C. difficile* (Poilane et al. 2000). Subsequently, several studies have validated the correlation of this method for susceptibility testing of anaerobes, and it has been shown that the results obtained with this method correlate well with the reference method (Citron et al. 1991; Wust and Hardegger 1992; Appelbaum et al. 1994; Pierard et al. 1996; Poilane et al. 2000), although strict adherence to proper anaerobic incubation conditions is essential for metronidazole (Wust and Hardegger 1992; Rosenblatt and Gustafson 1995; Spangler et al. 1995; Comican et al. 1996). The Etest is a simple agar diffusion susceptibility testing method that holds promise as being accurate and flexible enough for use in most laboratories (Sanchez and Jones 1993). This FDA-approved method continues to be used as a relatively easy but somewhat expensive test to determine MIC values for individual anaerobic isolates. Despite its high degree of correlation, the Etest has not been specifically identified in CLSI documents, including those testing anaerobes, because of the exclusion of proprietary products from their documents (Hecht 2002).

Pulsed-Field Gel Electrophoresis (PFGE). PFGE was first described in 1984 as a tool for examining the chromosomal DNA of eukaryotic organisms (Schwartz and Cantor 1984). Subsequently, PFGE has proven to be a highly effective molecular typing technique for many different bacterial species (Goering 1993; Maslow et al. 1993; Swaminathan and Matar 1993; Arbeit 1995). The bacterial genome, which typically is 2,000-5,000 kilo base pairs in size, is digested with a restriction enzyme that has relatively few recognition sites and thus generates approximately 10 to 30 restriction fragments ranging from 10 to 800 kb. Essentially all of these fragments can be resolved as a pattern of distinct bands by PFGE, using a specially designed chamber that positions the agarose gel between three sets of electrodes that form a hexagon around the gel. Instead of applying an electric current to the gel in a single direction, as is done in conventional electrophoresis, in PFGE, the current is applied first in one direction from one set of electrodes, then shifts to the second set of electrodes for a short period of time (a pulse), and then shifts to the third set of electrodes. Thus the electric field that causes the DNA to migrate in the gel is provided in pulses that alternate from three sets of electrodes. This causes the DNA to wiggle through the gel, and the back-and-forth movement results in a high level of fragment resolution (Tenover et al. 1997). All species are typeable by PFGE, although the isolation of intact chromosomal DNA is technically difficult for some species (Tenover et al. 1997). As noted above, the chromosomal DNA of some strains of *C. difficile* spontaneously degrades during the cell lysis procedure, making this typing approach impractical (Kato et al. 1994). PFGE has been applied successfully to a wide range of bacterial species, both Gram-positive (for example, staphylococci, enterococci, and mycobacteria) and Gram-negative (for example, *E. coli*, other *Enterobacteriaceae*, and pseudomonads) (Tenover et al. 1995). In general, PFGE is one of the most reproducible and highly discriminatory typing techniques available and is currently the typing method of choice for many species. The major difficulties associated with PFGE relate to the technical demands of the procedure and initial cost of the equipment. Preparation of suitable genomic DNA requires 1 to 3 days, depending on the organisms tested, and the equipment required (including the electrophoresis apparatus and transilluminator) costs between $10,000 and $20,000. However, once the method is operational in a laboratory, it can be applied readily to a wide range of species with only minimal modifications (Tenover et al. 1997). The interpretation of PFGE gels is relatively straightforward, and consensus guidelines for correlating variations in restriction profiles with epidemiological relatedness have been reported (Tenover et al. 1995).

Repetitive Extragenic Palindromic-Polymerase Chain Reaction (rep-PCR). PCR, which has been used for several years for the direct detection of many types of infections agents in clinical samples (Persing 1993), has been adapted for use as a typing tool for many bacteria (Welsh and McClelland 1990; Williams et al. 1990; van Belkum 1994). The hallmark of PCR is the ability to produce millions of copies of a particular DNA segment with high fidelity within 3 to 4 hours time. The procedure requires template DNA (or RNA if a reverse transcriptase step is used initially), which may be present in the sample in minute quantities; two oligonucleotide primers, which flank the sequences on the template DNA to be amplified (thus defining the starting points for DNA polymerase activity); and a heat-stable DNA polymerase. Efficient amplification is accomplished readily for templates of less than 2,000 base pairs, although templates as large as 35 kilo base pairs now can be amplified by using newer polymerases. A typical PCR assay requires approximately 3 hours to complete 30 cycles, where each cycle consists of a heat denaturation phase, in which double-stranded DNA is melted into single strands; an annealing phase, in which the primers bind to the target sequences on the single strands; and an extension phase, in which DNA synthesis proceeds from the primers along each strand of the template DNA, thereby generating two new double-stranded copies of the original template. After 30 such cycles, a single initial copy of template DNA theoretically can be amplified to 1 billion copies (Tenover et al. 1997).

Repeated sequences are present in the genomes of all organisms (Lupski and Weinstock 1992). Bacterial chromosomes contain multiple interspersed repetitive sequences that occupy intergenic regions at sites dispersed throughout the genome. Such blocks of noncoding, repetitive sequences can serve as multiple genetic targets for oligonucleotide probes, enabling the generation of unique DNA profiles or fingerprints for individual bacterial strains. DNA fingerprinting requires the resolution of differently sized DNA fragments derived from chromosomal or plasmid DNA by restriction endonuclease-mediated digestion and/or DNA amplification to yield a band pattern that serves as a unique identifier. These unique “barcodes” or DNA fingerprintings define each bacterial chromosome without the need for measuring gene expression or enzyme function (Versalovic et al 1998). Interspersed repetitive sequences are characterized as relatively short (usually <500 bp), non-coding, intercistronic, and dispersed elements in bacterial genomes (Lupski and Weinstock 1992).

The prokaryotic interspersed repetitive sequence that was first described and most intensively studied is the repetitive extragenic palindromic (rep), initially found in *Escherichia coli* and *Salmonella* Typhimurium (Stern et al 1984). The rep sequence is about 38 nucleotides long, includes an inverted repeat, can occur singly or in multiple adjacent copies, and can form a stable stem-loop structure with a 5 base pair variable loop in the center of the consensus sequence (Stern et al 1984). As rep is different between species, it is necessary to define rep on the basis of general characteristics of their internal structure and on their genomic distribution.

For a sequence to be considered as rep, the following criteria should be met (Tobes and Ramos 2005): (1) it should be extragenic, (2) palindromic, (3) has a length between 21 and 65 bases, and (4) should constitute more than 0.5% of the total extragenic space. This interspersed repetitive element is conserved in diverse genera of bacteria and therefore, enables a single primer set to be used for DNA fingerprinting (Versalovic et al 1994). Locations and distances between rep sequences vary between strains, leading to diverse DNA fingerprint patterns following PCR amplification with primers complementary to rep sequence (Versalovic et al 1991). Genomic fingerprinting with interspersed repetitive sequence-based probes distinguishes unrelated organisms because individual bacterial strains vary with respect to the distances between the repetitive sequences. DNA probes containing repetitive sequences were used to effectively highlight a subset of chromosomal DNA fragments generated by restriction enzyme-meditated digestion. Because these sequences are repeated multiple times in individual bacterial genomes, multiple DNA fragments were highlighted by the repetitive sequence probes. Indeed, several repeated DNA elements were first discovered by hybridization of defined DNA probes with genomic DNA in Southern blots (Lupski and Weinstock 1992). Minimum length probes which yielded DNA fingerprint patterns after autoradiography were subsequently sequenced to uncover the exact nature of the repeats (Versalovic et al. 1994). Versalovic et al. (1991) first introduced the use of rep sequences to fingerprint bacterial genome. By combining the advantages of DNA amplification (polymerase chain reaction; PCR) with the application of repetitive sequence-based oligonucleotide primers, one can differentiate bacterial strains based on the fact that outwardly facing oligonucleotide primers complementary to interspersed repetitive sequences will enable the amplification of differently sized DNA fragments consisting of sequences lying between these elements. Multiple amplicons of different sizes can be fractionated by gel electrophoresis and enable the establishment of unique bacterial barcodes or DNA fingerprint patterns specific for individual bacterial clones.

2.3 Antimicrobial-Associated Risk Factors for CDAD

Ecosystem of the Human Colon. After birth, the development of a normal microflora in the digestive tracts is of great importance for survival. With a well-composed flora, an individual is well protected against oral contamination with numbers of pathogenic enteric organisms. The gastrointestinal tract of the newborn usually becomes colonized in a systematic pattern with numerous types of microorganisms within a few days after birth (van der Waaij 1989; Guarner and Malagelada 2003). Initially, the type of delivery (passage through the birth canal versus caesarean section) and the type of diet (breast milk versus formula feeding) might affect the colonization pattern (Long and Swenson 1977; Yoshioka et al. 1983; Gronlund et al. 1999; Harmsen et al. 2000). Other environmental factors also have a major role since differences exist between infants born in developed countries and those born in developing countries, and between infants from different hospital wards (Simhon et al. 1982; Lundequist et al. 1985; Adlerberth et al. 1991). Pioneer bacteria can modulate expression of genes in host epithelial cells (Hooper et al. 2001), thus creating a favorable habitat for themselves, and can prevent growth of other bacteria introduced later in the ecosystem. Initial colonization is therefore very relevant to the final composition of the permanent flora in adults (van der Waaij 1989). In healthy adults, the colon contains as many as 10^{12} CFU per gram of contents (Simon and Gorbach 1984; Donskey 2004); with obligate anaerobes outnumbering facultative organisms by approximately 1000:1 (Guarner and Malagelada 2003; Donskey 2004; Flint et al. 2007). Approximately 500 species of indigenous microflora colonize the human gastrointestinal tract, producing disease only when normal anatomic or immunologic defenses are abrogated (Swartz 2002). The genera

Bacteroides, *Bifidobacterium*, *Eubacterium*, *Clostridium*, *Peptococcus*, *Peptostreptococcus*, and *Ruminococcus* are predominant in humans (Simon and Gorback 1984; Salminen et al. 1998), whereas facultative anaerobes such as *Escherichia*, *Enterobacter*, *Enterococcus*, *Klebsiella*, *Lactobacillus*, and *Proteus* are among the subdominant genera (Guarner and Malagelada 2003). The principle invasive intestinal bacterial pathogens of food animal origin are *Campylobacter*, *Salmonella*, *Listeria*, *Escherichia coli* 0157 (and other Shiga toxin- and enterotoxin-producing strains of *E. coli*), *Yersinia*, and *Vibrio*. Nearly all are common commensals in swine, cattle, and poultry that sometimes cause invasive infection in animals and humans (except for *E. coli* 0157, a colonizer of cattle). *Vibrio* is found in seawater and shellfish. Other microorganisms of food animal origin, such as *Enterococcus* species and *E. coli* strains that produce neither Shiga toxin nor enterotoxin, also may enter and mix with commensal bacteria in the human gastrointestinal tract (Swartz 2002). The composition of an individual's flora can fluctuate under some circumstances, for instance acute diarrheal illnesses, antibiotic treatment, or to lesser extent dietary interventions, but individual's flora composition pattern usually remain constant (Simon and Gorbach 1984; Salminen et al. 1998). The indigenous microflora of the colon provide an important host defense by inhibiting colonization by and overgrowth of pathogens including *C. difficile* (Wilson 1993; Vollaard and Clasener 1994; Hurley and Nguyen 2002; Donskey 2004). Antimicrobial therapy can disrupt this host defense (Donskey 2004). A study by Mai et al. (2006) showed that in patients with diarrhea, the diversity of the colonic microflora decreases because of overgrowth of certain types of bacteria. In a series of molecular phylogenetic analyses, Young and Schmidt (2004) reported the first direct evidence of changes in the bacterial population in stool samples from a patient without CDAD who had antimicrobial-associated diarrhea. They found that antimicrobial use caused decreases in bacterial diversity and the prevalence of butyrate-producing organisms. Most of these changes resolved within 2 weeks after the cessation of therapy. The disruption of the indigenous flora by antimicrobials may increase the risk of CDAD during therapy and for the days to weeks required for the intestinal microflora to return to normal levels (Owens et al. 2008).

Risk Factors for CDAD. The major risks for infection with *C. difficile* are antibiotic exposure, advanced age, impaired host immunity, and prolonged hospitalization (Gerding 1989; Fekety and Shah 1993; Kelly et al. 1994; Kyne et al. 2000; Garey et al. 2008). Administration of antibiotics is the most significant and most frequently reported predisposing risk factor (Brown et al. 1990; Cartmill et al. 1994; Kelly et al. 1994; Owens et al. 2008). One of the main defenses against *C. difficile* colonization/infection is the maintenance of a normal bowel ecosystem. Even when it is present in the colon, the organism is suppressed by other components of the intestinal flora, so called “colonization resistance” (Borriello 1998) and usually produces no symptoms (Borriello 1990). It is not known which components of the intestinal flora are essential for excluding *C. difficile* (Borriello and Wilcox 1998). The use of antibiotics increases the chances of developing CDAD. Treatment with antibiotics alters the normal levels of beneficial bacteria found in the intestine and colon. When there are fewer of these beneficial bacteria, *C. difficile* can over grow and produce toxins that can cause an infection. Although most antibiotics have been associated with predisposition to *C. difficile* infection (Zwiener et al. 1989; Gerding et al. 1995; Delaney, et al. 2007), the most commonly implicated have been clindamycin (Worsley 1998; Freeman and Wilcox 1999; Johnson et al. 1999b), cephalosporins (Zwiener et al. 1989; Gerding et al. 1995; Spencer 1998; Worsley 1998; Freeman and Wilcox 1999; Loo et al. 2005; Bartlett 2006), ampicillin, and amoxicillin (Zwiener et al. 1989; Nelson et al. 1994; Gerding et al. 1995; Groschell 1996; Worsley 1998; Johnson et al. 1999a). However, the association between CDAD and clindamycin has declined following the decline in the clinical use of clindamycin, and the disease is now particularly associated with the use of broad-spectrum antibiotics (Spencer 1998; Garey et al. 2008). Recently, quinolones and fluoroquinolones are now major inducing agents in the development of CDAD (McCusker et al. 2003; Loo et al. 2005; Pepin et al. 2005; Bartlett 2006; Brook 2008; Spigaglia et al. 2008). Agents implicated as causes of PMC or CDAD are summarized in Table 2.1. A prolonged course of antibiotic treatment or the use of multiple antibiotics in combination increases the risk of CDAD (Gerding et al. 1986; Gorbach 1999). Depletion of the indigenous gut microflora by antibiotic therapy has long been established as a major factor in the disease (Starr and Impallomeni 1997; Freeman and Wilcox 1999). However, the direct influence of antimicrobials upon virulence mechanisms such as toxin production and adhesion in the bowel, and the exact mechanisms by which the organism causes disease remain to be elucidated (Freeman and Wilcox 1999) The most likely pathway by which antibiotics promote CDAD is by altering the colonic microflora and ecosystem (Spencer 1998). However, some reports have described infants (Zwiener et al. 1989) and adults (Larton and Price 1977; Johnson and Gerding 1998) with severe PMC associated with *C. difficile* toxin in the stools without previous antibiotic exposure.

Table 2.1 Agents implicated as causes of *Clostridium difficile* pseudomembranous colitis or diarrhea*

General classification and specific agent: Mode of action	Spectrum of Activity
Penicillins: Inhibit synthesis of bacterial cell wall	
Nafcillin, Oxacillin, Cloxacillin, Dicloxacillin	Gram-positive bacteria

Table 2.1 (con't) Agents implicated as causes of *Clostridium difficile* pseudomembranous colitis or diarrhea*

General classification and specific agent: Mode of action	Spectrum of Activity
Penicillin G, Penicillin V	Gram-positive bacteria, treatment for skin infection
Ampicillin	broad spectrum
Amoxicillin	Gram-positive and some Gram-negative bacteria
Cephalosporins: Inhibit synthesis of bacterial cell wall	
First generation- Cephalothin, Cefazolin,	Gram-positive bacteria, treatment for bacterial infections of skin
Cephradine, Cephalexin	Gram-positive bacteria, treatment for bacterial infections of skin (staphylococci, streptococci)
Second generation- Cephalosporins,	Gram-positive bacteria, anaerobic bacteria
Cefamandole	Gram-positive bacteria, anaerobic bacteria
Cefoxitin	Gram-positive bacteria, anaerobic bacteria
Third generation- Moxalactam, Cefotamine,	Gram-positive and negative bacteria
Cefoperazone, Imipenem	Gram-positive and negative bacteria
Lincosamides: Inhibit protein synthesis	
Lincomycin	*Actinomyces*, Mycoplasma, *Plasmodium*

Table 2.1 (con't) Agents implicated as causes of *Clostridium difficile* pseudomembranous colitis or diarrhea*

General classification and specific agent: Mode of action	Spectrum of Activity
Clindamycin	aerobic Gram-positive cocci bacteria, anaerobic Gram-negative rod bacteria, protozoa (malaria)
Macrolides/tetracyclines: Inhibit protein synthesis	
Erythromycin	Gram-positive bacteria, Mycoplasma
Spiramycin	protozoa (toxoplasmosis)
Tetracycline	broad spectrum
Fluoroquinolones: Inhibit DNA replication and transcription	
Gatifloxacin	treatment for respiratory tract infection, conjunctivitis
Levofloxacin	Gram-positive cocci bacteria, treatment for urinary tract infection
Moxifloxacin	treatment for conjunctivitis
Misc antimicrobials	
Chloramphenicol: Inhibit protein synthesis	broad spectrum, treatment for conjunctivitis
Rifampin: Inhibit protein synthesis	broad spectrum, treatment for tuberculosis

*Adapted from George 1980; Barlett 1981; Cudmore et al. 1982; George et al. 1982; Silva et al. 1984; Freeman and Wilcox 1999; Johnson et al. 1999a; McDonald et al. 2005;

2.4 Antibiotic Use in Livestock Production and Its Impact

Antibiotic is defined as a substance produced by organisms that inhibits or kills other microorganisms. Synthetic antimicrobial substances are referred to as chemotherapeuticals. The word “antimicrobial” (as a noun) is often used to encompass any substance of natural, semi synthetic or synthetic origin that kills or inhibits the growth of a microorganism (Edqvist and Pedersen 2002). Antibiotics have been used in animal agriculture since shortly after their discovery, in the 1950s (Fuller 1989; Revington 2002). They represent an extremely important tool in the efficient production of pork, beef, poultry, and other animal products. When used at low (subtherapeutic) levels in feeds, antibiotics improve growth rate and efficiency of feed utilization (growth promoters), reduce mortality and morbidity, and improve reproductive performance. Antibiotics are also used at intermediate levels to prevent disease, and at high levels (therapeutic) to treat disease in animals (Cromwell 2002). Diseases requiring the most extensive use of antimicrobial drugs for treatment or prophylaxis are respiratory and enteric diseases in pigs and cattle, and mastitis in dairy cattle (Johnson 1998; National Research Council 1998). The most commonly used antimicrobial drugs in food animals are from one of five major classes: beta-lactams, tetracyclines, macrolides, aminogylcosides, and sulphonamide (McDermott et al. 2002) However, the use of antibiotics, particularly as growth promotants, has received increasing attention as a contributory factor in the international emergence of antibiotic resistant bacteria in human health and agriculture (Fuller 1989; Tollefson et al. 1997; Wegener et al. 1998; Wegener et al. 1999a; Turnidge et al. 1999; Aarestrup 1999; Aarestrup et al. 2000; Wray and Davies 2000; McDermott et al. 2002; World Health Organization,WHO 2003; Turnidge 2004). The concern regarding antimicrobial use in food animals and subsequent negative human health implications was initiated by the release of the Swann Committee report in the United Kingdom in 1969 (McDermott et al. 2002). They recommended that antimicrobials be used to treat animals only when prescribed by a veterinarian and that penicillin and tetracycline no longer be used in “subtherapeutic” doses to promote growth of food animals (Swartz 2002). Since 1969, other advisory committees have endorsed the Swann Committee report, numerous reports have been published (Table 2.2), and similarly recommended that antimicrobial drugs used to treat human disease not be used as growth promoters in food animals (WHO 1997; WHO 2001). In some countries, such as Sweden and Denmark, the use of antimicrobials as growth promoters (AGPs) is prohibited (Ewing and Cole 1994). Sweden banned all growth promoting antibiotics used for farm animal in 1986 (WHO 2003). Denmark banned avoparcin and virginiamycin in 1995 and withdrawn AGPs from use in broilers, cattle and finisher pigs in February 1998 (WHO 2003). Virtually no AGPs have been used in Denmark since the end of 1999 (WHO 2003). The Danish Integrated Antimicrobial Monitoring and Research Programme (DANMAP) defined AGPs as “antimicrobials for growth promotion include only those agents approved by the European Union (EU) as feed additives which currently are avilamycin, flavomycin and the ionophores salinomycin and monensin” (DANMAP 2002). The EU banned the use of antibiotics for growth promotion in January 1, 2006, whereas the United States has not (Swartz 2002; Ferber 2003). The United States Food and Drug Administration (FDA), the government agency that regulates antibacterial use in animals, does not have accurate information about what kinds, how much and where antibiotics are being used across the country. It is estimated that animals raised for food in the United States are given 25 million pounds of antibiotics annually just to promote growth and over 10 million pounds of antibiotics in swine production alone (Gilchrist et al. 2007). Bacterial pathogens of animal and human origin are becoming increasingly resistant to most frontline antimicrobials, including expanded-spectrum cephalosporins, aminoglycosides, and even fluoroquinolones (McDermott et al. 2002) The emergence of resistant bacteria and resistance genes following the use of antimicrobial agents is relatively well documented (Aarestrup 1999; McDermott et al. 2002; Frye and Fedorka-Cray 2007).

Table 2.2 Selected reports on the use of antimicrobials in animals and associated public health implications*

Year	Report
1969	Swann Committee Report - Joint committee on the use of antibiotics in animal husbandry and veterinary medicine
1969	National Academy of Sciences - The use of drugs in feed animals
1977	U.S. General Accounting Office Report - Need to establish safety and effectiveness of antibiotics used in animal feeds
1980	Institute of Medicine Report – The effects on human health of subtherapeutic use of antimicrobials in animal feeds
1981	Council for Agriculture Science and Technology – Antibiotics in Animal feeds
1989	Institute of Medicine Report – Human health risks with the subtherapeutic use of penicillin or tetracyclines in animal feed
1995	American Society for Microbiology Task Force Report
1997	World Health Organization – The medical impact of the use of antimicrobials in food animals
1998	World Health Organization – Fluoroquinolone use in food animals

Table 2.2 (con't) Selected reports on the use of antimicrobials in animals and associated public health implications*

Year	Report
1998	Ministry of Agriculture, Fisheries, and Food – A review of antimicrobial resistance in the food chain
1998	National Research Council – Use of drugs in food animals: Benefits and risk
1999	U.S. General Accounting Office Report – The agriculture use of antibiotics and its implications for human health
2001	EU Scientific Committee on Animal Nutrition Report – Opinion of the Scientific Committee on Animal Nutrition on the criteria for assessing the safety of microorganisms resistant to antibiotics of human clinical and veterinary importance
2001	U.S. Food and Drug Administration, Center for Veterinary Medicine Report – Human health impact of fluoroquinolone resistant *Campylobacter* attributed to the consumption of chicken.
2002	World Health Organization – Monitoring antimicrobial usage in food animals for the protection of human health
2002	FAAIR Report – The need to improve antimicrobial use in agriculture: Ecological and human health consequences.
2003	World Health Organization - Impacts of antimicrobial growth promoter termination in Denmark
2008	World Health Organization – Critically important antimicrobials

*Adapted from McDermott *et al.* 2002

Antimicrobial resistant bacterial pathogens not only pose a health risk in animals, they also affect public health when transmitted to humans as foodborne contaminants (Gonzalez and Blanco 1989; Farina et al. 1996; Piddock 1996; Johnson 1998; National Research Council 1998; Welton et al. 1998; Witte 1998; Fey et al. 2000; McDermott et al. 2002; Phillips et al. 2004). Contamination of food by animal intestinal bacteria has long been a concern of the meat and dairy industry (Pell 1997; Salyers 2002). This concern has now spread beyond the diarrheal pathogens to include antibiotic resistant bacteria that might colonize the human intestinal tract or pass on resistance genes to the normal residents of the human intestine (Swartz 2002). The danger from being colonized by resistant bacteria is that these normally nonpathogenic bacteria can cause life-threatening infections (Salyers 2002). A person colonized with multiple resistant bacteria would be at higher risk for infection that would be difficult or impossible to treat.

Several types of evidence might link the risks of humans becoming infected with antimicrobial-resistant pathogens to use of such drugs in food animals, including (1) direct epidemiologic studies, (2) emergence of resistance among bacteria associated with animals before the emergence of resistance among closely related pathogens associated with humans, (3) additional circumstantial evidence linking antimicrobial use in food animals to resistance among foodborne pathogens that do not tend to be transmitted between individuals, (4) trends in antimicrobial resistance among *Salmonella* isolates, (5) trends in antimicrobial resistance among other pathogens such as *Campylobacter jejuni* and *E. coli* 0157:H7 isolates, and (6) studies suggesting that farmers and family members may be more likely than the general population to acquire antimicrobial resistance bacteria of food animal origin (Swartz 2002).

Although many different mechanisms are responsible for antimicrobial resistance, two main genetic events generate the majority of antimicrobial resistance currently observed: the mutation of native genes to resistant alleles; and the acquisition of foreign genes (either chromosomal or extra chromosomal) that confer a resistant phenotype (Davies 1994; Martinez and Baquero 2000) These genetic changes alter the defensive functions of the bacteria by changing the target of the drug, by detoxifying or ejecting the antibiotic, or by routing metabolic pathways around the disrupt point (Davies 1994). Mead (2000) indicated that administration of antibiotics alone may eliminate the infection, but leaves animals open to re-infection once the treatment has ceased. Wegener et al. (1999b) also found a close association exists between the amount of antibiotics used and the levels of resistance observed. The potential risks of selecting resistant bacteria among food producing animals was first discussed in 1955 (National Research Council 1956) but no data on selection of resistance were available (Aarestrup 1999). Most classes of antibiotics used in animals have human analogues, and are capable of selecting for resistance to human antibiotics. In response to continued pressure, the EU adopted the precautionary principle and suspended the use of the AGPs in-feed antibiotics (avoparcin, virginiamycin, spiramycin, tylosin and bacitracin) because of their ability to select for resistance to antimicrobials of human importance. The United States has taken a different approach. The Center for Veterinary Medicine at the FDA has preferred instead to apply the principle of proof, gathering evidence that a problem has emerged before taking action. The center has withdrawn a fluoroquinolone from use in poultry based on this principle (Turnidge 2004).

Although it is true that there is little direct proof that agricultural use of antibiotics is making a major, or even a minor, contribution to the resistance problems plaguing human medicine, there are enough gaps in the existing body of scientific evidence to make denying that there is a problem a risky response to increasing public concern. The degree of impact that antimicrobial use in food animals has on human health is still debated (McDermott et al. 2002; Frye and Fedorka-Cray 2007).

Literature Cited

Aarestrup, F.M. (1999) Association between the consumption of antimicrobial agents in animal husbandry and the occurrence of resistant bacteria among food animals. *Int J Antimicrob Agents* **12**, 279-285.

Aarestrup, F.M., Bager, F., and Andersen, J.S. (2000) Association between the use of avilamycin for growth promotion and the occurrence of resistance among *Enterococcus faecium* from broilers: Epidemiological study and changes over time. *Microb Drug Resist Mec Epidemiol Dis* **6**, 71-75.

Adlerberth, I., Carlsson, B., de Man P., Jalil, F., Khan, S.R., Larsson, P., Mellander, L., Svanborg, C., Wold, A.E., and Hanson, L.A. (1991) Intestinal colonization of enterobacteriaceae in Pakistani and Swedish hospital delivered children. *Acta Paediatr Scand* **80**, 602-610.

Aktories, K., Selzer, J, Hofmann, R., and Just, I. (1997) Molecular mechanism of action of *Clostridium difficile* toxins A and B. In *The Clostridia Molecular Biology and Pathogenesis* ed. Rood, J.I., McGlane, B.A., Songer, J.G., and Titball, R.W. pp.393-407. Cambridge, Great Britain: Academy Press.

Al Saif, N. and Brazier, J.S. (1996) The distribution of *Clostridium difficile* in the environment of South Wales. *J Med Micro* **45**, 133-137.

Alonso, R., Martin, A. Pelaez, T., Marin, M., Rodriguez-Creixems, M., and Bouza, E. (2005) Toxigenic status of *Clostridium difficile* in a large Spanish teaching hospital. *J Med Micro* **54**, 159-162.

Anand, A. and Glatt, A.E. (1993) *Clostridium difficile* infection associated with antineoplastic chemotherapy: A review. *Clin Infect Dis* **17**, 109.

Appelbaum, P.C., Spangler, S.K., Cohen, M., and Jacobs, M.R. (1994) Comparison of the Etest and conventional agar dilution methods for susceptibility testing of Gram negative anaerobic rods. *Diagn Microbiol Infect Dis* **18**, 25-30.

