Clostridium difficile สายพันธุ์ที่สร้าง Toxin A จากทารกคลอดก่อนกำหนด ทารกคลอดครบกำหนด เด็กที่ได้รับยาต้านมะเร็ง และผู้ใหญ่ที่มีอาการอุจจาระร่วง

นางสาว ศรีวรรณา หัทยานานนท์

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TOXIN A-PRODUCING *Clostridium difficile* ISOLATED FROM FECES OF PREMATURE INFANT, CHILDREN WITH ANTICANCER DRUG AND ADULT WITH DIARRHEA

Miss Sriwanna Huttayananont

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Thesis Title	Toxin A-Producing *Clostridium difficile* Isolated from Feces of Premature Infant, Full-Term Infant, Children with Anticancer Drug and Adult with Diarrhea
By	Sriwanna Huttayananont
Inter-Department	Medical Microbiology
Thesis Advisor	Associate Professor Pintip Pongpech, Ph.D.
Co- Advisor	Siripan Wongwanich, M.Sc.

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---------- Dean of Graduate School
(Professor Suchada Kiranandana, Ph. D.)

THESIS COMMITTEE

---------- Chairman
(Anan Chongthaleong, M.D.)

---------- Thesis Advisor
(Associate Professor Pintip Pongpech, Ph.D.)

---------- Co-Advisor
(Siripan Wongwanich, M.Sc.)

---------- Member
(Associate Professor Chertsak Dhiraputra, M.D., M.Sc.)

---------- Member
(Assistant Professor Ariya Chindamporn, Ph.D.)

ศรีวรรณา หัทยานานนท์ : *Clostridium difficile* สายพันธุ์ที่สร้าง toxin A จากทารกคลอดก่อนกำหนด ทารกคลอดครบกำหนด เด็กที่ได้รับยาต้านมะเร็ง และผู้ใหญ่ที่มีอาการอุจจาระร่วง (Toxin A-Producing *Clostridium difficile* Isolated from Feces of Premature Infant, Full-Term Infant, Children with Anticancer Drug and Adult with Diarrhea) อ. ที่ปรึกษา : รศ. ดร. พิณทิพย์ พงษ์เพ็ชร, อ. ที่ปรึกษาร่วม : นาง ศิริพรรณ วงศ์วานิช, 114 หน้า, ISBN 974-346-357-7

อัตราการพบเชื้อ *Clostridium difficile* ในตัวอย่างอุจจาระ 446 ตัวอย่างจากประชากร 4 กลุ่มในโรงพยาบาลศิริราช ในระยะเวลา 7 เดือน (ต.ค. 2541 ถึง เม.ย. 2542) เป็น 15.38% ในทารกคลอดก่อนกำหนด 10.59%ในทารกคลอดครบกำหนด 21.05% ในเด็กที่ได้รับยาต้านมะเร็ง และ 25% ในผู้ใหญ่ที่มีอาการอุจจาระร่วง

เชื้อที่พบในทารกคลอดก่อนกำหนด 2 รายและผู้ใหญ่ที่มีอาการอุจจาระร่วง 18 รายเป็นสายพันธุ์ที่ตรวจพบ toxin A gene ด้วยวิธี polymerase chain reaction (PCR) สำหรับการใช้วิธี enzyme immunoassay (EIA) ไม่สามารถตรวจพบ toxin A จากสายพันธุ์ที่ได้แยกได้จากทารกคลอดก่อนกำหนดทั้ง 2 ราย และ จากผู้ใหญ่ที่มีอาการอุจจาระร่วง 5 ราย เมื่อนำเชื้อสายพันธุ์ที่มี toxin A gene ทั้ง 20 สายพันธุ์มาจำแนกสายพันธุ์โดยการตัดโครโมโซมด้วยเอนไซม์ตัดจำเพาะ *Sma* I และแยกชิ้นส่วนโครโมโซมโดยการใช้ pulsed field gel electrophoresis (PFGE) พบว่า สามารถจำแนกรูปแบบพันธุ์กรรมได้ 4 แบบ คือ A, B, C และ D โดยสายพันธุ์ที่พบในผู้ใหญ่ที่มีอาการอุจจาระร่วง 11 ราย อยู่ใน type A ส่วนสายพันธุ์ที่พบในทารกคลอดก่อนกำหนด 2 รายและผู้ใหญ่ที่มีอาการอุจจาระร่วงอีก 5 รายเป็น type B

จากการศึกษานี้พบว่า การใช้วิธี PCR เป็นวิธีที่เหมาะสมในการตรวจหาเชื้อ *Clostridium difficile* สายพันธุ์ที่สร้างสารพิษได้อย่างถูกต้องและการใช้ PFGE สามารถแสดงให้เห็นว่าเชื้อ type A เป็นสายพันธุ์หลักที่เกี่ยวข้องกับการเกิดอุจจาระร่วงในโรงพยาบาล ซึ่งข้อมูลเหล่านี้สามารถนำมาใช้เป็นประโยชน์ต่อการป้องกันและควบคุมโรคติดเชื้อ *Clostridium difficile*

ภาควิชา สหสาขาวิชาจุลชีววิทยาทางการแพทย์ ลายมือชื่อนิสิต .....

สาขา จุลชีววิทยาทางการแพทย์ ลายมือชื่ออาจารย์ที่ปรึกษา .....

ปีการศึกษา 2543 ลายมือชื่ออาจารย์ที่ปรึกษาร่วม .....

4075249530 : MAJOR MEDICAL MICROBIOLOGY
KEY WORD: Toxin A / *Clostridium difficile* / Premature / Diarrhea

Sriwanna Huttayananont : Toxin A-Producing *Clostridium difficile* Isolated from Feces of Premature Infant, Full-Term Infant, Children with Anticancer Drug and Adult with Diarrhea. Thesis Advisor : Assoc. Prof. Pintip Pongpech, Ph. D., Thesis co-Advisor : Siripan Wongwanich, M.Sc. 114 pp. ISBN 974-346-357-7

The prevalence of *C. difficile* in 4 groups of 443 patients (446 stool specimens) at Siriraj Hospital during the 7 month (October 1998-April 1999) of the study were 15.38% of premature infants, 10.59% of full-term infants, 21.05% of children with anticancer drugs and 25% of the adult patients with diarrhea. It was shown by polymerase chain reaction (PCR) that only 2 isolates from the premature infants and 18 isolates from the adult patients with diarrhea had toxin A gene while enzyme immunoassay (EIA) could not detect toxin A production in both 2 isolates from the premature infants and 5 out of 18 isolates from the adult patients. In order to perform the genotyping, the chromosomal DNA from all 20 enterotoxigenic *C. difficile* isolates were digested with *Sma* I restriction endonuclease and separated by pulsed field gel electrophoresis (PFGE). Four different pulsotypes ; A, B, C and D were obtained. Fourteen isolates from 11 adult patients with diarrhea were pulsotype A which was the major pulsotype. Seven isolates from 5 adult patients with diarrhea and from 2 premature infants which were only toxin A gene positive by PCR but not toxin A positive by EIA were all pulsotype B or its subtype.

PCR has been shown to be a sensitive and useful tool in the detection of the toxigenic *C. difficile*. PFGE results indicated that the pulsotype A strain was the predominant type and could be related to nosocomial diarrhea. The results obtained should be beneficial for patients in both prevention and treatment of this infection.

Inter-Department	Medical Microbiology	Student's signature	Sriwanna Huttayananont
Field of study	Medical Microbiology	Advisor's signature	Pintip Pongpech
Academic year	2000	Co- advisor's signature	Siripan Wongwanich

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ABBREVIATIONS

A	adenine or adenosine
AAC	antibiotic associated colitis
AAD	antibiotic associated diarrhea
ADP	adenosine 5-diphosphate
AIDS	acquired immune deficiency syndrome
bp	base pair
C	cytosine or cytidine
ca	calculation
cfu	colony forming units
CHEF-DRII	Contour-clamped homogenous electric field apparatus
D	dalton
DNA	deoxyribonucleic acid
DW	distilled water
ed (s)	editor (s)
EDTA	ethylenediaminetetraacetic acid
EIA	enzyme immunoassay
et al.	et alli
g	gram
G	guanine or guanosine
hr	hour (s)
Ig	immunoglobulin
kb	kilobase
kD	kilodalton (s)
M, mM, μM	molar (s), millimolar (s), micromolar (s)
mg, ml, mm	milligramme (s), millilitre (s), millimetre (s)
min	minute (s)
M_r	relative molecular weight
MW	molecular weight

NEC	necrotizing enterocolitis
nm	nanometre (s)
No	number
°C	degree (s) celcius
OD_{450}	optical density at 450 nanometre
ORF	open reading frame
PCR	polymerase chain reaction
PFGE	pulsed field gel electrophoresis
pg	picogramme
PMC	pseudomembranous colitis
RNase	ribonuclease
rpm	revolutions per minute
sec	second (s)
T	thymine or thymidine
TEMED	N, N, N, N-tetramethyl-ethylenediamine
USA	United States of America
UV	ultraviolet
v/v	volume by volume
VPI	Virginia Polytechnic Institute
wt/vol	weight by volume
%	percent
µg, µl, µm	microgramme (s), microlitre (s), micrometre (s),

CHAPTER I

INTRODUCTION

C. difficile, a Gram positive anaerobic rod, is a pathogen causes gastrointestinal infections such as antibiotic-associated diarrhea (AAD), antibiotic-associated colitis (AAC), pseudomembranous colitis (PMC) in adults and children older than two years of age. At present, *C. difficile* is one of the most important cause of nosocomial infection (Larson *et al.*, 1978; Mc Farland *et* al., 1989). *C. difficile* is implicated in the pathogenesis of necrotizing enterocolitis (NEC) which is an important cause of morbidity and mortality in premature infant (Milner *et al.*, 1986). The toxins of *C. difficile* has been identified in the feces of patients with NEC. It has been suggested that *C. difficile* is either primary pathogen or secondary invader in such disease (Stark and Lee, 1982). Gastrointestinal pathogenesis of adult and infants are similar. Clinical manifestations of infection with this organism vary from colonic perforation, to life-threatening haemorrhagic colitis. The elevation of white blood cell count, hypoalbuminemia, cramping abdominal pain, diarrhea, profuse diarrhea consisting of mucoid and watery stools, shock and eventual death could be observed (Fekety, 1997).

C. difficile produces two exotoxins, toxin A and toxin B, which are involved in the disease. Toxin A is enterotoxin causing the fluid accumulation associated with mucosal epithelium cell damage and hemorrhagic within two to eighteen hours in several animal models such as rabbit ileal and colonic loops, hamster cecal segments. Toxin B is cytotoxin cause cytopathic effect in tissue culture monolayers (ศิริพรรณ วงศ์วานิช, 2538; Lima *et al.*, 1988). Other virulence factors include adhesion, capsule, hydrolytic enzyme and chemotaxis, may also play a part in pathogenesis (Borriello *et al.*, 1990; Borriello, 1998).

Predisposing factors for *C. difficile* infection in adult and premature infant are different. Adult, with prolong anticancer drugs or antibiotics such as ampicillin, amoxycillin, cephalosporins, clindamycin administration are high risk to infection. Such agents could promote *C. difficile* associated disease by altering the colonic microflora and ecosystem, and allow *C. difficile* to become established and colonization. Premature infants also have high risk factors implicated in the *C. difficile* infection. These factors include low birth weight, intestinal immaturity, oral feeding of hyperosmolar solution, and respiratory distress syndrome (RDS). Besides these two high risk groups, the other potential risks have been shown in the elderly, immunocompromised hosts and the patients with the long length of hospital stay (McFarland *et al.*, 1990; Pothoulakis and La Mont, 1993; Spencer, 1998b).

The reviewed study by Bartlett (1994) showed that the toxigenic *C. difficile* implicated in 10-25% of the patients with antibiotic associated diarrhea, and in 50-75% of the patients with antibiotic-associated colitis. More than 90% of those with pseudomembranous colitis carried the toxigenic *C. difficile* in their feces. There was no report on the isolation of toxigenic *C. difficile* from feces of healthy adult.

Five to seventy percent of the healthy infants have toxigenic *C. difficile* in their feces but so few develop the disease. No one knows why infants are protected but there are a number of hypothesis including colostrum containing substances which neutralize toxins A and B (Kim *et al.*, 1984), immature nature of the intestinal flora and the lack of the toxin receptors in the intestine (Lyerly *et al.*, 1988). However, asymptomatic infants can spread this organism to healthcare workers and hospital environment, which could be the possible mode of nosocomial transmission.

The trend of increasing prevalence of *C. difficile* has been reported in Europe and United States during the past ten years. The economic impact of this disease is significant. There have been about 300,000 cases annually in the United States alone. A recent study estimated that the *C. difficile* disease

added, on average, more than two weeks of hospitalization at an additional cost of $ 10,000 per patient. In England and Wales, it has been almost 20 years since the connection between antibiotic-associated colitis and *C. difficile* was first established (Wilcox, 1998).

There has rarely been reported on *C. difficile* toxin detection in Thailand, eventhough it is valuable information for the treatment of *C. difficile* associated diseases. The knowledge on the role of toxin in disease development leads to the improvement in the treatment and preventative options. Various methods have been developed for toxin detection including the ileal loop in animal models for toxin A detection, tissue culture assay for toxin B detection, and enzyme immunoassay (EIA) for toxin A and/or toxin B detection. These assays requires expertise techniques, difficult, time-consuming and cost.

In this study, the polymerase chain reaction and enzyme immunoassay were used in detection of toxin A of *C. difficile*. In order to reduce the expense and time for toxin detection. Only toxin A detection was performed in this study because both toxins normally act in a synergistic manner to cause *C. difficile* associated disease. There has been no report on isolation of *C. difficile* from patients that could produce only toxin A or toxin B alone (Rupnik *et al.*, 1998). In addition, another reason that only toxin A detection was performed is that enterotoxin rather than the cytotoxin causes the diarrhea.

C. difficile infections have been recognized as a nosocomial acquisition. Typing methods would be important in epidemiologic investigation of nosocomial infections. In this study all enterotoxigenic *C. difficile* isolates was typed. The most appropriated typing method used was the detection of restricted chromosomal fragment patterns of the organism from pulsed field gel electrophoresis. This part of the study should be helpful in delineate the most important source, spread of the organism, and defining the epidemiology of *C. difficile* in the hospital.

CHAPTER II

OBJECTIVES

1. To detect toxin A-producing *C. difficile* strains isolated from fecal specimens of premature infants, full-term infants, children with anticancer drugs and adult with diarrhea by using polymerase chain reaction and enzyme immunoassay kit (Premier *C. difficile* toxin A test, Meridian Diagnostic, USA).

2. To perform the DNA typing of toxin A-producing *C. difficile* by Pulsed-Field Gel Electrophoresis (PFGE).

CHAPTER III

REVIEW OF LITERATURES

1. Historical perspectives

Clostridium difficile was first isolated in 1935 by Hall & O' Toole from the meconium and stools of healthy newborn infants. These investigators referred to the microorganism as *Bacillus difficilis* because of the difficulty in the isolation. In 1937, these investigators along with Snyder, preformed the follow-up study to further characterize the toxin from this microorganism and showed that it was a thermolabile toxin with lethal toxic effect particularly when injected into the laboratory animals. It was almost four decades after the first isolation of *C. difficile* when the pathogenic potential of this organism, primarily in the elderly, was to become apparent. In 1974, Tedesco *et al.* demonstrated an association between the occurrence of pseudomembranous colitis (PMC) and the clindamycin administration in patients. Few years later, Larson *et al.* (1977) was the first group of investigators who made a hypothesis on the role of *C. difficile* toxin in PMC. These investigators showed that cytotoxin could be detected in the stools from five out of the six patients with histologically proven of PMC. In the same year, Bartlett *et al.* (1977), used clindamycin to induce enterocolitis in hamster and found that intra caecal material containing *C. difficile* transferred the disease from affected animals to healthy ones. Rifkin *et al.* (1977) simultaneously reported that stool filtrates from humans with PMC were lethal to hamsters and also produced edema, hemorrhage, and increased vascular permeability in rabbit skin. The filtrate also possesed cytotoxic activity which could be neutralized by *C. sordellii* antitoxin. These investigators concluded that *C. sordellii* might be a cause of PMC, even though, it was never been isolated from patients with the disease. In 1978, Bartlett *et al.* and George *et al.*, demonstrated that toxigenic strains of *C. difficile* was readily isolated from the stool of patients with PMC. It had been believed that the cytotoxic factor was the only toxin produced by *C.*

difficile. Two years later, Bartlett *et al.* reported that there was a second protein which could also be separated by ion-exchange chromatography. This substance had enterotoxic effect. At that time, there were two different aspects on *C. difficile* toxins; the cytotoxin and the enterotoxin. Some groups of investigators worked primarily only on the cytotoxin whereas the others were worked mostly on the enterotoxin. Finally, Taylor *et al.* (1981) and Banno *et al.* (1981) concluded that *C. difficile* produced two distinct toxins; toxin A or enterotoxin and cytotoxin designated as toxin B.