Arbeit, R.D. (1995) Laboratory procedures for the epidemiologic analysis of microorganisms. In *Manual of Clinical Microbiology* 6th edn ed. Murray, P.R., Baron, E.J., Pfaller, M.A., Tenover, F.C., and Yolken, R.H. pp.190-208. Washington, DC: American Society for Microbiology.

Banno, Y., Kobayashi, T., Watanabe, K., Ueno, K., and Nozawa, Y. (1981) Two toxins (D-1 and D-2) of *Clostridium difficile* causing antibiotic-associated colitis: purification and some characterization. *Biochem Int* **2**, 629-635.

Banno, Y., Kobayashi, T., Kono, H., Watanabe, K., Veno, K., and Nozawa, Y. (1984) Biochemical characterization and biological action of two toxins (D-1 and D-2) from *Clostridium difficile*. *Rev Infect Dis* **6**, S11-S20.

Barbut, F. and Petit, J.C. (2001) Epidemiology of *Clostridium difficile*-associated infections. *Clin Microbiol Infect* **7**, 405-410.

Bartlett, J.G. (1981) Antimicrobial agents implicated in *Clostridium difficile* toxin-associated diarrhea or colitis. *Johns Hopkins Med J* **149**, 6-9.

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

Bartlett, J.G. (1996) Management of *Clostridium difficile* infection and other antibiotic-associated diarrhoes. *Eur J Gastroenterol Hepatol* **8**, 11054-11061.

Bartlett, J.G. (2006) The new epidemic of *Clostridium difficile*-associated enteric disease. *Ann Intern Med* **145**, 758-764.

Bliss, D.Z., Johnson, S., Clabots, C.R., Savik, K., and Gerding, D.N. (1997) Comparison of cycloserine-cefoxitin-fructose agar (CCFA) and taurocholate-CCFA for recovery of *Clostridium difficile* during surveillance of hospitalized patients. *Diagn Microbiol Infect Dis* **29**, 1-4.

Blossom, D.B. and McDonald, L.C. (2007) The challenges posed by reemerging *Clostridium difficile* infection. *CID* **45**, 222-227.

Bongaerts, F.P. and Lyerly, D.M. (1994) Role of toxins A and B in the pathogenesis of *Clostridium difficile* disease. *Microb Pathog* **17**, 1-12.

Borriello, S.P. and Honour, P. (1981) Simplified procedure for the routine isolation of *Clostridium difficile* from faeces. *J Clin Pathol* **34**, 1124-1127.

Borriello, S.P. (1990) The influence of the normal flora on *Clostridium difficile* colonization of the gut. *Ann Med* **22**, 61-67.

Borriello, S.P. and Dodson, A.P. (1996) *Clostridium difficile* infection of the gut. *J Clin Patho* **49**, 529-532.

Borriello, S.P. (1998) Pathogenesis of *Clostridium difficile* infection. *J Antimicrob Chemother* **41**(Suppl.C), 13-19.

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

Brazier, J.S. (1998) The diagnosis of *Clostridium difficile*-associated disease. *J Antimicrob Chemother* **41**(suppl.C), 29-40.

Brazier, J.S. and Borriello, S.P. (2000) Microbiology, epidemiology and diagnosis of *Clostridium difficile* infection. In *Clostridium difficile* ed. Aktories, K. and Wilkins, T.D. pp. 1-33. Germanny: Springer.

Brook, I. (2008) Pseudomembranous colitis. In *Anaerobic Infections Diagnosis and Management* ed. Brook, I. pp. 325-330. New York: Informa Healthcare USA, Inc.

Brown, E., Talbot, G.H., Axelrod, P., Provencher, M., and Hoegg, C. (1990) Risk factors for *Clostridium difficile* toxin-associated diarrhea. *Infect Control Hosp Epidemiol* **11**, 283-290.

Buggy, B.P., Hawkins, C.C., and Fekety, R. (1985) Effect of adding sodium taurocholate to selective media on the recovery of *Clostridium difficile* from environmental surfaces. *J Clin Microbiol* **21**(4), 636-637.

Burdon, D.W. (1982) *Clostridium difficile*: the epidemiology and prevention of hospital-acquired infection. *Infection* **10**, 203-204.

Carey, J.B. and Watson, C.J. (1955) Isolation of deoxycholic acid from normal human feces. *J Biol Chem* **216**, 847-850.

Cartmill, T.D.I., Panigrahi, H., Worsley, M.A., McCann, D.C., Nice, C.N., and Keith, E. (1994) Management and control of a large outbreak of diarrhea due to *Clostridium difficile*. *J Hosp Infect* **27**, 1-15.

Chow, A.W., Cheng, N., and Bartleet, K.H. (1985) In vitro susceptibility of *Clostridium difficile* to new β–lactam and quinolone antibiotics. *Antimicrob Agents Chemother* **17**, 695-698.

Citron, D.M., Ostovari, M.I., Karlsson, A., Goldstein, E.J. (1991) Evaluation of the Etest for susceptibility testing of anaerobic bacteria. *J Clin Microbiol* **29**, 2197-2203.

Clabots, C.R., Gerding, S.J., Olson, M.M., Peterson, J.R., and Gerding, D.N. (1989) Detection of asymptomatic *Clostridium difficile* carriage by an alcohol shock procedure. *J Clin Microbiol* **27**(10), 2386-2387.

Clinical and Laboratory Standards Institute. (2007) Methods for antimicrobial susceptibility testing of anaerobic bacteria; Approved standard. 7th edn. Document M11 A7. Wayne, PA: Clinical and Laboratory Standards Institute.

Cohen, S.H., Tang, Y.J., Hansen, B., and Silva, J., Jr. (1997) Isolation of a toxin B-deficient mutant strain of *Clostridium difficile* in a case of recurrent *C. difficile*-associated diarrhea. *Clin Infect Dis* **26**, 410-412.

Cormican, M.G., Erwin, M.E., and Jones, R.N. (1996) False resistance to metronidazole by Etest among anaerobic bacteria investigations of contributing test conditions and medium quality. *Diagn Microbiol Infect Dis* **24**, 117-119.

Cromwell, G.L. (2002) Why and how antibiotics are used in swine production. *Anim Biotechnol* **13**(1), 7-27.

Cudmore, M.A., Silva, J., Jr., Fekety, R., Liepman, M.K., and Kim, K.-H. (1982) *Clostridium difficile* colitis associated with cancer chemotherapy. *Arch Intern Med* **142**, 333-335.

Curtis, H. and Barnes, N.S. (1994) Digestion. In *Invitation to Biology* ed. Curtis, H. and Barnes, N.S. pp. 524-537. New York, NY: Worth Pub.

DANMAP (2002) Use of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from animals, foods and humans in Denmark: a report by The Danish Integrated Antimicrobial Resistance Monitoring and Research Programme. ISSN 1600-2032 http://www.danmap.org/pdffiles/danmap_2002.pdf

Davies, J. (1994) Inactivation of antibiotics and the dissemination of resistance genes. *Science* **264**, 375-382.

Dei, R. (1989) Observations on phage-typing of *Clostridium difficile*: preliminary evaluation of a phage panel. *Eur J Epidemiol* **5**, 351-354.

Delaney, J.A.C., Dial, S., Barkun, A., and Suissa, S. (2007) Antimicrobial drugs and community-acquired *Clostridium difficile*-associated disease, UK. *Emerg Infect Dis* **13**(5), 761-763.

Delmee, M. and Avesani, V. (1988) Correlation between serogroup and susceptibility to choroamphenicol, clindamycin, erythromycin, rifampin and tetracycline. *J Antimicrob Chemother* **22**, 325-331.

Delmee, M. Avesani, V., Delferriere, N., and Burtonboy, G. (1990) Characterization of flagella of *Clostridium difficile* and their role in serogrouping reactions. *J Clin Microbiol* **28**, 2210-2214.

Depitre, C., Delmee, M., Avesani, V., Roels, A., Popoff, M., and Corthier, G. (1993) Serogroup F strains of *Clostridium difficile* produce toxin B but not toxin A. *J Med Microbiol* **38**, 434-441.

Donskey, C.J. (2004) The role of the intestinal tract as a reservoir and source for transmission of nosocomial pathogens. *Clin Infect Dis* **39**, 219-226.

Dzink, J. and Bartlett, J.G. (1980) In vitro susceptibility of *Clostridium difficile* isolates from patient with antibiotic-associated diarrhea or colitis. *Antimicrob Agents Chemother* **17**, 695-698.

Edqvist, L.E. and Pedersen, K.B. (2002) Antimicrobials as growth promoters: resistance to common sense. In *Late Lessons from Early Warnings: the precautionary principle 1896-2000* ed. Harremo, P., Gee, D., MacGarvin, M., Stirling, A., Keys, J., Wynne, B., Vaz, S.G. pp. 93-1000. Copenhagen: Office for Official Publications of the European Communities.

Enevold, F.E., Bertil, K.B., Lars, N.L., Borje, N.B., and Svedhem, A. (1980) *Clostridium difficile* in relation to enteric bacterial pathogens. *J Clin Microbiol* **12**, 297-300.

Ewing, W.N. and Cole, D.J.A. (1994) The use of antibiotics. In *The living gut* ed. Ewing, W.N. and Cole, D.J.A. pp.75-89. Tyrone, Ireland: A context publication.

Farina, C., Goglio, A., Conedera, G., Minelli, F., and Caprioli, A. (1996) Antimicrobial susceptibility of *Escherichia coli* 0157 and other Enterohaemorrhagic *Escherichia coli* isolated in Italy. *Eur J Clin Microbiol Infect Dis* **15**, 351-353.

Fedorka-Cray, P.J., Englen, M.D., Gray, J.T., Hudson, C., and Headrick, M.L. (2002) Programs for monitoring antimicrobial resistance. *Anim Biotechnol* **13**, 43-55.

Fedorka-Cray, P.J., Dargatz, D., Petersen, K., and Tollefson, L. (2005) National Antimicrobial Resistance Monitoring System (NARMS). In *Veterinary strains* ed. U.S. Food and Drug Administration, Center for Veterinary Medicine.

Fekety, R., Kim, K.H., Brown, D., Batts, D.H., Cudmore, M., and Silva, J. (1981) Epidemiology of antibiotic associated colitis: isolation of *Clostridium difficile* from the hospital environment. *Am J Med* **70**, 906-908.

Fekety, R. and Shah, A.B. (1993) Diagnosis and treatment of *Clostridium difficile* colitis. *JAMA* **269**, 255-257.

Ferber, D. (2003) Antibiotic resistance: WHO advises kicking the livestock antibiotic habit. *Science* **301**, 1027.

Fey, P.D., Safranek, T.J., Rupp, M.E. Dunne, E.F., Ribot, E., Iwen, P.C., Bradford, P.A., Angulo, F.J., and Hinrichs, S.H. (2000) Ceftriazone-resistant *Salmonella* infection acquired by a child from cattle. *N Engl J Med* **17**, 1242-1249.

Finegold, S.M. (1986) Clinical considerations in the diagnosis of antimicrobial agent-associated gastroenteritis. *Diagn Microbiol Infect Dis* **4**, 87S-91S.

Flint, H.J., Duncan, S.H., Scott, K.P., and Louis, P. (2007) Interactions and competition within the microbial community of the human colon: links between diet and helath. *Env Microb* **9**(5), 1101-1111.

Florin, I. and Thelestan, M. (1991) ADP-ribosylation in *Clostridium difficile* toxin-treated cells is not related to cytopathogenicity of toxin B. *Biochem Biophys Acta* **1091**, 51-54.

Freeman, J. and Wilcox, M.H. (1999) Antibiotics and *Clostridium difficile*. *Microbes Infect* **1**, 377-384.

Frye, J.G. and Fedorka-Cray, P.J. (2007) Prevalence, distribution and characterization of ceftiofur resistance in *Salmonella enterica* isolated from animals in the USA from 1999-2003. *Int J Antimicrob Agents* **30**, 134-142.

Fuller, R. (1989) Probiotics in man and animals. *J Appl Bacteriol* **66**, 365-378.

Garey, K.W., Dao-Tran, T.K., Jiang, Z.D., Price, M.P., Gentry, L.O., and DuPont, H.L. A (2008) Cinical risk index for *Clostridium difficile* infection in hospitalized patients receiving broad-spectrum antibiotics. *J Hosp Infect* **70**, 142-147.

George, R.H., Symonds, J.M., Dimock, F., Brown, J.D., Arubi, Y., Shinagawa, N., Keighley, M.R., Alexander-Williams, J., and Burdon, D.W. (1978) Identification of *Clostridium difficile* as a cause of Pseudomembranous colitis. *BMJ* **1**, 695.

George, W.L., Sutter, V.L., Citron, D., and Finegold, S.M. (1979) Selective and differential medium for isolation of *Clostridium difficile*. *J Clin Microbiol* **9**(2), 214-219.

George, W.L. (1980) Antimicrobial agent-associated colitis and diarrhea. *West J Med* **133**, 115-123.

George, W.L., Rolfe, R.D., and Finegold, S.M. (1982) *Clostridium difficile* and its cytotoxins in feces of patients with antimicrobial agent-associated diarrhea and miscellaneous conditions. *J Clin Microbiol* **15**, 1049-1053.

George, W.L. (1984) Antimicrobial agent-associated colitis and diarrhea: historical background and clinical aspect. *Rev Infect Dis* **6**(Suppl. 1), S208-S213.

Gerding, D.N., Olson, M.M., Peterson, L.R., Teasley, D.G., Gebhard, R.L., Schwartz, M.L., and Lee, J.T. Jr. (1986) *Clostridium difficile*-associated diarrhea and colitis in adults. A prospective case-controlled epidemiologic study. *Arch Intern Med* **146**, 95-100.

Gerding, D.N. (1989) Disease associated with *Clostridium difficile* infection. *Ann Intern Med* **110**, 255-257.

Gerding, D.N., Johnson, S., Peterson, L.R., Mulligan, M.E., and Silva, J., Jr. (1995) *Clostridium difficile*-associated diarrhea and colitis. *Infect Control Hosp Epidemiol* **16**, 459-477.

Geric, B., Johnson, S., Gerding, D.N., Grabnar, M., and Rupnik, M. (2003) Frequency of binary toxin genes among *Clostridium difficile* strains that do not produce large clostridial toxins. *J Clin Microbiol* **41**, 5227-5232.

Gilchrist , M., Greko, C., Wallinga, D., Beran, G., Riley, D., and Thorne, P. (2007) The potential role of concentrated animal feeding operations in infectious disease epidemics and antibiotic resistance. *Environ Health Perspect* **115**(2), 313-316.

Goering, R.V. (1993) Molecular epidemiology of nosocomial infection: analysis of chromosomal restriction fragment patterns by pulsed-field gel electrophoresis. *Infect Control Hosp Epidemiol* **14**, 595-600.

Gonzales, E.A. and Blanco, J. (1989) Serotypes and antibiotic resistance of verotoxigenic (VTEC) and necrotizing (NTEC) *Escherichia coli* strains isolated from calves with diarrhea. *FEMS Microbiol Lett* **51**, 31-36.

Goorhuis, A., van der Kooi, T., Vaessen, N., Dekker, F.W., van den Berg, R., Harmanus, C., van den Hof, S., Notermans, D.W., and Kuijper, E.J. (2007) Spread and epidemiology of *Clostridium difficile* polymerase chain reaction ribotype 027/toxinotype III in the Netherlands. *CID* **45**, 695-703.

Gorbach, S.L. (1999) Antibiotics and *Clostridium difficile. N Engl J Med* **341**, 1690-1691.

Groschell, D.H. (1996) *Clostridium difficile* infection. *Crit Rev Clin Lab Sci* **33**, 203-245.

Gronlund, M.M. Lehtonen, O.P., Eerola, E., and Kero, P. (1999) Fecal microflora in healthy infants born by different methods of delivery: permanent changes in intestinal flora after cesarean delivery. *J Pediatr Gastroenterol Nutr* **28**, 19-25.

Guarner, F. and Malagelada, J.R. (2003) Gut flora in health and disease. *Lancet* **361**, 512-519.

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

Harmsen, H.J., Wildeboer-Veloo, A.C., Raangs, G.C., Wagendorp, A.A., Klijn, N., Bindels, J.G., and Welling, G.W. (2000) Analysis of intestinal flora development in breast-fed and formula-fed infants by using molecular identification and detection methods. *J Pediatr Gastroenterol Nutr* **30**, 61-67.

Hecht, D.W. (2002) Evolution of anaerobe susceptibility testing in the United States. *Clin Infect Dis* **35**(1), S28-S35.

Hecht, D.W. (2006) Anaerobes: antibiotic resistance, clinical significance, and the role of susceptibility testing. *Anaerobe* **12**, 115-121.

Henriques, A.O. and Moran, C.P.J. (2007) Structure, assembly, and function of the spore surface layers. *Annu Rev Microbiol* **61**, 555-588.

Hilbert, D.W. and Piggot, P.J. (2004) Compartmentalization of gene expression during *Bacillus subtilis* spore formation. *Microbiol Mol Biol Rev* **68**, 234-262.

Hirschhorn, L.R., Trnka, Y., Onderdonk, A., Lee, M.L., and Platt, R. (1994) Epidemiology of community-acquired *Clostridium difficile*-diarrhea. *J Infect Dis* **169**, 127-133.

Holst, E., Helin, I., and Mardh, P. (1981) Recovery of *Clostridium difficile* from children. *Scand J Infect Dis* **13**, 41-45.

Hooper, L.V., Wong, M.H., Thelin, A., Hansson, L., Falk, P.G., and Gordon, J.I. (2001) Molecular analysis of commensal host-microbial relationships in the intestine. *Science* **291**, 881-884.

Hubert, B., Loo, V.G., Bourgault, A.-M., Poirier, L., Dascal, A., Fortin, E., Dionne, M., and Lorange, M. (2007) A portrait of the geographic dissemination of the *Clostridium difficile* North American Pulsed-Field type 1 strain and the epidemiology of *C. difficile*-associated disease in Quebec. *CID* **44**, 238-244.

Hunter, P.R. and Gaston, M.A. (1988) Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity. *J Clin Microbiol* **26**, 2465-2466.

Hunter, P.R. (1990) Reproducibility and indices of discriminatory power of microbial typing methods. *J Clin Microbiol* **28**(9), 1903-1905.

Hurley, B.W. and Nguyen, C.C. (2002) The spectrum of pseudomembranous enterocolitis and antibiotic-associated diarrhea. *Arch Intern Med* **162**, 2177-2184.

Irie, R., Fujita, Y., and Kobayashi, M. (1996) Nucleotide sequence and gene organization of the gerK spore germination locus of *Bacillus subtilis* 168. *J Gen Appl Microbiol* **42**, 141-153.

Johnson, S., Clabots, C.R., Linn, F.V., Olson, M.M., Peterson, L.R., and Gerding, D.N. (1990) Nosocomial *Clostridium difficile* colonization and disease. *Lancet* **336**, 97-100.

Johnson, A.M. (1998) Use of antimicrobial drugs in veterinary practice. *Br Med J* **317**, 665-667.

Johnson, S. and Gerding, D.N. (1998) *Clostridium difficile*-associated diarrhea. *Clin Infect Dis* **26**, 1027-1036.

Johnson, S., Samore, M.H., and Farrow, K.A. (1999a) Epidemics of diarrhea caused by a clindamycin-resistant strain of *Clostridium difficile* in four hospitals. *N Engl J Med* **341**, 1645-1651.

Johnson, S., Samore, M.H., Farrow, K.A., Killgore, G.E., Tenover, F.C., Jyras, D., Rood, J. I., DeGirolami, P., Baltch, A.L., Rafferty, M.E., Pear, S.M., and Gerding, D.N. (1999b) Epidemics of diarrhea caused by a clindamycin-resistant strain of *Clostridium difficile* in four hospitals. *N Engl J Med* **341**, 1645-1651.

Jones, E.M., Kirkpatrick, B.L., Feeney, R., Reeves, D.S., and Macgowan, A.P. (1997) Hospital-acquired *Clostridium difficile* diarrhea. *Lancet* **349**, 1176-1177.

Joseph, R., Demeyer, D., Vanrenterghem, D., van den, B.R., Kuijper, E., and Delmee, M. (2005) First isolation of *Clostridium difficile* PCR ribotype 027, toxinotype III in Belgium. *Euro Surveill* **10**, E051020.

Jump, R.L.P., Pultz, J., and Donskey, C.J. (2007) Vegetative *Clostridium difficile* survives in room air on moist surfaces and in gastric contents with reduced acidity: a potential mechanism to explain the association between proton pump inhibitors and *C. difficile*-associated diarrhea? *Antimicrob Agents Chemother* **51**, 2883-2887.

Kato, H., Cavallaro, J.J., Kato, N., Bartley, S.L., Killgore, G.E., Watanabe, K., and Ueno, K. (1993) Typing of *Clostridium difficile* by western immunoblotting with 10 different antisera. *J Clin Microbiol* **31**, 413-415.

Kato, H., Kato, N., Watanabe, K. Ueno, K., Ushijima, H., Hashira, S., and Abe, T. (1994) Application of typing by pulsed-field gel electrophoresis to the study of *Clostridium difficile* in a neonatal intensive care unit. *J Clin Microbiol* **32**, 2067-2070.

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

Kamaras, J. and Murrell, W.G. (2001) Intestinal epithelial damage in SIDS babies and it similarily to that caused by bacterial toxins in the rabbit. *Pathology* **33**, 197-203.

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

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

Kelly, C.P., Pothoulakis, C., and LaMont, J.T. (1994) *Clostridium difficile* colitis. *N Engl J Med* **330**, 257-262.

Kuijper, E.J., Coignard, B., and Tull, P. (2006a) Emergence of *Clostridium difficile*-associated disease in North America and Europe. *Clin Microbiol Infect* **12**(Suppl.6), 2-18.

Kuijper, E.J., Renate, van den Berg, J., Debast, S., Visser, C., Veenendaal, D., Troelstra, A., van der Kooi, T., van den Hof, S., and Notermans, D.W. (2006b) *Clostridium difficile* Ribotype 027, Toxinotype III, the Netherlands. *Emerg Infect Dis* **12**(5), 827-830.

Kuijper, E.J. and Surawicz, C.M. (2008) *Clostridium difficile* infection. *Lancet* **371**, 1486-1488.

Kyne, L., Merry, C., O'Connell, B., Keane, C., and O'Neill, D. (1998) Community-acquired *Clostridium difficile* infection. *J Infect* **36**, 287-288.

Kyne, L., Warny, M., Qamar, A., and Kelly, C.P. (2000) Asymptomatic carriage of *Clostridium difficile* and serum levels of IgG antibody against toxin A. *N Engl J Med* **342**, 390-397.

Larson, H.R., Price, A.B., Honour, P, and Borriello, S.P. (1978) *Clostridium difficile* and the aetiology of Pseudomembranous colitis. *Lancet* **1**,1063-1066.

Larton, H.E. and Price, A.B. (1977) Pseudomembranous colitis: presence of clostridial toxin. *Lancet* **2**, 1312-1314.

Letournel-Glomaud, C., Houssaye, S., Milhaiha, L., and Ghnassia, J.C. (2003) E-test antibiotics susceptibility of strict anaerobic bacteria. *Anaerobe* **9**, 281-284.

Lima, A.A., Lyerly D.M., Wilkins, T.D., Innes, D.J., and Guerrant, R.L. (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.

Locksley, R.M. Cohen, M.I. Quinn, T.C., Tompkins, L.S., Coyle, M.B., Kirihara, J.M., and Counts, G.W. (1982) Multiply antibiotic-resistant *Staphylococcus aureus*: introduction, transmission, and evolution of nosocomial infection. *Ann Intern Med* **97**, 317-324.

Long, S.S. and Swenson, R.M. (1977) Development of anaerobic fecal flora in healthy newborn infants. *J Pediatr* **91**, 298-301.

Loo, V.G., Poirier, L., Miller, M.A., Oughton, M., Libman, M.D., Michaud, S., Bourgault, A.M., Nguyen, T., Frenette, C., Kelly, M., Vibien, A., Brassard, P., Fenn, S., Dewar, K., Hudson, T.J., Horn, R., Rene, P., Monczak, Y., and Dascal, A. (2005) A predominantly clonal multi-institutional outbreak of *Clostridium difficile*-associated diarrhea with high morbidity and mortality. *N Eng J Med* **353**, 2442-2449.

Lundequist, B., Nord, C.E., and Winberg, J. (1985) The composition of the faecal microflora in breastfed and bottle fed infants from birth to eight weeks. *Acta Paediatr Scand* **74**, 45-51.

Lupski, J.R. and Weinstock, G.M. (1992) Short, interspersed repetitive DNA sequences in prokaryotic genomes. *J Bacteriol* **174**(14), 4525-4529.

Lyerly, D.M., Krivan, H.C., and Wilkins, T.D. (1988) *Clostridium difficile* its disease and toxins. *Clin Microbiol Rev* **1**, 1-18.

Lyerly, D.M., Barroso, L.A.,Wilkins, T.D., Depitre, C., and Corthier, G. (1992) Characterization of a toxin A-negative, toxin B-positive of *Clostridium difficile*. *Infect Immun* **60**, 4633-4639.

Mai, V., Braden, C.R., Heckendorf, J., Pironis, B., and Hirshon, J.M. (2006) Monitoring of stool microbiota in subjects with diarrhea indicates distortions in composition. *J Clin Microbiol* **44**, 4550-4552.

Makita, M. and Wells, W.W. (1963) Quantitative analysis of fecal bile acids by gas-liquid chromatography. *Anal Biochem* **5**, 523-530.

Martinez, J.L. and Baquero, F. (2000) Mutation frequencies and antibiotic resistance. *Antimicrob Agents Chemother* **44**, 1771-1777.

Maslow, J.N., Slutsky, A.M., and Aebeit, R.D. (1993) The application of pulsed-field gel electrophoresis to molecular epidemiology. In *Diagnostic Molecular Microbiology: Principle and Applications*. ed. Smith, T.F., Tenover, F.C., and White, T.J. pp. 563-572. Washington, DC: American Society of Microbiology.

Mead, G.C. (2000) Prospects for "Competitive Exclusion" treatment to control salmonellas and other foodborne pathogens in poultry. *Vet J* **159**, 111-123.

McBee, R.H. (1960) Intestinal flora of some Antarctic birds and mammals. *J Bact* **79**, 311-312.

McCusker, M.E., Harris, A.D., Perencevich, E., and Roghmann, M.-C. (2003) Fluoroquinolone use and *Clostridium difficile*-associated diarrhea. *Emerg Infect Dis* **9**(6), 730-733.

McDermott, P.R., Zhao, S., Wagner, D.D., Simjee, S., Walker, R.D., and White, D.G. (2002) The food safety perspective of antibiotic resistance. *Anim Biotechnol* **13**, 71-84.

McDonald, L.C., Killgore, G.E., Thompson, A., Owens, R.C., Kazakova, S.V., Sambol, S.P. Johnson, S., and Gerding, D.N. (2005) An epidemic, toxin gene-variant strain of *Clostridium difficile*. *N Engl JMed* **353**, 2433-2441.

McFarland, L.V. and Stamm, W.E. (1986) Review of *Clostridium difficile*-associated disease. *Am J Infect Control* **14**, 99-109.

McFarland, L.V., Elmer, G.W., and Suravicz, C.M. (2002) Breaking the cycle: treatment Strategies for 163 cases of recurrent *Clostridium difficile* disease. *Am J Gastroenterol* **97**, 1769-1775.

Mickelsen, P.A., Plorde, J.J., Gordon, K.P., Hargiss, C., McClure, J., Schoenknecht, F.D., Condie, F., Tenover, F.C., and Tompkins, L.S. (1985) Instability of antibiotic resistance in a strain of prosthetic valve endocarditis. *J Infect Dis* **152**, 50-58.

Moir, A., Lafferty, E., and Smith, D.A. (1979) Genetic analysis of spore germination mutants of *Bacillus subtilis* 168: the correlation of phenotype with map location. *J Gen Microbiol* **124**, 165-180.

Moncrief, J.S. and Wilkins, T.D. (2000) Genetics of *Clostridium difficile* toxins. In *Clostridium difficile* ed. Aktories, K. and Wilkins, T.D. pp. 34-49. Germanny: Springer.

Moore, R., Pothoulakis, C., LaMont, J.T., Carlson, S., and Madara, J.L. (1990) *Clostridium difficile* toxin A increases intestinal permeability and induces Cl- secretion. *Am J Physiol* **259**, G165-G172.

Mulligan, M.E., Peterson, L.R., Kwok, R.Y., Clabots, C.R., and Gerding, D.N. (1988) Immunoblots and plasmid fingerprints compared with serotyping and polyacrylamide gel eletrophoresis for typing *Clostridium difficile*. *J Clin Microbiol* **26**, 41-46.

Nakamura, S., Nakamura, M., Kojima, T., and Yoshida, H. (1989) *gyrA* and *gyrB* mutations in Quinolone resistant strains of *Escherichia coli*. *Antimicrob Agents Chemother* **33**, 254-255.

Nash, J.Q., Chattopadhyay, B., Honeycombe, J., and Tabaqchali, S. (1982) *Clostridium difficile* and cytotoxin in routine faecal specimens. *J Clin Pathol* **35**, 561-565.