2. *Clostridium difficile*

2.1 Bacteriology

C. difficile is an anaerobic Gram-positive rod, with dimension of 0.5-1.9 by 3.0-16.9 μm (Hatheway, 1990). The bacterium forms oval subterminal spore. It can motile by peritrichous flagella. The colonies on blood agar are large, circular to irregular, flat, with rhizoid edge, translucent, gray, nonhemolytic and the odour liked elephant or horse manure. Once this odour has been recognized, it becomes a quick and easy aid for the bacterial identification (Freeman, 1985). *C. difficile* can be isolated on selective medium such as cycloserine-cefoxitin-fructose agar which was developed by George *et al.* (1979). This selective medium can detect as low as 2,000 cell of *C. difficile* in a 6×10^{10} total bacteria per g (wet weight) of feces. The culture yield may be enhanced by the use of heat or alcohol shock as a means of selection for *C. difficile* spores, but the clinical necessity for using these techniques on diarrheal specimens is not clear (Gerding and Brazier, 1993). C. *difficile* hydrolyzes gelatin but is nonproteolytic with respect to milk and meat proteins. It ferments fructose, glucose, mannitol, mannose, and usually xylose. It is negative for lecithinase and lipase production. It produces acetic, isobutyric, isovaleric, valeric, isocaproic, formic, and lactic acids in peptone-yeast extract-glucose. This microorganism grows under strict anaerobic

conditions after 36-72 hour incubation, with the optimum temperature of 35°C (Sutter *et al.*, 1985).

2.2 Factors affecting toxin production

The medium used for the growth of the organism also affects the amounts of toxins produced. The organism grows well in a complex medium such as brain heart infusion broth and produces high level of both toxin A and toxin B (Sullivan *et al.*, 1982). Lyerly *et al.* (1988) found that toxin concentration in supernatant fluid paralleled the growth of the organism and toxin is released during the late logarithmic or early stationary phase of growth, suggesting that toxin release might be an active process. Alternatively, Ketley *et al.* (1984) reported that toxins A and B are released after the stationary phase arguing for their release after cell lysis. Kamiya *et al.* (1992) recently demonstrated that the organism released cytotoxin during sporulation. On the other hand, stressful events such as an increased oxygen tension, elevated temperature, limited nutrients and the presence of small amounts of antibiotics had been observed to increase toxin production *in vitro* (Nakamura *et al.*, 1982).

2.3 Pathogenesis

C. difficile is the bacterial pathogen identified as the cause of pseudomenbranous colitis and is principally responsible for nosocomial antibiotic-associated diarrhea and antibiotic-associated colitis. Most toxigenic isolates produce both toxins while about 5-25% of the *C. difficile* isolates produce neither toxin A nor toxin B (Fekety, 1997). Infection is associated with antibiotic use because the normal stable gut microflora has been disrupted by such agents before *C. difficile* could become established and produce toxins. *C. difficile* diarrhea is caused primarily by elaboration within the intestinal lumen of both toxin A and toxin B during its multiplication.

These toxins bind to the colonic mucosa and then exert their damaging effects upon it including the disruption of the cell-cell tight junction (Bartlett, 1994; Mitty and LaMont, 1994; Johnson and Gerding, 1996). Direct damage is further exacerbated by activity of recruited neutrophils. The serum albumin-rich fluid being lost into the lumen may compete with host proteases and protect the toxins from proteolytic degradation. Released nutrients, and possibly locally induced anoxia, may in turn stimulate *C. difficile* growth and toxin production. Successive cycle of pathogen replication, toxin production and neutrophil recruitment would ultimately lead to cell detachment and apoptosis, local necrosis and the pseudomembranes associated with the diarrhea seen in this infection (Borriello, 1998). It is possible that the apparent higher incidence of infection in the elderly is in part the result of their poorer colonization resistance (Borriello *et al.*, 1986). The other age group in which there is a poor barrier effect is neonate, as they have still to develop a complex gut microbiota that can exclude *C. difficile*; this age group commonly has *C. difficile* present in the faeces (Larson *et al.*, 1982), as well as *C. difficile* toxins (Libby *et al.*, 1983). That so many infants are colonized with toxigenic *C. difficile* and that so few develop the disease indicate that the manner in which they are protected is quite effective, otherwise, more infants would die from the infection (Lyerly *et al.*, 1988). No one knows why infants are protected, but there are a number of the hypothesis. The protection may well result from a combination of several factors. Colostrum contains substances (perhaps secretory antibody) which neutralize toxins A and B (Kim *et al.*, 1984), and these substances probably help to protect the infant from the toxins, but it is more complicated than this since infants who are not breast-fed do not get the disease. Fetal intestinal cells are reported to be much less sensitive to the toxins than adult intestinal cells (Chang *et al.*, 1986), and this may contribute to the resistance. Another hypothesis is that infants may lack the toxin receptors in their intestines. The chemical composition of the receptor to

which toxin A binds contains Gal α1-3 Gal β1-4 Glc Nac trisaccharide. The carbohydrate chains on infant intestinal cells may exist in an immature form which is not recognized by toxin A. The carbohydrate sequences would subsequently develop into the active receptor containing the Gal α1-3 Gal β1-4 Glc Nac trisaccharide in adults, and this could prevent the toxin from binding to its receptor. Alternatively, the receptors on the infants cells may be covered by a thicker layer of mucins and this could prevent to toxin from binding to its receptor (Lyerly *et al.*, 1988).

2.3.1 *C. difficile* toxins

At least five toxic factors of *C. difficile* have been described, though only two of them, toxins A and B, have been studied in details with good evidences of involvement in disease (Borriello *et al.*, 1990). The designations A and B refer to the elution pattern of the toxin on anion-exchange resins. Toxin A binds less lightly on the rasin than toxin B and elutes before toxin B (Banno *et al.*, 1981; Banno *et al.*, 1984). Toxin A is a 308 kD enterotoxin capable of causing extensive mucosal damage, hemorrhagic and fluid accumulation activity in experimental animals and it is also cytotoxic for certain cell lines in culture, (Banno *et al.*, 1984). Toxin A is chemoattractant for human neutrophils and is an activator of macrophages, neutrophils and mast cells. This caused them to produce various inflammatory mediator (Johnson and Gerding, 1996) and systemic manifestations of sepsis (Kelly *et al.*, 1994a). Toxin A causes actin disaggregation and intracellular calcium release, and also appears to damage neurons. Toxin B is a 270 kD cytotoxin that appears to involve depolymerization of filamentous actin, causing a destructuring of the cell cytoskeleton and, thus, a rounding of the cell (Hatheway, 1990). Toxin B was first detected by virtue of its potent cytopathic effect in cell culture monolayer. This toxin was originally considered of little importance in causing colitis in humans because it was not cytotoxic to the

colonic mucosa of various animal species (Fekety, 1997). Recent evidence indicates that toxin B can disrupt the actin cytoskeleton, and is also a necrotizing enterotoxin 10 times more potent than toxin A in causing damange to human colonic mucosa in cell culture (Riegler *et al.*, 1995). It could also induce the release of inflammatory mediators from human monocytes and increase cell-mediated cytotoxicity (Flegel *et al.*, 1991; Siffert *et al.*, 1993). Both toxins A and B have monoglucosyltransferase activity which catalyses in corporation of glucose into a variety of substrate proteins. These include the small GTP-binding proteins (Rho, Rac and Cdc 42Hs) which are involved in regulation of the actin cytoskeletor, specifically in the formation of actin stress fibres and focal adhesions. Both toxins glucosylate threonine at position 37 on Rho. Disruption of the activities of these proteins leads to the shift from F-actin to G-actin and the resultant cell rounding which is characteristic of the toxin-induced cytopathic effects. For both toxins this activity is located towards the N-terminus (Borriello, 1998). Even though, both toxins are cytotoxic and lethal but toxin B is considerably more cytotoxic than toxin A to most tissue culture cells and is often referred to as the cytotoxin. On the other hand, toxin A is active in the intestinal tract, whereas toxin B is not. Consequently, toxin A is referred to as the enterotoxins and is believed to be responsible for most of the clinical symptoms (Lyerly *et al.*, 1988).

The other three toxins are a second, unstable, nonhemorrhagic, enterotoxic protein (Banno *et al.*, 1984), a high molecular weight protein that causes changes in electrical potential in isolated segments of rabbit intestine (Justus *et al.*, 1982) and an actin-specific ADP-ribosyltransferase (Popoff *et al.*, 1988). Giuliano *et al.* (1988) described a nonhemorrhagic enterotoxin reported to be distinct from toxin A and to have a molecular weight of 45,000 by SDS-PAGE.

2.3.1.1 Physicochemical of toxins

Both toxins A and B are extremely large. Native toxin A has an M_r in the range of 400,000 to 600,000, and native toxin B has an M_r in the range of 360,000 to 500,000. Under denaturing condition both toxins have an M_r in excess of 250,000 and consist of a single large polypeptide (Lyerly *et al.*, 1988). It has been reported that toxin B consists of subunits which have an estimated M_r of 50,000, but these findings have not been confirmed and do not agree with finding by other investigators (Banno *et al.*, 1984; Lyerly *et al.*, 1986a). Toxin A and toxin B are digested into more than 30 peptides by chymotrypsin (Lyerly *et al.*, 1986b), pronase (Banno *et al.*, 1984), papain, and proteinase K (Lyerly *et al.*, 1986b). In the early report, toxin A was described to be sensitive to the digestion by trypsin (Banno *et al.*, 1984) while another study reported the trypsin resistant property of toxin A (Taylor *et al.*, 1981). It was subsequently shown that highly purified trypsin did not inactivate toxin A but that crude trypsin contaminated with chymotrypsin did (Torres and Lonnroth, 1988). These toxins can be rapidly inactivated by freezing and thawing, and they are both stable at 37°C and inactivated at 56°C-60°C (Lyerly *et al.*, 1986b; Banno *et al.*, 1984). Unlike toxin A, which is stable at pH 4 and 10 toxin B is inactivated in more acidic and alkaline environments (Taylor *et al.*, 1981). They both are inactivated by oxidizing agents and can be protected by the addition of a reducing agents such as dithiothreital and 2-mercaptoethanol. The oxidizing agents affect a number of amino acids, so it is difficult to determine the specific amino acids which are modified. Both toxins contain high amounts of the amino acids aspartate, glutamate, and glysine, and low amounts of histidine, methionone and cystein (Lyerly *et al.*, 1986b; Lyerly *et al.*, 1988). Both of the toxins are acidic molecules. Reported isoelectric points range from 3.5 to 5.7 for toxin A and from 4.1 to 4.8 for toxin B (von Eichel-Streiber *et al.*, 1987; Lyerly *et al.*, 1988).

2.3.1.2 Biological activity

C. difficile toxin A has both enterotoxic and cytopathic activities, while toxin B is an extremely potent cytotoxin. Toxin A causes fluid accumulation associated with mucosal damage in several animal models such as rabbit ileal and colonic loops, hamster cecal segments, mouse and rat intestine (Lima *et al.*, 1988). Both toxins cause an increase in the vascular permeability, and the erythematous and hemorrhagic lesions in rabbit and guinea pig skin (Borriello, 1998). The rhesus monkeys receiving small amounts of either toxin through a variety of routes die (Lyerly *et al.*, 1988). The biggest difference between the toxins is that toxin A causes fluid accumulation in various animal models, whereas toxin B does not (Borriello, 1998). When administered intragastrically to hamsters, toxin A produces hemorrhage and fluid accumulation in the cecum and small intestine with diarrhea and eventual death. Toxin B also has no effect. However, when toxin B was given with the small amount of toxin A or given to hamster with bruised ceca, the animals died without antecedent symptom (Lyerly *et al.*, 1985b). Lima *et al.* (1988) demonstrated that *C. difficile* toxin A initially causes a viscous (at 2 hr) and then hemorrhagic fluid secretion that steadily increases over 18 hr in ligated rabbit small intestinal segments. These segments usually showed macroscopically hemorrhagic mucosa by 6 hr. These effects were time and dose dependent. Toxin B alone did not cause intestinal tissue damage or fluid accumulation and had a synergistic effect only when used at high doses (>10 μg/ml) with toxin A (>3 μg/ml) in the small intestine. In certain animal models, the disruption of the epithelium by toxin A probably facilities the entrance of toxin B (Lyerly *et al.*, 1985b).

Mitchell *et al.* (1986) investigated the effects of toxins A and B on both rabbit ileum and colon and found that only toxin A was active. The ileum was more vulnerable morphologically and more prone to secrete fluid than the colon, and that damage progressed from localized destruction of the villus tips

to entire destruction of villi with hemorrhage in the ileum. These finding led to hypothesize that fluid accumulation results primarily from cell disruption at the basement membrane (Lima *et al.*, 1989).

There has been limited evidence that *C. difficile* toxins alone are secretagogues. In rabbit ileal loops, toxin A causes a significant net accumulation of sodium, chloride, potassium, and total protein at 6 hr; but such fluid secretion was observed only in the presence of significant mucosal damage (Lima *et al.*, 1988). This is in contrast to the previous report by Hughes *et al.* (1983), who suggested that a crude extract of *C. difficile* toxins could abolish net sodium absorption and induced net chloride secretion in the absence of visible histological damage in Ussing chambers.

Moore *et al.* (1990) demonstrated that *in vitro* toxin A could correlate the perturbation in intercellular tight junctions of the epithelial cells from guinea pig ileum with increased chloride secretion and diminished sodium absorption in Ussing chambers. It is notable that permeability was increased in systems that included intact lamina propria that were relatively devoid of neutrophils. Similarly, in a human intestinal, perturbations of barrier function of these epithelial cells line and increased tight junction permeability were associated with alteration of the cytoskeletal structure (Hecht *et al.*, 1988). Thus, it appears that toxin A may alter intestinal barrier function directly through its effects on tight junction permeability *in vitro*; it may also elicit a secretory response indirectly activating an inflammation response.

2.3.1.3 Immuno characterization of toxins A and B

Toxins A and B are largely immunologically distinct. Specific antisera raised against toxin A that neutralize its biological activity do not affect toxin B. Similarly, antibodies that specifically block the activity of toxin B have no effect on toxin A (Banno *et al.*, 1984; Taylor *et al.*, 1981). Kamiya *et al.* 1991 identified a monoclonal antibody (37B5) that neutralized the enterotoxicity but

not the hemagglutination activity of toxin A and concluded that the epitopes associated with the enterotoxicity and hemagglutination activity of toxin A were differents entities. Another monoclonal antibody, PCG-4, actually precipitates the toxin, has no effect on cytotoxicity but neutralizes its enterotoxicity (Lyerly, 1985). The antibody blocks the binding of the toxin to the Gal α1-3 Gal β1-4 Glc Nac receptor, indicating that the antibody recognizes the binding and not the active site on toxin A. The antibodies that neutralizes the enterotoxic and the cytotoxic activities of toxin A are distinct (Lyerly *et al.*, 1986a).

2.3.2 Toxin genes

The toxin genes are located next to each other, with a small open reading frame (ORF) in between. Hammond and Johnson (1995) defined the region of the *C. difficile* VPI 10463 chromosome containing the toxin genes as the toxigenic element. These toxin genes are arranged on a 19.6 kb toxigenic element and have their own promoters and ribosome binding sites, although they can be expressed from a single common promoter. This toxigenic element has been shown to be common only in the toxigenic strains. In non-toxigenic strains, there is a stretch of 127 base pairs of non-coding DNA instead of this region. The toxigenic element is comprised of five ORF, three small ORF, toxin A and B genes. One of the small ORFs is located upstream of toxin B (*orftxe* 1 in Figure 1, which has been designated as *txe* R. The *txe* R gene encodes for a 22 kD protein which contains a helix-turn-helix motif with sequence identity to DNA-binding-regulatory gene. Further more, the *txe* R could also positively regulate the expression of the toxin A gene. The extent to which *orftxe* 2 can positively regulate toxin A, and the extent which *orftxe* 3, which is transcribed in the opposite direction, can down-regulate either toxin (Hammond and Johnson, 1995 ; Hammond *et al.*, 1997 ; Moncrief *et al.*, 1997).

The toxin B gene is 7,098 bp long, codes for 2,366 amino acids. The gene contains 27.4 mol% G+C and a deduced polypeptide molecular weight of 269,696 D. The 3' end of this sequence is contiguous with the 5' flanking region of the small ORF which is located between toxins A and B (Barroso *et al.*, 1990). A small ORFs (ca. 500 bp) is located 122 base downstream from the stop codon of the toxin B gene (Dove *et al.*, 1990). Toxin A gene has 8,133 bp and contains 26.9 mol% G+C. The gene encodes a protein with a deduced molecular weight of 308,000 which consists of 2,710 amino acids (Dove *et al.*, 1990). The amino acids sequence at the N-terminal end of toxin A has been determined by microanalysis after electrophoresis under denaturing conditions. The first 10 amino acids agreed with the first 10 deduced amino acids of the toxin A ORF, indicating that there was no posttranslational modifications involving a signal peptide (Matsudaira, 1987). This gene has the repeating sequences at the 3' end. A total of 2,551 nucleotides, or 31.5% of the gene, are in 38 contiguous repeating units. The only strongly hydrophobic region in the deduced protein is from residues 1,050 to 1,100. There appears to be a periodicity in the hydropathic index within each repeat region. However, the repeat region is the most hydrophilic part. At the 3' end of the gene has been used to express the major antigenic and carbohydrate binding sites of toxin. In fact, antiserum against this portion of protein neutralizes the enterotoxicity of toxin A. Further evidence indicated that the repeating units represent the binding portion which constituted about one third of the entire toxin molecule. The repeats were highly hydrophilic. It would be interesting to determine the spatial distribution of these repeats in the native protein and whether a periodicity on the surface of the toxin molecule confers certain unique biological properties to the protein (Dove *et al.*, 1990). When the genes have been sequenced, it was shown that the deduced amino acid sequences contain 49% identity and 63% similarity when conserved substitutions and considered, suggesting that the toxins arose by gene

duplication. Moreover, the proteins share a number of similar structural features, including a putative nucleotide binding site, a central hydrophobic region, four conserved cysteines, and a long series of repeating units at their carboxyl ends (von Eichel-Streiber, 1992; von Eichel-Streiber *et al.*, 1992).

2.3.3 Toxin receptor

Toxin A has a series of repeats at the COOH-terminus that represent the binding of the molecule. Toxin A binds to specific glycoconjugate receptors. The carbohydrate receptors have been identified on rabbit erythrocytes, hamster brush border membrane, human intestinal epithelial and rabbit ileal brush border and are likely present on human granulocytes. It was further deduced that the receptor on rabbit erythrocytes and brush border membrane of the hamster included the trisaccharide Gal α1-3 Gal β1-4 Glc Nac, the full sequence of which is necessary for recognition by the toxin (Krivan *et al.*, 1986; Clark *et al.*, 1987). The terminal alpha galactose residue is very important. Toxin A does not bind to erythrocytes treated with either alpha galactosidase, which enzymatically cleaves the terminal alpha galactose. The entire trisaccharide sequence is needed for recognition by the toxin (Lyerly *et al.*, 1988). The carbohydrate receptors Gal α1-3 Gal β 1-4 Glc Nac identified by Krivan *et al.* (1986) was not present on normal human cells and therefore likely had no role in the pathogenesis of *C. difficile* toxin A-mediated disease in human. Three carbohydrate antigens, I, X and Y, that do exist on human intestinal epithelium were found to bind toxin A (Tucker and Wilkins, 1991). These all shared the disaccharide Gal β1-4 Glc Nac, the minimum carbohydrate structure bound by the toxin A. Furthermore, it has been noted that human granulocytes express large amounts of the X antigen (Spooncer *et al.*, 1984). As granulocytes likely participate in the pathogenesis of *C. difficile* associated diarrhea and colitis, there is increasing speculation that toxin A produces colonic damage not only through its direct effects on epithelial cells

but also via inflammatory mediators released from granulocytes (Triadafilopoulos *et al.*, 1991).

Toxin B has been reported to bind to human erythrocytes ghosts and to rabbit erythrocytes. Although, the toxin B also acts on tissue outside of intestine but the receptor for the toxin and the target tissue have not been identified to date (Lyerly *et al.*, 1988).

2.3.4 Toxins interactions with the immune system

The final pathogenic mechanisms in *C. difficile* mediated colitis and diarrhea likely involve some interplay between the direct effects of its toxins on intestinal epithelium and their indirect effects via activation of the host inflammatory response. Toxin A is ability to elicit the acute inflammatory response by activation of macrophages, mast cells and mobilization of neutrophils. The signaling mechanism involved in this inflammatory response are quite complex and involving the release of the potent proinflammatory mediators and cytokines, including prostaglandin E2 (PG E2), leukotriene B4 (LT B4), leukotriene C4 (LT C4), platelet activating factors (PAF), interleukin-1 (IL-1), interleukin-8 (IL-8), and histamine (Pothoulakis and LaMont, 1993). Borriello and Bhatt (1995) provided evidence that there was a requirement for recruited neutrophils in order to generate tissue damage. A possible mechanism for this includes an apparent toxin A stimulation of macrophage cytokine production (tumour necrosis factor alpha, interleukin-1 beta, leukotrienes) which leads to the neutrophil migration (infiltration) and consequent inflammation (Ohguchi *et al.*, 1996). The stimulation of mucosal mast cells resided in the intestinal lamina propria causes the release of several mediators such as PGD2, LTC4, and E4, PAF, histamine, serotonin, and protease II (Pothoulakis *et al.*, 1993). Previous studies indicated that histamine PGs, LTs, and PAF were involved in the cellular mechanisms of fluid secretion and diarrhea (Powell, 1991). Mucosal mast cells also participated in

the induction and amplification of intestinal inflammatory responses (Perdue *et al.*, 1990). More recent studies suggested a stimulatory effect of toxin A on granulocytes. Toxin A directly activated human granulocytes *in vitro*, inducing a transient rise in unbound cytosolic calcium that was rapid and dose-dependent while promoting chemotaxic and chemokinesis (Pothoulakis *et al.*,1988; Triadafilopoulos *et al.*, 1991). Toxin B also induced the release of inflammatory mediators from human monocytes and increases cell-mediated cytotoxicity (Flegel *et al.*, 1991; Siffert *et al.*1993). Flegel *et al.* (1991) showed that toxin B was more potent in the activation of human monocytes, inducing production of interleukin-1, interleukin-6, and tumour necrosis factor than toxin A. Hence, in human, toxin B may first activate monocytes, thereby promoting inflammation, and then kill these cells.

Libby *et al.* (1982) found that in order to protect hamster from colonization with highly toxigenic strains of *C. difficile*, vaccination with both toxins was necessary. In contrast, Kim *et al.* (1987) showed that vaccination against toxin A alone was protective in hamsters. Corthier *et al.*1991 reported that in gnotobiotic mice, passive immunity conferred by monoclonal antibodies to toxin A was protective against pseudomembranous colitis. Although *C. difficile* is not invasive, its toxins are capable of inducing an antibody response by the host, suggesting an ability to cross the mucosal barrier. Individual serum response to toxin A widely in a clinical study characterizing toxin A-specific serum and secretory antibody responses in patients with *C. difficile* colitis. The patients with *C. difficile*-associated diarrhea had dramatic increases in convalescent titers, especially IgA antibody while the others had no change. Though the statistical analysis was hampered by small numbers, it appeared that the serum antibody response to natural infection did not effectively protect against relapse (Johnson *et al.*, 1992).

2.4 Other virulence factors

Other factors, such as production of proteolytic and hydrolytic enzymes, expression of fimbriae and flagella, chemotoxis, adhesion to gut receptors and production of capsule, may all play roles in pathogenesis by facilitating colonization or by directly contributing to tissue damage, or both.

2.4.1. Hydrolytic enzyme

The most detailed study of hydrolytic enzymes that may be involved in the breakdown of connective tissue was undertaken by Seddon and colleagues (1990), who showed that most of the strains examined had hyaluronidase, chondroitin-4-sulphatase and heparinase activity, though the heparinase activity was generally weak. Highly virulent strains were more active than less virulent strains. Collagenase activity was also present, but was generally weak and more restricted to highly virulent strains.

2.4.2. Adhesin, fimbriae and flagella

Adhesion to host tissue is important for full expression of virulence for many pathogens. In hamster model disease, a highly virulent toxigenic strain adhered better than a poorly virulent strain, and both strains adhered better than an avirulent non-toxigenic strain. In all case adherence was most pronounced in the terminal ileum and cecum, in keeping with the pathology of ileocaecitis. A number of factors can be involved in binding to mucus and cells. There have been several attempts to identify adhesins of *C. difficile*. Fimbriae were detected in 1988 and shown to be polar, 4-9 nm in diameter and 6 μm in length. However, their roles in colonization was unclear, especially as no correlation could be found between presence of fimbriae and the relative ability of different strains of *C. difficile* in adhering to hamster gut mucus. Many strains of *C. difficile* are motile and have flagella, but it is not known whether these flagella also serve as adhesion (Borriello *et al.*, 1988a; Borriello

et al., 1988b). Physiochemical properties of microorganisms may also contribute to adhesion. *C. difficile* cell surfaces are moderately hydrophobic, even when grown in *ex-vivo* conditions, and carry a net positive charge. This charge is evenly distributed and resides predominantly in the cell wall. Charge interactions with negatively charged host cells may contribute to gut colonization (Krishma *et al.*, 1996; Borrillo, 1998).

2.4.3. Capsule

C. difficile requires opsonization for significant phagocytosis, suggesting that there might be an anti-phagocytic factor on its cell surface. Removal of the cell surface carbohydrates did not affect the degree of phogocytosis, suggesting that *C. difficile* might have a polysaccharide capsule (Dailey *et al.*,1987; Borriello *et al.*, 1990).

2.4.4. Chemotaxis

Borriello (1998) reported that the degree of chemotaxis correlated positively with the relative virulence of the strains examined in a hamster model, indicating chemotaxis was impossible without motility.

3. *C. difficile*-associated disease

3.1 Epidemiology

C. difficile has been shown to be implicated in 15-25% of patients with antibiotic-associated diarrhea, in 50-75% of the patients with antibiotic-associated colitis, in more than 90% of those with pseudomembranous colitis and in 5-70% of healthy neonates (Bartlett *et al.*, 1980; Bartlett, 1994). Prevalence studies in infants have shown that healthy newborns frequently become colonized with *C. difficile* during the first two weeks of life. Acquisition frequencies of 0-63% have been reported. After the first two

weeks of life, colonization rate remains constant until about 1-2 years of age. In this age range, prevalence has been reported to be between 7% and 60% (Spencer, 1998a). Holst *et al.* (1981) showed that 64% of 218 healthy neonates with the age group between 1 to 8 months carried this organism. The reported carrier rate in children with more than 2 years of age is less than 4% (Stark *et al.*, 1982; Svedhem *et al.*, 1982). El Mohandes *et al.* (1993) investigated 50 preterm babies housed in an intensive care facility over the first 34 days of life, sampling at weekly intervals. They found that 15% of the babies had culture positive during the first week. The rate was increased to 33% at the second week and was maintained at this rate throughout the study. Isolates were also tested for toxin B production. These investigators showed that between 71% and 100% of isolates produced toxin B over the sampling period. Such marked differences probably reflect different degrees of nosocomial environmental exposure in different nurseries, rather than differences in diet or rates of maternal colonization.

C. difficile is presumably acquired from the environment or from a colonized birth canal (Tabaqchali *et al.*, 1984b; McFarland *et al.*, 1989). Approximately 3% of healthy adults harbor this organism as a component of the normal flora, although it is not known if this represents transient colonization or a component of the stable flora (Viscidi *et al.*, 1981). Alternatively, the patient may acquire *C. difficile* from an environmental source. However, others have reported that the carriage rates in healthy adults were vary from 0-3% in Europe (Dodson and Borriello, 1996) and up to 15% in Japan (Nakamura *et al.*, 1981a).

C. difficile disease occurs both sporadically and in clusters or outbreak in hospitals, nursing homes and chronic care facilities, but the frequency of the disease is much lower in the community. *C. difficile* has been increasingly recognized as an important nosocomial enteric pathogen associated with outbreaks of diarrhea and colitis in hospitalized adults receiving antimicrobial

therapy (Brown *et al.*, 1990). It may also be found in the stool of 10% or more of hospitalized adults without diarrhea who have received antibiotic or cancer chemotherapeutic agent (Viscidi *et al.*, 1981; McFarland *et al.*, 1989).

The frequency and incidence of *C. difficile* disease varies widely not only geographically but within different institutions in the same area, and depends on patterns of the antimicrobial use, on the antimicrobial resistant patterns of the prevalent *C. difficile* isolates, on the epidemiologic factors favoring transmission of the organism, on patients risk factors, on clinicians index of suspicion, and especially on the frequency with which endoscopy and/or various laboratory tests for the presence of toxins A and B in stools performed on patients with antibiotic-associated disease (Fekety, 1997).

3.2 Reservoirs

The two major potential reservoirs of *C. difficile* in hospitals are infected human (symptomatic and asymptomatic) and inanimate objects. *C. difficile* , either as vegetative organisms or as spores, can be isolated from the stools of asymptomatic patients as well as from patients with diarrhea. Patients with *C. difficile* diarrhea, however, normally excrete larger numbers of organisms in feces, and bacterial spores have been found in abundance in the environment of individuals with disease. The organism has also been found on the hands of healthcare workers dealing with affected patients (Kaplan *et al.*, 1996). While the intestinal tract has been considered the most important endogenous reservoir of *C. difficile*, the organism was also isolated from urethra and vagina, respectively, from 100% of men and 71% of women attending a sexually transmitted disease clinic (Hafiz *et al.*, 1975). Tabaqchali *et al.* (1984b) detected the *C. difficile* vaginal carriage in 11-18% of pregnant women. However, Larson *et al.* (1982) were unable to detect urogenital carriage of *C. difficile*. Thus, the frequency of extraintestinal carriage of this

organism has not yet been clearly defined. C. *difficile* is able to survive for long periods of time in the hospital environment in the form of heat-resistant spores. Fekety *et al.* (1981) had cultured *C. difficile* from floors, hoppers, toilets, beddings, mops, scales, and furniture. Spores of *C. difficile* can survive for months on the floors of the hospital rooms, long after symptomatic patients have been discharged. The organism was also cultured from the medical apparatus and fingers of asymptomatic hospital personnel. It has been convincingly shown that an existing reservoir of *C. difficile* is not a prerequisite for symptomatic infection and that the disease causing organism may be acquired from exogenous sources. Arguing for the importance of environmental acquisition of *C. difficile* are the number of outbreaks that have been reported in hospitals, long-term care facilities, and day care centers (Kim *et al.*, 1983; Delmee *et al.*, 1986a). In addition, *C. difficile* is found in many sources outside the hospital. Hafiz and Oakley (1976) cultured the organism from soil, mud and sand and several animals. Carriage rates in household pets such as dogs and cats ranges from 20-40%. Riley *et al.* (1991) proposed that the organism might be zoonotically acquired.

3.3 Risk factors

The elderly and the debilitated are most susceptible to *C. difficile* infection, although other groups at high risk have been identified including the patients with severe underlying disease and chronic illness patients in hospital (McFarland *et al.*, 1990). However, the most pre-eminent risk factors is the use of antibiotics and anticancer drugs. The use of antibiotics can disrupt the normal ecosystem and allow *C. difficile* to become established, colonize the empty bowel and produce toxins. Although most antibiotics have been associated with the predisposition to *C. difficile* infection but the most commonly implecated are clindamycin, cephalosporins and ampicillin (Spencer, 1998). Less frequently incriminated are erythromycin,

aminoglycosides, fluoroquinolones, sulfamethoxazole-trimethoprim, quinolones, ureidopenicillins, rifampicin and perhaps surprisingly, both vancomycin and metronidazole, which are the drugs of choice for treatment of *C. difficile* diarrhea (Mitty and LaMont, 1994). A survey in Canada by Gurwith *et al.* (1977) showed that diarrhea occurred in 5% of ampicillin recipients and 18% of clindamycin recipients, but PMC was diagnosed in 0.3% and 2.0% of these recipients, respectively. In addition, *C. difficile* disease has been associated with several antineoplastic agents including adriamycin, cyclosphospamide, 5-fluorouracils and methotrexate, all of which have antibacterial activity (Cudmore *et al.*, 1982; Barc *et al.*, 1992). Hospitalization for longer than 4 weeks significantly increased the risk of *C. difficile* associated diarrhea (Anand *et al.*, 1994). It is possible that the elderly are at risk because they are subjected to higher-risk procedures, such as frequent antimicrobial administration and longer hospitalization. There has been a lot of evidences for a long time that patients on antibiotics would have a higher risk of infection when they were more than 60 years old because gut flora of the elderly had a less effective colonization resistance capability. The implication of this is that a smaller effect of antibiotics may be needed to induce suscepibility to colonization by *C. difficile* than is the case for younger patients (Borriello, 1998). The strong association between *C. difficile* carriage and old age, increasing degrees of underlying illness and increased length of stay probably reflect the fact that much patients have more opportunitics for acquisition of *C. difficile* by virtue of longer hospitalizations and more frequent contacts with healthcare workers (McFarland *et al.*, 1990).