National Research Council. (1956) *Proceedings first international conference on the use of antibiotics in agriculture*. Washington DC: National Academy of Sciences.

National Research Council. (1998) *The use of drugs in food animals: Benefits and risks.* Washington DC: National Academy Press.

Nelson, D.E., Auerbach, S.B., Baltch, A.L. Desjarardin, E., Beck-Sague, C., Rheal, C., Smith, R.P., and Jarvis, W.R. (1994) Epidemic *Clostridium difficile*-associated diarrhea: role of second- and third-generation cephalosporins. *Infect Control Hosp Epidemiol* **15**, 88-94.

Nusrat, A., Giry, M., Turner, J.R., Colgan, S.P., Parkos, C.A., Carnes, D., Lemichez., E., Boquet, P., and Madara, J.L. (1995) Rho protein regulates tight junctions and perijunctional actin organization in polarized epithelia. *Proc Natl Acad Sci USA* **92**, 10629-10633.

O'Farrell, S., Wilks, M., Nash, J.Q., and Tabaqchali, S. (1984) A selective enrichment broth for the isolation of *Clostridium difficile*. *J Clin Pathol* **37**, 98-99.

Owens, R.C. Jr., Donskey, C.J., Gaynes, R.P., Loo, V.G., and Muto, C.A. (2008) Antimicrobial-associated risk factors for *Clostridium difficile* infection. *Clin Infect Dis* **46**(Suppl. 1), S19-S31.

Paredes, C.J., Alsaker, K.V., and Papoutsakis, E.T. (2005) A comparative genomic view of clostridial sporulation and physiology. *Nat Rev Microbiol* **3**, 969.

Pell, A.N. (1997) Manure and microbes: public and animal health problem? *J Dairy Sci* **80**, 2673-2681.

Pepin, J., Valiquettte, L., and Alary, M.E. (2004) *Clostridium difficile*-associated diarrhea in a region of Quebec from 1991-2003: a changing pattern of disease severity. *CMAJ* **171**, 466-472.

Pepin, J., Saheb, N., Coulombe, M.A., Alary, M.E., Corriveau, M.P., Authier, S., Lebranc, M., Rivard, G., Bettez, M., Primeau, V., Nguyen, M., Jacob, C.E., and Lanthier, L. (2005) Emergence of fluoroquinolones as the predominant risk factor for *Clostridium difficile*-associated diarrhea: a cohort study during an epidemic in Quebec. *Clin Infect Dis* **41**, 1254-1260.

Persing, D.H. (1993) In vitro nucleic acid amplification techniques. In *Diagnostic Molecular Microbiology: Principles and Applications* eds. Persing, D.H., Smith, T., Tenover, F.C., White, T. pp. 51-87. Washington, DC: American Society for Microbiology.

Phillips, I., Casewell, M., Cox, T., De Groot, B., Friss, C., Jones, R., Nightingale, C., Preston, R., and Waddell, J. (2004) Does the use of antibiotics in food animals pose a risk to human health? A critical review of published data. *J Antimicrob Chemother* **53**, 28-52.

Piddock, L.J.V. (1996) Does the use of antimicrobial agents in veterinary medicine and animal husbandry select antibiotic-resistant bacteria that infect man and compromise antimicrobial chemotherapy. *J Antimicrob Chemother* **38**, 1-3.

Pierard, D., De Meyer, A., Rosseel, P., and Lauwers, S. (1996) Use of the Etest for determining antimicrobial susceptibility of anaerobic bacteria. *Path Biol* **44**, 358-362.

Pirs, T., Ocepek, M., and Rupnik, M. (2008) Isolation of *Clostridium difficile* from food animals in Slovenia. *J Med Microbiol* **57**, 790-792.

Poilane, I., Cruaud, P., Torlotin, J.C., and Collignon, A. (2000) Comparison of the Etest to the reference agar dilution method for antibiotic susceptibility testing of *Clostridium difficile*. *Clin Microbiol Infect* **6**(3), 154-156.

Popoff, M.R., Rubin, E.J., Gill, D.M., and Boquet, P. (1988) Actin-Specific ADP-Ribosyltransferase produced by a *Clostridium difficile* strain. *Infect Immun* **56**, 2299-2306.

Power, E.G.M. (1996) RAPD typing in microbiology: a technical review. *J Hosp Infect* **34**, 247-265.

Poxton, I.R., McCoubrey, J.M. and Blair, G. (2001) The pathogenicity of *Clostridium difficile*. *Clin Microbiol Infect* **7**, 421-427.

Poxton I.R. (2006) *Clostridium difficile*-new challenges for hyper-virulent strains. *Culture* **27**(1), 4-8.

Poutanen, S.M. and Simor, A.E. (2004) *Clostridium difficile*-associated diarrhea in adults. *CMAJ* **171**(1), 51-58.

Price, A.B. and Davies, D.R. (1977) Pseudomembranous colitis. *J Clin Pathol* **30**, 1-12.

Rao, A., Jump, R.L., Pultz, N.J., Pultz, M.J., and Donskey, C.J. (2006) In vitro killing of nosocomial pathogens by acid and acidified nitrite. *Antimicrob Agents Chemether* **50**, 3901-3904.

Redelings, M.D., Sorvillo, F., and Mascola, F. (2007) Increase in *Clostridium difficile*-related Mortality rates, United States, 1999-2004. *Emerg Infect Dis* **13**(9), 1417-1419.

Revington, B. (2002) *Feeding poultry in the post-antibiotic era*. Multi-state Poultry Meeting May 14-16, 2002.

Ridlon, J.M., Kang, D., and Hylemon, P.B. (2006) Bile salt biotransformations by human intestinal bacteria. *J Lipid Res* **47**, 241-259.

Riley, T.V., Brazier, J.S., Hamimah, H., Williams, K, and Phillips, K.D. (1987) Comparison of alcohol shock enrichment and selective enrichment for the isolation of *Clostridium difficile*. *Epidem Inf* **99**, 355-359.

Rodriguez-Palacios, A., Stampfli, H.R., Duffield, T., Peregrine, A.S., Trotz-Williams, L.A., Arroyo, L.G., Brazier, J.S., and Weese, J.S. (2006) *Clostridium difficile* PCR ribotypes in calves, Canada. *Emerg Infect Dis* **12**(11), 1730-1736.

Rodriguez-Palacios, A., Stampfli, H.R., Duffield, T. and Weese, J.S. (2007) *Clostridium difficile* in retail ground meat, Canada. *Emerg Infect Dis* **13**(3), 485-487.

Rosenblatt, J.E. and Gustafson, D.R. (1995) Evaluation of the Etest for susceptibility testing of anaerobic bacteria. *Diagn Microbiol Infect Dis* **22**, 279-284.

Rupnik, M., Kato, N., Grabnar, M. and Kato, H. (2003) New types of toxin A-negative, toxin B-positive strains among *Clostridium difficile* isolates from Asia. *J Clin Microbiol* **41**, 1118-1125.

Rutala, W.A., Gergen, M.F., and Weber, D.J. (1993) Inactivation of *Clostridium difficile* spores by disinfectants. *Infect Control Hosp Epidemiol* **14**, 36-39.

Salminen, S. Bouley, C., Boutron-Ruault, M.C., Cummings, J.H., Franck, A., Gibson, G.R., Isolauri, E., Moreau, M.C., Roberfroid, M., and Rowland, I. (1998) Function food science and gastrointestinal physiology and function. *Br J Nutr* **80**(Suppl. 1), S147-171.

Salyers, A.A. (2002) An overview of the genetic basis of antibiotic resistance in bacteria and its implications for agriculture. *Animal Biotech* **13**, 1-5.

Sanchez, M.L. and Jones, R.N. (1993) Etest, an antimicrobial susceptibility testing method with broad clinical and epidemiologic application. *Antimicrob Newslett* **8**, 1-8.

Samuel, P., Holtzman, C.M., Meilman, E., and Sekowski, I. (1973) Effect of neomycin and other antibiotics on serum cholesterol levels an don 7α-dehydroxylation of bile acids by the fecal bacterial flora in man. *Circ Res* **33**, 393-402.

Schwartz, D.C. and Cantor, C.R. (1984) Separation of yeast chromosome-sized DNAs by pulsed-field gel electrophoresis. *Cell* **37**, 67-75.

Sebaihia, M., Wren, B.W., Mullany, P., Fairweather, N.F., Minton, N., Stabler, R., Thomson, N.R., Roberts, A.P., Cerdeno-Tarraga, A.M., Wang, H., Holden, M.T.G., Wright, A., Churcher, C., Quail, M.A., Baker, S., Bason, N., Brooks, K., Chillingworth, T., Cronin, A., Davis, P., Dowd, L., Fraser, A., Feltwell, T., Hance, Z., Holroyd, S., Jagels, K., Moule, S., Mungall, K., Price, C., Rabbinowitsch, E., Sharp, S., Simmonds, M., Stevens, K., Unwin, L., Whithead, S., Dupuy, B., Dougan, G., Barrell, B., and Parkhill, J. (2006) The multidrug-resistant human pathogen *Clostridium difficile* has a highly mobile, mosaic genome. *Nat Genet* **38**, 779-786.

Sell, T.L., Schaberg, D.R., and Fekety, F.R. (1983) Bacteriophage and bacteriocins typing scheme for *Clostridium difficile*. *J Clin Microbiol* **17**, 1147-1152.

Setlow, P. (2003) Spore germination. *Curr Opin Microbiol* **6**, 550.

Silva, J., Fekety, R., Werk, C., Ebright, J., Cudmore, M., Batts, D., Syrgamaki, C., and Lukens, J. (1984) Inciting and etiologic agents of colitis. *Rev Infect Dis* **6**(suppl.), S214-S221.

Simango, C. (2006) Prevalence of *Clostridium difficile* in the environment in a rural community in Zimbabwe. *Trans R Soc Trop Med Hyg* **100**, 1146-1150.

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

Simhon, A., Douglas, J.R., Drasar, B.S., and Soothill, J.F. (1982) Effect of feeding on infants' faecal flora. *Arch Dis Child* **57**, 54-58.

Simon, G.L. and Gorbach, S.L. (1984) Intestinal flora in health and disease. *Gastroenterology* **86**, 174-193.

Smith, A. (2005) Outbreaks of *Clostridium difficile* infection in an English hospital linked to hypertoxin-producing strains in Canada and the US. *Euro Surveill* **10**, E050630.

Smith, L.D.S. and King, E.O. (1962) Occurrence of *Clostridium difficile* in infections of man. *J Bacteriol* **84**, 65-67.

Songer, J.G. (1996) Clostridial enteric diseases of domestic animals. *Clin Microbiol Rev* **9**, 216-234.

Songer, J.G., Post, K.W., Larson, D.J., Jost, B.H., and Glock, R.D. (2000) Infection of neonatal swine with *Clostridium difficile*. *J Swine Health Prod* **8**, 185-189.

Songer, J.G. (2004) The emergence of *Clostridium difficile* as a pathogen of food animals. *Anim Health Res Rev* **5**(2), 321-326.

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

Sorg, J.A. and Sonenshein, A.L. (2008) Bile salts and glycine as cogerminants for *Clostridium Difficile* spores. *J Bacteriol* **190**(7), 2505-2512.

Spangler, S.K., Jacobs, M.R., and Appelbaum, P.C. (1995) Susceptibilities of 201 anaerobes to erythromycin, azithromycin, clarithromycin, and roxithromycin by oxyrase agar dilution and Etest methodologies. *J Clin Microbiol* **33**, 1366-1367.

Spencer, R.C. (1998) The role of antimicrobial agents in the aetiology of *Clostridium difficile*-associated disease. *J Antimicrob Chemother* **41**(Suppl. C), 21-27.

Spigaglia, P., Barbanti, F., Mastrantonio, P., Brazier, J.S., Barbut, F., Delmee, M., Kuijper, E., and Poxton, R. (2008) Fluoroquinolone resistance in *Clostridium difficile* isolates from a prospective study of *C. difficile* infections in Europe. *J Med Microb* **57**, 784-789.

Starr, J.M. and Impallomeni, M. (1997) Risk of diarrhea, *Clostridium difficlie* and cefotaxime in the elderly. *Biomed Pharmacother* **51**, 63-67.

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

Stevenson, J.P. (1966) The normal bacterial flora of the alimentary canal of laboratory stocks of the desert locust Schistocerca gregaria Forskal. *J Invertebr Pathol* **8**, 205-211.

Stern, M.J., Ames, G.F., Smith, N.H., Robinson, E.C., and Higgins, C.F. (1984) Repetitive extragenic Palindromic sequences: a major component of the bacterial genome. *Cell* **34**, 1015-1026.

Struelens, M.J., Mass, A., Nonhoff, C., Deplano, A., Rost, F., Serruys, E., and Delmee, M. (1991) Control of nosocomial transmission of *Clostridium difficile* based on sporadic case surveillance. *Am J Med* **91**(Suppl.3b), 1385-1445.

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

Sullivan, N.M., Pellett, S., and Wilkins, T.D. (1982) Purification and characterization of toxin A and B of *Clostridium difficile*. *Infect Immun* **35**, 1032-1040.

Sunenshine, R.H. and McDonald, L. (2006) *Clostridium difficile*-associated disease: New Challenges from an established pathogen. *Cleve Clin J Med* **73**(2), 186-197.

Swaminathan, B. and Matar, G.M. (1993) Molecular typing methods: definition, applications, and advantages. In *Diagnostic Molecular Microbiology: Principles and Applications* ed. Persing, D.H., Smith, T.F., Tenover, F.C., and White, T.J. pp. 26-50. Washington, DC: American Society for Microbiology.

Swartz, M.N. (2002) Human diseases caused by foodborne pathogens of animal origin. *Clin Infect Dis* **34**(Suppl. 3), S111-S122.

Tabaqchali, S., Holland, D., and O'Farell, S., and Silman, R. (1984) Typing scheme for *Clostridium difficile*. Its application in clinical and epidemiological studies. *Lancet* **1**, 935-938.

Tabaqchali, S. (1990) Epidemiologic markers of *Clostridium difficile*. *Rev Infec Dis* **12**(Suppl.2), S192-S198.

Taylor, N. S., Thorne, G.M., and Bartlett, J.G. (1981) Comparison of two toxins produced by *Clostridium difficile*. *Infect Immun* **34**, 1036-1043.

Tedesco, F.J., Napier, J., Gamble, W., Chang, T.W., and Bartlett, J.G. (1979) Therapy of antibiotic-associated pseudomembranous colitis. *J Clin Gastroenterol* **1**, 51-54.

Tenover, F.C., Arbeit, R.D., Goering, R.V., Mickelsen, P.A., Murray, B.E., Persing, D.H., and Swaminathan, B. (1995) Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *J Clin Microbiol* **33**, 2233-2239.

Tenover, F.C. and McGowan, J.E. Jr. (1996) Reasons for the emergence of antibiotic resistance. *Am J Med Sci* **311**, 9-16.

Tenover, F.C., Arbeit, R.D., and Goering, R.V. (1997) How to select and interpret molecular strain typing methods for epidemiological studies of bacterial infections: a review for healthcare epidemiologists. *Infect Control Hosp Epidemiol* **18**(6), 426-439.

Thomas, L.A., Veysey, M.J., French, G., Hylemon, P.B., Murphy, G.M., and Dowling, R.H. (2001) Bile acid metabolism by fresh human colonic contents: a comparison of caecal versus faecal samples. *Gut* **49**, 835-842.

Tobes, R. and Ramos, J-L. (2005) REP code: defining bacterial identity in extragenic space. *Environ Microbiol* **7**(2), 255-228.

Tollefson, L., Altekruse, S.F., and Potter, M.E. (1997) Therapeutic antibiotics in animal feeds and antibiotic resistance. *Rev Sci Tech* **16**, 709-715.

Tollefson, L., Angulo, F.J., and Fedorka-Cray, P.J. (1998) National surveillance for antibiotic resistance in zoonotic enteric pathogens. *Vet Clin North Am Food Anim Pract* **14**, 141-150.

Tollefson, L., Fedorka-Cray, P.J., and Angulo, F.J. (1999) Public health aspects of antibiotic resistance monitoring in the USA. *Acta Vet Scand* **92**(Suppl.), 67-75.

Torres, J.F. (1991) Purification and characterization of toxin B from a strain of *Clostridium difficile* that does not produce toxin A. *J Med Microbiol* **35**, 40-44.

Toyokawa, M., Ueda, A., Tsukamoto, I.N., Horikawa, M., Sunada, A., and Asari, S. (2003) Pseudomembranous colitis caused by toxin A-negagive/toxin B-positive variant strain of *Clostridium difficile*. *J Infect Chemother* **9**, 351-354.

Turnidge, J., Barton, M., Blackman, N., Collignon, P., Doyle, K., Doust, T., Fairley, C., Grimes, T., Hall, R., Love, R., Nicholls, T., Turner, A., Valois, A., Wong, C., and O'Brien, E. (1999) The use of antibiotics in food-producing animals: antibiotic-resistant bacteria in Animals and humans. *Report of the Joint Expert Advisory Committee on Antibiotic Resistance (JETACAR), Commonwealth department of health and aged care, Commonwealth department of agriculture, fisheries and forestry*. Canberra: Biotext.

Turnidge, J. (2004) Antibiotic use in animals-prejudices, perceptions and realities. *J Antimicrob Chemother* **53**, 26-27.

van Belkum, A. (1994) DNA fingerprinting of medically important microorganisms by use of PCR . *Clin Microbiol Rev* **7**, 174-184.

van Belkum, A. and Meis, J. (1994) Polymerase chain reaction-mediated genotyping in microbial epidemiology. *Clin Infect Dis* **18**, 1018-1019.

van der Waaij, D. (1989) The ecology of the human intestine and its consequences for overgrowth by pathogens such as *Clostridium difficile*. *Annu Rev Microbiol* **43**, 69-87.

Versalovic, J., Koeuth, T., and Lupksi, J.R. (1991) Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. *Nucleic Acids Res* **19**, 6823-6831.

Versalovic, J., Schneider, M., de Bruijn, F.J., and Lupski, J.R. (1994) Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. *Methods Mol Cell Biol* **5**, 25-40.

Versalovic, J., de Bruijn, F.J., and Lupksi, J.R. (1998) Repetitive sequence-based PCR (rep-PCR) DNA fingerprinting of bacterial genomes In *Bacterial genomes physical structure and analysis*. ed. de Bruijn, F.J., Lupski, J.R., and Weinstock, G.M. pp. 38-48. New York, NY: Chapman&Hall Internatinal Thomson Pub.

Vlahcevic, Z.R., Heuman, D.M., and Hylemon, P.B. (1996) Physiology and pathophysiology of enterohepatic circulation of bile acids. In *Hepatology: a textbook of liver disease* ed. Zakim, D. and Boyer, T. pp. 376-417. Philadelphia, PA: Saunders Company.

Vollaard, E.J. and Clasener, H.A. (1994) Colonization resistance. *Antimicrob Agents Chemother* **38**, 409-414.

Voth, D.E. and Ballard, J.D. (2005) *Clostridium difficile* toxins: Mechanism of action and role in disease. *Clin Microbiol Rev* **18**(2), 247-263.

Wanahita, A., Goldsmith, E.A., Marino , B.J., and Musher, D.M. (2003) *Clostridium difficile* infection in patients with unexplained leukocytosis. *Am J Med* **115**, 543-546.

Warny, M., Pepin, J., Fang, A., Killgore, G., Thompson, A., Brazier, J., Frost, E., and McDonald, L.C. (2005) Toxin production by an emerging strain of *Clostridium difficile* associated with outbreaks of severe disease in North America and Europe. *Lancet* **366**, 1079-1084.

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

Weese, J.S., Staempfli, H.R., and Prescott, J.F. (2000) Isolation of environmental *Clostridium difficile* from a veterinary teaching hospital. *J Vet Diagn Invest* **12**, 449-452.

Wegener, H.C., Aarestrup, F.M., Jensen, L.B., Hammerum, A.M., and Bager, F. (1998) The association between the use of antimicrobial growth promoters and development of resistanct in pathogenic bacteria towards growth promoting and therapeutic antimicrobials. *J Anim Feed Sci* **7**, 7-14.

Wegener, H.C., Aarestrup, F.M., Jensen, L.B., Hammerum, A.M., and Bager, F. (1999a) Use of antimicrobial growth promoters in food animals and *Enterococcus faecium* resistance to therapeutic antimicrobial drugs in Europe. *Emerg Infect Dis* **5**, 329-335.

Wegener, H.C., Aarestrup, F.M., Gerner-Smidt, P. and Bager, F. (1999b) Transfer of antibiotic resistant bacteria from animals to man. *ACTA Veterinaria Scandinavica* **Suppl.92**, 51-57.

Welsh, J. and McClelland, M. (1990) Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Res* **18**, 7213-7218.

Welton, L.A., Thal, L.A., Perri, M.B., Donabedian, S., McMahon, J., Chow, J.W., and Zervos, M.J. (1998) Antimicrobial resistance in enterococci isolate from Turkey flocks fed virginiamycin. *Antimicrob Agents Chemother* **42**, 705-708.

Williams, J.G.K., Kubelik, A.R., Livak, K.J., Rafalski, J.A., and Tingey, S.V. (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acid Res* **18**, 6531-6535.

Willis, A.T. (1986) Historical aspects. In *Clostridium difficile*: Its role in intestinal disease ed. Rolfe, R. D. and Finegold, S.M. pp.15-28. San Diego, CA: Academic Press.

Wilson, K.H., Kenney, M.J., and Fekety, F.R. (1982) Use of sodium taurocholate to enhance spore recovery on a medium selective for *Clostridium difficile*. *J Clin Microbiol* **15**(3), 443-446.

Wilson, K.J. (1993) The microecology of *Clostridium difficile*. *Clin Infect Dis* **16**(Suppl. 4), S214-S218.

Witte, W. (1998) Medical consequences of antibiotic use in agriculture. *Science* **279**, 996-997.

World Health Organization. (1997) *The Medical Impact of the Use of Antimicrobials in Food Animals: report of a WHO meeting*, Document No. WHO/EMC/ZOO/97.4.WHO, Berlin, Germany.

World Health Organization. (2001) *WHO Global strategy for containment of antibiotic resistance.* http://www.who.int/drugresistance/guidance/en/index.html. Accessed November 27, 2007.

World Health Organization. (2002) *Monitoring antimicrobial usage in food animals for the protection of human health: report of a WHO consultation*, Document No. WHO/CDS/CSR/EPH/2002.11, Oslo, Norway.

World Health Organization. (2003) *Impacts of Antimicrobial Growth Promoter Termination in Denmark: the WHO international review panel's evaluation of the termination of the use of antimicrobial growth promoters in Denmark*: *report of a WHO meeting,* Document No. WHO/CDS/CPE/ZFK/2003.1. WHO, Foulum, Denmark.

World Health Organization. (2008) Joint FAO/WHO/OIE expert meeting on *Critically Important Antimicrobials.* Report of a meeting held in FAO, Rome, Italy, 26-30 November 2007. FAO, Rome, Italy, and WHO, Geneva, Switzerland.

Worsley, M.A. (1998) Infection control and prevention of *Clostridium difficile* infection. *J Antimicrob Chemother* **41** (Suppl.C), 59-66.

Wray, C. and Davies, R.H. (2000) Competitive Exclusion-an alternative to antibiotics. *Vet J* **159**, 107-108.

Wust, J. and Hardeggaer, U. (1992) Comparison of the Etest and a reference agar dilution method for susceptibility testing of anaerobic bacteria. *Eur J Clin Microbiol Infect Dis* **11**, 1169-1173.

Yoshioka, H., Iseki, K., and Fujita, K. (1983) Development and differences of intestinal flora in the neonatal period in breast-fed and bottle-fed infants. *Pediatrics* **72**, 317-321.

Young, V.B. and Schmidt, T.M. (2004) Antibiotic-associated diarrhea accompanied by large-scale alterations in the composition of the fecal microbiota. *J Clin Microbiol* **42**, 1203-1206.

Zar, F.A., Bakkanagari, S.R., Moorthi, K.M., and Davis, M.B. (2007) A comparison of vancomycin and metronidazole for the treatment of *Clostridium difficile*-associated diarrhea, stratified by disease severity. *Clin Infect Dis* **45**, 302-307.

Zwiener, R.J., Belknap, W.M., and Quan, R. (1989) Severe pseudomembranous enter colitis in a child: case report and literature review. *Pediatr Infect Dis J* **8**, 876-882.

CHAPTER 3

PREVALENCE AND COMPARISON OF CULTURAL AND ISOLATION METHODS FOR *CLOSTRIDIUM DIFFICILE* FROM PORCINE AND BOVINE FECES[1]

---

[1] Thitaram, S.N., Fedorka-Cray, P.F., Frank, J. F., Siragusa, G.R., Lyon, S. A., and Bailey, J.S. To be submitted to *Journal of Food Protection*.

Abstract

Two isolation methods were compared for their recovery of *C. difficile* from food animal feces and environment. One method used selective enrichment in cycloserine-cefoxitin fructose broth supplemented with 0.1% sodium taurocholate (TCCFB) followed by alcohol shock and plated onto tryptic soy agar supplemented with 5% sheep blood (BA) and cycloserine-cefoxitin fructose agar (CCFA); single alcohol shock. The other used alcohol shock prior to and after selective enrichment in TCCFB and plated onto BA and CCFA; double alcohol shock. A total of 55 (15.9%) of 345 swine fecal samples, 32 (2.4%) of 1,325 dairy cattle fecal samples, and 32 (8.6%) of 371 dairy cattle environmental samples were positive for *C. difficile* by either single or double alcohol shock. Double alcohol shock was significantly better than single alcohol shock for the recovery of *C. difficile* in swine feces. In environmental samples, double alcohol shock plating to CCFA was found to be a method of choice for recovery of *C. difficile* compared to single alcohol shock. However, there was no significant difference for the recovery of *C. difficile* between any combination of isolation methods and media observed in dairy cattle feces. This study suggests that swine is a major reservoir of *C. difficile* and different isolation methods may be needed for different types of samples.

Introduction

Clostridium difficile is an anaerobic, Gram-positive, spore-forming bacillus that is commonly associated with a spectrum of disease referred to as *C. difficile*-associated disease (CDAD), which can range from uncomplicated mild diarrhea (George et al. 1977; Bliss et al. 1998; Brazier 1998; Gorbach 1999; Barbut and Petit 2001) to life-threatening toxic megacolon and pseudomembranous colitis (PMC) (George et al. 1978; Bartlett 1992; Kelly et al. 1994; Voth and Ballard 2005; Kuijpers and Surawicz 2008). The infection is generally believed to be acquired nosocomially (Simango 2006). However, community-acquired CDAD is being increasingly reported (Hirschhorn et al. 1994; Kyne et al. 1998; Kuijper et al 2006).

Few studies have been directed at the role of *C. difficile* as a potential cause of diarrhea outside hospital environments (O'Neill et al. 1993; Simango and Mwakurudza 2008; Pirs et al. 2008). *C. difficile* has been isolated from domestic pets (Borriello et al. 1983; Arroyo et al. 2005a; Lefebvre et al. 2006), food animals (Rodriguez-Palacios et al. 2006; Keel et al. 2007; Pirs et al. 2008; Simango and Mwakurudza 2008), and retail meat (Rodriguez-Palacios et al. 2007). Moreover, molecular typing of *C. difficile* isolates from calves has shown similarities in PCR ribotypes in humans including two PCR ribotypes associated with outbreaks of severe disease in humans in Canada (Rodriguez-Palacios et al. 2006), North America, and Europe (Warny et al. 2005). It is not clear whether these animals are a possible source of human CDAD. However, recent detection of *C. difficile* in retail meat markets along with a high degree of molecular similarity is cause for concern.

Several diagnostic tests have been developed that detect the enterotoxin (toxin A) and/or cytoxin (toxin B) produced by *C. difficile*. However, a combination of *C. difficile* detection tests and culturing methods should be performed as the optimal laboratory approach. The culturing of

C. difficile is not commonly performed because of its limited clinical value and the difficulties inherent in working with this obligate anaerobe. As a result, isolates are not routinely available for molecular typing, antimicrobial susceptibility testing, toxin gene detection, or identification of virulence factors. The lack of *C. difficile* isolates limits the ability of researchers to investigate any apparent changes in the epidemiology or clinical presentation that may develop over time. This information would be a valuable avenue of infection control and preventive strategies of CDAD.

Several selective media and isolation procedures have been developed during the past three decades. Many of these procedures rely on an enrichment step to enhance the recovery of spores. The use of alcohol shock procedure effectively reduces the number of *C. difficile* vegetative cells and other organisms (Koransky et al. 1978; Clabots et al. 1989). The objectives of this study were to determine the prevalence of *C. difficile* in swine fecal samples, dairy cattle fecal samples and dairy cattle environmental samples, and to compare single alcohol shock method using selective enrichment in cycloserine-cefoxitin fructose broth supplemented with 0.1% sodium taurocholate (TCCFB) followed by alcohol shock and plate onto tryptic soy agar supplemented with 5% sheep blood (BA) and cycloserine-cefoxitin fructose agar (CCFA), with double alcohol shock method, using alcohol shock prior to and after selective enrichment in TCCFB and plate onto BA and CCFA, for the isolation of *C. difficile* from feces of swine, dairy cattle and from dairy cattle environments.