3.4 Transmission

Transmission of *C. difficile* requires the organism or its spores to reach the patients gastrointestinal tract, either by ingestion or by direct inoculation into the bowel via contaminated equipment. Outbreaks in hospitals suggest

that, generally, transmission is either via staff's hands or by direct contact with affected patients, contaminated surfaces or formites (Cartmill *et al.*, 1994). Personnel transient hand carriage probably accounts for the majority of hospital transmission of *C. difficile* (McFarland *et al.*, 1989). In addition to the convincing evidence for horizontal transmission in the hospital setting, there has been some suggestions that vertical (maternal-child) transmission might occur. As noted earlier, neonates frequently experience asymptomatic acquisition of *C. difficile*. Hafiz *et al.* (1975) reported that 71% of women attending a clinic for sexually transmission disease had positive vaginal culture but other investigators could not confirm this finding and provided alternative evidence for infant acquisition from other external sources. Al-Jumaili *et al.* (1984) suggested that the same mode of nosocomial transmission was operative in the neonatal unit as in other parts of the hospital frequently.

3.5 Clinical manifestations

The symptoms of *C. difficile* disease usually appear after 5-10 days of antibacterial treatment, but they may develop as early as the first day of therapy or as late as 10 weeks after the cessation of therapy (Tedesco, 1982). The clinical manifestations of infection with this organism have ranged from asymptomatic carriage to mild self-limited diarrhea, to severe, PMC, toxic megacolon, and fulminant colitis. Most frequently, *C. difficile* disease is manifested by diarrhea, which may be brief and self-limited or cholera-like, resulting in more than 20 stools per day (McFarland and Stamm, 1986). Many patients with *C. difficile* diarrhea have fever that exceeds 40°C and leukocytosis as high as 50,000 per mm; in fact, leukemoid reactions in range of 100,000 per mm^3 have been reported (Kelly *et al.*, 1994b). Although colitis can occur throughout the colon, it is usually most severe in the distal colon and rectum. When patients develop colitis localized to the cecum and right side of the colon, they may have little or no diarrhea. Instead, fever, marked

right-sided lower abdominal pain and tenderness marked leukocytosis, and decreased intestinal motility may be the only clues to the disease. *C. difficile* colitis rarely presents without diarrhea as an acute abdominal syndrome or toxic megacolon. Although, this presentation is rare but is especially serious, in part because diagnosis and treatment may be delayed because of the lack of diarrhea (Triadifopoulos and Hallstone, 1991). Unrecognized and untreated, *C. difficile* colitis may be fatal. Death rates of 10-20% have been reported in elderly and debilitated untreated patients. With specific-therapy, the mortality rate is less than 2 % (Morris *et al.*, 1990). Other disease entilies associated with *C. difficile* include septicaemia, wound infections, splenic abscess, osteomyelitis, peritonitis and urogenital tract infection (Levett, 1986).

3.6 Treatment

The treatment options for *C. difficile* infection remain limited, although promising agents are currently being assessed. Two specific treatments of *C. difficile* infection are available, namely oral metronidazole and vancomycin (Wilcox, 1998). Therapy given by the oral route is always preferred, because *C. difficile* remain within the colonic lumen without invading the colonic mucosa, and the toxins produced by the organism within the intestinal lumen cause disease only after binding to specific receptors on the colonic mucosa (Cleary, 1998). Oral therapy with metronidazole (250 mg four times a day for 10 days) is the recommended first-line therapy because of its lower cost and the absence of metronidazole-resistant strains. Vancomycin is also effective, but its use must be limited to decrease the development of vancomycin-resistant organism such as enterococci. Vancomycin (125-500 mg four times a day for 10 days) limited to those who cannot tolerate or have not responded to metronidazole, or when metronidazole use is contraindicated, as in the first trimester of pregnancy. A therapeutic response within a few days is usual. Bacitracin, teicoplanin, and nonabsorbable anion binding resins such as

cholestyranine may be given orally for treatment of mild *C. difficile* diarrhea, but these agents are neither as reliable nor as rapidly effective as metronidazole or vancomycin (Wilcox, 1998; Fekety, 1997; Surawicz and McFarland, 1999). Occasionally, patients with *C. difficile* infection are unable to take oral medication. Either metronidazole or vancomycin can be given via a nasogastric tube or by intravenous in such cases (Wilcox, 1998).

3.7 Recurrence

Recurrence (relapse or reinfection) of diarrhea or colitis are recognized when there is a return of typical symptoms, signs and positive diagnostic tests a few weeks to months after discontinuation of successful antibiotic therapy for *C. difficile* infection. Because the patients had previously responded, these occurrences should not be thought of as treatment failures. Although nearly all patients respond to therapy with oral metronidazole or vancomycin, but many patients recurrence occur in about 15-25% of patients (Fekety *et al.*, 1989; Wilcox and Spencer, 1992). The cause of the recurrence is the failure to eradicate the organism that occurs because metronidazole and vancomycin could not reliably kill the spore forms of the organism (Delmee *et al.*, 1986a). Recurrences may be caused either by persistences of the original strain of *C. difficile* or by reinfection with the same or a different strain (Tabaqchali *et al.*, 1984b). However, it is now clear that the majority of these episodes is due to the reinfection with new *C. difficile* strains, not the relapse caused by the original bacterium (Wilcox, 1998). Relapsing *C. difficile* infection is a serious, difficult, and still unsolved management problem, especially when patients have experienced three or more episodes (Tabaqchali *et al.*, 1984b). There has been evidence of a poor immune response to *C. difficile* toxins in elderly patients, and it is known that antibodies to *C. difficile* are reduced in these patients. Elderly patients and those who have recently undergone abdominal surgery are more prone to relapse (Young *et al.*, 1986).

Relapses of *C. difficile* colitis are common and can be more difficult to treat. Tedesco *et al.* (1985) reported that tapering and pulsed or intermittent therapy with metronidazole or vancomycin was effective in curing multiple relapses. Another exciting approach to treat the relapses is the use of biotherapy. Biotherapy aims to restore the commensal gut flora and hence colonization resistance against *C. difficile* (Wilcox, 1998). Biotherapy included *Lactobacillus* GG (Gorbach *et al.*, 1987), *Enterococcus faecium* SF68 (Lewenstein *et al.*, 1979), non-toxigenic *C. difficile* (Seal *et al.*, 1987), Yoghurt (Siitonen *et al.*, 1990) and nonpathogenic yeast (*Saccharomyces boulardii*) (McFarland *et al.*, 1994). The use of competing microorganism have been devised for relapsing disease. Immunotherapy for *C. difficile* infection has also been considered. The nature and role of the immune response to *C. difficile* infection is an area of interest which may ultimately lead to the development of effective vaccines or other forms of immunotherapy (Torres *et al.*, 1995; Ryan *et al.*, 1997). Another possible approach to passive immunotherapy is the use of bovine colostrum from dairy cow immunized with *C. difficile*. Bovine colostrum is rich in IgG, unlike human colostrum which is enriched with IgA, and level of specific IgG antibodies can be increased markedly following immunization with *C. difficile* toxoids. A bovine immunoglobulin concentrate has been reported to inhibit the cytotoxicity and enterotoxicity of *C. difficile* toxin (Kell *et al.*, 1996). In children with various immunoglobulin, immunotherapy with intravenous immune globulin has appeared effective in prevention of recurrences in anecdotal reports (Larson *et al.*, 1982; Kim *et al.*, 1983). Relapsing episodes do not necessarily progress in severity, and standard therapy with metronidazole or vancomycin is usually successful. Preventing further recurrences is a more complex problem, and some patients develop a protracted, chronic illness (Fekety, 1997; Fekety *et al.*, 1997).

3.8 Prevention and control

In the prevention and control of *C. difficile*, it is important to develop the ways to interrupt transmission within hospitals, nursing homes, and to reduce the risks factor of disease. Medical personnel should carefully wash their hands before and after contact with all patients. The use of disposable gloves and stool isolation precautions when contacting the patients with *C. difficile* diarrhea or who are carriers of this organism (Kim *et al.*, 1981). The hospital environment such as commodes, baby baths and electronic thermometers (stethoscopes, blood pressure caffs) should be cleaned after use and disinfected or sterilized according to the local infection control policy (Worsley, 1998). Alkaline glutaraldehyde, sodium hypochlorite, and ethylene oxide are effective in killing the spores as well as the vegetative forms of *C. difficile* that persist on fomites, instruments and contaminated surfaces. None of these are satisfactory for handwashing, which is still best carried out using ordinary disinfectant soaps or chlorhexidine, with limited power to kill *C. difficile* spores (McFarland *et al.*, 1989; Fekety, 1996). Hospitalization and intensive exposure to antibiotics are important risk factor for acquisition of *C. difficile*. Avoidance of the unnecessary use of antimicrobial drugs is of obvious importance. The use of narrow-spectrum antibiotic and regular prescription review to ensure that antimicrobial are discontinued as soon as possible are also suggested (Fekety, 1997; Worsley, 1998). In addition, some authorities justifiably recommend isolation and formal enteric precautions in patients with known *C. difficile* diarrhea to prevent its spread (Wilcox and Spencer, 1992). It is important that everyone involved with patient care in hospitals, nursing homes, and at home be educated about the organism and its epidemiology, about rational approaches to the treatment and care of patients with *C. difficile* diarrhea, about the importance of handwashing between contact with patients, about the use of gloves when caring for a patient with *C.*

difficile diarrhea, and about the avoidance of the unnecessary use of antimicrobials (Fekety, 1997).

4. Laboratory diagnosis

4.1 Detection of *C. difficile* toxins

4.1.1 Tissue culture assay

Tissue culture assay for the detection of the cytotoxin activity in stool from patients with antibiotic associated colitis (AAC) was the initial observation leading to the discovery of *C. difficile* as the causative agent of this infection. Virtually all *C. difficile* produce either both toxins or neither toxin, permitting assay for cytotoxic activity as reliable diagnostic test for *C. difficile*-associated diarrhea, regardless of whether toxin A or toxin B, is the primary agent to be involved in the pathophysiology of this disease (Gerding *et al.*, 1995). The assay has still been considered as the gold standard for *C. difficile* toxin B in stool specimens because of its high sensitivity (1pg of toxin B is sufficint to cause the rounding of the cells) and specificity (neutralization of the cytopathic effect only by a specific *C. difficile* antiserum (Lyerly *et al.*, 1988). On the other hand, this method is time-consuming (up to 24 hr of incubation), requires expertise in tissue culture techniques, specimen-handling, and lacks of the standardization technique. Thus, these is a need for alternative rapid, accurate, and easy to perform screening tests (Barbut *et al.*, 1993).

4.1.2 Counterimmunoelectrophoresis assay (CIE)

The counterimmunoelectrophoresis (CIE) has been recommended to be another method for detection of cytotoxin of *C. difficile*. This test is considerably faster than tissue culture assays (1.5 hr compared to overnight). Thus, the antiserum used in the clinical trials was prepared against culture filtrates of *C. difficile* and the immunoprecipitin bands detected in most stool specimens do not represent the toxins. Nontoxigenic strains of *C. difficile*, as

well as strains of *C. sordellii*, *C. bifermentans*, react with the antiserum and give false-positive reactions in the assay. The cross-reaction are most likely occur due to the cell surface antigens shared by these species (Lyerly *et al.*, 1988; Brazier, 1993).

4.1.3 Latex particle agglutination assay

The latex particle agglutination test was originally designed to detect toxin A. This assay is simple, rapid, and inexpensive. However, subsequent studies have demonstrated that it also detected a nontoxic enzyme (glutamate dehydrogenase) not toxin A. This enzyme is produced by both toxigenic and nontoxigenic strains of *C. difficile* as well as other microbes and it appears to play no role in the pathogenic of *C. difficile* diarrhea. Despite its rapidity and convenience, this assay is not sufficiently sensitive to justify the routine use (Lyerly *et al.*, 1991; Fekety, 1997).

4.1.4 Enzyme immunoassay (EIA)

The enzyme immunoassay is based on the detection of toxins A and /or B with specific monoclonal or polyclonal antibobies. In 1981, Yolken *et al.* evaluated an EIA in the detection of *C. difficile* toxin in fecal samples. From the 277 specimens examined, the 84 positives by EIA were also positive for cytotoxin. The authors reported discrepant result in stool from three patients who had history of treatment for *C. difficile* associated diarrhea, which ever EIA positive but cytotoxin negative and made a conclusion that the method did not give false positive results. The excellent results of 100% sensitivity and 98.4% specificity paved the way forward for the other studies using this method. There have been numerous publications regarding the efficacy of commercial EIA kits (Borriello *et al.*, 1992; Knapp et al., 1993; Brazier, 1998b). Most of the kits are designed to detect toxin A, but a few are designed to detect both toxins. They are rapidly performed and relatively inexpensive

when done in batch, and they are probably the most widely used laboratory aids in the United States in diagnosing *C. difficile* diarrhea. The EIA tests are more specific than they are sensitive. The sensitivity of the commercial EIA kits used for toxins A and B detection has varied widely when evaluated in different laboratories using the same kits but different criteria for a positive endpoint. Therefore, the sensitivities have ranged from as low as 70% to as high as 95% while the specificity has been shown to be generally very good. On average, EIA tests for toxins A and/or B failed to detect about 10% (range 5 to 33%) or more of cases from *C. difficile* diarrhea diagnosed clinically and by endoscopy with biopsy or by use of toxin A assays in cell culture (Fekety, 1997). In 1988, Premier EIA test for toxin A was the first EIA based on monoclonal antibodies against toxin A to be approved by the Food and Drug Administrated (Barbut *et al.*, 1993). This commercial kit has been studied most extensively and has demonstrated acceptable performance for toxin detection, with a mean test sensitivity of 77.5% (range, 65 % to 88 %) and a mean specificity of 98.6% (range, 95% to 100%) (Gerding *et al.*, 1995). Although, not as sensitivity as the tissue culture assay, EIA provide speed with reasonable accuracy, sensitivity, specificity, and cost.

4.1.5 Molecular methods; Polymerase chain reaction (PCR)

The detection of the toxin A and B genes of *C. difficile* by using PCR technique offer the potential for increased speed, sensitivity and specificity. The PCR protocol used by Wren *et al.* (1990) amplified 63 bp tandem repeat nucleotide sequences of the *C. difficile* toxin A gene. Although they could distinguish toxigenic *C. difficile* strains from nontoxigenic *C. difficile* strains by the PCR assay, the positive amplification was also seen with DNA of *C. sordellii.* Kato *et al.* (1991) also used PCR methodology to detect both non-repeating and repeating segments of the toxin A gene in *C. difficile* isolates. They could differentiate 26 nontoxigenic from 35 toxigenic strains, and

demonstrate no adverse cross-reactions with other clostridia. McMillin *et al.* (1991) used the multiplex PCR to amplified a 1050 bp fragment of the toxin B gene and a 1217 bp fragment of the toxin A gene of *C. difficile*. This multiplex PCR procedure could to differentiate 42 toxigenic from 10 nontoxigenic strains. Wren *et al.* (1993) used a PCR method to amplify the tandem repeat sequence of the toxin A gene and a non-repeat sequence of toxin B gene and could be able to distinguish between the toxigenic and the nontoxigenic strains. The toxins A and B specific primers used could distinguish between 184 toxigenic and 34 nontoxigenic strains. The result obtained was consistent with those from the other techniques included ELISA and cytotoxic assay. The authors also claimed that this method could detect as few as 20 cfu of toxigenic bacteria (toxin A) or 50 cfu of toxigenic bacteria (toxin B)/PCR sample. However, the test also had a few disadvantages such that the toxin A primer could also give a positive result with toxigenic strains of *C. sordellii* and toxin B primer occasionally produced the amplified produce incorrect size. Alonso *et al.* (1997) reported the use of a nested PCR method to detect the toxin B gene in 59 clinical isolates. A 322 bp product indicated the presence of the toxin B gene and these workers claimed a specificity of 100% and sensitivity of 100% as compared with cytotoxin detection. The seminested PCR method was used by Karasawa *et al.* (1999) for the detection of toxin A gene and toxin B gene in *C. difficile* isolates. From 58 toxigenic strains the investigators could amplify a 236 bp fragments of the toxin A gene and 862 bp of the toxin B gene. No PCR product could be obtained from 40 nontoxigenic strains and eight other clostridial species. Karasawa *et al.* claimed that the detection by PCR assay was compatibly to the tissue culture assay in the diagnosis of toxigenic *C. difficile*.

The next logical step was to bypass the need for culture and to apply molecular methodology directly to stool samples in order to obtain the rapid diagnosis. Kato *et al.* (1993) applied their earlier experience to amplify a

segment of the toxin A gene in stool specimens, but experienced difficulty with PCR inhibitory substances which required prior treatment by an ion-exchange column. In the 39 stool specimens examined in this report, PCR results agreed with both culture and cytotoxin results. Gumerlock *et al.* (1993) designed a primer which amplified a 399 bp sequence of toxin B gene and applied it to stool sample. In a small study of 18 cytotoxin positive specimens, they reported a 100% agreement by PCR and claimed an increased in the sensitivity to be as high as 100-fold over tissue culture methodology, detecting as little as 1 pg of DNA. In two out of 18 cytotoxin negative samples, they obtained a positive PCR result and claimed that this was a result of the increased sensitivity of the test because these two samples were obtained from the two patients who had the typical symptoms of CDAD. Boondeekhun *et al.* (1993) also developed a PCR method to apply directly to stools and they could amplify a 63 bp repetitive sequence of the toxin A gene. They reported a 94% correlation with stools yielding a positive cytotoxin result but also found one discrepant result. Arzese *et al.* (1995) used a PCR method to detect the toxin A gene fragments in feces from patients on a long-term care ward, and claimed that they could improve the detection of toxigenic strains. Alonso *et al.* (1999) applied the nested PCR technique to detect the toxin B gene in 102 stool specimens. A 322 bp product indicated the presence of the toxin B gene. The authors claimed a specificity of 100% and sensitivity of 96.3% as compared with cytotoxin detection.

4.2 *C. difficile* typing Methods

4.2.1 Methods based on phenotypic characteristics

4.2.1.1 Serogrouping, serotyping

The serogrouping method is based on slide agglutination of formol-treated cells with various rabbit antisera. Nakamura *et al.* (1981) were the first to perform the agglutination test using three antisera raised against *C. difficile*

to differentiate four serovars amongst the 79 isolates from healthy carriers. Delmee *et al.* (1985) developed a rapid slide agglutination method using the six absorbed antisera. They showed that some serogroups are homogenous, but the others, particularly serogroup A, are heterogenous and consisted of the strains with at least 12 different electrophoretic patterns.

Serotyping has been applied extensively in epidemiologic studies. Immunologic results from various laboratories in which different antisera were used were difficult to compare because of the lack of standardized reagents. The virulence of 10 *C. difficile* serogroups in hamsters was studied by Delmee and Avesani (1996) who reported that types A, C, H and K killed the challenged hamsters, while type G and nontoxigenic strains of serogroups B, D, I and X resulted in fecal colonization without disease. Serogroup F has been identified as producing toxin B, but not toxin A.

4.2.1.2 Bacteriophage and Bacteriocin typing

This system was based on the susceptibility of *C. difficile* to varies bacteriophages or bacteriocins. This technique was first developed by Sell *et al.* (1983) and has been successfully applied in the epidemiologic study. The number of these sensitivity markers has been expanded, and at least 40 different patterns have been observed (Tabaqchali, 1990). This method is not typeable for some strains, and is not available widely (Dei, 1989).

4.2.1.3 Antibiogram

Antibiogram has been used to differentiate among the strains of *C. difficile* but is of limited value in the epidemiologic study. Results of the *in vitro* studies have shown that the MICs of most antibiotics for *C. difficile* fall within a narrow range and that the strains are either highly sensitive or highly resistant, although some strains have variable resistance. Burdon *et al.* (1982) found a distinct pattern of resistance to three antibiotics in isolates from cases

on a surgical ward compared with isolates in the rest of the hospital. This method is, at best only rudimentary.

4.2.1.4 Gel Electrophoretic methods

Several electrophoretis methods based on cellular and surface protein patterns have been developed to be the very useful investigative tools; these include polyacrylamide gel electrophoresis combined with the radiolabeling or immunoblotting.

(a) Radio-polyacrylamide gel electrophoresis or PAGE of (^{35} S) methionine-labeled proteins

This method is based on the incorporation of (^{35} S) methionine into cellular proteins, the separation of these proteins on SDS-PAGE, and subsequent autoradiography. (Tabaqchali *et al.*, 1984a)

(b) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE of whole cell proteins was applied to the 79 isolates in an outbreak investigation by Costas *et al.* (1994). Their method yielded approximately 40 bands ranging from 18 to 100 kD; 60 of the 79 isolates were still indistinguishable.

4.2.2 Methods based on genotypic characteristics

4.2.2.1 Plasmid analysis

This method may be useful in following up the strains harboring specific plasmid patterns. However, it has the drawback that 30-60% of isolates may be plasmid negative (Clabots *et al.*, 1988). A multiple approach was described by Mahony *et al.* (1991) who used bacteriocin, bacteriophage

and plasmid analysis, but all three methods combined could type only 84% of 114 *C. difficile* isolates so this approach has little to be commended.

4.2.2.2 Restriction endonuclease analysis (REA)

REA of the genomic DNA using the restriction enzyme has been proven to be an excellent system capable of a highly discriminatory power and reproducibility. The main disadventage of REA is that the comparison of the complex pattern of DNA fragments usually requires comparing isolates that are run on the same gel. To compare the isolates from different institution, maintenance of a large type library was required. Another problem with REA is that frequently-cutting enzymes, such as *Hind* III, *Eco* RI, *Cfo* I or *Bam* HI produce such complex digestion products (>50 bands) that comparisons by eye are extremely difficult. This would be overcome by the use of modern image analysis equipment and computer software packages (Brazier, 1998b).

4.2.2.3 Restriction fragment length polymorphism (RFLP)

RFLP analysis involves initial restriction endonuclease digestion followed by gel electrophoresis, Southern blotting and hybridization with selected labeled nucleic acid probes to highlight specific restriction site heterogeneity. This method was first applied to *C. difficile* by Bowman *et al.* (1991). O'Neill *et al.* (1993) compared RFLP to REA in the study on the molecular characterization of *C. difficile* isolates from humans, animals and their environments. This study neatly illustrated the difference in discrimination between these methods as, from a total of 116 isolates, REA could differentiate 34 types compared with only six by RFLP analysis. Thus, RFLP analysis, in addition to being very labour-intensive, offers little advantage as a typing scheme.

4.2.2.4 Arbitraly primed PCR (AP-PCR)

AP-PCR permits the detection of polymorphisms without prior knowledge of the target nucleotide sequence. The 53 bp oligonucleotide primers are usually used singly, and have a non-specific sequence. Killgore and Kato (1994) used this approach to type 41 isolates from an outbreak of antibiotic associated diarrhea that had been previously typed by immunoblotting. There was agreement for 33/34 strains with a further seven isolates that were untypeable by immunoblotting but could be grouped by AP-PCR.

4.2.2.5 Random amplified polymorphic DNA (RAPD)

This method was closely related to the AP-PCR method. RAPD are usually used 2 oligonucleotide primers which are shorter in length (ca.10 bp) with arbitrary sequence. Barbut *et al.* (1994) evaluated a RAPD method using two 10 bp primers, AP4 and AP5, in an investigation of AAD in AIDS patients. They found the same PCR profile in 25 isolates from 15 patients, suggesting infection with the same strain, and claimed that RAPD was easy to perform and an effective way of distinguishing between isolates of *C. difficile*.

4.2.2.6 PCR ribotyping

PCR ribotyping uses specific primers complementary to sites within the RNA operon. This method was first applied to *C. difficile* by Gurtler (1993), who targeted the amplification process at the spacer region between the 16s and 23s rRNA regions. This region is very heterogeneous, in contrast to the rRNA genes themselves which are highly conserved. There is variation in spacer length not only between strains but also between different copies in the same genome. Gurtler could distinguish 14 different PCR ribotypes from 24 isolates. This method hence provided good discrimination and, because the primers were fixed and the targets were known, good theoretical reproducibility could be obtained. However, it required a long (18-96 hr) and

complicated denaturing PAGE procedure because it yield large PCR fragments of 800-1200 bp.

4.2.2.7 Pulsed-Field Gel Electrophoresis (PFGE)

PFGE differs from the conventional agarose electrophoresis in that it can separate very large DNA fragments by alternating the direction of the current between two sets of electrodes at an obtuse angle. This allows the whole chromosome to be analysed after digestion with rare-cutting restriction endonucleases, such as *Sma* I, *Sac* II or *Nru* I, which produce about 10 DNA fragments per strain. Consequently, analysis and comparisons of PFGE gels may be relatively simple. PFGE is highly discriminatory, uncomplicated to interpret and reproducible method (Brazier, 1998b). However, the limitations of this method relate primarily to the relatively long processing time required to isolate genomic DNA in agarose, the specialize equipment required to perform the electrophoresis and approximately 5% of isolates cannot be typed due to DNA degradation (Kristjansson *et al.*, 1994).

PFGE has been applied to many different genera. Kato *et al.* (1994) used PFGE to type 55 *C. difficile* isolates from a neonatal intensive care unit. The results were compared with those of immunoblot analysis. There was a good correlation between the two methods, and their results indicated the nosocomial spread of nontoxigenic *C. difficile* infection in the neonatal intensive care unit. Chachaty *et al.*, (1994) used the 30 unrelated strains of *C. difficile* belonging to 8 serotypes in the comparative study using PFGE, RAPD and PCR ribotyping. Their results indicated that the PFGE was more discriminatory than PCR ribotype assay, the ribotype assay was more discriminatory than RAPD and the three genotyping techniques were more discriminatory than serotyping. Thus, the RAPD were poorly reproducible. Kristjansson *et al.* (1994) compared the results from REA, PCR ribotyping and PFGE when they were used to differentiate 16 nosocomial isolates plus 17

other nosocomial isolates and 13 community acquired isolates. This work concluded that REA and PFGE have comparable discriminatory powers for epidemiologic typing of *C. difficile* isolates and the PCR ribotyping was appreciably less discriminatory. Talon *et al.* (1995) investigated 22 isolates of *C. difficile* from an outbreak in an elderly care facility and 30 epidemiologically unrelated isolates. Serogrouping was also performed and 2 epidemic serogroups, C and K were identified amongst the outbreak strains. Two different PFGE patterns were found among serogroup C isolates and three in serogroup K. All of the unrelated isolates had different PFGE profiles, illustrating the high degree of discrimination by this method. Kato *et al.* (1996) combined PFGE and immunoblotting to differentiate there infection from relapse in a 10 year old child who had suffered four episodes of CDAD. There was a good correlation between the two methods, and their results indicated that the second episode (after a 17 day tapering course of vancomycin) represented a relapse with the original strain, but that the third and fourth episodes were infection with the other different strains. They also typed five separate colonies from each episode to show that infection with multiple strains probably did not occur.

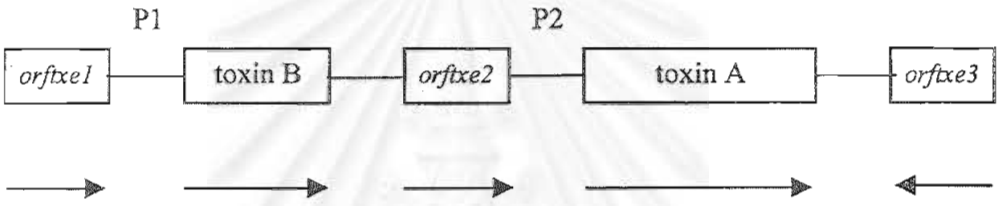
Van Dijck *et al.* (1996) used 56 isolates known to belong to the serogroup C and compared the performance of RAPD and PCR ribotyping. PFGE was found to be the most discriminatory among the three methods and a combination of RAPD and PFGE recognized 13 genotypes within serogroup C. Whilst PFGE is very discriminatory, drawbacks include the initial cost of the equipment, the slowness of the electrophoresis procedure and its complexity. These workers have also noted that some strains were repeatedly untypeable by PFGE because of the degradation during DNA the extraction. Later study showed that these PFGE untypeable strains belong to serogroup G (Kato *et al.*, 1996).

Figure 1 Diagrammatic representation of the 19.6 kb toxicon of *C. difficile* strain VPI 10463.

O*rftxe* 1, 2 and 3are small open reading frames.

P1 and P2 are toxin B and toxin A promoters respectively.

The arrows indicate the direction of transcription.



CHAPTER IV
MATERIALS AND METHODS

1. Stool specimen

Stool specimens were collected from 4 different groups of patients at Siriraj Hospital, Bangkok, Thailand, during the 7 month period (October 1998 to April 1999). The groups of patients were as followed:

1.1 Infants

1.1.1 Premature infants : Sixty-five freshly stool specimens were obtained from 65 premature infants.

1.1.2 Full-term infants : One hundred and seventy freshly stool specimens were obtained from 170 full-term infants.

1.1.3 Children : Seventy-six freshly stools were obtained from 76 children on anticancer drugs.

1.2 Adult patients : One hundred and thirty-five freshly stool specimens were obtained from 132 adult patients who developed diarrhea during their hospital stays. There were 2 patients whose stool were collected more than one time according to the development of chronic diarrhea during their long hospital stays.

Definition : diarrhea was defined as six or more unformed stools in 36 hour (Gerding *et al.*, 1986)

2. Isolation and Identification of *C. difficile*

Approximately 0.1 mg of stool specimens were inoculated on the selective medium cycloserine-cefoxitin fructose agar (CCFA) and blood agar. The plates were incubated at 35-37°C for 36-48 hours in an anaerobic atmosphere. The organisms were further identified on the basic of morphology (Table 1, Figure 2-3), Gram's stain (Table 2, Figure 4) and typical odor. *C. difficile*-like colonies were subcultured on the aerobic and anaerobic blood agar plates in

order to determine their obligate anaerobic characteristic. The organisms were tested for the production lecithinase and lipase by inoculating on egg yolk agar and incubated at 35-37°C for 36-48 hours in an anaerobic conditions. Definitive identification of *C. difficile* by biochemical profiles obtained with commercial test system (Rapid ID 32 A, bio Merieux).

Definitive identification using the Rapid ID 32 A

The Rapid ID 32 A is an identification system for anaerobic bacteria using the standardized and miniaturized enzymatic tests with a specially adapted database. Briefly, one colony of *C. difficile*-like colonies was subcultured onto blood agar plate, incubated at 35-37°C for 36-48 hours in an anaerobic conditions. This culture was suspended into 2 ml of the suspension medium using a sterile swab to make the turbidity equal to McFarland No 4. Fifty-five µl of the bacterial suspension was inoculated into each capule of the strip. Two drops of mineral oil was added to URE capule. The lid was placed on the strip which was then incubated at 37°C for 4 hours in aerobic conditions. The result was read and interpreted by using automatic ATB instruments, or visually.

3. Reference *C. difficile* strains

Reference toxigenic (GAI 10029) and nontoxigenic (CDT 603) *C. difficile* strains were kindly provided from Dr. Naoki and Dr. Haru Kato, Anaerobic Bacteria Branch, Hospital Infection Program, Japan.

4. Toxin A detection

4.1 Enzyme immunoassay

Enzyme immunoassay kits for *C. difficile* toxin A detection (Premier *C. difficile* toxin A test, Meridian Diagnostics, USA) were used in this study. The assay kit composes of the microtiter wells coated with a polyclonal antibody to

toxin A and an enzyme conjugated monoclonal antibody to toxin A. Briefly, a single colony of *C. difficile* was inoculated into 1 ml reduced brain heart infusion broth, incubated at 35-37°C for 36-48 hours in an anaerobic atmosphere. Half millilitre of the culture was transferred into a microcentrifuge tube and centrifuged at 8,000 rpm for 3 minutes. Fifty µl of the supernatant was transferred into the new microcentrifuge tube containing 200 µl sample diluent and mixed with micropipette for 15 seconds. Fifty µl of monoclonal antibody against *C. difficile* toxin A, conjugating with horseradish peroxidase, was added in to an assay well which was coated with polyclonal capture antibody. Fifty µl of the mixture sample, or one drop of a positive or negative control was added last. The wells were then gently rotated and tapped for 30 seconds. The wells were slabed with plastic tape, incubated at 37°C for 2 hours. At the end of the incubation period, the well were emptied and washed manually 5 times with wash buffer. Next, one free-flowing drop of the 2 reagents including a buffered solution of urea peroxide (substrate A), and the solution of tetramethylbinzidine (substrate B), were added in to each well and incubated for 10 minutes at ambient temperature. The reaction was stopped by adding one drop of 2N sulfuric acid (stop solution). The plate was read by using the spectrophotometer (Whitoker Bioproducts EIA 400AT reader, Wathersvill) at a single wavelength (450 nm) or a dual-wavelength reading at 450 and 630 nm.

The optimum density of the positive control should read less than 2.00 but greater than 0.600 at either 450 nm or 450/630 nm. The positive control should have a definite yellow color when read visually. The negative control should read less than 0.100 at 450 nm and less than 0.070 at 450/630 nm but greater than 0.00.

For a single wavelength, 450 nm reading, the optimum density of a positive test result was 0.150 or more, indeterminate was reading between 0.100 and 0.149, and a negative test result was a reading of less than 0.100.

For a dual wavelength reading, a positive test result was an OD450/630 reading of 0.100 or more, an indeterminate 0.07 to 0.099, and a negative test result was a reading of less than 0.070. Alternatively, the samples could be read visually, with a definite yellow being read as positive, a faint yellow as indeterminate, and a colorless reaction as negative.