Materials and Methods

Samples. Three hundred forty-five swine fecal samples, 1,325 dairy cattle fecal samples and 371 environmental samples from dairy cattle farms were aseptically collected in whirl pak bags, shipped on ice packs, and sent overnight to the laboratory from 17 states of the United

States to be investigated for the presence of *C. difficile*. All samples were processed immediately upon receipt.

Culturing Procedures. Two different methods (single and double alcohol shock) and 2 different plating media (tryptic soy agar supplemented with 5% sheep blood, BA; Remel and cycloserine-cefoxitin fructose agar; CCFA) were employed to isolate *C. difficile*. All media and broth were pre-reduced in an anaerobic chamber (Bactron Anaerobic, Model BacII, Sheldon Manufacturing; 5% hydrogen, 5% CO_2, balanced nitrogen) 24 hr prior to use. All steps, except incubation, were done aerobically.

Single alcohol shock method (Arroyo et al., 2005b): One fecal/environmental swab was placed into 9.0 ml of cycloserine-cefoxitin fructose broth supplemented with 0.1% sodium taurocholate (TCCFB) in screw-capped tubes. After 7 days of aerobic incubation at 37^{o}C, 3.0 ml was transferred into a 15-ml conical tube and mixed with an equal amount of absolute ethanol and left at room temperature for 60 min. After 60 min, the sample was centrifuged at 4,600 x g for 30 min at 4^{o}C. The supernatant fluid was discarded, and a sterile swab was pressed into the resulting pellet. The well-mixed pellet was then plated onto BA and CCFA and incubated anaerobically at 37^{o}C for 72 hr.

Double alcohol shock method: Two g or 2 ml of feces or environmental samples were mixed with three times volume of absolute ethanol and left at room temp for 60 min. The samples were then centrifuged at 3,800 x g for 10 min at 4^{o}C. The resulting pellet was swabbed and placed into 9.0 ml TCCFB in screw-capped tubes and incubated aerobically at 37^{o}C for 7 days. After incubation, 3.0 ml was transferred into a conical tube and mixed with an equal amount of absolute ethanol and left at room temperature for 60 min. After 60 min, the samples were centrifuged at 4,600 x g for 30 min at 4^{o}C. The supernatant fluid was discarded, and a sterile swab was pressed into the resulting pellet. A well-mixed pellet was then plated onto BA and CCFA and incubated anaerobically at 37°C for 72 hr.

Confirmation of *Clostridium difficile*. Plates were examined for typical *C. difficile* colonies after 72 hr anaerobic incubation at 37°C by the following criteria: (1) fluoresced yellow/green under long wavelength UV light and (2) produced a horse manure odor. For each media type and/or isolation method, suspect colonies were subcultured to CCFA and incubated anaerobically at 37°C for 72 hr for purity prior to further tests. After incubation, the isolates were identified as *C. difficile* by the following criteria: (1) they showed a flat, ground glass like surface with irregular edges on CCFA, (2) they had a distinct horse manure odor, (3) they fluoresced yellow/green under long wavelength UV light, (4) they were Gram-positive and posed long, thin, straight rods usually possessing many sub-terminal to terminal spores under 1000X light microscope, (5) they showed a production of L-proline aminopeptidase (Pro-Disc; Remel, Carr-Scarborough Microbiologicals, Inc., Decatur, GA), and (6) they were positive by a 16S rDNA PCR detection as described by Kikuchi et al. (2002). All positive isolates were kept aerobically in 10 ml cooked meat medium in parafilmed screw-capped tubes at room temp after 48 hr anaerobic growth initiation at 37°C.

Statistical Analysis. A Yates-corrected chi-square test was used for the analysis of proportions. If a cell value was less than 5 in the two-by-two contingency table, Fisher's exact test was used. All P values were two-sided. Logistic regression model was used to analyze the recovery rate of *C. difficile* in each combination of isolation methods and media. The results were presented in term of odds ratio (OR) with 95% confidence intervals (CI). Analyses were performed with the use of SAS software (PROC FREQ) (SAS 9.1, 2006; SAS Institute Inc., Cary, NC). All statements of statistical significance were based on $P<0.05$.

Results

The results are presented in Table 3.1. Of the 345 swine feces analyzed, 55 (15.9%) were positive for *C. difficile* by at least one of the two previously described methods (regardless of culture media). Of these 55 positive samples, 21 were detected by single alcohol shock, and 50 by the double alcohol shock. Of the 1,325 dairy cattle feces analyzed, 32 (2.4%) were positive for *C. difficile* by at least one the two methods (regardless of culture media). Of these 32 positive samples, 22 were detected by single alcohol shock, and 14 by the double alcohol shock. Of the 371 dairy cattle environmental samples analyzed, 32 (8.6%) were shown to have *C. difficile* present by at least one of the two methods described (regardless of media). Of these 32 positive samples, 14 were detected by single alcohol shock and 27 by the double alcohol shock.

In swine feces, double alcohol shock produced significantly more *C. difficile* positive samples than did single alcohol shock regardless of media tested (OR=0.3824; CI=0.2243 to 0.6521; *P*=0.0004). In environmental samples, double alcohol shock plating to CCFA provided significantly better recovery of *C. difficile* compared to single alcohol shock plating to BA (OR=0.2662; CI=0.1136 to 0.6234; *P*=0.0017). Double alcohol shock plating to both BA and CCFA was significantly better than single alcohol shock plating to BA (OR=0.2450; CI=0.1053 to 0.5701; *P*=0.0006) and to CCFA (OR=0.4627; CI= 0.2348 to 0.9115; *P*= 0.0334) for the recovery of *C. difficile*. Double alcohol shock plating to BA did not produce significantly more *C. difficile* positive samples than single alcohol shock regardless of media tested. While in dairy cattle feces, there was no significant difference for the recovery of *C. difficile* between any combination of isolation methods and media.

Discussion

This study determined the prevalence of *C. difficile* in food animals and environmental samples collected from dairy cattle farms. As a spore former organism, *C. difficile* can survive for long periods in the environment, and can therefore be ingested by animals or humans through contaminated food and water. The results suggested that swine is a major reservoir of *C. difficile*, where it was isolated in 15.9% of fecal samples. Detection of *C. difficile* in environmental samples (8.6%) from dairy cattle farms also demonstrates the potential of the environment as a source of infection. Shedding of *C. difficile* in environment is common regardless whether they have enteric disease.

Double alcohol shock was significantly better than single alcohol shock for the recovery of *C. difficile* from swine feces regardless of plating media used indicating double alcohol shock method affected the chance of getting more *C. difficile* positive samples. In environmental samples, double alcohol shock plating to CCFA produced significantly more *C. difficile* positive samples than single alcohol shock plating to BA. Single alcohol shock plating to CCFA may be the optimal method of choice for the recovery of *C. difficile* from environmental samples when time and resources are limited. Despite the difference were not statistically significant, double alcohol shock plating to CCFA gave almost twice as many as *C. difficile* positive samples than single alcohol shock plating to CCFA. Double alcohol shock plating to CCFA and to both media were not significantly different from each other, using double alcohol shock plating to CCFA alone would be more cost effective than using both media. Therefore, double alcohol shock with plating on CCFA was the method of choice for the recovery of *C. difficile* from environmental samples from dairy cattle farms. The selection of spores by alcohol shock greatly diminishes competing flora which enhances both the isolation, and provides for easier recognition of

C. difficile on the plating media. The addition of bile salts to the medium is believed to enhance the recovery of spores by inducing the germination of spores from environmental samples or feces after alcohol shock (Brazier 1998). Enrichment steps may greatly facilitate recovery in studies of the carriage and environmental distribution of *C. difficile* where similarly low numbers of organisms may be present in samples. Double alcohol treatment reduces the number of *C. difficile* vegetative cells and other organisms, and enhances sporulation thus resulting in a smaller inoculum and less competitive growth from other clostridia species.

However, there was no significant difference for the recovery of *C. difficile* between any methodological combinations applied to dairy cattle feces. This may indicate that alcohol shock step and plating media did not have a significant effect on the recovery of *C. difficile* in dairy cattle. It is unclear why additional alcohol shock step failed to improve the recovery of *C. difficile* in dairy cattle. However, the low prevalence rate may suggest that *C. difficile* is not a primary pathogen in dairy cattle. In addition, the concentration of *C. difficile* in the intestinal tract may not correlate with the concentration of spore in feces and the distribution of bacteria in fecal samples is not uniform. Quantitative culture is required to test these hypotheses.

Microorganisms other than *C. difficile* frequently grew on BA in large numbers, making isolation of *C. difficile* in pure culture difficult because of the necessity for repeated subculture. On the other hand, *C. difficile* could readily be distinguished from the other organisms that occasionally grew on CCFA. Colonies of *C. difficile* were easier to detect on CCFA than on that of BA because of less competitive growth. CCFA is the medium of choice to isolate *C. difficile* from feces when compared to BA.

Because *C. difficile* is an important infectious cause of antibiotic-associated diarrhea and life-threatening PMC, laboratories should be able to isolate the organism to be available for further testing. Our efforts have been directed towards improving techniques for the recovery of *C. difficile* from food animal feces. Although the isolation of *C. difficile* does not constitute a diagnosis of PMC or antibiotic-associated diarrhea, the ability to isolate this organism is important for the study of *C. difficile* in food animals that are a potential source of infection. The findings from this study suggest that different isolation methods may be needed for different types of samples.

The presence of *C. difficile* spores in swine and dairy cattle feces supports the potential risk for contamination of meat and milk products during slaughter and milking process. Although proper cooking of meat and proper pasteurization of milk are emphasized for reducing the risk for contamination, the fact that *C. difficile* is a spore former complicates this issue. Characterization and molecular typing studies are needed to establish the relationship between these strains and those from human strains and their clinical relevance.

Table 3.1 Prevalence and comparison of isolation methods for recovery of *Clostridium difficile*

Isolation method	Sample type [%positive (no. positive/no. total] *		
MEDIA	swine feces	dairy cattle feces	dairy cattle environments
Single alcohol shock			
BA	5.80(20/345)a	1.13(15/1,325)a	1.89(7/371)a
CCFA	4.93(17/345)a	1.43(19/1,325)a	3.51(13/371)a,b
BA/CCFA	3.49(21/345)a	1.66(22/1,325)a	3.77(14/371)a,b,c
Double alcohol shock			
BA	13.04(45/345)b	0.75(10/1,325)a	3.51(13/371)a,b
CCFA	12.75(44/345)b	1.06(14/1,325)a	6.67(25/371)b,c
BA/CCFA	14.50(50/345)b	1.06(14/1,325)a	7.28(27/371)c

*Values within a column followed by same lower-case do not differ significantly ($P<0.05$)

References

Arroyo, L.G., Kruth, S.A., Willey, B.M., Staempfli, H.R., Low, D.E., and Weese, J.S. (2005a) PCR ribotyping of *Clostridium difficile* isolates originating from human and animal sources. *J Med Microbiol* **54**, 163-166.

Arroyo, L.G., Rousseau, J., Willey, B.M., Low, D.E., Staempfli, H., McGeer, A., and Weese J.S. (2005b) Use of a selective enrichment broth to recover *Clostridium difficile* from stool swabs stored under different conditions. *J Clin Microbiol* **43**(10), 5341-5343.

Barbut, F. and Petit, J.C. (2001) Epidemiology of *Clostridium difficile*-associated infections. *Clin Microbiol Infect* **7**, 405-410.

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

Bliss, D., Johnson, S., Savik, K., Clabots, C.R., Willard, K., and Gerding, D.N. (1998) Acquisition of *Clostridium difficile* and *Clostridium difficile*-associated diarrhea in hospitalized patients receiving tube feeding. *Ann Intern Med* **129**, 1012-1019.

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

Brazier, J.S. (1998) The epidemiology and typing of *Clostridium difficile*. *J Antimicrobiol Chemother* **41**(Suppl C), 47-57.

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

George, W.L., Sutter, V.L., and Finegold, S.M. (1977) Antimicrobial agent-induced diarrhea-a bacterial disease. *J Infect Dis* **136**, 822-828.

George, R.H., Symonds, J.M., Dimock, F., Brown, J.D., Arubi, Y., Shinagawa, N., Keighley, M.R., Alexander-Williams, J., and Burdon, D.W. (1978) Identification of *Clostridium difficile* as a cause of Pseudomembranous colitis. *BMJ* **1**, 695.

Gorbach, S.L. (1999) Antibiotics and *Clostridium difficile*. *N Engl J Med* **341**, 1690-1692.

Hirschhorn, L.R., Trnka, Y., Onderdonk, A., Lee, M.L., and Platt, R. (1994) Epidemiology of community-acquired *Clostridium difficile*-diarrhea. *J Infect Dis* **169**, 127-133.

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

Kelly, C.P., Pothoulakins, C., and LaMont, J.T. (1994) *Clostridium difficile* colitis. *N Engl J Med* **330**, 257-262.

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

Koransky, J.R., Allen, S.D., and Dowell, V.R., Jr. (1978) Use of ethanol for selective isolation of sporeforming microorganisms. *Appl Environ Microbiol* **35**, 762-765.

Kuijper, E.J., Coignard, B., and Tull, P. (2006) Emergence of *Clostridium difficile*-associated disease in North America and Europe. *Clin Microbiol Infect* **12**(Suppl.6), 2-18.

Kuijper, E.J. and Surawicz, C.M. (2008) *Clostridium difficile* infection. *Lancet* **371**, 1486-1488.

Kyne, L., Merry, C., O'Connell, B., Keane, C., and O'Neill, D. (1998) Community-acquired *Clostridium difficile* infection. *J Infect* **36**, 287-288.

Lefebvre, S.L., Arroyo, L.G., and Weese, J.S. (2006) Epidemic *Clostridium difficile* strain in hospital visitation dog. *Emerg Infect Dis* **12**(6), 1036-1037.

O'Neill, G., Adams, J.E., Bowman, R.A., and Riley, T.V. (1993) A molecular characterization of *Clostridium difficile* isolates from human, animals and their environments. *Epidemiol Infect* **111**, 257-264.

Pirs, T., Ocepek, M., and Rupnik, M. (2008) Isolation of *Clostridium difficile* from food animals in Slovenia. *J Med Microbiol* **57**, 790-792.

Rodriguez-Palacios, A., Stampfli, H.R., Duffield, T., Peregrine, A.S., Trotz-Williams, L.A., Arroyo, L.G., Brazier, J.S., and Weese, J.S. (2006) *Clostridium difficile* PCR ribotypes in calves, Canada. *Emerg Infect Dis* **12**(11), 1730-1736.

Rodriguez-Palacios, A., Stampfli, H.R., Duffield, T., and Weese, J.S. (2007) *Clostridium difficile* in retail ground meat, Canada. *Emerg Infect Dis* **13**(3), 485-487.

Simango, C. (2006) Prevalence of *Clostridium difficile* in the environment in a rural community in Zimbabwe. *Trans R Soc Trop Med Hyg* **100**, 1146-1150.

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

Voth, D.E. and Ballard, J.D. (2005) *Clostridium difficile* toxins: Mechanism of action and role in disease. *Clin Microbiol Rev* **18**(2), 247-263.

Warny, M., Pepin, J., Fang, A., Killgore, G., Thompson, A., Brazier, J., Frost, E., and McDonald, L.C. (2005) Toxin production by an emerging strain of *Clostridium difficile* associated with outbreaks of severe disease in North America and Europe. *Lancet* **366**, 1079-1084.

CHAPTER 4

AUTOMATED REPETITIVE EXTRAGENIC PALINDROMIC-POLYMERASE CHAIN REACTION (REP-PCR) AS A MOLECULAR TYPING METHOD FOR *CLOSTRIDIUM DIFFICILE*[1]

---

[1] Thitaram, S.N., Fedorka-Cray, P.F. and Frank, J. F. To be submitted to *Journal of Applied Microbiology*

Abstract

Molecular typing of *Clostridium difficile* isolates from food animals and humans may be useful to gain information on interspecies transmission and epidemiology. The Diversilab system for automated microbial strain typing using repetitive extragenic palindromic-polymerase chain reaction (rep-PCR) was evaluated for typing *C. difficile* and compared to results obtained by pulsed-field gel electrophoresis (PFGE). A total of 221 isolates of *C. difficile* from swine (n=94), dairy cattle (n=40), environmental samples (n=54), and human (n=33) were typed by both methods. One hundred nineteen and 82 distinct patterns were identified by rep-PCR and PFGE, respectively. PFGE was not able to type one dairy cattle environmental isolate. Rep-PCR and PFGE results were concordant for 32 isolates. Clustering of the remaining 188 isolates differed. Overall, 20 (9%) isolates from animals were indistinguishable from one human isolate by rep-PCR whereas 40 (18%) isolates from animals were indistinguishable from one or more human isolates by PFGE. No isolates matched patterns for hypervirulent strains by either method. A high percentage of indistinguishable isolates of human and animal origin was observed. This suggests that interspecies or other forms of animal-to-human transmission may occur. Rep-PCR findings were comparable to those obtained by PFGE. The Diversilab system was found to be more rapid, user-friendly and less labor-intensive than PFGE and may be an alternate to PFGE for determining strain relatedness of *C. difficile* isolates.

Introduction

Outbreaks of nosocomial infections continue to occur among patients in a variety of healthcare settings. Investigations of presumed outbreaks of bacterial infections in hospitals often require strain typing data to identify outbreak-related strains. *Clostridium difficile* is an important nosocomial pathogen that is associated with a spectrum of disease referred to as *C. difficile*-associated disease (CDAD), which can range from uncomplicated mild diarrhea (George et al. 1977; Bliss et al. 1998; Brazier 1998; Gorbach 1999; Barbut and Petit 2001) to life-threatening pseudomembranous colitis (PMC) (George et al. 1978; Bartlett 1992; Kelly et al. 1994; Voth and Ballard 2005; Kuijpers and Surawicz 2008). Since 2003 there has been an increase in incidence and severity of CDAD (Pepin et al. 2004; Loo et al. 2005; Kuijper et al. 2006a; Mutlu et al. 2007) including higher mortality, increased risk of relapse, and more complications (Kuijper et al. 2006a). These changes in the incidence and severity of CDAD may be associated with a new hypervirulent strain referred to as North America pulsed-field gel electrophoresis (NAP) type 1 (NAP1), polymerase chain reaction (PCR) ribotype 027, restriction endonuclease analysis (REA) type BI, and toxinotype III (McDonald et al. 2005; Warny et al. 2005; Kuijper et al. 2006b; Hubert et al. 2007).

However, it is often not clear what the epidemiological relationship is among isolates retrieved from outside hospitals. *C. difficile* also appears to be an important cause of enteric disease in animals (Songer et al. 2000; Songer 2004; Songer and Anderson 2006; Keel et al. 2007) and has been isolated from a variety of food animals including poultry (Al Saif and Brazier 1996; Simango 2006; Simango and Mwakurudza 2008), calves (Rodriguez-Palacios et al. 2006), cattle (Simango 2006; Pirs et al. 2008), and pigs (Waters et al. 1998; Songer et al. 2000; Simango 2006; Songer and Anderson 2006; Keel et al. 2007; Pirs et al. 2008). Recent reports indicate that human and animal isolates are often indistinguishable (Arroyo et al. 2005; McDonald et al. 2005; Kuijper et al. 2006b) and that PCR ribotype 027 has been isolated from dairy calves, beef calves, and cattle in Canada (Rodriguez-Palacios et al. 2006). Molecular typing of *C. difficile* isolates from food animals and humans may be useful for evaluation of the possibility for interspecies transmission and for understanding whether food animals could be a source of human CDAD.

As an epidemiological tool, strain typing is used to assist in tracking the spread of food contamination and hospital-associated diseases. Rapid and accurate typing methods can significantly reduce costs associated with treatment, containment, and decontamination. The rep-PCR method uses primers that target noncoding repetitive sequences interspersed throughout the bacterial genome. It is an established approach for subspecies classification and strain delineation of bacteria. Automated detection and analysis by using microfluidics chips (LabChip device; Caliper Technologies, Inc.) and digitization of the obtained information in a software package allows simple data archiving and retrieval. Objectives of this study were to investigate the relationship between food animal isolates and human clinical isolates and to determine the reliability of the Diversilab system for typing *C. difficile* by comparing results to those obtained by the reference method pulsed-field gel electrophoresis (PFGE).

Materials and Methods

Automated rep-PCR. Modifications of PCR chemistry and PCR cycling parameters to increase reproducibility and decrease time and cost are shown in Table 4.1. Reaction mixture volumes were reduced from 50 to 25 μl, and the quantity of template DNA was reduced from 100 to 50 ng per reaction mixture. The Gitschier buffer was replaced with rep-PCR buffer plus AmpliTaq buffer (Applied Biosystems, Foster City, CA). Cycling parameters were modified to increase reaction stringency, decrease amplicon sizes and cycling times, improve assay reproducibility and ease detection. Amplicon detection was automated with a microfluidics-based BioAnalyzer (Agilent, Palo Alto, CA) and LabChip device, reducing setup time from 1 hr to 15 min and decreasing total run time from 6 hr to 1 hr.

Experimental isolates. A total of 221 *C. difficile* strains were used in the study. A total of 188 *C. difficile* strains were isolated from healthy food animals and environmental samples (94 from swine feces, 40 from dairy cattle feces, and 54 from environmental samples from dairy cattle farms). Twenty *C. difficile* human clinical isolates and 13 NAP type strains (Their characteristics are provided in Table 4.2) were kindly provided by Dr. Joyce Rousseau (Department of Clinical Studies, Ontario Veterinary College, University of Guelph, Canada), Dr. Angela Thompson (Center for Disease and Prevention Control; CDC, Atlanta, GA), and Dr. Mark Wise (Bacterial Barcodes, Inc., Athens, GA), respectively. All human clinical isolates and NAP type strains are toxigenic (except CD10) and 2 isolates are hypervirulent strain; NAP1/027/BI/III (NAP1 and CD3).

The strains from food animal species and environmental samples were identified as *C. difficile* by the following criteria: (1) they exhibited a flat, ground glass like surface with irregular edges on cycloserine-cefoxitin fructose agar, (2) they had a distinct horse manure odor, (3) they fluoresced yellow/green under long wavelength UV light, (4) they were Gram-positive, long, thin, straight rods usually possessing many sub-terminal to terminal spores when viewed using a 1000X light microscope, (5) they produced L-proline aminopeptidase (Pro-Disc; Remel, Carr-Scarborough Microbiologicals, Inc., Decatur, GA), and (6) they were positive by a 16S rDNA PCR detection as described by Kikuchi et al. (2002). All isolates were stored aerobically in 10 ml cooked meat medium in parafilmed screw-capped tubes at room temp after 48 hr anaerobic growth initiation at 37°C.

DNA extraction. An UltraClean Microbial DNA isolation kit (MoBio Laboratories, Solana Beach, CA) was used to extract DNA. According to the manufacturer's instructions, a single-well isolated colony was sub-cultured onto tryptic soy agar supplemented with 5% sheep blood; BA (Remel). Following incubation anaerobically at 37°C for 48 hr, one loop full of bacterial culture was mixed with 1.8 ml molecular grade water in a 2 ml microcentrifuge tube. The bacterial suspension was centrifuged twice for 30 sec at 10,000 x g to wash and to remove any excess water. The pellet was resuspended in 300 µl of MicroBead solution. The resuspended pellet was mixed (Vortex Genie 2, Bohemia, NY) and transferred to MicroBead tube. Fifty µl of solution MD1 was added to the MicroBead tube. Solution MD1 contains SDS and disruption agents required for cell lysis. The MicroBead tubes containing cell suspension were heated in a heating block for 30 min at 70°C (Dri-Bath Thermolyne, Model D816525, Dubuque, IA). Then the MicroBead tubes containing cell suspension were shaken at maximum speed (10) for 30 min using the MoBio vortex adapter (MoBio Laboratories, Solana Beach, CA) on a vortex (Vortex Genie 2, Bohemia, NY). The cell suspension was centrifuged for 30 sec at 10,000 x g. The supernatant fluid was then transferred to a 2 ml microcentrifuge tube containing 100 µl of solution MD2, mixed thoroughly and incubated at -20°C overnight. Solution MD2 contains a reagent to precipitate non-DNA organic and inorganic material including cell debris and proteins. Following incubation, the suspension was centrifuged for 1 min at 10,000 x g. Then the supernatant fluid was transferred to a 2 ml microcentrifuge tube containing 900 µl of solution MD3. Solution MD3 is a highly concentrated salt solution necessary to bind DNA to the Spin Filter membrane in the following step. Seven hundred µl of the well-mixed supernatant was loaded into the Spin Filter device (MoBio Laboratories, Solana Beach, CA) and centrifuged for 30 sec at 10,000 x g. The flow through liquid was discarded. Three hundred µl of solution MD4 was added to the Spin Filter and centrifuged for 30 sec at 10,000 x g. Solution MD4 is an ethanol based wash solution used to further clean the DNA that is bound to the silica filter membrane in the Spin Filter. The flow through was discarded and the Spin Filter was centrifuged again for 1 min at 10,000 x g to remove all traces of wash solution. The Spin Filter was placed in a new 2 ml microcentrifuge tube. Thirty five µl of solution MD5 was added to the center of the filter membrane and left at room temp for 5 min to ensure the release of bound DNA. Solution MD5 is an elution buffer (10mM Tris pH 8). The filter membrane was centrifuged for 30 sec at 10,000 x g to release bound DNA from the filter membrane into the collection tube. All DNA were standardized to a concentration of 25-50 ng/µl and kept at -20^{o}C until further testing.

rep-PCR methodology. The extracted DNA was amplified using the *Clostridium* DiversiLab DNA Fingerprinting kit (DiversiLab; Athens, GA), according to the manufacturer's instructions. For each sample reaction, 18 µl of rep-PCR mastermix, 2.5 µl of 10X PCR buffer (Applied Biosystems), 2 µl of rep-PCR primer mixture, 0.5 µl of AmpliTaq DNA polymerase, and 2 µl of genomic DNA, were added for a total of 25 µl per reaction mixture. The thermal cycling parameters were as follows: initial denaturation of 94^{o}C for 2 min; 35 cycles of denaturation at 94^{o}C for 30 sec; annealing at 45^{o}C for 30 sec; extension at 70^{o}C for 90 sec; and a final extension at 70^{o}C for 3 min.

Automated rep-PCR DNA fingerprinting. The DNA amplicons were separated with microfluidics chips (LabChip device; Caliper Technologies, Inc.) on a Bioanalyzer (model B 2100, Agilent Technologies, Inc., Palo Alto, CA). DNA standard markers (used for normalization of sample runs) and 1 µl of the Chip Kit molecular weight ladder (consisting of concentrations of 5 ng/µl of each 200-, 400-, 600-, 800-, 1,000-, 2,000-, 3,000-, and 4,000-bp DNA marker) were used. Analyses were performed with the Diversilab system software (version 3.3) with the Kullback-Leibler Distance method (Jeffrey's divergence) to determine distance matrices and the unweighted-pair group method with arithmetic mean (UPGMA) to create dendrograms. Reports were automatically generated and included the dendrogram (Figure 4.1), electropherograms, virtual gel images (Figure 4.1), scatter plots (Figure 4.4), and selectable demographic fields to aid in interpretations of the data.

PFGE methodology. To obtain a fresh 24 hr culture, a single-well isolated colony was sub-cultured onto BA. Following incubation anaerobically at 37^{o}C for 24 hr, the culture was then inoculated in pre-reduced 2 ml protein yeast glucose broth and incubated anaerobically at 37^{o}C for 7 hr. Then 1000 µl of bacterial culture was transferred into a microcentrifuge tube for each agarose plug to be made and centrifuged for 5 min at 13.5 rpm to obtain the bacterial pellet. The supernatant was discarded and the pellet was mixed (Vortex Genie 2, Bohemia, NY) to emulsify in 300 µl of Gram-positive lysis buffer (6 mM Tris HCl pH 8, 1 M NaCl, 100 mM EDTA pH 8, 0.5% polyoxyethylene 20 cetry ether, 0.2% sodium deoxycholate, and 0.5% sodium laurylsarcosine). The resuspended pellet was warmed to 37^{o}C in a water bath for 5 min. The 1.8% SeaKem Gold agarose was melted and equilibrated to 65^{o}C. Three hundred fifty µl of agarose was added to the resuspended pellet and mixed thoroughly. The mixture was transferred to reusable plug molds and the agarose was allowed to solidify at 4^{o}C for 15 min. The solidified plug was incubated in 3 ml Gram-positive lysis buffer with Rnase and lysozyme solution (Gram-positive lysis buffer plus 1ml/ml Rnase solution and 5mg/ml lysozyme) in water bath overnight at 35^{o}C. Gram-positive lysis buffer with Rnase and lysozyme solution was removed and the plug was then incubated with 3 ml proteinase K with SDS solution (0.5 M EDTA pH 8, 1% sodium dodecyl sulfate, and 2% proteinase K) in a water bath overnight at 50-55 °C. Proteinase K with SDS solution was removed and the plug was then washed 4 times in 4 ml 0.01 M Tris-EDTA buffer (pH 8) with gentle agitation (rotating on each wash for 30 min). After inactivating the proteinase K, the plug was digested for 3 hr at room temp with *Sma*I (Roche). DNA fragments were separated in 1% SeaKem Gold agarose in 0.5 Tris-Borate-EDTA buffer for 17.5 hr at 14 °C in a Chef Mapper XA apparatus (BioRad) at 6V/cm with initial and final pulse times of 5 sec and 40 sec, respectively. DNA fragments were stained using ethidium bromide, captured under UV light (Quantity One, BioRad), and analyzed using Bionumerics (version 4.0, Applied Maths, Kortrijk, Belgium). Chromosomal DNA of global standard *Salmonella* Braenderup was run one in every 6 plugs on comb using 50U *XbaI* (Roche) for molecular standard markers.