4.2 Polymerase chain reaction

Polymerase chain reaction was prepared by the method from that by Kato *et al.* (1991).

4.2.1 DNA isolation

Genomic DNAs from the microorganisms were prepared by the method of Kato *et al.* (1991), except that the organisms were culture on brain heart infusion agar, in stead of broth culture. Briefly as followed :

A quarter of a colony was suspended in 500 µl of lysis buffer I {(TES (10mM Tris HCl pH8.0, 5mM EDTA, 10mM NaCl) containing 25% sucrose and lysozyme (5 mg/ml)} in an eppendorf microcentrifuge. The tube was then incubated at 37°C for 15 minutes. The suspension was centrifuged at 14,000 rpm for 2 minutes after that the supernate was removed. The bacterial pellet was resuspended in 200 µl of lysis buffer II {(TEN (10mM Tris HCl pH 7.5, 1mM EDTA, 10mM NaCl) containing 0.8% Sarkosyl and proteinase K (100 µ l/ml)}, and incubated at 56°C for 60 minutes. The mixture was centrifuged at 14,000 rpm for 2 minutes. One hundred µl (DNA concentration ~0.10 µg/µl) of the supernatant was transferred to a new Eppendorf microcentrifuge tube and stored at -20°C, ready for the PCR analysis.

4.2.2 DNA primers and PCR amplification

The primers NK2 (5'-CCC AAT AGA AGA TTC AAT ATT AAG CTT- 3', position 2479-2505) and NK3 (5'-GGA AGA AAA GAA CTT CTG GCT CAC TCA GGT- 3', position 2254-2283) were kindly provided by Dr.

Naoki and Dr. Haru Kato from Anaerobic Bacteria Branch, Hospital Infection Program, Japan. The presence of toxin A gene was assayed by PCR amplification of the 252 bp fragmant. PCR was done in a total volume of 30µl containing 0.3 µl of the target DNA preparation, 0.15 µg of each primer, the four deoxynucleoside triphosphates (200 µM each), 10mM Tris HCl pH 8.3, 2.5 mM $MgCl_2$, 50 mM KCl and 0.75 units of Taq polymerase (Promega, USA). The reaction mixture was overlaid with 50 µl of mineral oil (Sigma), to prevent evaporation. The thermal profile was done for 35 cycles in an automated thermal cycler (Perkin-Elmer Cetus) using the conditions of 95°C for 95 sec, ramping to 55°C in 90 sec; 55°C for 30 sec, ramping to 60°C in 30 sec; and 60°C for 120 sec, ramping to 95°C in 75 sec.

4.2.3 Gel electrophoresis and visualization

PCR products was analyzed in a 5% polyacrylamide gel and electrophoresed in 1x TBE running buffer. The gel was run at 125 constant voltage for 30 min and then stained with ethidium bromide (0.5 µg/ml of 1x TBE) for 15 min. After that, it was rinsed and destained with deionized water for 15 min followed by photographing under UV illumination. Phl x 174 DNA/ *Hae* III (Promega) was used as the molecular standard marker.

5. Analysis of restricted fragments of chromosomal DNA by Pulsed-Field Gel Electrophoresis (PFGE)

DNA extraction and PFGE were performed according to the method described by Wongwanich *et al.* (2000). Briefly as followed :

5.1 Culture plug preparation

A single colony of *C. difficile* from 48 hours anaerobic blood agar plate was inoculated into 5 ml reduced brain heart infusion broth. The culture media were incubated under anaerobic condition for 36-48 hours at 37°C. Organisms

were harvested by centrifugation at 3,500-3,800 rpm at 4°C for 10 min. The cells were then washed with 5 ml TES buffer and then centrifuged. The supernatant was then discarded. The washing step was repeated 3 times. Cells were resuspended in the TES buffer and adjusted the turbidity to McFarland No 4. This suspension was then mixed with an equal volume of 1.6% low-melting-point agarose and poured into each well of the disposable plug molds (BioRad, USA). It was allowed to solidify in the refrigerator for 30 min.

5.2 DNA extraction

Plugs were pulled out and incubated in 3 ml fresh lysis solution I [100 units of mutanolysin in stock lysis buffer A (10mM Tris HCl pH7.6, 10mM EDTA)] overnight at 37°C on gentle shaking water bath. The plug was then transferred into the new tube contained 3 ml of fresh lysis solution I (1% SDS, 0.5M EDTA and 200 µg/ml of RNase), incubated overnight at 37°C on gentle shaking water bath. After that, the plug was transferred into 3 ml of fresh lysis solution II (1% Sarkosyl, 0.5M EDTA and 2 mg/ml of Protease) on the new tube, incubated on gentle shaking water bath at 37°C overnight. The protease was inactivated by the treatment with 3 ml of phenylmethylsulfonyl fluoride two times, 1 hour each, at room temperature. The plugs were then washed with 3 ml 0.5x TE buffer, at room temperature, 3 times and were kept in 0.5x TE buffer at 4°C.

5.3 Restriction enzyme digestion

One plug of each strain was placed on the plate and covered with 150 µl of 1x restriction enzyme *Sma* I buffer (BioRad, USA), and incubated at room temperature for 30 min. After that the plug was cut into half with sterile surgical blade and were added into the microcentrifuged tube containing 150 µl of the restriction enzyme *Sma* I solution (1x restriction enzyme *Sma* I

buffer, and 100 units of *Sma* I). The tube was then incubated overnight at 25°C and then stored at 4°C.

5.4 Gel preparation and gel running

The running agarose gel was prepared by dissolving 1 g of Ultrapure high-melting temperature agarose (1% wt/vol, BioRad) in 100 ml of 0.5x TBE buffer, melted by microwave and then cool at 56°C. The plug was poured out into the sterile plate and washed the plug with 0.5x TE buffer. One half of the plug was placed in the microcentrifuged tube containing 1 ml of 0.5x TE buffer. The another half was placed onto the horizontal side of the comb. Buffer around the plug was absorbed with sterile tissues. The comb was then adjusted to the vertical line on the gel block. One percent running agarose gel was poured into the block and let gel to solidify for 30 min at room temperature. After the gel has hardened, the comb was removed. The gel was placed in the PFGE box containing 0.5x TBE buffer enough to cover the gel to a depth of about 1 mm or just until the tops of the wells are submerged. CHEF DNA size standards Lambda ladder (BioRad, USA) was used for molecular standard markers. PFGE was performed at 200 v constant voltage by using a contour-clamped homogenous electric field apparatus (CHEF-DRII system) with an initial switch time of 5 sec and a final switch time of 50 sec for 20 hours.

5.5 Gel visualization

The gel was stained with 0.5 µg/ml of ethidium bromide for 30 min. After that it was rinsed and destained with deionized water for 30 min. The gel was then photographed under UV illumination.

Result was interpreted by according to Tenover *et al.* (1995).

Table 1 Colony characteristics of *Clostridium difficile* on various media

Organism	Media	Colony characteristics
Clostridium difficile	Cycloserine-cefoxitin fructose agar	Colonies are approimately 5 to 8 mm in diameter, yellowish circular to irregular, and flat, with rhizoid edge , ground -glass appearance and the odour linked elephant or horse manure
	Blood agar	Colonies are approximately 2 to 4 mm in diameter, gray, nonhemolytic, circular to irregular, and flat, with rhizoid edge , translucent and the odour linked elephant or horse manure

Table 2 Microscopic characteristics of *Clostridium difficile*

Organism	Gram stain	Characteristics
Clostridium difficile	Gram positive rod	The rod is thin even sided, 0.5 μm wide by 3 to 16 μm long with oval subterminal spores

Figure 2 Colony characteristics of *C. difficile* on blood agar

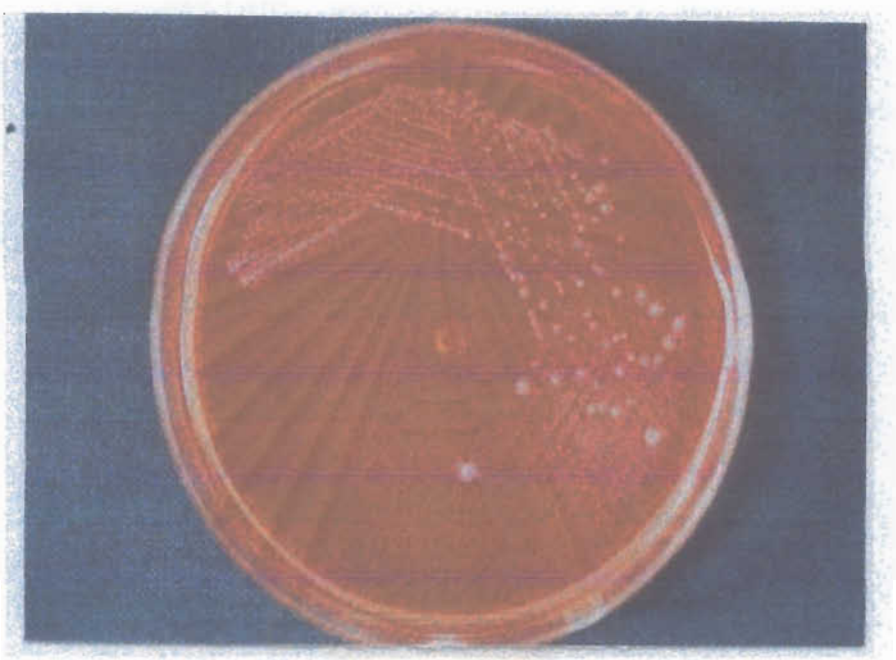


Figure 3 Colony characteristics of *C. difficile* on cycloserine cefoxitin fructose agar

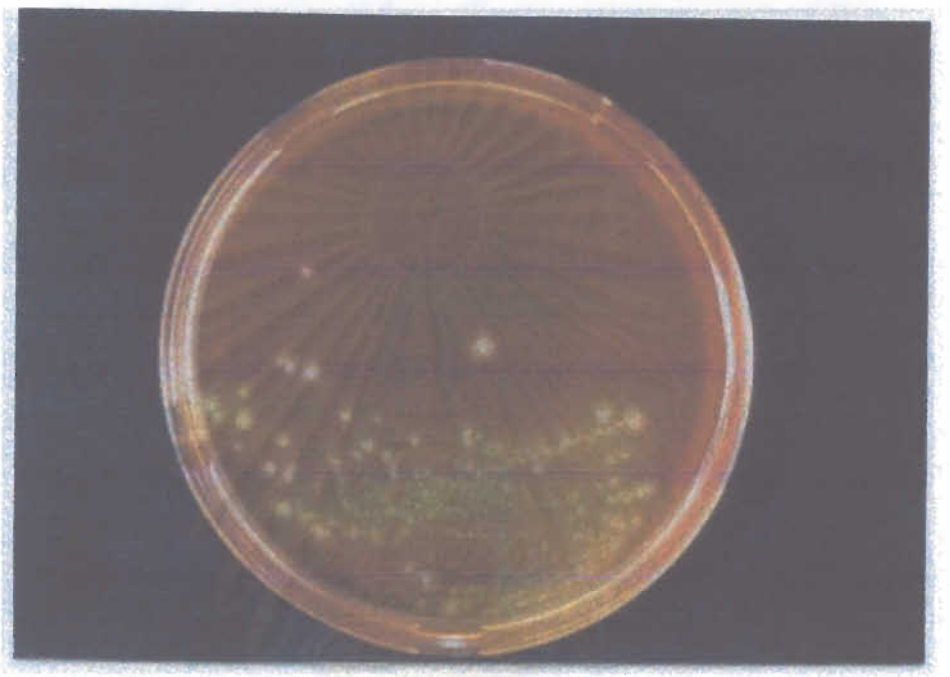
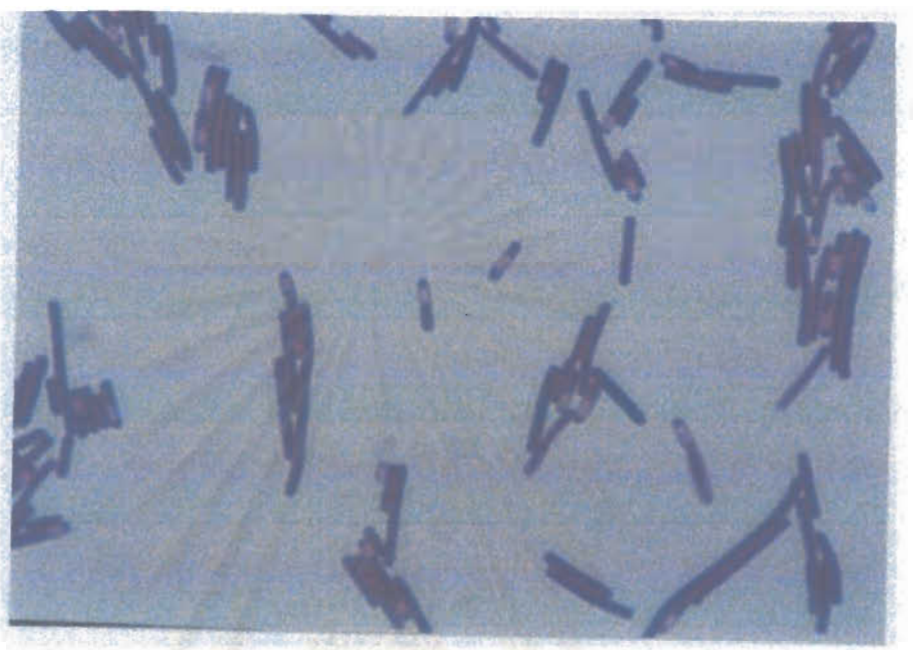


Figure 4 Microscopic characteristic of *C. difficile*



CHAPTER V
RESULTS

C. difficile in four groups of patients

The total of 77 patients harbored *C. difficile* in their stools, including 10 out of the 65 premature infants (15.38%), 18 out of the 170 full-term infants (10.59%), 16 out of the 76 children with anticancer drugs (21.05%) and 33 out of 132 adults with diarrhea (25%) as shown in table 3. The number of *C. difficile* positive among the four groups of patients were significant different ($p = 0.009$).

The detection of toxin A gene and toxin A in all *C. difficile* isolates were performed using polymerase chain reaction (PCR) and enzyme immunoassay (EIA). It was shown that only 2 out of the 65 premature infants (3.08%) and 18 out of the 132 adult patients with diarrhea (13.64%) were toxin A gene positive by PCR. However, the toxin A could not be detected by the use of EIA from all 2 isolates from the premature infants and 5 out of 18 isolates from the adult patients with diarrhea. The statistical analysis of the data was also performed. It was shown that the number of patients with toxin A positive *C. difficile* were significant difference among the four groups of patients (** $p = 0.00000004$; *** $p = 0.00000065$) (Table 4).

Risk factors and the occurrence of *C. difficile* and its toxin A gene in premature infants

The correlation between risk factors including respiratory distress syndrome (RDS), feeding and age, and the occurrence of *C. difficile* in premature infants was determined as shown in table 5-8.

Among the 45 premature infants with respiratory distress syndrome, 8 of them (17.78%) had *C. difficile* positive in their stools while 2 out of the 20 premature infants with no sign of respiratory distress syndrome (10%) had

stool positive culture as shown in table 5 (* p = 0.422). However, only 2 isolates from premature infants; one had respiratory distress syndrome and another did not have any sign of respiratory distress syndrome, were toxin A gene positive by PCR (** p = 0.796).

The correlation between feeding and the occurrence of *C. difficile* and its toxin A gene in the premature infants were determined as shown in table 6. Six out of the 48 breast fed premature infants (12.5%) had stool culture positive while as high as 4 out of 17 (23.53%) of the formula fed infants carried *C. difficile* in stool (* p = 0.279). Only 2 isolates from the breast feeding infant were toxin A gene positive by PCR (** p = 0.393).

It was also shown in the table 7 that there was not much different in the occurrence of *C. difficile* in the premature infants at different ages within the first 3 month after birth (* p = 0.811). However, at the age between 1 week to 1 month the highest prevalence was observed. There were 2 premature infants at this age range had isolates with toxin A gene positive (** p = 0.368).

As shown in the table 8 the summary on the correlation between all 3 risk factors and the occurrence of *C. difficile* and its toxin A gene in the premature infants. The highest number of *C. difficile* positive stool culture were obtained from the premature infants with respiratory distress syndrome who were on formula feeding and in the age group between 1week to 1 month. Two toxigenic *C. difficile* isolates were obtained from the premature infants at the age between 1week to 1 month who were on breast feeding. Between the two infants, only one had respiratory distress syndrome.

Risk factors and the occurrence of *C. difficile* and its toxin A gene in full-term infants

No correlation between risk factors including respiratory distress syndrome, feeding and age and the occurrence of *C. difficile* in the full-term infants were observed as shown in the table 9-12.

Only one out of 10 full-term infants with respiratory distress syndrome (10%) had *C. difficile* positive in the stool culture. There was no different from those obtained from 160 full-term infants with no sign of respiratory distress syndrome who 17 of them (10.63%) had *C. difficile* in their stool (* p = 0.950). Surprisingly, none of *C. difficile* isolates had toxin A gene (Table 9).

There were as high as, 14 out of the 82 breast fed full-term infants (17.07%) who had *C. difficile* positive stool culture while only 4 out of 88 (4.55%) of the formula fed infants carried *C. difficile* in stool as shown in table 10 (* $p = 0.008$).

It was shown in table 11 that there was no different in the occurrence of *C. difficile* in the full-term infants at different ages within the first 1 month after birth. However, the recovery of *C. difficile* from the stool of full-term infants at the age between 1 week to 1 month was slightly higher than that in the younger infants. In addition, most of the full-term infants were discharged from the hospital within one month after birth. Therefore only 3 infants at the age more than one month were included in the study (* $p = 0.398$).

Table 12 summarized the results obtained after blending all 3 risk factors with the occurrence of *C. difficile* in stools from the full-term infants. Most of the full-term infants were less than one week old (152 out of 170 infants) The 146 out of 152 infants had no sign of respiratory distress syndrome. Among these, 12 out of the 63 breast fed infants had *C. difficile* in their stool while only 3 out of 83 formula fed infants were stool positive. When the infants grew older, very low number of them stayed in the hospital, so the extended study in the older infants on the effects of all 3 risk factors could not be determined. None of the isolates were toxin A gene positive.

***C. difficile* in children with anticancer drugs**

As shown in the table 3, 16 out of 76 children with anticancer drugs (21.05%) had *C. difficile* positive in their stool. Unfortunately, all the patients did not develop diarrhea during the time of study. Among the 16 *C. difficile*

positive patients, the therapeutic regimen could be obtained in 13 patients. Most of these children (9 out of 13 patients) received combined anticancer and antimicrobial agents as shown in the table 13.

C. difficile in adult patients with diarrhea according to age range

The recovery rate of *C. difficile* from the adult patients with diarrhea increased according to age. The highest number of both *C. difficile* isolates and toxin A gene positive were observed in patients at more than 60 years of age (* $p = 0.287$; ** $p = 0.254$) (Table 14).

The adult patients with diarrhea in this study could be divided into 2 groups according to the physician's diagnosis. The first group was the patients who developed diarrhea during their hospital stays. The other group was the patients who were the suspected cases of antibiotic associated diarrhea by physicians. The 13 out of 84 patients with diarrhea (15.48%) and 20 out of 48 patients with antibiotic associated diarrhea (41.67%) had *C. difficile* in their stools (** $p = 0.001$). Toxin A gene positive was detected from the 8 *C. difficile* isolates of patients with diarrhea (9.52%) while the 10 *C. difficile* isolates of patients with suspected antibiotic associated diarrhea had toxin A gene (20.83%) (*** $p = 0.069$) (Table 15).

Gonotyping of toxin A positive _C. difficile_ by pulsed field gel electrophoresis

Two toxigenic *C. difficile* isolates from the premature infants and 18 toxigenic isolates from the adults with diarrhea were typed by PFGE (Table 16). Among all 20 isolates, there were 4 different pulsotypes; A, B, C and D, and 3 subtype A1, A2 and B1. Both strains from the premature infants were type B. Among 11 strains from the adults with diarrhea, 9 isolates were in type A and 2 isolates were in subtype A1 and A2, respectively. There were 5 isolates from the adults with diarrhea which were in type B and its subtype. The last 2 isolates were type C and D, respectively.

Time course and the pulsotypes from two individual patient

There were 2 patients who stayed in the hospital longer than 2 months. They both developed chronic diarrhea during the time. Pulsotype was not changes among the isolates obtained from the serial specimens from these patients. Sequential isolates changed only in subtype in one patient (after 2 months and 20 days). Another patient, pulsotype A strain was persisted throughout the time of the hospital stay (4 months and 20 days) (Figure 7).

Table 3 Number of *C. difficile* culture positive among four groups of patients

Population group	No. of patients (n = 443)	No.(%) of *C. difficile* positive culture (n = 77) *
Premature infants	65	10 (15.38)
Full-term infants	170	18 (10.59)
Children with anticancer drugs	76	16 (21.05)
Adults with diarrhea	132	33 (25)

* $\chi^2 = 11.692$, p-value = 0.009

Table 4 Toxin detection by PCR and EIA

Population groups	No. of patients	No. (%) of toxin A gene positive by PCR**	No. (%) of toxin A positive by EIA***
Premature infants	65	2 (3.08)	0
Full-term infants	170	0	0
Children with anticancer drugs	76	0	0
Adults with diarrhea	132	18 (13.64)	13* (9.85)

* All isolates were toxin A gene positive by PCR

** $\chi^2 = 37.42$, p-value = 0.00000004

*** $\chi^2 = 31.55$, p-value = 0.00000065

Table 5 *C. difficile* positive in premature infants with respiratory distress syndrome and no sign of respiratory distress syndrome

	No. of infants	No. (%) of *C. difficile* positive culture*	No. (%) of toxin A gene Positive**
RDS	45	8 (17.78)	1 (2.2)
no sign of RDS	20	2 (10)	1 (5)

RDS = respiratory distress syndrome

* $\chi^2 = 0.634$, p-value = 0.422

** $\chi^2 = 0.067$, p-value = 0.796

Table 6 *C. difficile* positive in premature infants with different feeding

Feeding	No. of infants	No. (%) of *C. difficile* positive culture*	No. (%) of toxin A gene positive**
Breast-fed	48	6 (12.5)	2 (4.2)
Formula-fed	17	4 (23.53)	0

* $\chi^2 = 1.173$, p-value = 0.279

** $\chi^2 = 0.731$, p-value = 0.393

Table 7 *C. difficile* in premature infants at different age ranges

Age ranges	Total no. of infants	No. (%) of *C. difficile* positive culture*	No. (%) of toxin A gene positive**
after birth - 1 week	17	2 (11.76)	0
> 1 week - 1 month	33	6 (18.18)	2 (6.06)
> 1 month - 3 months	15	2 (13.33)	0

* $\chi^2 = 0.418$, p-value = 0.811

** $\chi^2 = 2.001$, p-value = 0.368

Table 8 *C. difficile* positive in premature infants with respiratory distress syndrome and no sign of respiratory distress syndrome at different age ranges and feeding

Age ranges	RDS						No sign of RDS					
	Breast- fed			Formula- fed			Breast- fed			Formula- fed		
	No. of infants	No. of *C. difficile* positive culture	No. of toxin A gene positive	No. of infants	No. of *C. difficile* positive culture	No. of toxin A gene positive	No. of infants	No. of *C. difficile* positive culture	No. of toxin A gene positive	No. of infants	No.of *C. difficile* positive culture	No. of toxin A gene positive
after birth - 1 week	8	0	0	3	2	0	3	0	0	3	0	0
> 1 week - 1 month	20	4	1	2	1	0	10	0	1	1	1	0
> 1 month - 3 months	6	0	0	6	1	0	1	1	0	2	0	0

Table 9 *C. difficile* positive in full-term infants with respiratory distress syndrome and no sign of respiratory distress syndrome

	No. of infants	No. (%) of *C. difficile* positive culture*	No. (%) of toxin A gene positive
RDS	10	1 (10)	0
no sign of RDS	160	17 (10.63)	0

RDS = respiratory distress syndrome

* $\chi^2 = 0.004$, p-value = 0.950

Table 10 *C. difficile* positive in full-term infants with different feeding

Feeding	No. of infants	No. (%) of *C. difficile* positive culture*	No. (%) of toxin A gene positive
Breast-fed	82	14 (17.07)	0
Formula-fed	88	4 (4.55)	0

* $\chi^2 = 7.037$, p-value = 0.008

Table 11 *C. difficile* positive in full-term infants at different age ranges

Age ranges	Total no. of infants	No. (%) of *C. difficile* positive culture*	No. (%) of toxin A gene positive
after birth - 1 week	152	15 (9.87)	0
> 1 week - 1 month	15	2 (13.33)	0
> 1 month - 6 months	3	1 (33.33)	0

* $\chi^2 = 1.842$, p-value = 0.398

Table 12 *C. difficile* positive in full-term infants with respiratory distress syndrome and no sign of respiratory distress syndrome at different age range and feeding

Age range	RDS						No sign of RDS					
	Breast-fed			Formula-fed			Breast-fed			Formula-fed		
	No. of infants	No. of *C. difficile* positive culture	No. of toxin A gene positive	No. of infants	No. of *C. difficile* positive culture	No. of toxin A gene positive	No. of infants	No. of *C. difficile* positive culture	No. of toxin A gene positive	No. of infants	No. of *C. difficile* positive culture	No. of toxin A gene positive
after birth - 1 week	4	0	0	2	0	0	63	12	0	83	3	0
> 1 week - 1 month	1	0	0	0	0	0	12	1	0	2	1	0
> 1 month - 6 months	2	1	0	1	0	0	0	0	0	0	0	0

Table 13 Therapeutic regimens in 13 *C. difficile* positive patients children with anticancer drug*

	No. of children
Anticancer only	4
Anticancer +Antimicrobial	9

* Therapeutic regimens in 3 other *C. difficile* positive patients could not be obtained

Table 14 *C. difficile* positive in adult patients with diarrhea according to age ranges

Age ranges (years)	No. of patients	No. (%) of *C. difficile* positive culture*	No. (%) of toxin A gene positive**
20-40	34	6 (17.65)	3 (8.8)
41-60	41	9 (21.95)	4 (9.75)
> 60	57	18 (31.59)	11 (19.3)

* $\chi^2 = 2.499$, p-value = 0.287

** $\chi^2 = 2.744$, p-value = 0.254

Table 15 *C. difficile* positive in adult patients with diarrhea (according to diagnosis*)

	No. of patients	No. (%) of *C. difficile* positive culture**	No. (%) of toxin A gene positive***
Patients with diarrhea	84	13 (15.48)	8 (9.52)
Patients with AAD	48	20 (41.67)	10 (20.83)

AAD = antibiotic-associated diarrhea

* according to physician's diagnosis

** $\chi^2 = 11.175$, p-value = 0.001

*** $\chi^2 = 3.317$, p-value = 0.069

Table 16 Pulsotype of toxin A positive *C. difficile*

Population groups	No. of patients of *C. difficile* isolates	Result of PCR	Result of EIA	Pulsotype A			Pulsotype B		Pulsotype C	Pulsotype D
				A	A1	A2	B	B1		
Premature infants	2	positive	negative				2			
Adults with diarrhea	13	positive	positive	9*(#)	1(#)	1			1	1
	5	positive	negative				4	1		

* Three strains of pulsotype A were obtained from one patient

\# Two isolates were obtained from one patient ; one was pulsotype A and another one was pulsotype A1

Figure 5 Polyacrylamide gel electrophoresis of PCR products from *Clostridium difficile* with toxin A gene primers NK2-NK3.

lane 1 : *Hae* III-digested ϕX174 (DNA marker);

lanes 2, 3, 4, 5 and 6 : *Clostridium difficile* isolates from patients; lane 7 : reference strain of toxigenic *Clostridium difficile* (GAI 10029); lane 8 : reference strain of nontoxigenic *Clostridium difficile* (CDT 603); lane 9 : DNA of toxin A gene of *Clostridium difficile* used as positive control;

lane 10 : negative control

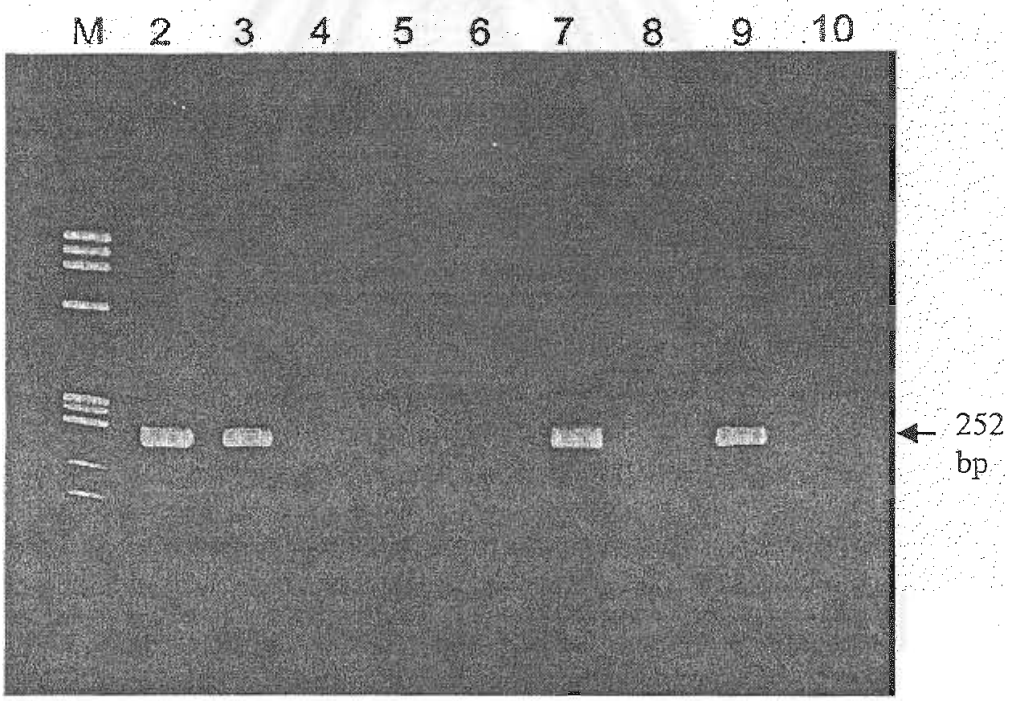


Figure 6 Typing patterns of enterotoxigenic *Clostridium difficile* isolates as determine by PFGE of *Sma*I-digested DNA.
lanes 1,5 and 10 : the lambda ladder marker; lane 2 pulsotype A; lane 3 pulsotype A1; lane 4 pulsotype A2; lane 6 pulsotype B; lane 7 pulsotype B1; lane 8 pulsotype C; lane 9 pulsotype D