Results

A total of 221samples from swine (n=94), dairy cattle (n=40), dairy cattle environment (n=54), human clinical isolates (n=20) and NAP type strains (n=13) were analyzed. One dairy cattle isolate was not included in the rep-PCR analysis due to poor DNA quality. Fifty-six and 119 sub-group, and 82 distinct patterns were identified by rep-PCR (Figure 4.1, Table 4.3) and PFGE (Table 4.3), respectively. Swine, dairy cattle, dairy cattle environment, human, and NAP type strains were identified by rep-PCR into to 59, 17, 29, 20, and 12 groups, respectively. The most common profile by rep-PCR was group 42-10, representing 18.2% while group 28 was the most common by PFGE (10.45 %) (Table 4.4 and 4.5).

Overall, 20 (9%) isolates from animals were indistinguishable from one human isolate by rep-PCR (Table 4.4) whereas 40 (18%) isolates from animals were indistinguishable from one or more human isolates by PFGE (Table 4.5). No isolates matched the hypervirulent strains (NAP1 and CD3) by both methods. However, *C. difficile* isolated from food animals and environment appeared to have a high degree of similarity to human isolates by both rep-PCR and PFGE.

Typeability. PFGE was not able to type all *C. difficile* strains examined (221 isolates). The DNA of one dairy cattle environment appeared consistently degraded during the analysis. On the other hand, all *C. difficile* isolates (220 isolates) could be analyzed by rep-PCR. However, one dairy cattle isolate appeared consistently contaminated with residues of salt during DNA extraction. This sample was subsequently omitted from rep-PCR analysis.

Reproducibility. Reproducibility of the Diversilab system was evaluated. Of 220 samples, 76 were randomly selected for analysis. Seventy six *C. difficile* isolates (a combination of all sample groups) were examined by repeating the entire process of typing (different operator, laboratory, and equipment). The profile obtained by all replications was highly consistent with respect to clustering and band patterns (Figure 4.2). However, there were small differences in fluorescence intensity of the bands obtained (Figure 4.3).

Discriminatory power. The discriminatory powers of the different techniques refer to the number of distinct typing groups and subgroups identified among isolates examined and the total number of distinct patterns detected (Hunter 1990). Among the 220 isolates examined, PFGE discerned fewer groups than rep-PCR (swine; 41 versus 59, dairy cattle environment; 24 versus 29 and human; 17 versus 20, respectively) (Table 4.6).

Ease of interpretation. PFGE restriction profiles comprised 6-12 distinct, well-resolved fragments and were relatively easy to interpret and compare. Rep-PCR yielded 1-5 intense fragments and another 1-10 less intense fragments and was easily identified. The rep-PCR fingerprints were analyzed with accompanying Diversilab software. Results were displayed in a customizable report format containing dendrogram, virtual gel images (Figure 4.1), sample graphs (Figure 4.3), scatter plot (Figure 4.4), sample graph overlay (Figure 4.5) and demographic information (data not shown). These tools help aid visualization of sample clustering and interpretation.

Ease of performance. The PFGE is a tedious and time-consuming process (8 hr for 12 samples). One strain of *C. difficile* remained nontypeable because the chromosomal DNA was consistently degraded during analysis. On the other hand, rep-PCR by the Diversilab system provided a simple, user friendly platform and required less time to perform than PFGE (1 hr for 12 samples).

Comparison of results obtained by rep-PCR and PFGE. Results for all groups are summarized in Table 4.4 (rep-PCR) and Table 4.5 (PFGE). Overall interpretations of rep-PCR and PFGE were concordant for 32 isolates. Results were identical in both rep-PCR and PFGE in 3 swine isolates (group 50 in rep-PCR or group 9 in PFGE). These 3 isolates were considered indistinguishable by both rep-PCR and PFGE. The remaining 29 isolates were in 29 groups and considered different by both methods (data not shown). The clustering of the remaining 188 isolates differed.

The following are examples of discrepancies between the 2 methods. The 2 virulent strains (NAP1 and CD3) were grouped together by rep-PCR (group 52, 98.5% similarity) whereas they were separately grouped by PFGE (group 2 and 14). In swine, 17 isolates within 4 groups were not discriminated by PFGE (Table 4.5; 5 in group 3; 5 in group 16; 4 in group 21; and 3 in group 82) but were considered different by rep-PCR. Ten isolates were considered different by PFGE but indistinguishable by rep-PCR within 4 groups (Table 4.4; 2 in group 13; 2 in group 41; 2 in group 44; and 4 in group 47-1). In dairy cattle environment, 13 isolates within 3 groups were not discriminated by PFGE (Table 4.5; 3 in group 13; 3 in group 10; and 7 in group

29) but were considered different by rep-PCR. Nine isolates were considered different by PFGE but indistinguishable by rep-PCR within 2 groups (Table 4.4; 4 in group 3-1; and 5 in group 6-1). In human isolates, 6 isolates within 3 groups were not discriminated by PFGE (Table 4.5; 2 in group 2; 2 in group 14; and 2 in group 28) but were considered different by rep-PCR. In dairy cattle, 7 isolates within 2 groups were not discriminated by PFGE (Table 4.5; 5 in group 10 and 2 in group 7) but were considered different by rep-PCR. Six isolates were considered different by PFGE but indistinguishable by rep-PCR within 2 groups (Table 4.4; 4 in group 3-1; and 2 in group 6-3).

Discussion

PCR is used for the direct detection of many types of infectious agents in clinical samples (Persing 1993) and has been adapted for use as a typing tool for many bacteria (Welsh and McClelland 1990; Williams et al. 1990; van Belkum 1994). Repeated sequences are present in the genomes of all organisms (Lupski and Weinstock 1992). Bacterial chromosomes contain multiple interspersed repetitive sequences that occupy intergenic regions at sites dispersed throughout the genome. Such blocks of noncoding, repetitive sequences can serve as multiple genetic targets for oligonucleotide probes, enabling the generation of unique DNA profiles or fingerprints for individual bacterial strains. These unique “barcodes” or DNA fingerprints define each bacterial chromosome without the need for measuring gene expression or enzyme function (Versalovic et al 1998). Palindromic units, or rep elements, and enterobacterial repetitive intergenic consensus (ERIC) sequences are the most commonly used targets for DNA typing (Gilson et al. 1984; Stern et al. 1984; Hulton et al. 1991)

Manual rep-PCR compares favorably to PFGE for microbial strain typing (Malathum et al. 1998) but lack of reproducibility, sub-optimal turnaround times, and difficulties with interpretation have limited its acceptance (Healy et al. 2005). Another potential problem associated with manual rep-PCR was the apparent need to optimize the primers (Malathum et al. 1998; Johnson and Clabots 2000). The automated rep-PCR by the Diversilab system has addressed these issues. DNA fingerprinting kits are commercially available with primers optimized based on each genus (Healy et al. 2005) and analysis is simplified by the use of web-based software.

PFGE is a tedious and time-consuming analysis which required 3 days to process a set of 12 samples. Positive aspects of the Diversilab system include rapid results (4 hr versus 3 days for PFGE), standardized reagents commercially available in kit form, and the web-based platform. Rep-PCR analysis for different organisms can be conducted using the same Microfluidics chip. Results are archived in the web based software, allowing for easier comparison of samples between laboratories and over time, making repeat testing of historical samples with new samples unnecessary. Bionumerics is available to aid in the interpretation and comparison of PFGE gel images, however, the software is expensive. In addition, the Diversilab system is less technically demanding than PFGE. The DNA extraction step, although technically simple, is the most labor intensive part of the rep-PCR, requiring 2-3 hr hands-on time (for 2 days), depending on the number of isolates processed. There are some drawbacks to the Diversilab system. The occurrence of electrical interference within the Bioanalyzer, the presence of dust, or minor vibrations on the laboratory bench during analysis can produce an electrical spike in the electropheregram requiring repeat testing of the amplified DNA. In addition, for the Diversilab system to be cost efficient, 13 samples must be tested, because all wells of the microfluidics chip must contain DNA marker and the gel-dye mix, even if no sample is being tested. The time involved with interpretation is difficult to calculate on a per sample basis. However, from this study, the web based Diversilab system requires considerably less time to determine relatedness of samples than PFGE. In summary, the automated rep-PCR by the Diversilab system has promise as a rapid, user-friendly, and less labor-intensive alternative to PFGE for determining strain relatedness of *C. difficile* isolates.

Despite some discrepancies between the 2 methods, the results obtained by rep-PCR were relatively comparable to those obtained by PFGE in term of typeability, reproducibility, discriminatory power, ease of interpretation, and ease of performance. Rep-PCR demonstrated a higher discriminatory power than PFGE in analyzing both animal and human *C. difficile* isolates. Among isolates from swine, dairy cattle, dairy cattle environment, human, and NAP strains, rep-PCR discriminated 59, 17, 29, 20, and 12 groups, respectively, whereas PFGE discriminated 41 swine, 24 dairy cattle environment, and 17 human isolates. The isolates that were untypeable by PFGE were recognized as belonging to group 18-2 by rep-PCR.

The results showed that isolates shared common groupings between human, animal, and environmental isolates. Because there was no reported contact between the animals and infected humans in this study, the lack of identifiable variation among some isolates of animal and human origin suggests that interspecies transmission of *C. difficile* may occur. There were also indistinguishable isolates between environment and animal by both rep-PCR and PFGE. Interestingly, several isolates (20; 9% by rep-PCR and 40; 18% by PFGE) from swine and dairy cattle environment were indistinguishable from human isolates. The fact that a high percentage of indistinguishable isolates between human and animal was found indicates the potential for transmission and infection of *C. difficile* from animals and environment.

Although *C. difficile* is recognized as a cause of disease in several animal species (Frazier et al. 1993; Songer et al. 2000; Weese et al. 2001; Marks et al. 2002; Songer 2004, Songer and

Anderson 2006; Keel et al. 2007), little investigation has been conducted on the potential for interspecies transmission of *C. difficile* to humans. Previous studies have suggested the possibility of *C. difficile* transmission between humans and domestic pets (O'Neill et al. 1993; Lefebvre et al. 2005), and few studies have examined the possible link between CDAD in food animals and humans (Arroyo et al. 2005; Jhung et al. 2008). Finding similarity between human and animal isolates in this study suggests at least 3 possible causes for community-acquired CDAD in human: 1) exposure of humans and animals to a common environmental source of *C. difficile*, 2) infection caused by transmission by means of indirect (through contaminated water, produce, or the environment, for example) or direct contact with infected live animals, and 3) infection linked to consumption of products from food producing animals. Both the genetic similarity of the human and animal isolates in this study and the apparent increasing importance of a hypervirulent strain (NAP1/027/BI/III) in human CDAD after their emergence in animals may suggest foodborne or other forms of animal-to-human transmission. Further epidemiological studies of the potential for *C. difficile* transmission between animals and humans are needed.

Table 4.1 General comparison of manual and automated rep-PCR[a]

Analysis/conditions	Manual rep-PCR	Automated rep-PCR[b]
Application:		
Reaction volume (μl)	50	25
Buffer	Gitschier[c] plus DMSO and BSA[d]	no DMSO or BSA[d]
Primer(s)	Oligonucleotide primers based on rep elements[c]	Oligonucleotide primers based on rep elements[c]
Annealing temp (°C)	38-60	50-70
Cycling parameters	95°C for 7 min, 35 cycles (for each cycle, 90°C for 30 sec, 45°C for 1 min, and 65°C for 8 min), 65°C for 16 min, and 4°C holding	94°C for 2 min, 35 cycles (for each cycle, 94°C for 30 sec, 45°C for 30 sec, and 70°C for 1.5 min), 70°C for 3 min, and 4°C holding
Cycle time (hr)	6.5	2
DNA fingerprinting:		
Amplicon separation	Agarose gel	Microfluidics chip[e]
Data capture	High-resolution digital camera; manually	Bioanalyzer[f]; automatically
Assay set-up time	1hr	15min
Total run time	6hr	1hr
Time for 12 samples	8hr	1hr
Analysis and reporting:		
Analysis software	BioNumerics	Diversilab[g]
Interface	Local or network	Internet based
Data upload	Manual	Automatic
Report	Manual	Automatic and multiple options

[a] Some conditions may vary depending on source

[b] Diversilab system (Bacterial Barcodes, Inc.)

[c] Versalovic et al. 1991

[d] DMSO=dimethyl sulfoxide; BSA=bovine serum albumin (ABI, Applied Biosystems, Inc.)

[e] LabChip device (Caliper Technologies, Inc.)

[f] Bioanalyzer (model B 2100, Agilent Technologies)

[g] Diversilab web based software (Diversilab system, Bacterial Barcodes, Inc.)

Table 4.2 Characteristics of *Clostridium difficile* strains from human

Strains[a]	Source	Toxin A	Toxin B	Binary toxin	Deletion gene	Toxinotype	Ribotype
C745	Human	pos[c]	pos	pos	neg[d]	n/a[g]	other[h]
S61	Human	pos	pos	neg	neg	n/a	other
N432	Human	pos	pos	pos	pos(18bp)[e]	n/a	027
L13	Human	pos	pos	neg	neg	n/a	other
Q676	Human	pos	pos	neg	neg	n/a	other
B1017	Human	pos	neg	neg	neg	n/a	other
K744	Human	pos	pos	pos	pos(39bp)[f]	n/a	078
E1002	Human	pos	pos	neg	neg	n/a	other
AK477	Human	pos	pos	neg	neg	n/a	other
AC345	Human	pos	pos	neg	neg	n/a	other
AA438	Human	pos	pos	neg	neg	n/a	other
H447	Human	pos	pos	neg	neg	n/a	other
AE978	Human	pos	pos	pos	pos(18bp)	n/a	027
AE765	Human	pos	pos	pos	neg	n/a	other
Y431	Human	pos	pos	pos	pos(18bp)	n/a	other
Z718	Human	pos	pos	neg	neg	n/a	other
W73	Human	pos	pos	neg	pos(18bp)	n/a	other
X57	Human	pos	pos	neg	neg	n/a	other
I927	Human	pos	pos	neg	neg	n/a	other
M680	Human	pos	pos	neg	neg	n/a	other
NAP1[b]	Human	pos	pos	pos	pos(18bp)	III	027
NAP2	Human	pos	pos	neg	neg	O	other
NAP3	Human	pos	pos	neg	neg	O	other
NAP4	Human	pos	pos	neg	neg	O	other
NAP5	Human	pos	pos	neg	neg	O	other
NAP6	Human	pos	pos	neg	neg	O	other
NAP7	Human	pos	pos	neg	pos(39bp)	V	078
NAP8	Human	pos	pos	pos	pos(39bp)	V	078
CD1	Human	pos	pos	neg	neg	n/a	other
CD3	Human	pos	pos	pos	pos(18bp)	III	027
CD6	Human	pos	pos	pos	pos(18bp)	n/a	other
CD8	Human	pos	pos	neg	neg	O	other
CD10	Human	n/a	n/a	neg	neg	O	other

[a] 20 human clinical strains were kindly provided by Dr. Joyce Rousseau (Department of Clinical Studies, University of Guelph, Canada), 8 NAP type strains by Dr. Angela Thompson (CDC, Atlanta, GA), and CD1-CD10 by Dr. Mark Wise (Bacterial Barcodes, Inc., Athens, GA)

[b] NAP=North America pulsed-field gel electrophoresis type strains

[c] Positive

[d] Negative

[e] Positive and posses an 18-base pair deletion gene

[f] Positive and posses an 39-base pair deletion gene

[g] Data are not available

[h] Patterns do not match 027 or 078

Table 4.3 Summary of overall grouping by rep-PCR[a] and PFGE[b]

rep-PCR			PFGE	
Group	Sub-group	No. of isolates per group	Group	No. of isolates per group
1	1	1	1	2
	2	1	2	3
	3	1	3	8
	4	1	4	4
	5	2	5	1
2	1	1	6	2
3	1	8	7	4
	2	1	8	1
	3	1	9	3
	4	1	10	11
4	1	1	11	2
	2	1	12	2
	3	1	13	12
5	1	1	14	3
	2	1	15	2
	3	1	16	6
6	1	21	17	1
	2	2	18	1
	3	3	19	1
	4	1	20	1
	5	1	21	5
	6	1	22	1
	7	1	23	1
	8	1	24	2
	9	1	25	2
	10	1	26	1
	11	1	27	1
	12	1	28	23
7	1	1	29	19
	2	1	30	2
	3	1	31	1
	4	1	32	1
	5	1	33	1
8	1	1	34	3
9	1	1	35	1
10	1	1	36	1
11	1	1	37	1
	2	1	38	1
	3	1	39	1
	4	1	40	1
	5	1	41	1
12	1	1	42	1
	2	1	43	1
	3	1	44	1
	4	1	45	1

Table 4.3 (Con't) Summary of overall grouping by rep-PCR[a] and PFGE[b]

rep-PCR			PFGE	
Group	Sub-group	No. of isolates per group	Group	No. of isolates per group
13	1	3	46	1
14	1	1	47	2
15	1	2	48	3
	2	1	49	2
16	1	1	50	2
17	1	1	51	2
18	1	2	52	19
	2	1	53	6
19	1	1	54	2
20	1	1	55	3
21	1	1	56	1
	2	1	57	1
22	1	1	58	1
23	1	1	59	1
	2	1	60	1
24	1	1	61	2
25	1	2	62	1
	2	1	63	1
	3	1	64	1
26	1	1	65	1
27	1	1	66	1
28	1	1	67	1
29	1	1	68	1
	2	1	69	1
30	1	1	70	1
31	1	1	73	1
32	1	1	75	2
	2	1	76	1
33	1	1	77	1
34	1	1	78	1
35	1	3	79	2
36	1	1	80	3
37	1	1	81	1
	2	1	82	3
	3	1	83	1
	4	1	84	1
	5	1	85	1
38	1	1		
	2	1		
	3	1		
	4	1		

Table 4.3 (Con't) Summary of overall grouping by rep-PCR[a] and PFGE[b]

	rep-PCR			PFGE	
	Group	Sub-group	No. of isolates per group	Group	No. of isolates per group
	39	1	1		
	40	1	1		
		2	1		
	41	1	2		
	42	1	1		
		2	1		
		3	2		
		4	1		
		5	2		
		6	1		
		7	1		
		8	3		
		9	1		
		10	40		
	43	1	6		
	44	1	2		
	45	1	2		
		2	3		
	46	1	1		
	47	1	5		
		2	1		
		3	1		
		4	1		
	48	1	1		
	49	1	1		
		2	1		
	50	1	3		
	51	1	1		
	52	1	3		
	53	1	1		
	54	1	1		
	55	1	1		
	56	1	1		
Total	56	119	220	82	220

[a] One dairy cattle isolate was not included in the interpretation due to poor DNA quality

[b] PFGE was not able to type one dairy cattle environment isolate

Table 4.4 Distribution and frequency of *Clostridium difficile* isolates recovered from swine, dairy cattle, dairy cattle environment, human, and NAP type strains according to rep-PCR grouping

rep-PCR	Swine	Dairy cattle[a]	Environment	Human	NAP strains[b]	Total
1-1	1	-	-	-	-	1
1-2	1	-	-	-	-	1
1-3	-	-	-	-	1	1
1-4	-	-	-	-	1	1
1-5	1	-	1	-	-	2
2	-	-	-	-	1	1
3-1	-	4	4	-	-	8
3-2	-	-	1	-	-	1
3-3	-	1	-	-	-	1
3-4	-	-	1	-	-	1
4-1	1	-	-	-	-	1
4-2	1	-	-	-	-	1
4-3	1	-	-	-	-	1
5-1	-	-	-	1	-	1
5-2	-	1	-	-	-	1
5-3	1	-	-	-	-	1
6-1	4	11	5	1	-	21
6-2	-	1	1	-	-	2
6-3	1	2	-	-	-	3
6-4	-	-	1	-	-	1
6-5	-	-	-	1	-	1
6-6	-	-	-	1	-	1
6-7	-	1	-	-	-	1
6-8	1	-	-	-	-	1
6-9	1	-	-	-	-	1
6-10	1	-	-	-	-	1
6-11	1	-	-	-	-	1
6-12	-	-	-	1	-	1
7-1	-	-	-	1	-	1
7-2	-	-	-	1	-	1
7-3	-	-	-	1	-	1
7-4	-	-	-	1	-	1
7-5	-	-	-	1	-	1
8	-	-	-	1	-	1
9	-	-	-	-	1	1
10	1	-	-	-	-	1
11-1	-	-	-	1	-	1
11-2	-	-	-	-	1	1
11-3	1	-	-	-	-	1
11-4	1	-	-	-	-	1
11-5	1	-	-	-	-	1
12-1	1	-	-	-	-	1
12-2	-	1	-	-	-	1
12-3	-	-	-	1	-	1
12-4	1	-	-	-	-	1
13	2	-	1	-	-	3

Table 4.4 (Con't) Distribution and frequency of *Clostridium difficile* isolates recovered from swine, dairy cattle, dairy cattle environment, human, and NAP type strains according to rep-PCR grouping

rep-PCR	Swine	Dairy cattle[a]	Environment	Human	NAP strains[b]	Total
14	1	-	-	-	-	1
15-1	1	-	1	-	-	2
15-2		-	1	-	-	1
16	1	-	-	-	-	1
17		-	-	-	1	1
18-1	1	-	1	-	-	2
18-2		-	1	-	-	1
19	1	-	-	-	-	1
20	1	-	-	-	-	1
21-1	-	-	-	-	1	1
21-2	1	-	-	-	-	1
22	1	-	-	-	-	1
23-1	-	-	1	-	-	1
23-2	-	-	1	-	-	1
24	-	1	-	-	-	1
25-1	1	-	1	-	-	2
25-2	-	-	1	-	-	1
25-3	1	-	-	-	-	1
26	3	-	-	-	-	3
27	-	-	1	-	-	1
28	-	1	-	-	-	1
29-1	1	-	-	-	-	1
29-2	-	-	1	-	-	1
30	1	-	-	-	-	1
31	1	-	-	-	-	1
32-1	1	-	-	-	-	1
32-2	1	-	-	-	-	1
33	-	-	-	1	-	1
34	1	-	-	-	-	1
35	3	-	-	-	-	3
36	1	-	-	-	-	1
37-1	-	-	1	-	-	1
37-2	-	-	1	-	-	1
37-3	1	-	-	-	-	1
37-4	1	-	-	-	-	1
37-5	1	-	-	-	-	1
38-1	-	-	-	-	1	1
38-2	1	-	-	-	-	1
38-3	1	-	-	-	-	1
38-4	-	-	-	-	1	1
39	-	-	1	-	-	1
40-1	-	-	1	-	-	1
40-2	1	-	-	-	-	1
41	2	-	-	-	-	2
42-1	-	-	-	-	1	1
42-2	1	-	-	-	-	1

Table 4.4 (Con't) Distribution and frequency of *Clostridium difficile* isolates recovered from swine, dairy cattle, dairy cattle environment, human, and NAP type strains according to rep-PCR grouping

rep-PCR	Swine	Dairy cattle[a]	Environment	Human	NAP strains[b]	Total
42-3	1	-	1	-	-	2
42-4	-	-	1	-	-	1
42-5	1	1	-	-	-	2
42-6	1	-	-	-	-	1
42-7	-	-	1	-	-	1
42-8	-	-	3	-	-	3
42-9	-	-	1	-	-	1
42-10	18	9	13	-	-	40
43	-	1	5	-	-	6
44	2	-	-	-	-	2
45-1	2	-	-	-	-	2
45-2	3	-	-	-	-	3
46	-	-	-	1	-	1
47-1	4	1	-	-	-	5
47-2	-	1	-	-	-	1
47-3	-	-	-	-	1	1
47-4	-	1	-	-	-	1
48	-	-	-	1	-	1
49-1	1	-	-	-	-	1
49-2	-	-	-	1	-	1
50	3	-	-	-	-	3
51	1	-	-	-	-	1
52	-	-	-	1	2	3
53	-	-	-	1	-	1
54	-	-	-	1	-	1
55	1	-	-	-	-	1
56	-	1	-	-	-	1
Total isolates	94	39	54	20	13	220

[a] One dairy cattle isolate was not included in the rep-PCR interpretation due to poor DNA quality

[b] North America pulsed-field gel electrophoresis (NAP) type strains

Table 4.5 Distribution and frequency of *Clostridium difficile* isolates recovered from swine, dairy cattle, dairy cattle environment, human, and NAP type strains according to PFGE grouping

PFGE	Swine	Dairy cattle	Environment[a]	Human	NAP strains[b]	Total
1	2	-	-	-	-	2
2	-	-	-	2	1	3
3	5	3	-	-	-	8
4	-	3	-	-	1	4
5	-	-	-	1	-	1
6	-	1	-	1	-	2
7	2	2	-	-	-	4
8	-	-	-	1	-	1
9	3	-	-	-	-	3
10	2	5	3	-	1	11
11	1	-	-	-	1	2
12	1	-	-	1	-	2
13	8	-	3	1	-	12
14	-	-	-	2	1	3
15	-	2	-	-	-	2
16	5	-	1	-	-	6
17	-	-	-	1	-	1
18	-	-	-	1	-	1
19	-	-	-	-	1	1
20	-	-	-	-	1	1
21	4	-	-	1	-	5
22	1	-	-	-	-	1
23	1	-	-	-	-	1
24	2	-	-	-	-	2
25	-	-	-	-	2	2
26	-	-	-	-	1	1
27	-	-	-	-	1	1
28	16	2	3	2	-	23
29	10	1	7	-	1	19
30	-	-	-	1	1	2
31	-	-	-	1	-	1
32	-	-	-	1	-	1
33	1	-	-	-	-	1
34	3	-	-	-	-	3
35	1	-	-	-	-	1
36	1	-	-	-	-	1
37	1	-	-	-	-	1
38	1	-	-	-	-	1
39	1	-	-	-	-	1
40	1	-	-	-	-	1
41	-	-	-	1	-	1
42	1	-	-	-	-	1
43	1	-	-	-	-	1
44	1	-	-	-	-	1
45	1	-	-	-	-	1
46	-	-	-	1	-	1

Table 4.5 (Con't) Distribution and frequency of *Clostridium difficile* isolates recovered from swine, dairy cattle, dairy cattle environment, human, and NAP type strains according to PFGE grouping

PFGE	Swine	Dairy cattle	Environment[a]	Human	NAP strains[b]	Total
47	-	-	2	-	-	2
48	-	1	2	-	-	3
49	-	-	2	-	-	2
50	-	1	1	-	-	2
51	2	-	-	-	-	2
52	-	9	10	-	-	19
53	-	3	3	-	-	6
54	1	-	1	-	-	2
55	-	-	2	1	-	3
56	-	-	1	-	-	1
57	-	-	1	-	-	1
58	1	-	-	-	-	1
59	-	-	1	-	-	1
60	1	-	-	-	-	1
61	1	1	-	-	-	2
62	1	-	-	-	-	1
63	-	1	-	-	-	1
64	-	1	-	-	-	1
65	-	-	1	-	-	1
66	-	-	1	-	-	1
67	1	-	-	-	-	1
68	-	-	1	-	-	1
69	-	-	1	-	-	1
70	1	-	-	-	-	1
73	1	-	-	-	-	1
75	-	-	2	-	-	2
76	1	-	-	-	-	1
77	1	-	-	-	-	1
78	-	-	1	-	-	1
79	-	-	2	-	-	2
80	-	3	-	-	-	3
81	1	-	-	-	-	1
82	3	-	-	-	-	3
83	-	1	-	-	-	1
84	-	-	1	-	-	1
85	1	-	-	-	-	1
Total	94	40	53	20	13	220

[a] One dairy cattle environment isolate was not included in the PFGE interpretation due to nontypeability

[b] North America pulsed-field gel electrophoresis (NAP) type strains

Table 4.6 Discriminatory powers of different molecular methods for typing *Clostridium difficile*

Source	No. of isolates	No. of groups identified by:	
		rep-PCR	PFGE
Swine	94	59	41
Dairy cattle	40	17	17
Dairy cattle environment	54	29	24
Human	20	20	17
NAP type stains*	13	12	12

*North America pulsed-field gel electrophoresis (NAP) type strains

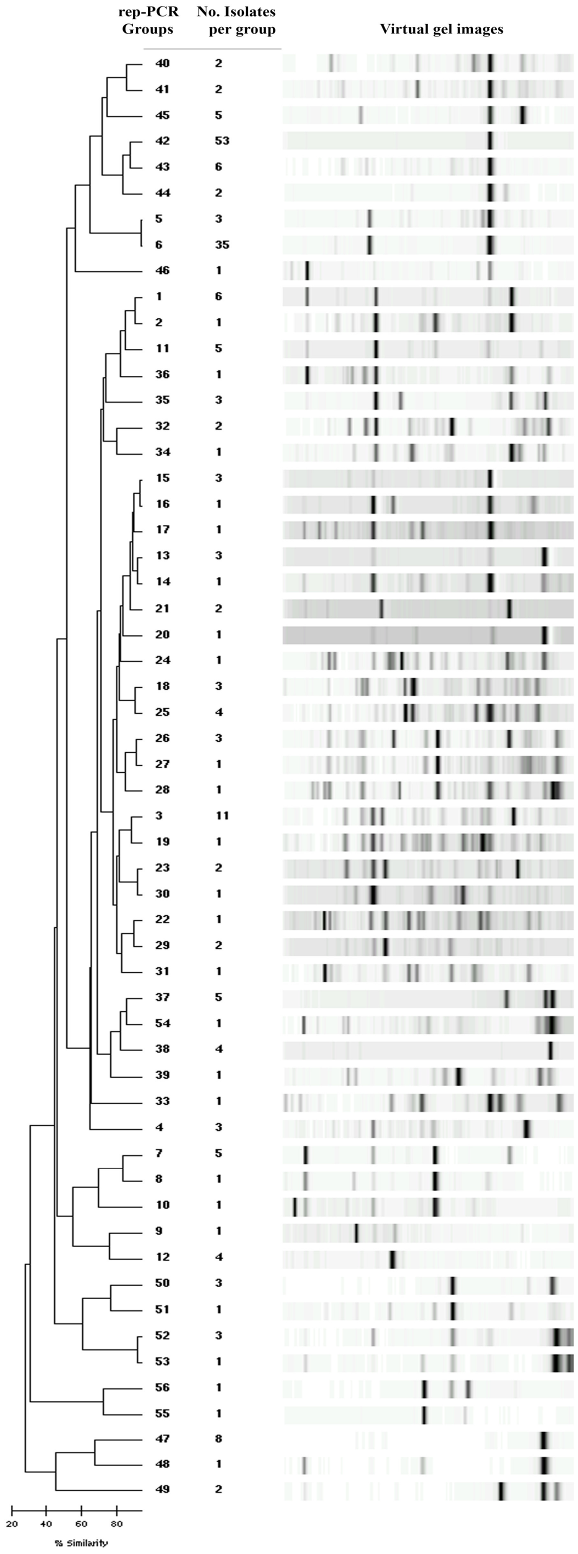

Figure 4.1 Fifty six distinct clusters of 220 *Clostridium difficile* isolates by rep-PCR

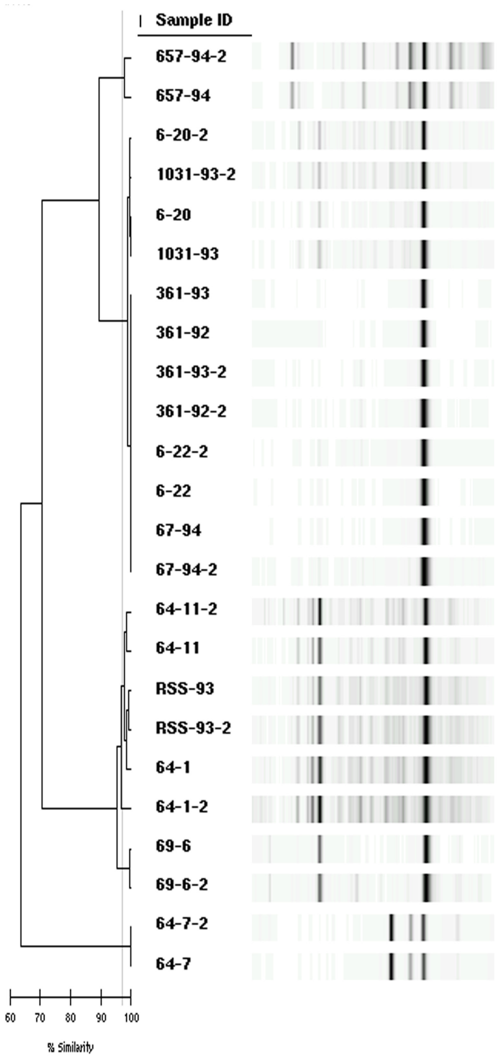

Figure 4.2 Virtual gel image of rep-PCR reproducibility and pattern stability of 12 *Clostridium difficile* isolates. A dendrogram and virtual gel images represent samples analyzed by 2 operators (2^{nd} operator denoted by "-2" after sample ID) using 2 separate Diversilab system during multiple days.

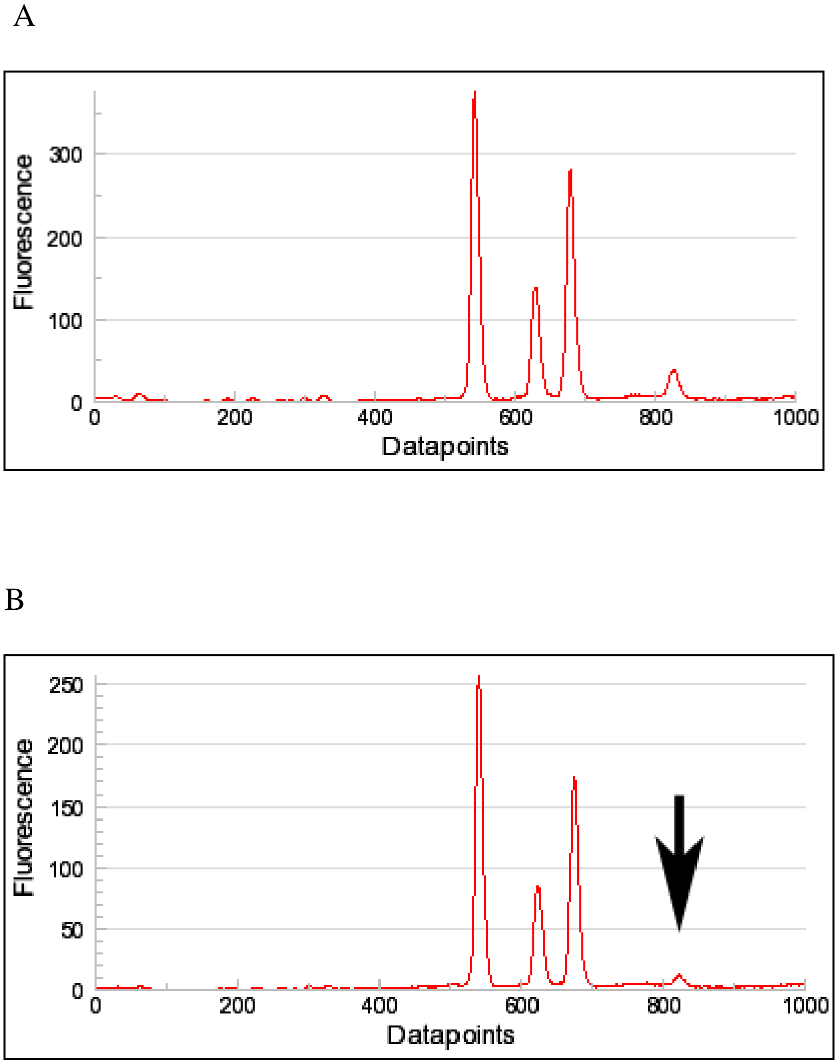

Figure 4.3 The two graphs show indistinguishable samples by rep-PCR that vary only in overall intensity. All peaks have proportionally different fluorescence units. Sample B has only a difference in the intensity of one peak (arrow) from sample A.

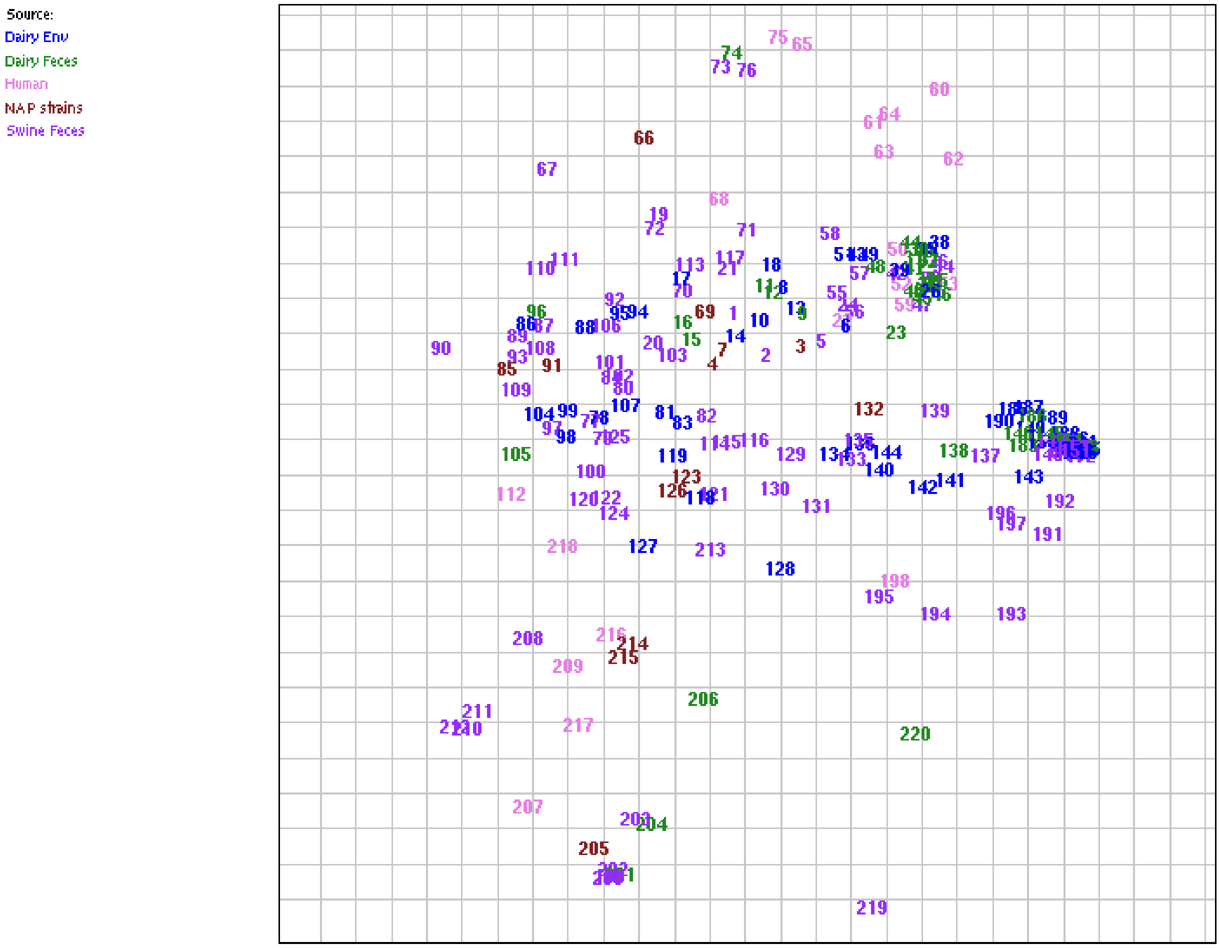

Figure 4.4 Scatter plot generated by the Diversilab software (version 3.3) represents the relationship of 220 *Clostridium difficile* isolates by rep-PCR.

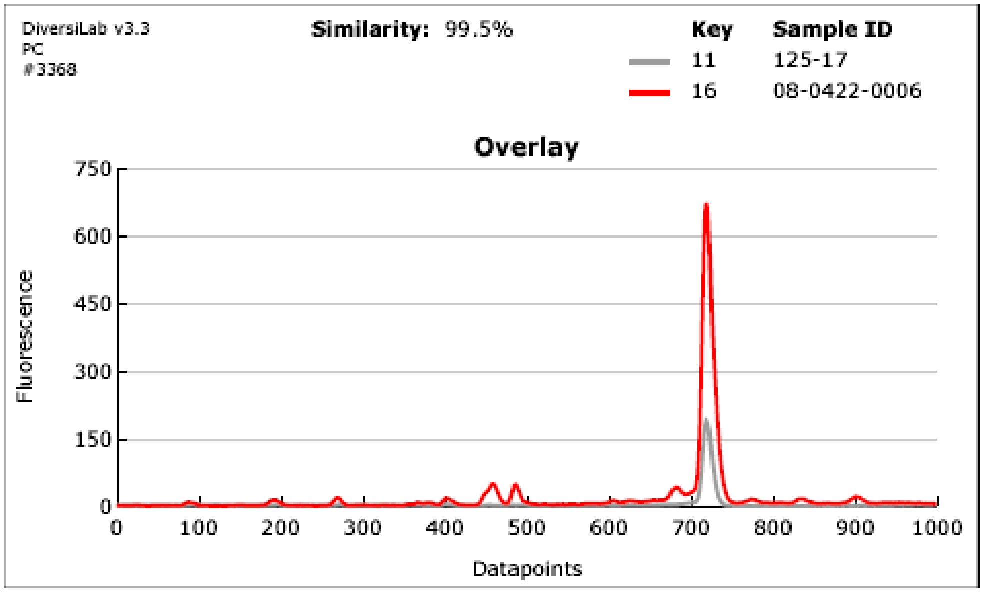

Figure 4.5 Sample graph overlay generated by the Diversilab software (version 3.3) allows two graphs to be placed on the same scale which aids in interpretation.

References

Al Saif, N. and Brazier, J.S. (1996) The distribution of *Clostridium difficile* in the environment of South Wales. *J Med Micro* **45**, 133-137.

Arroyo, L.G., Kruth, S.A., Willey, B.M., Staempfli, H.R., Low, D.E., and Weese, J.S. (2005) PCR ribotyping of *Clostridium difficile* isolates originating from human and animal sources. *J Med Microbiol* **54**, 163-166.

Barbut, F. and Petit, J.C. (2001) Epidemiology of *Clostridium difficile*-associated infections. *Clin Microbiol Infect* **7**, 405-410.

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

Bliss, D., Johnson, S., Savik, K., Clabots, C.R., Willard, K., and Gerding, D.N. (1998) Acquisition of *Clostridium difficile* and *Clostridium difficile*-associated diarrhea in hospitalized patients receiving tube feeding. *Ann Intern Med* **129**, 1012-1019.

Brazier, J.S. (1998) The diagnosis of *Clostridium difficile*-associated disease. *J Antimicrob Chemother* **41**(suppl.C), 29-40.

Frazier, K.S., Herron, A.J., Hines, M.E., II, Gaskin, J.M., and Altman, N.H. (1993) Diagnosis of enteritis and enterotoxemia due to *Clostridium difficile* in captive ostriches (*Struthio camelus*). *J Vet Diagn Invest* **5**, 623,625.

George, R.H., Symonds, J.M., Dimock, F., Brown, J.D., Arabi, Y., Shinagawa, N., Keighley, M.R., Alexander-Williams, J., and Burdorn, D.W. (1978) Identification of *Clostridium difficile* as a cause of Pseudomembranous colitis. *BMJ* **1**, 695.

George, W.L., Sutter, V.L., and Finegold, S.M. (1977) Antimicrobial agent-induced diarrhea-a bacterial disease. *J Infect Dis* **136**, 822-828.

Gilson, R., Clement, J.M., Brutlag, D., and Hofnung, M. (1984) A family of dispersed repetitive extragenic palindromic DNA swquences in *E. coli*. *EMBO J* **3**, 1417-1421.

Gorbach, S.L. (1999) Antibiotics and *Clostridium difficile*. *N Engl J Med* **341**, 1690-1691.

Healy, M., Huong, J., Bittner, T., Lising, M., Frye, S., Raza, S., Schrock, R., Manry, J., Renwick, A., Nieto, R., Woods, C., Versalovic, J., and Lupski, J.R. (2005) Microbial DNA typing by automated rep-PCR. *J Clin Microbiol* **42**, 4016-4024.

Hubert, B., Loo, V.G., Bourgault, A.-M., Poirier, L., Dascal, A., Fortin, E., Dionne, M., and Lorange, M. (2007) A portrait of the geographic dissemination of the *Clostridium difficile* North American Pulsed-Field type 1 strain and the epidemiology of *C. difficile*-associated disease in Quebec. *CID* **44**, 238-244.

Hulton, C.S.J., Higgins, C.F., and Sharp, P.M. (1991) ERIC sequences: a novel family of repetitive elements in the genomes of *Escherichia coli*, *Salmonella typhimurium* and other enterobacteria. *Mol Microbiol* **5**, 825-834.

Hunter, P.R. (1990) Reproducibility and indices of discriminatory power of microbial typing methods. *J Clin Microbiol* **28**(9), 1903-1905.

Jhung, M.A., Thompson, A.D., Killgore, G.E., Zukowski, W.E., Songer, G., Warny, M., Johnson, S., Gerding, D.N., McDonald, C., and Limbago, B.M. (2008) Toxinotype V *Clostridium difficile* in humans and food animals. *Emerg Infect Dis* **14**(7), 1039-1045.

Johnson, J.R. and Clabots, C. (2000) Improved repetitive-element PCR fingerprinting of *Salmonella enterica* with the use of extremely elevated annealing temperatures. *Clin Diagn Lab Immunol* **7**, 258-264.

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

Kelly, C.P., Pothoulakis, C., and LaMont, J.T. (1994) *Clostridium difficile* colitis. *N Engl J Med* **330**, 257-262.

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

Kuijper, E.J., Coignard, B., and Tull, P. (2006a) Emergence of *Clostridium difficile*-associated disease in North America and Europe. *Clin Microbiol Infect* **12**(Suppl.6), 2-18.

Kuijper, E.J., Renate, van den Berg, J., Debast, S., Visser, C., Veenendaal, D., Troelstra, A., van der Kooi, T., van den Hof, S., and Notermans, D.W. (2006b) *Clostridium difficile* Ribotype 027, Toxinotype III, the Netherlands. *Emerg Infect Dis* **12**(5), 827-830.

Kuijper, E.J. and Surawicz, C.M. (2008) *Clostridium difficile* infection. *Lancet* **371**, 1486-1488.

Lefebvre, S.L., Arroyo, L.G., and Weese, J.S. (2006) Epidemic *Clostridium difficile* strain in hospital visitation dog. *Emerg Infect Dis* **12**(6), 1036-1037.

Loo, V.G., Poirier, L., Miller, M.A., Oughton, M., Libman, M.D., Michaud, S., Bourgault, A.M., Nguyen, T., Frenette, C., Kelly, M., Vibien, A., Brassard, P., Fenn, S., Dewar, K., Hudson, T.J., Horn, R., Rene, P., Monczak, Y., and Dascal, A. (2005) A predominantly clonal multi-institutional outbreak of *Clostridium difficile*-associated diarrhea with high morbidity and mortality. *N Eng J Med* **353**, 2442-2449.

Lupski, J.R. and Weinstock, G.M. (1992) Short, interspersed repetitive DNA sequences in prokaryotic genomes. *J Bacteriol* **174**(14), 4525-4529.

Malathum, K., Singh, K.V., Weinstock, G.M., and Murray, B.E. (1998) Repetitive sequence-based PCR versus pulsed-field gel electrophoresis for typing of *Enterococcus faecalis* at the subspecies level. *J Clin Microbiol* **36**, 211-215.

Marks, S.L., Kather, E.J., Kass, P.H., and Melli, A.C. (2002) Genotypic and phenotypic characterization of *Clostridium perfringens* and *Clostridium difficile* in diarrheic and healthy dogs. *J Vet Intern Med* **16**, 533-540.

McDonald, L.C., Killgore, G.E., Thompson, A., Owens, R.C., Kazakova, S.V., Sambol, S.P. Johnson, S., and Gerding, D.N. (2005) An epidemic, toxin gene-variant strain of *Clostridium difficile*. *N Engl JMed* **353**, 2433-2441.

Mutlu, E., Wroe, A.J., Sanchez-Hurtado, K., Brazier, J.S., and Poxton, I.R. (2007) Molecular characterization and antimicrobial susceptibility patterns of *Clostridium difficile* strains isolated from hospitals in south-east Scotland. *J Med Microbiol* **56**, 921-929.

O'Neill, G., Adams, J.E., Bowman, R.A., and Riley, T.V. (1993) A molecular characterization of *Clostridium difficile* isolates from human, animals and their environments. *Epidemiol Infect* **111**, 257-264.

Pepin, J., Valiquettte, L., and Alary, M.E. (2004) *Clostridium difficile*-associated diarrhea in a region of Quebec from 1991-2003: a changing pattern of disease severity. *CMAJ* **171**, 466-472.

Persing, D.H. (1993) In vitro nucleic acid amplification techniques. In *Diagnostic Molecular Microbiology: Principles and Applications* eds. Persing, D.H., Smith, T., Tenover, F.C., White, T. pp. 51-87. Washington, DC: American Society for Microbiology.

Pirs, T., Ocepek, M., and Rupnik, M. (2008) Isolation of *Clostridium difficile* from food animals in Slovenia. *J Med Microbiol* **57**, 790-792.

Rodriguez-Palacios, A., Stampfli, H.R., Duffield, T., Peregrine, A.S., Trotz-Williams, L.A., Arroyo, L.G., Brazier, J.S., and Weese, J.S. (2006) *Clostridium difficile* PCR ribotypes in calves, Canada. *Emerg Infect Dis* **12**(11), 1730-1736.

Simango, C. (2006) Prevalence of *Clostridium difficile* in the environment in a rural community in Zimbabwe. *Trans R Soc Trop Med Hyg* **100**, 1146-1150.

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

Songer, J.G., Post, K.W., Larson, D.J., Jost, B.H., and Glock, R.D. (2000) Infection of neonatal swine with *Clostridium difficile*. *Swine Health Production* **8**, 185-189.

Songer, J.G. (2004) The emergence of *Clostridium difficile* as a pathogen of food animals. *Anim Health Res Rev* **5**(2), 321-326.

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

Stern, M.J., Ferro-Luzzi Ames,G., Smith, N.H., Robinson, E.C., and Higgins, C.F., (1984) Repetitive Extragenic palindromic sequences: a major component of the bacterial genome. *Cell* **37**, 1015-1026.

van Belkum, A. (1994) DNA fingerprinting of medically important microorganisms by use of PCR . *Clin Microbiol Rev* **7**, 174-184.

Versalovic, J., Koeuth, T., and Lupski, J.R. (1991) Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. *Nucleic Acids Res* **19**, 6823-6831.

Versalovic, J., de Bruijn, F.J. and Lupksi, J.R. (1998) Repetitive sequence-based PCR (rep-PCR) DNA fingerprinting of bacterial genomes In *Bacterial genomes physical structure and analysis*. ed. de Bruijn, F.J., Lupski, J.R., and Weinstock, G.M. pp. 38-48. New York, NY: Chapman&Hall Internatinal Thomson Pub.

Voth, D.E. and Ballard, J.D. (2005) *Clostridium difficile* toxins: Mechanism of action and role in disease. *Clin Microbiol Rev* **18**(2), 247-263.

Warny, M., Pepin, J., Fang, A., Killgore, G., Thompson, A., Brazier, J., Frost, E., and McDonald, L.C. (2005) Toxin production by an emerging strain of *Clostridium difficile* associated with outbreaks of severe disease in North America and Europe. *Lancet* **366**, 1079-1084.

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

Weese, J.S., Weese, H.E., Bourdeau, T.L., and Staempfli, H.R. (2001) Suspect *Clostridium difficile*-associated diarrhea in two cats. *J Am Vet Med Assoc* **218**, 1436-1421.

Welsh, J. and McClelland, M. (1990) Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Res* **18**, 7213-7218.

Williams, J.G.K., Kubelik, A.R., Livak, K.J., Rafalski, J.A., and Tingey, S.V. (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acid Res* **18**, 6531-6535.

CHAPTER 5

ANTIMICROBIAL SUSCEPTIBILITY OF *CLOSTRIDIUM DIFFICILE* ISOLATED FROM PORCINE AND BOVINE FECES[1]

---

[1] Thitaram, S.N., Fedorka-Cray, P.F. and Frank, J. F. To be submitted to *International Journal of Food Microbiology*

Abstract

A total of 188 isolates of *Clostridium difficile* isolated from healthy food animals and their environment, were examined for susceptibility to 9 antimicrobials; linezolid, amoxicillin-clavulanic acid, ampicillin, clindamycin, erythromycin, levofloxacin, metronidazole, rifampicin, and vancomycin. Amoxicillin-clavulanic acid, metronidazole, and vancomycin had excellent activity against all isolates of *C. difficile* with MIC_{90s} of 0.5, 0.25, and 1.5 µl/ml, respectively. Rifampicin showed good activity against isolates of *C. difficile* with only 1% of isolates being resistant with MIC_{90} of 0.25 µg/ml. All isolates were resistant to levofloxacin (MIC_{90} $\geq$32 µl/ml) and 135 (71.8%) were resistant to clindamycin ($\mathrm{MIC}_{90} = 24$ µl/ml). Eighteen isolates (13%) exhibited high clindamycin-resistance with a MIC of $\geq$256 µl/ml. Thirty (16%), 25 (13.3%) and 10 (5.3%) were resistant to ampicillin, erythromycin and linezolid with MIC_{90s} of 3, $\geq$256 and 3 µl/ml, respectively. Multiple antimicrobial resistances (two or more antimicrobials) were noted in 144 isolates. Almost half of the isolates (46%) were resistant to clindamycin and levofloxacin. The Etest proved simple to use and the MIC endpoints were easily read.

Introduction

Clostridium difficile is an anaerobic, Gram-positive, spore-forming bacillus that is commonly associated with a spectrum of disease referred to as *C. difficile*-associated disease (CDAD), which can range from uncomplicated mild diarrhea (George et al. 1977; Bliss et al. 1998; Brazier 1998; Gorbach 1999; Barbut and Petit 2001) to life-threatening toxic megacolon and pseudomembranous colitis (PMC) (George et al. 1978; Bartlett 1992; Kelly et al. 1994; Voth and Ballard 2005; Kuijpers and Surawicz 2008). The major risks for infection with *C. difficile* are antibiotic exposure, advanced age, impaired host immune and prolonged hospitalization (Gerding 1989; Fekety and Shah 1993; Kelly et al. 1994; Kyne et al. 2000; Garey et al. 2008). The administration of antibiotics is considered the most significant and most frequently reported predisposing risk factor (Brown et al. 1990; Cartmill et al. 1994; Kelly et al. 1994; Owens et al. 2008).

Antibiotic resistance among many pathogenic anaerobic bacteria has increased significantly over the past three decades (Letournel-Glomaud et al. 2003; Hecht 2006). Awareness of these disturbing findings is limited among both clinicians and microbiologists due to a number of confounding factors, including lack of isolation and standardization of susceptibility testing methods. The use of antibiotics can disrupt the normal ecosystem and allow *C. difficile* to become established and colonize an empty bowel. Also, improper uses of antibiotics can lead to the development of antibiotic resistant bacteria. Although most antibiotics have been associated with predisposition to *C. difficile* infection, the most commonly implicated have been clindamycin (Worsley 1998; Freeman and Wilcox 1999; Johnson et al. 1999b), cephalosporins (Zwiener et al. 1989; Nelson et al. 1994; Gerding et al. 1995; Spencer 1998; Worsley 1998; Freeman and Wilcox 1999; Loo et al. 2005; Bartlett 2006), amoxicillin and ampicillin (Zwiener et al. 1989; Nelson et al. 1994; Gerding et al. 1995; Groschell 1996; Worsley 1998; Johnson et al. 1999a). Recently, quinolones and fluoroquinolones have been implicated as important agents in the development of CDAD (McCusker et al. 2003; Loo et al. 2005; Pepin et al. 2005; Bartlett 2006; Brook 2008; Spigaglia et al. 2008).

Monitoring the development of antimicrobial resistance in bacteria isolated from animals as well as humans is necessary to ensure the proper use and prolonged lifespan of current antimicrobials (Tollefson et al. 1998; Tollefson et al. 1999; Fedorka-Cray et al. 2002; Fedorka-Cray et al. 2005) and may allow us to provide optimal therapy in difficult cases (Poilane et al. 2000). Because resistance is increasing, susceptibility testing is often necessary. The recent availability of alternative, simpler methods for susceptibility testing may have an important impact. The Etest (AB Biodisk, Sweden) has proved to be a reliable method for measuring the minimum inhibitory concentrations (MIC) of antimicrobial resistant *C. difficile* (Wust and Hardegger 1992; Olsson-Liljequist and Nord 1994; Rosenblatt and Gustafson 1995; Wong et al. 1999; Poilane et al. 2000). Susceptibility testing is rarely performed for *C. difficile* because of the complexity and cost. To the best of our knowledge, the *in vitro* susceptibility of *C. difficile* isolated from healthy swine, dairy cattle and their environment has not been determined. The objective of this study was to investigate the susceptibility of *C. difficile* isolated from feces of healthy swine, healthy dairy cattle and from the environment in dairy cattle farms to linezolid, amoxicillin-clavulanic acid, ampicillin, clindamycin, erythromycin, metronidazole, levofloxacin, rifampicin, and vancomycin by using Etest.

Materials and Methods

Experiment isolates. One hundred eighty-eight *C. difficile* strains were isolated from healthy food animals and environmental samples (94 from swine feces, 40 from dairy cattle feces, and 54 from dairy cattle farms).

They were identified as *C. difficile* by the following criteria: (1) they showed a flat, ground glass surface with irregular edges on cycloserine-cefoxitin fructose agar, (2) they had a distinct horse manure odor, (3) they fluoresced yellow/green under long wavelength UV light, (4) they were Gram-positive, long, thin, straight rods usually possessing many sub-terminal to terminal spores when viewed under a light microscope at 1000X, (5) they showed a production of L-proline aminopeptidase (Pro-Disc; Remel, Carr-Scarborough Microbiologicals, Inc., Decatur, GA), and (6) they were positive by a 16S rDNA PCR detection as described by Kikuchi et al. (2002). All isolates were kept aerobically in 10 ml cooked meat medium in parafilmed screw-capped tubes at room temp after 48 hr at 37^{o}C anaerobic growth initiation until analyzed.

Also, *Bacteroides thetaiotanmicron* ATCC 29741 and *C. difficile* ATCC 700057 were included as a control and aid in anaerobic condition indicator during experiment.

Susceptibility testing. Susceptibility to the following antibiotics were tested: linezolid (LZ), amoxicillin-clavulanic acid (XL), ampicillin (AM), clindamycin (CM), erythromycin (EM), levofloxacin (LE), metronidazole (MZ), rifampicin (RI), and vancomycin (VA). Susceptibility testing was carried out with Etest (AB Biodisk, Solna, Sweden). The Etest is a plastic strip with a predefined antibiotic gradient immobilized on one side and a minimum inhibitory concentration (MIC) interpretative scale printed on the other. The test was performed according to the recommendations of the manufacturer. The isolates were sub-cultured from 10 ml cooked meat medium onto tryptic soy agar supplemented with 5% sheep blood (BA, Remel) and incubated anaerobically 37°C for 48 hr. The inoculum was made in pre-reduced brucella broth. The Etest was carried out by inoculating the surface of pre-reduced 150 mm diameter brucella agar plates supplemented with 5% horse blood (Remel) with a 1 McFarland standard-matched inoculum. The inoculation was performed with sterile cotton-tipped swabs and Etest strips were applied to the agar surface according to the manufacturer's instructions. Following anaerobic incubation for 72 hr at 37°C, an ellipse of inhibition and the MIC was determined.

Determination of MICs. The lowest concentration of antimicrobial which affected growth on the test plate constituted the MIC. MIC_{50} and MIC_{90} are the lowest concentration at which 50% and 90%, respectively, of growth was inhibited. MICs were read where the inhibition ellipse intersected the scale. The Etest MIC values that were between standard two-fold dilutions were rounded to the next higher value. A categorical discrepancy was defined as a > 2-dilution difference that resulted in a change of interpretation from susceptible to resistant or vice versa. The test was then repeated. Resistance breakpoints (Table 5.1) for amoxicillin-clavulanic acid, ampicillin, clindamycin, and metronidazole were set according to Clinical and Laboratory Standards Institute; CLSI (CLSI 2007). There are no current established CLSI breakpoints for linezolid, erythromycin, levofloxacin, rifampicin, and vancomycin. For these antimicrobials the breakpoints used by Zheng et al. (2007), Drudy et al. (2007), Martin et al. (2008), O' Conner et al. (2008) and Indra et al. (2008) were used for linezolid, erythromycin, levofloxacin, rifampicin, and vancomycin, respectively (Table 5.1).

Results

A total of 188 isolates were studied. The MICs of quality control strains (*Bacteroides thetaiotanmicron* ATCC 29741 and *C. difficile* ATCC 700057) were within acceptable limits as established by the CLSI (CLSI 2007). The MIC distributions for all antimicrobials are provided in Table 5.2 and the MIC range, $MIC_{50,}$ MIC_{90}, % resistance are shown in Table 5.3.Amoxicillin-clavulanic acid, metronidazole, and vancomycin had excellent activity against all isolates of *C. difficile* with MIC_{90}s of 0.5, 0.25, and 1.5 µg/ml, respectively. Rifampicin also showed good activity against isolates of *C. difficile* with only 1% of isolates being resistant with MIC_{90} of 0.25 µg/ml. Resistance against levofloxacin was found in all isolates tested (MIC_{90} $\geq$32 µl/ml). One hundred thirty-five (71.8%) isolates were resistant to clindamycin with MIC_{90} = 24 µl/ml. Of these 135 resistant isolates, 18 (13%) exhibited high-level clindamycin resistance with MIC $\geq$256 µg/ml. The MIC of clindamycin for the remaining 117 isolates was 8-32 µg/ml. Of interest, 30 (16%) and 25 (13.3%) were resistant to ampicillin and erythromycin with MIC_{90s} of 3 and $\geq$256 µg/ml, respectively. Ten isolates (5.3%) were resistant to linezolid with MIC_{90} = 3 µl/ml.

Susceptibility profiles of *C. difficile* isolates grouped by sample type are shown in Table 5.4. Interestingly, resistance to levofloxacin followed by clindamycin was observed in all sample groups. Erythromycin was the third most resistant in swine feces (23.4%) whereas ampicillin was among dairy cattle feces (25%) and dairy cattle environment (11.1%). In dairy cattle, all isolates from feces were susceptible to erythromycin and low level of resistance was found in environment (5.6%).

Analysis of individual isolates in relation to multiple resistances is shown in Table 5.5. A total of 7 isolates were resistant to 4 antimicrobials (6 resistant to ampicillin, clindamycin, erythromycin and levofloxacin; 1 resistant to linezolid, clindamycin, erythromycin and levofloxacin). Thirty eight isolates were resistant to combinations of 3 antimicrobials. Of 38 isolates, 16 isolates were resistant to ampicillin, clindamycin, and levofloxacin whereas 15 isolates were resistant to clindamycin, erythromycin, and levofloxacin. Ninety nine isolates were resistant to combinations of 2 antimicrobials. Of these 99 isolates, 87 isolates were resistant to clindamycin and levofloxacin.

Discussion

C. difficile is an important nosocomial pathogen that causes antibiotic-associated diarrhea (George et al. 1977; Bliss et al. 1998; Brazier 1998; Gorbach 1999; Barbut and Petit 2001) and PMC (George et al. 1978; Bartlett 1992; Kelly et al. 1994; Voth and Ballard 2005; Kuijpers and Surawicz 2008). Mortality rate ranges from 15-30% (Chang 1985). Recent studies have shown the presence of *C. difficile* in food animals such as chickens (Simango and Mwakurudza 2008), pigs (Songer et al. 2000; Songer and Anderson 2006), cattle (Simango 2006) and calves (Rodriguez-Palacios et al. 2006). *C. difficile* has been isolated in ground beef and ground veal in retail markets (Rodriguez-Palacios et al. 2007). Studies that compared human and animal *C. difficile* isolates revealed the presence of *C. difficile* in foods of animal origin suggest that animal reservoirs and transmission via foods are possible sources for community-acquired human infections (Rupnik 2007). Therefore, the possiblilty of *C. difficile* as an emerging foodborne pathogen is a concern.

Antibiotics are used extensively in both humans and animals. The majority of the problems arising from antibiotic resistance are due to the over-use of antibiotics in human medicine and agriculture. Examples of animal diseases requiring the most extensive use of antibiotics for therapy or prophylaxis are respiratory and enteric disease, especially of swine and beef cattle, and mastitis in dairy cattle (Johnson 1998). The treatment of food producing animals, especially swine is frequently on a group or herd basis (Walton 1983). The main concern regarding the use of antibiotics in humans and animals is the emergence of antibiotic-resistant bacteria that could infect humans either directly or by the transfer of resistant genes to human pathogens. Antibiotic resistance among many pathogenic anaerobic bacteria has increased significantly over the past three decades (Letournel-Glomaud et al. 2003; Hecht 2006). It is therefore prudent to be abreast of the susceptibility pattern of food animal isolates to the antimicrobials that appears to be an important risk factor for colonization by *C. difficile* and that are available for the treatment of CDAD.

The data from this study showed that all of the food animal isolates were susceptible to metronidazole (MIC 0.047-0.25 µg/ml) and vancomycin (MIC 0.5-3 µg/ml). This finding may indicate that resistance to these 2 antimicrobials has yet to develop in food animal species. Metronidazole and vancomycin are the first-line of drugs used in the treatment of human CDAD (Kelly et al. 1994; McFarland et al. 2002; Zar et al. 2007). However, vancomycin is primarily used in patients who do not respond to metronidazole or in relapsing CDAD cases (Kink and Williams 1998). In most studies, isolates of *C. difficile* have generally been found to be susceptible to these drugs (Jamal et al. 2002; Drummond et al. 2003; Pituch et al. 2005; Aspevall et al. 2006; Bourgault et al. 2006; Simango 2006; Simango and Mwakurudza 2008). However, some studies have reported strains resistant to metronidazole (Wong et al. 1999; Ferreira et al. 2004; Bishara et al. 2006; Martin et al. 2008) or with reduced susceptibility to vancomycin (Brazier et al. 2001; Pelaez et al. 2005). The first UK isolate of *C. difficile* with reduced susceptibility to metronidazole was reported in 2001 (Brazier et al. 2001). Despite these reports, the incidence of metronidazole resistant strains remains very low. Metronidazole and vancomycin are equally effective for treatment of CDAD (Kelly et al. 1994; Olson et al. 1994). Metronidazole showed excellent in vitro activity, has lower cost and fewer side effects than vancomycin (Simango and Mwakurudza 2008). In addition, concern regarding the emergence of vancomycin-resistant enterococci (Kelly et al. 1994; Olson et al. 1994; Fekety 1997; Simango

2006) supports its choice as a preferred drug. All isolates were susceptible to amoxicillin-clavulanic acid (MIC 0.125-2 µg/ml), a finding similar to some reports (Jamal et al. 2002; Letournel-Glomaud et al. 2003; John and Brazier 2005). Rifampicin is an alternative for the treatment of CDAD (Bourgault et al. 2006; O'Connor et al. 2008). However, in this study 2 isolates (1%) were shown to have high resistant to rifampicin (MIC ≥32 µg/ml), similar findings were reported by Hecht et al. (2007) and Johnson et al. (2007). Our finding may suggest that metronidazole should remain the drug of choice for the treatment of CDAD.

All *C. difficile* strains from swine feces, dairy cattle feces and dairy cattle environment were resistant to levofloxacin (third generation of fluoroquinolone) with high MIC (≥32 µg/ml). This result is consistent with previous studies for bovine-derived strains (Rodriguez-Palacios et al. 2006; Rodriguez-Palacios et al. 2007). High fluoroquinolone MICs have been a shared characteristic of virulent strain (NAP1/BI/027/III) and may, in part, explain why quinolone and fluoroquinolone use has been implicated in recent CDAD outbreaks (McCusker et al. 2003; Loo et al. 2005; Pepin et al. 2005; Bartlett 2006; Brook 2008; Spigaglia et al. 2008). Quinolone and fluoroquinolone are not used in swine and dairy production and the source of quinolone resistance in *C. difficile* isolates in our study is uncertain.

Clindamycin and ampicillin have been implicated in CDAD (Zwiener et al. 1989; Nelson et al. 1994; Gerding et al. 1995; Groschell 1996; Worsley 1998; Freeman and Wilcox 1999; Johnson et al. 1999a; Johnson et al. 1999b). Almost 72% of isolates tested were resistant to clindamycin. However, a small percentage (16%) of isolates tested was resistant to ampicillin.

Multiple resistance data revealed that almost half of the isolates tested (46%) were resistant to clindamycin and levofloxacin. Resistance to 4 antimicrobials (6 resistant to ampicillin, clindamycin, erythromycin and levofloxacin; 1 resistant to linezolid, clindamycin, erythromycin and levofloxacin) was exclusively observed in the swine feces. This may suggest the relationship between animal species and resistance grouping.

The methodology for antimicrobial susceptibility testing of anaerobes has not been standardized to the same extent as for aerobic organisms. Although published guidelines from the CLSI do exist (CLSI 2007), there are still unresolved problems. These include the complicated methodology (agar and broth dilution methods) which is not easily performed by a routine clinical laboratory, choice of optimal medium for testing, inoculum preparation, criteria for defining MIC breakpoints, as well as reading of endpoints. The Etest provides a simple and viable alternative to MIC determination of anaerobic bacteria for the routine laboratory, as it agrees very well with MIC determined by the standard agar dilution method (Wust and Hardegger 1992; Olsson-Liljequist and Nord 1994; Rosenblatt and Gustafson 1995; Wong et al. 1999; Poilane et al. 2000). In this study, reading the endpoint for *C. difficile* was generally not difficult with a clear intersection of the zone of inhibition with the strip. The only drawback is the relatively high cost of the strips, which can be an issue for smaller laboratories.

Table 5.1 Antimicrobials used in susceptibility testing and their properties

Antimicrobials	Class	Catagories	Reason to test	Range tested (µg/ml)	Resistance breakpoint, µg/ml (References)
Linezolid	Oxazolidinone	both	risk factor for CDAD	0.016-256	≥4 (Zheng et al. 2007)
Amoxicillin-Clavulanic acid	β-lactam	bactericidal	risk factor for CDAD	0.016-256	≥16 (CLSI 2007)
Ampicillin	Penicillins	bactericidal	risk factor for CDAD	0.016-256	≥2 (CLSI 2007)
Clindamycin	Lincosamine	bacteriostatic	risk factor for CDAD	0.016-256	≥8 (CLSI 2007)
Erythromycin	Macrolide	bacteriostatic	risk factor for CDAD	0.016-256	≥256 (Drudy et al. 2007)
Metronidazole	Imidazole	bactericidal	treatment for CDAD	0.016-256	>32 (CLSI 2007)
Levofloxacin	Quinolone	bactericidal	risk factor for CDAD	0.002-32	≥8 (Martin et al. 2008)
Rifampicin	Rifamycin	bactericidal	treatment for CDAD	0.002-32	≥32 (O' Conner et al. 2008)
Vancomycin	Glycopeptide	bactericidal	treatment for CDAD	0.016-256	≥32 (Indra et al. 2008)

Table 5.2 Distribution of antimicrobials tested against 188 *Clostridium difficile* isolated from swine, dairy cattle and dairy cattle environment

% of isolates inhibited at antimicrobial concentration (µg/ml)	Antimicrobial*							% of isolates inhibited at antimicrobial concentration (µg/ml)	Antimicrobials*	
	LZ	XL	AM	CM	EM	MZ	VA		LE	RI
≤0.032								≤0.004	89	
0.047-0.064						7		0.006-0.008		
0.094-0.125		3				59		0.012-0.016		
0.19-0.25		38	0.5			98		0.023-0.032		
0.38-0.5		90	34		3	100	25	0.047-0.064		
0.75-1	2	97	78		35		86	0.094-0.125		
1.5-2	68	100	89	2	82		97	0.19-0.25		
3-4	99		94	17	85		100	0.38-0.5		
6-8				58	87			0.75-1		
12-16				89				1.5-2		
24-32				90				3-4		
48-64			99					6-8		
96-128								12-16		
192-≥256	100		100	100	100			24-≥32	100	100

* LZ=Linezolid, XL=Amoxicillin-Clavulanic acid, AM=Ampicillin, CM=Clindamycin, EM=Erythromycin, MZ=Metronidazole, LE=Levofloxacin, RI=Rifampicin, VA=Vancomycin

Table 5.3 Range of MIC values and resistance rates from 188 *Clostridium difficile* isolated from swine, dairy cattle and dairy cattle environment

Antimicrobials	MIC range (µg/ml)	MIC_{50} (µg/ml)	MIC_{90} (µg/ml)	Resistance (%)
Linezolid	1-≥256	2	3	5.3
Amoxicillin-Clavulanic acid	0.125-2	0.38	0.5	0
Ampicillin	0.25-≥256	0.75	3	16
Clindamycin	2-≥256	8	24	71.8
Erythromycin	0.38-≥256	1.5	≥256	13.3
Metronidazole	0.047-0.25	0.125	0.25	0
Levofloxacin	≥32	≥32	≥32	100
Rifampicin	≤0.002-≥32	≤0.002	0.25	1
Vancomycin	0.5-3	0.75	1.5	0

Table 5.4 Susceptibility profiles of 188 *Clostridium difficile* isolates grouped by sample type

	Antimicrobials*								
	LZ	XL	AM	CM	EM	MZ	LE	RI	VA
Swine feces (n=94)									
MIC_{50} (µg/ml)	2	0.38	0.75	8	1.5	0.125	>32	<0.002	0.75
MIC_{90} (µg/ml)	3	0.75	3	>256	>256	0.25	>32	2	1.5
Resistance (%)	8.5	0	14.9	62.8	23.4	0	100	2.1	0
Dairy cattle feces (n=40)									
MIC_{50} (µg/ml)	2	0.38	1	8	1.5	0.125	>32	<0.002	0.75
MIC_{90} (µg/ml)	3	0.5	6	12	2	0.19	>32	0.004	1.5
Resistance (%)	2.5	0	25	75	0	0	100	0	0
Dairy cattle environment (n=54)									
MIC_{50} (µg/ml)	2	0.25	0.75	12	1.5	0.125	>32	<0.002	0.75
MIC_{90} (µg/ml)	3	0.5	2	12	2	0.19	>32	<0.002	1.5
Resistance (%)	1.9	0	11.1	85.2	5.6	0	100	0	0

* LZ=Linezolid, XL=Amoxicillin-Clavulanic acid, AM=Ampicillin, CM=Clindamycin, EM=Erythromycin, MZ=Metronidazole, LE=Levofloxacin, RI=Rifampicin, VA=Vancomycin

Table 5.5 Antimicrobial resistance profile of 188 multiple resistant *Clostridium difficile* isolates

	Number of isolates			
Resistance groups*	Swine feces	Dairy cattle feces	Dairy cattle environment	Total
AM, CM, EM, LE	5	0	1	6
LZ, CM, EM, LE	1	0	0	1
AM, CM, LE	3	8	5	16
CM, EM, LE	13	0	2	15
CM, RI, LE	1	0	0	1
LZ, CM, LE	3	1	1	5
LZ, AM, LE	1	0	0	1
AM, LE	3	2	0	5
CM, LE	29	21	37	87
EM, LE	3	0	0	3
LZ, LE	3	0	0	3
RI, LE	1	0	0	1
LE	28	8	8	44
Total	94	40	54	188

* LZ=Linezolid, XL=Amoxicillin-Clavulanic acid, AM=Ampicillin, CM=Clindamycin, EM=Erythromycin, MZ=Metronidazole, LE=Levofloxacin, RI=Rifampicin VA=Vancomycin

References

Aspevall, O., Lundberg, A., Burman, L.G., Akerlund, T., and Svenungsson, B. (2006) Antimicrobial susceptibility pattern of *Clostridium difficile* and its relation to PCR Ribotypes in a Swedish university hospital *Antimicrob Agents Chemother* **50**(5), 1890-1892.

Barbut, F. and Petit, J.C. (2001) Epidemiology of *Clostridium difficile*-associated infections. *Clin Microbiol Infect* **7**, 405-410.

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

Bartlett, J.G. (2006) The new epidemic of *Clostridium difficile*-associated enteric disease. *Ann Intern Med* **145**, 758-764.

Bishara, J., Bloch, Y., Garty, M., Behor, J., and Samra, Z. (2006) Antimicrobial resistance of *Clostridium difficile* isolates in a tertiary medical center, Israel. *Diag Microbiol Infect Dis* **54**, 141-144.

Bliss, D., Johnson, S., Savik, K., Clabots, C.R., Willard, K., and Gerding, D.N. (1998) Acquisition of *Clostridium difficile* and *Clostridium difficile*-associated diarrhea in hospitalized patients receiving tube feeding. *Ann Intern Med* **129**, 1012-1019.

Bourgault, A.-M., Lamothe, F., Loo, V.G., Poirier, L., and the CDAD-CSI Study Group. (2006) In vitro susceptibility of *Clostridium difficile* clinical isolates from a multi-institutional outbreak in Southern Quebec, Canada. *Antimicrob Agents Chemother* **50**(10), 3473-7475.

Brazier, J.S. (1998) The epidemiology and typing of *Clostridium difficile*. *J Antimicrobiol Chemother* **41**(Suppl C), 47-57.

Brazier, J.S., Fawley, W., Freeman, J., and Wilcox, M.H. (2001) Reduced susceptibility of *Clostridium difficile* to metronidazole. *J Antimicrob Chemother* **48**, 741-742.

Brook, I. (2008) Pseudomembranous colitis. In *Anaerobic Infections Diagnosis and Management* ed. Brook, I. pp. 325-330. New York: Informa Healthcare USA, Inc.

Brown, E., Talbot, G.H., Axelrod, P., Provencher, M., and Hoegg, C. (1990) Risk factors for *Clostridium difficile* toxin-associated diarrhea. *Infect Control Hosp Epidemiol* **11**, 283-290.

Cartmill, T.D.I., Panigrahi, H., Worsley, M.A., McCann, D.C., Nice, C.N., and Keith, E. (1994) Management and control of a large outbreak of diarrhea due to *Clostridium difficile*. *J Hosp Infect* **27**, 1-15.

Chang, T.W. (1985) Antibiotic-associated injury to the gut. In *Bockus gastroenterology* ed. Berk, J.E. pp. 2583-2592. Philadelphia, PA: WB Saunders.

CLSI. (2007) Methods for antimicrobial susceptibility testing of anaerobic bacteria; Approved standard. 7th edn. Document M11 A7. Wayne, PA: Clinical Laboratory Standard Institute.

Drudy, D., Harnedy, N., Fanning, S., Hannan, M., and Kyne, L. (2007) Emergence and control of fluoroquinolone-resistant, toxin A-negative, toxin-B positive *Clostridium difficile*. *Infect Control Hosp Epidemiol* **28**(8), 932-940.

Drummond, L.J., McCoubrey, J., Smith, D.G., Starr, G., and Poxton, J.M. (2003) Changes in sensitivity patterns to selected antibiotics in *Clostridium difficile* in geriatric in-patients over an 18-month period. *J Med Microbiol* **52**, 259-263.

Fedorka-Cray, P.J., Englen, M.D., Gray, J.T., Hudson, C., and Headrick, M.L. (2002) Programs for monitoring antimicrobial resistance. *Anim Biotechnol* **13**, 43-55.

Fedorka-Cray, P.J. Dargatz, D., Petersen, K., and Tollefson, L. (2005) National Antimicrobial Resistance Monitoring System (NARMS). In *Veterinary strains* ed. U.S. Food and Drug Administration, Center for Veterinary Medicine.

Fekety, R. and Shah, A.B. (1993) Diagnosis and treatment of *Clostridium difficile* colitis. *JAMA* **269**, 255-257.

Fekety, R., (1997) Guidelines for the diagnosis and management of *Clostridium difficile* associated diarrhoea and colitis, American college of gastroenteroloty, Practice Parameter committee. *Am J Gastroenterol* **92**, 739-750.

Ferreira, C.E.A., Nakano, V., and Avila-Campos, M.J. (2004) Cytotoxicity and antimicrobial susceptibility of *Clostridium difficile* isolated from hospitalized children with acute diarrhea. *Anaerobe* **10**, 171-177.

Freeman, J. and Wilcox, M.H. (1999) Antibiotics and *Clostridium difficile*. *Microbes Infect* **1**, 377-384.

Garey, K.W., Dao-Tran, T.K., Jiang, Z.D., Price, M.P., Gentry, L.O., and DuPont, H.L. (2008) A clinical risk index for *Clostridium difficile* infection in hospitalized patients receiving broad-spectrum antibiotics. *J Hosp Infect* **70**, 142-147.

George, W.L., Sutter, V.L., and Finegold, S.M. (1977) Antimicrobial agent-induced diarrhea-a bacterial disease. *J Infect Dis* **136**, 822-828.

George, R.H., Symonds, J.M., Dimock, F., Brown, J.D., Arubi, Y., Shinagawa, N., and Keighley, M.R., Alexander-Williams, J., and Burdon, D.W. (1978) Identification of *Clostridium difficile* as a cause of Pseudomembranous colitis. *BMJ* **1**, 695.

Gerding, D.N. (1989) Disease associated with *Clostridium difficile* infection. *Ann Intern Med* **110**, 255-257.

Gerding, D.N., Johnson, S., Peterson, L.R., Mulligan, M.E. and Silva, J., Jr. (1995) *Clostridium difficile*-associated diarrhea and colitis. *Infect Control Hosp Epidemiol* **16**, 459-477.

Gorbach, S.L. (1999) Antibiotics and *Clostridium difficile*. *N Engl J Med* **341**, 1690-1692.

Groschell, D.H. (1996) *Clostridium difficile* infection. *Crit Rev Clin Lab Sci* **33**, 203-245.

Hecht, D.W. (2006) Anaerobes: antibiotic resistance, clinical significance, and the role of susceptibility testing. *Anaerobe* **12**, 115-121.

Hecht, D.W., Galang, M.A., Sambol, S.P., Osmolski, J.R., Johnson, S., and Gerding, D.N. (2007) In vitro activities of 15 antimicrobials agents against 110 toxigenic *Clostridium difficile* clinical isolates collected from 1983-2004. *Antimicrob Agents Chemother* **51**, 2716-2719.

Indra, A., Schmid, D., Huhulescu, S., Hell, M., Gattringer, R., Hasenberger, P., Fiedler, A., Wewalka, G., and Allerberger, F. (2008) Characterization of clinical *Clostridium difficile* isolates by PCR ribotyping and detection of toxin genes in Austria, 2006-2007. *J Med Microbiol* **57**, 702-708.

Jamal, W.Y., Mokaddas, M., Verghese, T.L., and Rotimi, V.O. (2002) In vitro activity of 15 antimicrobial agents clinical isolates of *Clostridium difficile* in Kuwait. *Int J Antimicrob Agents* **20**, 270-274.

John, R. and Brazier, J.S. (2005) Antimicrobial susceptibility of polymerase chain reaction ribotypes of *Clostridium difficile* commonly isolated from symptomatic hospital patients in the UK. *J Hosp Infect* **61**, 11-14.

Johnson, A.M. (1998) Use of antimicrobial drugs in veterinary practice. *BMJ* **317**, 665-667.

Johnson, S., Samore, M.H., and Farrow, K.A. (1999a) Epidemics of diarrhea caused by a clindamycin-resistant strain of *Clostridium difficile* in four hospitals. *N Engl J Med* **341**, 1645-1651.

Johnson, S., Samore, M.H., Farrow, K.A., Killgore, G.E., Tenover, F.C., Jyras, D., Rood, J. I., DeGirolami, P., Baltch, A.L., Rafferty, M.E., Pear, S.M., and Gerding, D.N. (1999b) Epidemics of diarrhea caused by a clindamycin-resistant strain of *Clostridium difficile* in four hospitals. *N Engl J Med* **341**, 1645-1651.

Johnson, S., Schriever, C., Galang, M., Kelly, C.P., and Gerding, D.N. (2007) Interruption of recurrent *Clostridium difficile*-associated diarrhea episodes by serial therapy with vancomycin and rifaximin. *Clin Infect Dis* **44**, 846-848.

Kelly, C.P., Pothoulakis, C., and LaMont, J.T. (1994) *Clostridium difficile* colitis. *N Engl J Med* **330**, 257-262.

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

Kink, J.A. and Williams, J.A. (1998) Antibodies to recombinant *Clostridium difficile* toxins A and B are effective treatment and prevent relapse of *C. difficile*-associated disease in a hamster model of infection. *Infect Immun* **66**, 2018-2025.

Kuijper, E.J. and Surawicz, C.M. (2008) *Clostridium difficile* infection. *Lancet* **371**, 1486-1488.

Kyne, L., Warny, M., Qamar, A., and Kelly, C.P. (2000) Asymptomatic carriage of *Clostridium difficile* and serum levels of IgG antibody against toxin A. *N Engl J Med* **342**, 390-397.

Letournel-Glomaud, C., Houssaye, S., Milhaiha, L., and Ghnassia, J.C. (2003) E-test antibiotics susceptibility of strict anaerobic bacteria. *Anaerobe* **9**, 281-284.

Loo, V.G., Poirier, L., Miller, M.A., Oughton, M., Libman, M.D., Michaud, S., Bourgault, A.M., Nguyen, T., Frenette, C., Kelly, M., Vibien, A., Brassard, P., Fenn, S., Dewar, K., Hudson, T.J., Horn, R., Rene, P., Monczak, Y., and Dascal, A. (2005) A predominantly clonal multi-institutional outbreak of *Clostridium difficile*-associated diarrhea with high morbidity and mortality. *N Eng J Med* **353**, 2442-2449.

Martin, H., Willey, B., Low, D.E., Staempfli, H.R., McGeer, A., Boerlin, P., Mulvey, M., and Weese, J.S. (2008) Characterization of *Clostridium difficile* strains isolated from patients In Ontario, Canada, from 2004-2006. *J Clin Microbiol* **46**(9), 2999-3004.

McCusker, M.E., Harris, A.D., Perencevich, E., and Roghmann, M.-C. (2003) Fluoroquinolone use and *Clostridium difficile*-associated diarrhea. *Emerg Infect Dis* **9**(6), 730-733.

McFarland, L.V., Elmer, G.W., and Suravicz, C.M. (2002) Breaking the cycle: treatment strategies for 163 cases of recurrent *Clostridium difficile* disease. *Am J Gastroenterol* **97**, 1769-1775.

Nelson, D.E., Auerbach, S.B., Baltch, A.L., Desjarardin, E., Beck-Sague, C., Rheal, C., Smith, R.P., and Jarvis, W.R. (1994) Epidemic *Clostridium difficile*-associated diarrhea: role of second- and third-generation cephalosporins. *Infect Control Hosp Epidemiol* **15**, 88-94.

O' Conner, J.R., Galang, M.A., Sambol, S.P., Hecht, D.W., Vedantam, G., Gerding, D.N., and Johnson, S. (2008) Rifampin and rifaximin resistance in clinical isolates of *Clostridium difficile*. *Antimicrob Agents Chemo* **52**(8), 2813-2817.

Olson, M.M., Shanholtzer, C.J., Lee, Jr., and Gerding, D.N. (1994) Ten years of prospective *Clostridium difficile* associated-disease surveillance and treatment at the Minneapolis VA medical center, 1982-1991. *Infect Control Hosp Epidemiol* **15**, 371-381.

Olsson-Liljequist, B. and Nord, C.E. (1994) Methods for susceptibility testing of anaerobic bacteria. *Clin Infect Dis* **18**(Suppl 4), S293-S296.

Owens, R.C. Jr., Donskey, C.J., Gaynes, R.P., Loo, V.G., and Muto, C.A. (2008) Antimicrobial-associated risk factors for *Clostridium difficile* infection. *Clin Infect Dis* **46**(Suppl. 1), S19-S31.

Pelaez, T., Alcala, L., Alonso, R., Martin-Lopez, A., Garcia-Arias, V., Marin, M., and Bouza, E. (2005) In vitro activity of ramoplanin against *Clostridium difficile*, including strains with reduced susceptibility to vancomycin or with resistance to metronidazole. *Antimicrob Agents Chemother* **49**, 1157-1159.

Pepin, J., Saheb, N., Coulombe, M.A., Alary, M.E., Corriveau, M.P., Authier, S., Lebranc, M., Rivard, G., Bettez, M., Primeau, V., Nguyen, M., Jacob, C.E., and Lanthier, L. (2005) Emergence of fluoroquinolones as the predominant risk factor for *Clostridium difficile*-associated diarrhea: a cohort study during an epidemic in Quebec. *Clin Infect Dis* **41**, 1254-1260.

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

Poilane, I., Cruaud, P., Torlotin, J.C., and Collignon, A. (2000) Comparison of the Etest to the reference agar dilution method for antibiotic susceptibility testing of *Clostridium difficile*. *Clin Microbiol Infect* **6**(3), 154-156.

Rodriguez-Palacios, A., Stampfli, H.R., Duffield, T., Peregrine, A.S., Trotz-Williams, L.A., Arroyo, L.G., Brazier, J.S., and Weese, J.S. (2006) *Clostridium difficile* PCR ribotypes in calves, Canada. *Emerg Infect Dis* **12**(11), 1730-1736.

Rodriguez-Palacios, A., Stampfli, H.R., Duffield, T., and Weese, J.S. (2007) *Clostridium difficile* in retail ground meat, Canada. *Emerg Infect Dis* **13**(3), 485-487.

Rosenblatt, J.E. and Gustafson, D.R. (1995) Evaluation of the Etest for susceptibility testing of anaerobic bacteria. *Diagn Microbiol Infect Dis* **22**, 279-284.

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

Simango, C. (2006) Prevalence of *Clostridium difficile* in the environment in a rural community in Zimbabwe. *Trans R Soc Trop Med Hyg* **100**, 1146-1150.

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

Songer, J.G., Post, K.W., Larson, D.J., Jost, B.H., and Glock, R.D. (2000) Infection of neonatal swine with *Clostridium difficile*. *J Swine Health Prod* **8**, 185-189.

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

Spencer, R.C. (1998) The role of antimicrobial agents in the aetiology of *Clostridium difficile*-associated disease. *J Antimicrob Chemother* **41**(Suppl. C), 21-27.

Spigaglia, P., Barbanti, F., Mastrantonio, P., Brazier, J.S., Barbut, F., Delmee, M., Kuijper, E., and Poxton, R. (2008) Fluoroquinolone resistance in *Clostridium difficile* isolates from a prospective study of *C. difficile* infections in Europe. *J Med Microb* **57**, 784-789.

Tollefson, L., Angulo, F.J., and Fedorka-Cray, P.J. (1998) National surveillance for antibiotic resistance in zoonotic enteric pathogens. *Vet Clin North Am Food Anim Pract* **14**, 141-150.

Tollefson, L., Fedorka-Cray, P.J., and Angulo, F.J. (1999) Public health aspects of antibiotic resistance monitoring in the USA. *Acta Vet Scand* **92**(Suppl.), 67-75.

Voth, D.E. and Ballard, J.D. (2005) *Clostridium difficile* toxins: Mechanism of action and role in disease. *Clin Microbiol Rev* **18**(2), 247-263.

Walton, J.R. (1983) Antibiotics, animal, meat and milk. *Zentralbl Vet A* **30**, 81-92.

Wong, S. S.-Y., Woo, P.C.-Y., Luk, W.-K., and Yuen, K.-Y. (1999) Susceptibility testing of *Clostridium difficile* against metronidazole and vancomycin by disk diffusion and Etest. *Diagn Microbiol Infect Dis* **34**, 1-6.

Worsley, M.A. (1998) Infection control and prevention of *Clostridium difficile* infection. *J Antimicrob Chemother* **41** (Suppl.C), 59-66.

Wust, J. and Hardegger, U. (1992) Comparison of the Etest and a reference dilution method for susceptibility testing of anaerobic bacteria. *Eur J Clin Microbiol Infect Dis* **11**, 1169-1173.

Zar, F.A., Bakkanagari, S.R., Moorthi, K.M., and Davis, M.B. (2007) A comparison of vancomycin and metronidazole for the treatment of *Clostridium difficile*-associated diarrhea, stratified by disease severity. *Clin Infect Dis* **45**, 302-307.

Zheng, L., Citron, D.M., Genheimer, C.W., Sigmon, S.F., Carman, R.J., Lyerly, M., and Goldstein, J.C. (2007) Molecular characterization and antimicrobial susceptibilities of extra intestinal *Clostridium difficile* isolates. *Anaerobe* **13**, 114-120.

Zwiener, R.J., Belknap, W.M., and Quan, R. (1989) Severe pseudomembranous enterocolitis in a child: case report and literature review. *Pediatr Infect Dis J* **8**, 876-882.

CHAPTER 6

CONCLUSIONS

Prevalence of *C. difficile* in food animals and environment

Clostridium difficile is commonly associated with a spectrum of disease referred to as *C. difficile*-associated disease (CDAD), which can range from uncomplicated mild diarrhea to life-threatening pseudomembranous colitis (PMC). While exposure to antibiotics is a prerequisite in most cases of CDAD, exposure to *C. difficile* is essential. It is interesting to speculate where such exposure might have occurred in the case of community-acquired CDAD. The hospital environment is an acknowledged risk factor for exposure to *C. difficile*, and it has been postulated that food animals may be a potential reservoir of infection.

In this study, swine was found to be a major reservoir of *C. difficile* compared to dairy cattle (2.4%), where it was isolated in 15.9% of fecal samples. Detection of *C. difficile* (8.6%) in dairy cattle farms also demonstrates potential of environmental sources of infection. Shedding of *C. difficile* in animal environments is common regardless whether they have enteric disease.

Comparison of cultural and isolation methods for *C. difficile* from porcine and bovine feces

Double alcohol shock was significantly better than single alcohol shock for the recovery of *C. difficile* from swine feces regardless of plating media used. Double alcohol shock with plating on CCFA was found to be the method of choice for the recovery of *C. difficile* from environment. However, there was no significant difference for the recovery of *C. difficile* between any combination of isolation methods and media observed in dairy cattle feces. Different isolation methods may be needed for different types of samples.

Colonies of *C. difficile* were easier to detect on CCFA than on that of BA because of less competitive growth from other organisms. CCFA is the medium of choice to isolate *C. difficile* from feces.

Automated rep-PCR as a molecular typing method for *C. difficile*

Rep-PCR findings were relatively comparable to those obtained by PFGE in terms of typeability, reproducibility, discriminatory power, ease of interpretation, and ease of performance. Rep-PCR showed a higher discriminatory power than PFGE in analyzing animal and human *C. difficile* isolates. Both methods demonstrated wide genetic variation among *C. difficile* strains.

Indistinguishable isolates between human, animals and environment were found by both rep-PCR and PFGE. Because there was no reported contact between the animals and infected humans in this study, the lack of identifiable variation among some isolates of animal and human origin suggests that there is potential for foodborne or other forms of animal-to-human transmission. Further epidemiological studies of the potential for *C. difficile* transmission between food animals and humans are needed.

Antimicrobial susceptibility of *C. difficile* isolated from porcine and bovine feces

All isolates (188) were susceptible to amoxicillin-clavulanic acid, metronidazole, and vancomycin with MIC_{90}s of 0.5, 0.25, and 1.5 µg/ml, respectively. Rifampicin showed good activity against isolates of *C. difficile* with only 1% of isolates being resistant with MIC_{90} of 0.25 µg/ml. Almost all of isolates (71.8%) were resistant to clindamycin with MIC_{90} = 24 µl/ml. Thirty (16%), 25 (13.3%), and 10 (5.3%) isolates were resistant to ampicillin, erythromycin, and linezolid with MIC_{90s} of 3, $\geq$256, and 3 µg/ml, respectively.

Resistance to levofloxacin followed by clindamycin was observed in all sample types. Erythromycin was shown to be the third most resistant in swine feces (23.4%) whereas ampicillin was among dairy cattle feces (25%) and dairy cattle environment (11.1%). In dairy cattle, all isolates from feces were susceptible to erythromycin and low level of resistance was found in environment (5.6%).

A total of 7 isolates were resistant to 4 antimicrobials (6 resistant to ampicillin, clindamycin, erythromycin and levofloxacin; 1 resistant to linezolid, clindamycin, erythromycin and levofloxacin). Thirty eight isolates were resistant to combinations of 3 antimicrobials. Ninety nine isolates were resistant to combinations of 2 antimicrobials. Eighty seven isolates were resistant to clindamycin and levofloxacin.

Metronidazole and vancomycin are the first-line of drugs used in the treatment of human CDAD. The data from this study showed that resistance to these 2 antimicrobials has yet to develop in swine and dairy cattle. Resistance against levofloxacin was found in all isolates tested. Quinolone and fluoroquinolone are not used in swine and dairy production and the source of quinolone resistance in *C. difficile* isolates in our study is unknown.

Antimicrobial susceptibility in relation to rep-PCR grouping

Five predominant groups had similar resistant characteristics. Of these 5 groups, they were most resistant to levofloxacin and followed by clindamycin. All isolates in one group were resistant to levofloxacin and clindamycin. The two hypervirulent strains had identical resistant patterns (they were resistant to clindamycin, erythromycin, and levofloxacin) and were in the same group. All isolates in 5 groups showed identical resistant characteristic. Despite the low discriminatory power of antimicrobial susceptibility testing, data showed some similarity and agreement with rep-PCR grouping. Antimicrobial resistance patterns in relation to rep-PCR grouping are detailed in appendix C.

APPENDIX A

MEDIA FORMULATIONS

Cycloserine-Cefoxitin Fructose broth supplemented with 0.1% sodium taurocholate (TCCFB)

	g/L
Proteose peptone	40
Na_2HPO_4	5
KH_2PO_4	1
$MgSO_4 \cdot H_2O$	0.1
NaCl	2
Fructose	6
0.1% Sodium taurocholate	1
Lysed horse blood	70ml
Clostridium difficile CDMN supplement (SR0173E, Oxoid)	2 vials
Deionized H_2O	1000ml

Cycloserine-Cefoxitin Fructose agar

(CCFA)

	g/L
Proteose peptone	40
Na_2HPO_4	5
KH_2PO_4	1
$MgSO_4 \cdot H_2O$	0.1
NaCl	2
Fructose	6
0.1% Sodium taurocholate	1
Cefoxitin	0.016
Agar	15
Lysed horse blood	70ml
Clostridium difficile selective supplement (SR0096E, Oxoid)	2 vials
Deionized H_2O	1000ml

Peptone Yeast Extract Medium with Glucose

(PYG)

	g/L
Glucose	10
Yeast extract	10
Peptone	5
Pancreatic digest of casein	5
L-cysteine·HCl·H_2O	0.5
Salts solution	40ml
Hemin solution	10ml
Resazurin solution	4ml
Vitamin K_1 solution	0.2ml
Deionized H_2O	945.8ml

Salts solution:

	g/L
$NaHCO_3$	10
NaCl	2
K_2HPO_4	1
KH_2PO_4	1
$CaCl_2$, anhydrous	0.2
$MgSO_4$	0.2
Distilled/Deionized H_2O	1000ml

Hemin solution:

	g/100ml
Hemin	0.05
NaOH (1N solution)	1ml
Distilled/Deionized H_2O	99ml

Resazurin solution:

	g/44ml
Resazurin	0.044
Distilled/Deionized H_2O	44ml

Vitamin K_1 solution:

	g/30ml
Vitamin K_1	0.15
95% Ethanol	30ml

APPENDIX B

CHARACTERIZATION DATA

sample ID	Source	rep-PCR group	PFGE group	Antimicrobial resistance[±]
20-94	Dairy Env	1-5	13	CM LE
751-93	Dairy Env	3-1	66	CM LE
RSS-94	Dairy Env	3-1	47	LE
70-96	Dairy Env	3-2	47	LE
RSS-93	Dairy Env	3-1	10	LE
594-95	Dairy Env	3-4	75	AM CM LE
594-96	Dairy Env	3-1	75	CM LE
11-91	Dairy Env	6-1	54	CM LE
624-94	Dairy Env	6-1	13	LE
669-96	Dairy Env	6-1	48	LE
RSS-91	Dairy Env	6-1	84	AM LE
758-94	Dairy Env	6-2	55	CM LE
61-93	Dairy Env	6-1	49	CM LE
647-93	Dairy Env	6-4	13	AM CM LE
658-91	Dairy Env	13	50	AM CM LE
296-95	Dairy Env	15-1	29	CM LE
23-93	Dairy Env	15-2	55	CM LE
410-93D	Dairy Env	18-1	29	LZ CM LE
250-93	Dairy Env	18-2	nontypeable	CM LE
575-91	Dairy Env	23-1	65	CM LE
751-95	Dairy Env	23-2	48	CM LE
758-95	Dairy Env	25-1	56	CM LE
22-92	Dairy Env	25-2	69	CM EM LE
22-94D	Dairy Env	27	78	CM EM LE
17-93	Dairy Env	29-2	57	AM CM LE
20-92	Dairy Env	37-1	68	CM LE
20-93	Dairy Env	37-2	59	AM CM EM LE
339-93	Dairy Env	39	10	AM CM LE
657-94	Dairy Env	40-1	52	CM LE
296-93	Dairy Env	42-3	29	CM LE
513-96	Dairy Env	42-4	28	CM LE
250-94	Dairy Env	42-7	29	CM LE
15-96	Dairy Env	42-8	28	CM LE
15-94	Dairy Env	42-8	29	CM LE
276-95	Dairy Env	42-8	28	CM LE
17-91	Dairy Env	42-9	52	CM LE
350-91	Dairy Env	42-10	52	CM LE
17-94	Dairy Env	42-10	79	CM LE
361-92	Dairy Env	42-10	53	CM LE
361-93	Dairy Env	42-10	53	CM LE
207-92	Dairy Env	42-10	29	LE
361-94	Dairy Env	42-10	53	CM LE
67-93	Dairy Env	42-10	52	CM LE
67-91	Dairy Env	42-10	52	LE
67-94	Dairy Env	42-10	52	CM LE
67-95	Dairy Env	42-10	52	CM LE

sample ID	Source	rep-PCR group	PFGE group	Antimicrobial resistance$^{\pm}$
23-92	Dairy Env	42-10	79	CM LE
376-96	Dairy Env	42-10	10	LE
186-95	Dairy Env	42-10	49	CM LE
1031-93	Dairy Env	43	52	CM LE
350-95	Dairy Env	43	52	CM LE
791-96	Dairy Env	43	16	CM LE
350-94	Dairy Env	43	52	CM LE
571-95	Dairy Env	43	29	CM LE
64-11	Dairy Feces	3-1	50	LE
64-35	Dairy Feces	3-1	53	LE
64-29	Dairy Feces	3-1	83	LE
64-1	Dairy Feces	3-1	80	CM LE
116-14	Dairy Feces	3-3	80	AM CM LE
64-27	Dairy Feces	5-2	48	AM CM LE
50-33	Dairy Feces	6-1	6	LZ CM LE
9-5	Dairy Feces	6-1	4	CM LE
9-7	Dairy Feces	6-1	4	CM LE
69-6	Dairy Feces	6-1	61	LE
40-4	Dairy Feces	6-1	3	CM LE
40-2	Dairy Feces	6-1	3	LE
96-32	Dairy Feces	6-1	10	AM CM LE
9-11	Dairy Feces	6-1	3	LE
143-11	Dairy Feces	6-1	63	AM CM LE
74-34	Dairy Feces	6-1	7	AM CM LE
250-2	Dairy Feces	6-2	7	CM LE
15-27D	Dairy Feces	6-3	10	AM LE
89-1	Dairy Feces	6-3	64	AM CM LE
9-27	Dairy Feces	6-1	4	CM LE
96-4	Dairy Feces	6-7	10	CM LE
21-28	Dairy Feces	12-2	15	AM LE
89-3	Dairy Feces	24	10	AM CM LE
89-9	Dairy Feces	28	10	CM LE
52-35	Dairy Feces	42-5	28	LE
54-20	Dairy Feces	42-10	52	CM LE
54-30	Dairy Feces	42-10	52	CM LE
6-4	Dairy Feces	42-10	52	CM LE
54-26	Dairy Feces	42-10	52	CM LE
IDA-26D	Dairy Feces	42-10	29	CM LE
6-22	Dairy Feces	42-10	52	CM LE
54-18	Dairy Feces	42-10	52	CM LE
54-34	Dairy Feces	42-10	53	CM LE
6-14-2	Dairy Feces	42-10	52	CM LE
6-20	Dairy Feces	43	52	CM LE
21-34	Dairy Feces	47-1	15	CM LE
28-32	Dairy Feces	47-2	52	CM LE
102-13	Dairy Feces	47-4	28	AM CM LE
64-7	Dairy Feces	56	80	LE

sample ID	Source	rep-PCR group	PFGE group	Antimicrobial resistance$^{\pm}$
2-27	Dairy Feces	n/d*	53	CM LE
W73	Human	5-1	13	LE
L13	Human	6-1	28	LE
X57	Human	6-5	8	CM LE
M680	Human	6-6	6	LE
I927	Human	6-12	28	CM LE
Q676	Human	7-1	31	LE
H447	Human	7-2	18	LE
AK477	Human	7-3	5	EM LE
B1017	Human	7-4	17	LE
E1002	Human	7-5	41	EM LE
C745	Human	8	14	LE
A765	Human	11-1	14	LE
Y431	Human	12-3	55	LE
K744	Human	33	30	EM LE
Z718	Human	46	32	CM EM LE
S61	Human	48	21	LE
AA438	Human	49	46	LZ CM EM LE
AE978	Human	52	2	CM EM LE
N432	Human	53	2	EM LE
AC345	Human	54	12	EM LE
NAP6	NAP strains	1-3	4	CM LE
NAP2	NAP strains	1-4	11	CM EM LE
CD10-BBC	NAP strains	2	25	CM EM LE
CD8-BBC	NAP strains	9	10	CM LE
CD6-BBC	NAP strains	11-2	19	LE
NAP3	NAP strains	17	25	CM EM LE
NAP4	NAP strains	21-1	20	CM LE
NAP8	NAP strains	38-1	30	CM EM LE
NAP5	NAP strains	38-4	27	LZ CM LE
NAP7	NAP strains	42-1	29	LE
CD1-BBC	NAP strains	47-3	26	LZ CM EM LE
CD3-BBC	NAP strains	52	14	CM EM LE
NAP1	NAP strains	52	2	CM EM LE
96-13	Swine Feces	1-1	7	AM CM LE
158-41	Swine Feces	1-2	61	LZ CM LE
158-25	Swine Feces	1-5	82	LE
114-5	Swine Feces	4-1	1	CM EM LE
114-9	Swine Feces	4-2	81	CM EM LE
235-17	Swine Feces	4-3	62	CM EM LE
158-33	Swine Feces	5-3	82	CM LE
216-25	Swine Feces	6-1	28	CM LE
96-1	Swine Feces	6-1	13	AM CM LE
801-37	Swine Feces	6-1	13	LZ LE
213-9	Swine Feces	6-1	3	LE
80-17	Swine Feces	6-3	10	AM LE
205-45	Swine Feces	6-8	11	LE
96-45	Swine Feces	6-9	13	CM LE
96-41	Swine Feces	6-10	13	LE

sample ID	Source	rep-PCR group	PFGE group	Antimicrobial resistance$^{\pm}$
96-25	Swine Feces	6-11	7	CM LE
61-57	Swine Feces	10	58	CM LE
225-29	Swine Feces	11-3	16	LE
244-17	Swine Feces	11-4	16	CM LE
59-25	Swine Feces	11-5	16	CM LE
59-5	Swine Feces	12-1	16	LE
217-33	Swine Feces	12-4	16	CM LE
212-49	Swine Feces	13	51	AM CM EM LE
195-21	Swine Feces	13	21	CM LE
43-29	Swine Feces	14	3	LE
125-37	Swine Feces	15-1	28	LE
118-5	Swine Feces	16	34	AM CM EM LE
61-25	Swine Feces	18-1	37	LE RI
61-41	Swine Feces	19	3	CM LE
96-37	Swine Feces	20	21	AM LE
213-1	Swine Feces	21-2	3	LE
61-13	Swine Feces	22	44	CM LE
61-29	Swine Feces	25-1	36	LE
61-1	Swine Feces	25-3	38	LE
801-17	Swine Feces	26	13	CM LE
27-1	Swine Feces	26	10	AM CM LE
96-5	Swine Feces	26	13	LZ AM LE
242-49	Swine Feces	29-1	28	CM EM LE
151-21	Swine Feces	30	43	CM EM LE
61-17	Swine Feces	31	40	CM LE RI
801-53	Swine Feces	32-1	13	LE
125-9	Swine Feces	32-2	28	CM LE
96-17	Swine Feces	34	13	LE
118-9	Swine Feces	35	34	AM CM EM LE
118-41	Swine Feces	35	34	AM CM EM LE
80-29	Swine Feces	35	60	AM CM EM LE
184-13	Swine Feces	36	42	LE CM LE
184-49	Swine Feces	37-3	23	CM EM LE
30-37	Swine Feces	37-4	45	CM EM LE
195-49	Swine Feces	37-5	21	AM LE
63-33	Swine Feces	38-2	35	CM EM LE
212-12	Swine Feces	38-3	51	CM EM LE
27-25	Swine Feces	40-2	29	CM LE
61-45	Swine Feces	41	76	LE
61-5	Swine Feces	41	70	LE
125-33	Swine Feces	42-2	28	CM LE
158-53	Swine Feces	42-3	28	CM LE
158-17	Swine Feces	42-5	28	LE
158-45	Swine Feces	42-6	28	CM LE
158-57	Swine Feces	42-10	28	LE
216-21	Swine Feces	42-10	28	LE
96-33	Swine Feces	42-10	29	CM LE
125-45	Swine Feces	42-10	28	CM LE
125-17	Swine Feces	42-10	33	LE

sample ID	Source	rep-PCR group	PFGE group	Antimicrobial resistance[±]
96-21	Swine Feces	42-10	29	LZ LE
125-1	Swine Feces	42-10	12	CM LE
125-53	Swine Feces	42-10	28	LE
213-17	Swine Feces	42-10	29	CM LE
158-29	Swine Feces	42-10	3	CM LE
216-5	Swine Feces	42-10	28	LZ CM LE
217-5	Swine Feces	42-10	29	CM LE
125-41	Swine Feces	42-10	1	CM LE
212-45	Swine Feces	42-10	67	LZ CM EM LE
158-13	Swine Feces	42-10	28	CM LE
125-57	Swine Feces	42-10	28	LE
125-49	Swine Feces	42-10	28	CM LE
213-49	Swine Feces	42-10	54	LE
61-21	Swine Feces	44	73	LE
61-49	Swine Feces	44	39	CM LE
217-25	Swine Feces	45-2	29	EM LE
217-9	Swine Feces	45-1	29	CM EM LE
217-41	Swine Feces	45-1	29	EM LE
217-13	Swine Feces	45-2	29	CM EM LE
217-29	Swine Feces	45-2	29	EM LE
63-45	Swine Feces	47-1	24	CM EM LE
195-37	Swine Feces	47-1	21	LE
196-45	Swine Feces	47-1	22	LE
158-5	Swine Feces	47-1	82	CM LE
184-45	Swine Feces	49	77	CM EM LE
213-45	Swine Feces	50	9	LE
213-41	Swine Feces	50	9	LE
231-57	Swine Feces	50	9	LE
80-25	Swine Feces	51	85	LZ LE
61-33	Swine Feces	55	24	CM LE

[±] LZ=Linezolid, XL=Amoxicillin-Clavulanic acid, AM=Ampicillin, CM=Clindamycin, EM=Erythromycin, MZ=Metronidazole, LE=Levofloxacin, RI=Rifampicin, VA=Vancomycin

[*] Did not determine

APPENDIX C

ANTIMICROBIAL SUSCEPTIBILITY IN RELATION TO REP-PCR DATA

Percentage of isolates resistant to antimicrobials based on rep-PCR grouping

Antimicrobials	rep-PCR group (no. per group)					
	42-10 (n=40)	6-1 (n=21)	3-1 (n=8)	43 (n=6)	47-1 (n=5)	Other (n=141)
Linezolid	7.5	9.5	0	0	0	4.9
Amoxicillin-Clavulanic acid	0	0	0	0	0	0
Ampicillin	0	24	0	0	0	16.3
Clindamycin	75	57	37.5	100	60	63.8
Erythromycin	2.5	0	0	0	20	25.5
Metronidazole	0	0	0	0	0	0
Levofloxacin	100	100	100	100	100	100
Rifampicin	0	0	0	0	0	1.4
Vancomycin	0	0	0	0	0	0

Antimicrobial resistance profile of 221 multiple resistant *Clostridium difficile* isolates based on rep-PCR grouping

	Number of isolates												
Resistance group*	42-10	6-1	3-1	43	47-1	6-3	13	26	35	42-8	45-2	50	52
AM, CM, EM, LE	0	0	0	0	0	0	1	0	3	0	0	0	0
LZ, CM, EM, LE	1	0	0	0	0	0	0	0	0	0	0	0	0
AM, CM, LE	0	4	0	0	0	1	1	1	0	0	0	0	0
CM, EM, LE	0	0	0	0	1	0	0	0	0	0	1	0	3
CM, RI, LE	0	0	0	0	0	0	0	0	0	0	0	0	0
LZ, CM, LE	1	1	0	0	0	0	0	0	0	0	0	0	0
LZ, AM, LE	0	0	0	0	0	0	0	1	0	0	0	0	0
AM, LE	0	1	0	0	0	2	0	0	0	0	0	0	0
CM, LE	28	7	3	6	2	0	1	1	0	3	0	0	0
EM, LE	0	0	0	0	0	0	0	0	0	0	2	0	0
LZ, LE	1	1	0	0	0	0	0	0	0	0	0	0	0
RI, LE	0	0	0	0	0	0	0	0	0	0	0	0	0
LE	9	7	5	0	2	0	0	0	0	0	0	3	0
Total	40	21	8	6	5	3	3	3	3	3	3	3	3

* LZ=Linezolid, XL=Amoxicillin-Clavulanic acid, AM=Ampicillin, CM=Clindamycin, EM=Erythromycin, MZ=Metronidazole, LE=Levofloxacin, RI=Rifampicin, VA=Vancomycin

BIOGRAPHY

Sutawee Narint Thitaram was born on May 29, 1976 in Khon Kaen, Thailand. She is the eldest daughter of Nat and Em-on Narint. She received her elementary education from Sanambin School and secondary education from the Satit Khon Kaen University School in Khon Kaen. After finishing high school in 1992, she attended Khon Kaen University in department of plant pathology, where she was awarded the student exchange scholarship from the Royal Thai government to work on isolation and identification soil fungi under the direction of Dr. Richard T. Hanlin in the Department of Plant Pathology at the University of Georgia. After receiving her Bachelor of Science in plant pathology from Khon Kaen University in 1996, she enrolled in the master program in the Department of Food Science and Technology at the University of Georgia in 2002 under the direction of Dr. Gregory R. Siragusa.

After receiving her Master of Science in food science from the University of Georgia in 2004, she continued in the doctoral program where she conducted her doctoral research under the guidance of Dr. Joseph F. Frank.