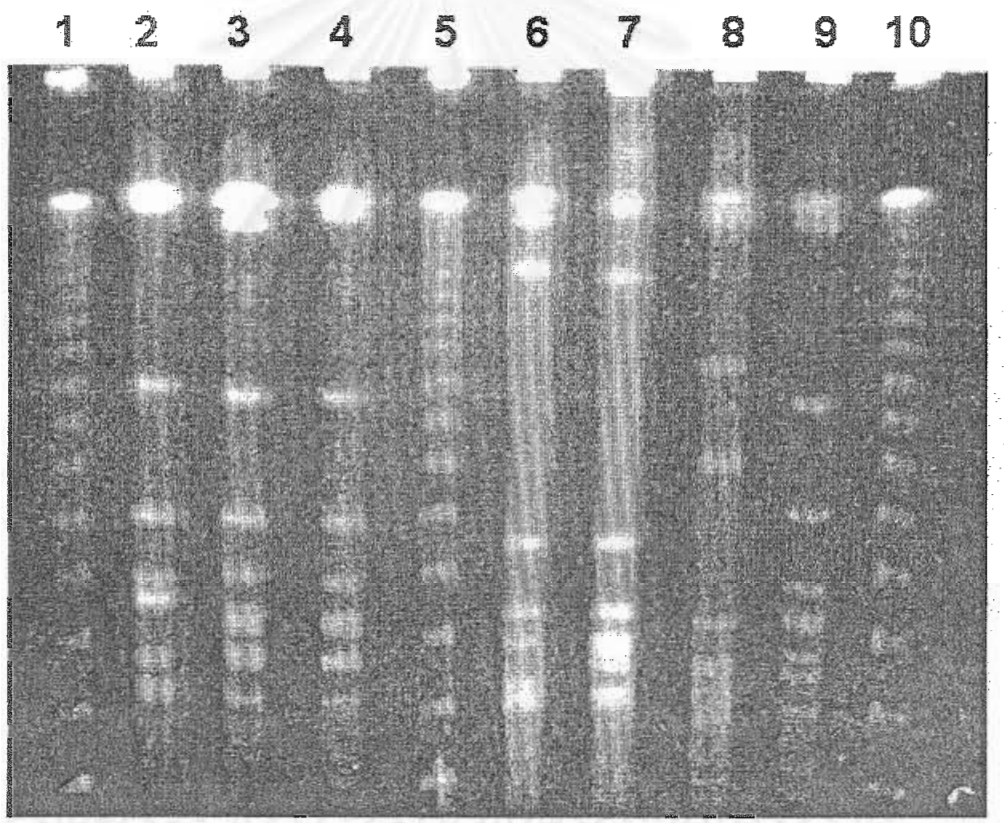
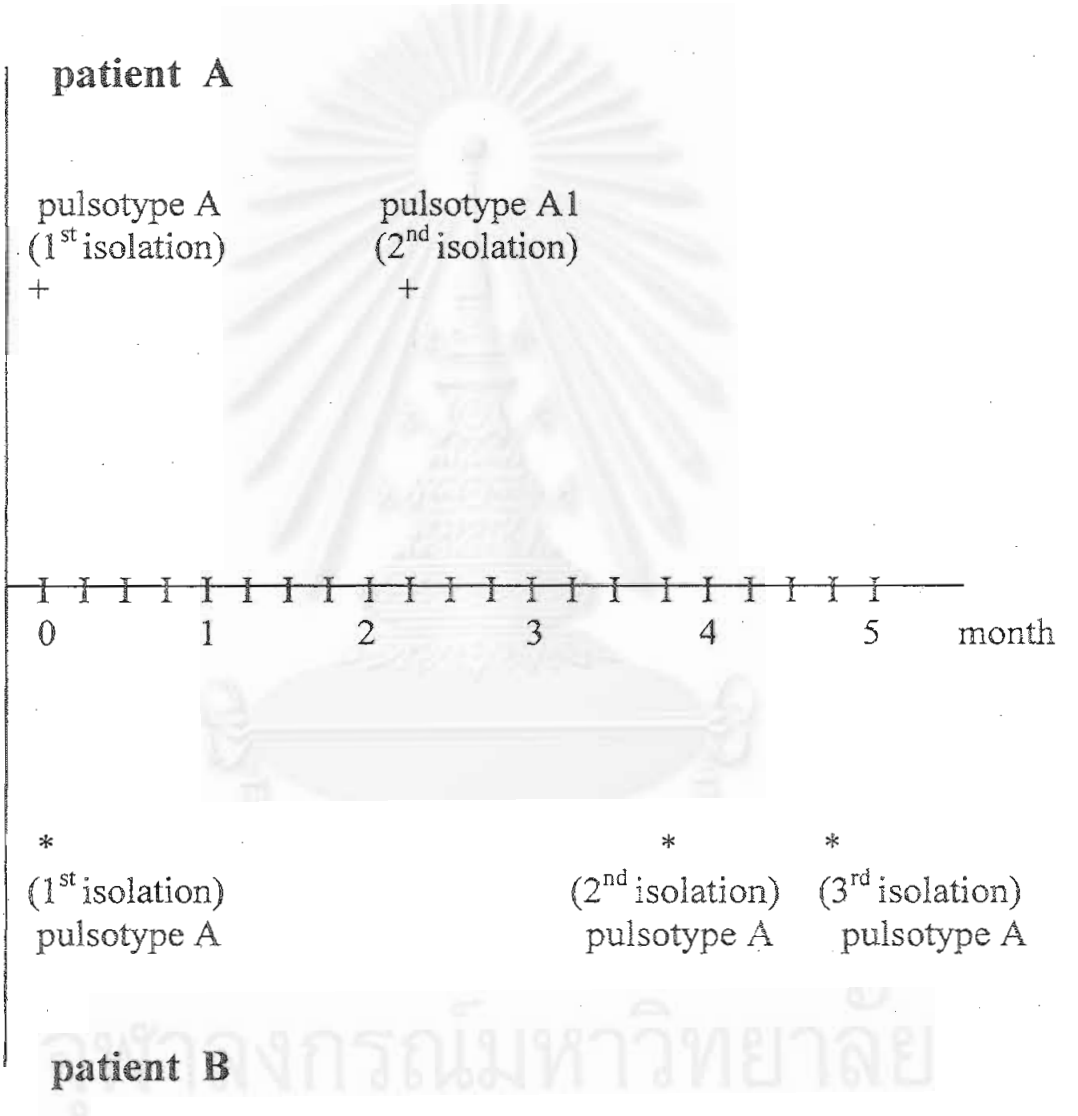


Figure 7 Time course and the pulsotypes of *Clostridium difficile* isolated from two individual patient



CHAPTER VI
DISCUSSION

The prevalence of *C. difficile* in 4 groups of patients were determined. The prevalence of the organism in the premature infants was slightly higher than that in the full-term infants which were 15.38% and 10.59%, respectively. This result agree with the recovery rates in previous reports. Bartlett (1994) indicated that very wide range (5-70%) of the healthy neonates had *C. difficile* in stools. Another study done by El Mohandes *et al.* (1993) shown that 15% of the premature infants at the age of 1 week had *C. difficile* in stools.

Dodson and Borriello (1996) reported that *C. difficile* carriage rate was up to 3% in healthy adults in Europe while in Japan, Nakamura *et al.*, (1981a) showed that this rate was as high as 15%. It has also been shown that *C. difficile* carriage rate in the healthy infant was higher that in the healthy adults. It was possible to suggest that the normal flora has been referred to as colonization resistance in neonates were poorly colonization resistance or gastrointestinal tract immaturity. They have still to develop a complex gut normal flora that can exclude *C. difficile*.

Viscidi *et al.* (1981) showed that *C. difficile* colonized up to 50% of human infants during the first few month of life after the other the flora had matured. The population of *C. difficile* dwindled from very few to undetectable level. Spencer (1998a) reviewed that healthy neonates frequently become colonized during the first 2 weeks of life. Holst *et al.* (1981) showed that *C. difficile* colonized up to 64% of healthy neonates during 1 to 8 months. However, Willson (1993) concluded that the size of *C. difficile* population was contrasted by the colonic microflora.

Tannock (1994) described that the acquisition of the normal flora began at, or soon after, birth. In the process of normal flora establishment in the intestinal tract, one group of organism after another dominated the ecosystem

in successive waves until the mature, predominant flora was permanently established.

However, in this study, only the isolates from the premature infants from all *C. difficile* isolates from both groups of infants had toxin A gene. The difference between the prevalence of *C. difficile* and its ability to produce toxin might be due to the gastrointestinal prematurity period (Wilson *et al.*, 1982). This could be the explanation that necrotizing enterocolitis (NEC) commonly occurred in premature infants but not common in full-term infants. This was agreeable with the result from the study by Brown and Sweet (1982) which indicated that NEC were found most in the premature infants.

The detection of toxin A gene by PCR seemed to correlate with the detection of toxin by EIA. However, toxin A from 7 *C. difficile* isolates with toxin A gene including 2 of *C. difficile* isolates from the premature infants and 5 of *C. difficile* isolates from adults with diarrhea could not be detected by EIA. Eventhough PCR and EIA test were repeated twice. It has been shown in various previous studies that the problem of EIA was its low sensitivity The review by Gesding (1995) showed that Premier EIA test for toxin A had a mean test sensitivity of 77.5% (range, 65% to 88%) and a mean specificity of 98.6% (range, 95% to 100%).

The results in this study clearly showed that non of *C. difficile* from the full-term infants was toxigenic isolate which contrast to the result from previous study by Bartlett (1994) and Cooperstock *et al.* (1983) who showed that *C. difficile* in infants could produce very high titre of toxins but still remain asymtomatic. Extended study should be performed on the presence of toxin A gene in larger number of *C. difficile* isolated from the full-term infants in order to confirm that the full term infants with *C. difficile* does not develop disease because of the inability to produce toxin.

There were several risk factors for the occurrence of *C. difficile* in the infants. These factors included respiratory distress syndrome, feeding and age (Hopkins *et al.*, 1970; Stark and Lee, 1982; Wilson *et al.*, 1982). It has already

shown in this study that all these risk factors had impact on the presence of *C. difficile* in premature infants. Higher percentage of premature infants with respiratory distress syndrome (17.78%) carried *C. difficile* in their stools as compare to the number of *C. difficile* positive in premature infants (10%) with no sign of respiratory distress syndrome. The number of breast feeding premature infants who had *C. difficile* positive stools (12.5%) was much lower than the number of *C. difficile* positive premature infants who were on formula fed (23.53%). However, the ability of *C. difficile* to produce toxin did not depend directly on risk factors. Thus, the high number of *C. difficile* positive in infant indirectly increase the number of toxigenic isolates. Unfortunately, the number of *C. difficile* positive premature infants in this study was not high enough to conclude about the difference between the number of the toxigenic strains isolated from the patients with and without respiratory distress syndrome.

Type of feeding has been suggested to be important risk factor for *C. difficile* infection. Kliegmen (1985) reported that almost all neonates with NEC had been fed with artificial formula by oral or enteric route. Formula fed was supported the growth of enteric bacteria. It has also shown in this study that the premature infants with formula fed had higher tendency to carry *C. difficile* in their stool than those with breast fed. Surprisingly, only 2 isolates from the breast fed premature infants were toxigenic isolates and none of these 2 premature infants develop NEC or *C. difficile* infection during the time of study. Breast fed could possible play role in the protection of infants from the toxigenic effect of the organism by containing substances perhaps antibody which could neutralize toxins (Kim *et al.*, 1984).

Premature infants at the age range between 1 week to 1 month seemed to carry higher number of *C. difficile* in their stools than those at the other age ranges. Only isolates from such group were toxigenic. This result was similar with the result studied by Willson *et al.* in 1982 who indicated that NEC onset in premature infants could occur only between 3 to 8 weeks after birth. They

explained that the infants were affect by neonate age which correspond to the gastrointestinal maturity period (Willson *et al.*, 1982).

Risk factors seemed to play no role on the presence of *C. difficile* in the full-term infants in this study. It was shown that there was no difference in the number of *C. difficile* positive full-term infants with RDS or without respiratory distress syndrome. In contrary to those previous study (Bartlett, 1994; Cooperstock *et al.*, 1983), in full-term infants higher number of *C. difficile* was found in breast fed than in formula fed.

From the immunological point of view, it could indicate only that maternal immunity might not play role against the presence of *C. difficile* in the full-term infants. One possibility is that the ecological conditions in the intestinal tract of the full-term infants are different from that of the premature infants.

Kamthan *et al.* (1992) showed an association between *C. difficile* disease and the use of antineoplastic chemotherapy. Cudmore *et al.* (1982) reported that anticancer drugs including adriamycin, cyclophosphamide, methotrexate and 5-fluorouracil were able to precipitate *C. difficile*-associated disease. These anticancer drugs have antibacterial activity and could also suppress the immunity. Sriuranpong and Voravut (1995) reported that the colitis developed in patients with 5-fluorouracil. The patients in this study received similar anticancer drugs including Ara C, adriamycin, methotrexate, vinblastin and vincristine as in other previous study (Cudmore *et al.*, 1982). These anticancer drugs could have side effect to the gastrointestinal tract. In children with anticancer drugs in this the study, *C. difficile* carriage rate was higher (21.05%) than those in premature infants and full-term infants, 15.38% and 10.59%, respectively. However, none of the isolates had toxin A gene and all the patients did not develop diarrhea during the time of the study. Extended study in the selected patients with anticancer drugs who developed diarrhea is needed in order to make more conclusive discussion.

Bartlett (1994) reviewed that *C. difficile* implicated in 15-25% of patients with antibiotic associated diarrhea while McFarland *et al.* (1990) showed that 7.8% of patients had *C. difficile* nosocomial diarrhea. In this study, as high as 25% of the adult patients with diarrhea at Siriraj hospital had *C. difficile* in their stools. There were 54.55% of *C. difficile* isolated from adult patients with diarrhea had toxin A gene positive by PCR.

Cartmill, *et al.* (1994) performed the study during the nosocomial outbreak of *C. difficile* infection in three hospitals in Manchester, UK. They showed that most patients (90%) were over 60 years old and the infection was thought to have contributed to 17 deaths. The great majority of *C. difficile* positive specimens reported to the Communicable Disease Surveillance Unit, Colindale (1998) originate from patients aged over 65 years (81% in 1997). The present study confirms that nosocomial *C. difficile* infection predominate in the patients with this age group. There were 31.59% of patients over 60 years of age who had *C. difficile* positive in stool. This was higher than the prevalence found in younger patients. The prevalence of the presence of toxin A gene in isolates from this group of patients was also higher. There has been some evidences that *C. difficile* was more likely to become established in the gut flora of elderly patients, although it was difficult to separate such increased susceptibility from that induced by exposure to antibiotics (Borriello, 1990). The specific reasons why increasing age may predispose to *C. difficile* colonization or infection are unknown. A reduced humoral response to the organism has been reported (Viscidi, *et al.*, 1983), while other believe that the elderly have impaired resistance to colonization by *C. difficile* (Borriello *et al.*, 1986).

Typing methods designed to distinguish between bacterial strains of the same species are often used in epidemiologic investigations of nosocomial infections. One pulsotype (A) was dominant in enterotoxigenic *C. difficile* isolated strains (toxin A positive both PCR and EIA). In *C. difficile* strains which were toxin A positive by PCR but negative by EIA were belonged to

pulsotype B. This study showed that *C. difficile* with pulsotype A was the common strain in Siriraj hospital. The result also indicated that PCR detection correlated well with the pulsotyping scheme. According to the low sensitively of EIA, it could possibly be that isolates with pulsotype B produced lower amount of toxin A not enough to be detected by EIA. The presence of symptoms with specific types of strains may be related in part to the ability of the strain to produce toxin A. The results in this part of study also suggested the persistence of strains isolated from individual patients. Pulsotype was not changes among isolates obtained from serial specimens from two adults with diarrhea. This suggested that the whole genome and not only the toxin genes of such strains are similar, which further supported their origination from a common ancestor. In addition, this result seemed to indicate that the patients carry single clone. There were two obvious explanations for the dominance and persistence of the strain. First, it may have a selective advantage *in vivo*. Another possibility is that pulsotype A has a survivability advantage in the environment. Numerous authors have already reported that nosocomial acquisition of *C. difficile* is facilitated by the persistence of its spores on environmental surfaces by spread through personnel or patient-to-patient contact, and by intensive antibiotic therapy (Johnson *et al.*, 1990; Kristjansson *et al.*, 1994; Simor *et al.*,1993)

This study was one of the very first study on the toxin A gene detected in Thailand. Eventhough, further study on larger population size is needed but this study has already shown many interesting results. First, all isolates from healthy full-term infants were nontoxigenic providing the explanation that disease did not developed in this population. Second, risk factors such as respiratory distress syndrome, feeding and ages seemed to play role in the increase in the recovery rate of *C. difficile* in stool of only the premature infants not the full-term infants. Third, *C. difficile* was also a common cause of nosocomial diarrhea in the inpatients. Forth, result showed that pulsotype A was the predominate type of *C. difficile* isolated from adult

with diarrhea indicating common strain in Siriraj hospital. The last result showed that nontoxigenic strain could be clearly differentiated from toxigenic strain by PCR assay. Thus, this technique is beneficial to the patient for treatment and helpful in controlling nosocomial infections as well as PFGE was the excellent method for typing which provided good discriminatory power and was ease to perform.

CHAPTER VII
CONCLUSIONS

The prevalence of *C. difficile* in premature infants, full-term infants, children with anticancer drugs and adult inpatients (at Siriraj hospital) with diarrhea were 15.38%, 10.59%, 21.05% and 25%, respectively.

Among these isolates, toxin A gene detection was performed using PCR. Only 2 out of 10 isolates from premature infants and 18 out of 33 isolates from adult inpatients with diarrhea were toxin A gene positive by PCR. EIA was also performed to detect toxin A production by the isolates. Therefore, EIA test was shown to be less sensitive than PCR technique.

PFGE could differentiate all toxin A gene positive into 4 different pulsotypes; A, B, C and D. Type A was the predominate pulsotype among all isolates (11 out of 20 isolates). Pulsotype of *C. difficile* isolated from individual patient persisted for than 2 months.

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APPENDICES

APPENDIX 1

Media, Solution and Identification procedures

Media

1. Blood agar

Base : yeast extract	0.5	g
trypticase soy base (Difco, USA)	40	g
distilled water	1000	ml
Supplement : vitamin K-hemin solution	10	ml
humen or sheep blood	50	ml

2. Brain heart infusion broth-supplemented

brain heart infusion broth (dehydrated) (Difco, USA)	37	g
yeast extract	5	g
resazurin solution	4	ml
distilled water	1000	ml
Supplement : cysteine HCl-H_2O	0.5	g
hemin solution	10	ml
vitamin K1	0.2	ml

3. Cycloserine cefoxitin fructose agar (CCFA) (Difco, USA)

Base : proteose peptone No. 2	40.0	g
disodium phosphate (Na_2HPO_2)	5.0	g
monopotassium phosphate (KH_2PO_4)	1.0	g
sodium chloride (NaCl)	2.0	g
magnesium sulfate anhydrous ($MgSO_4$)	0.1	g
fructose	6.0	g

neutral red	3 ml or 0.03	g
agar	20.0	g
distilled water	1000	ml
Supplement : cycloserine	0.5	g
cefoxitin	0.016	g

4. Egg yolk agar (modified)

tryticase (Difco, USA)	20	g
disodium phosphate (Na_2HPO_2)	2.5	g
sodium chloride (NaCl)	1.0	g
magnesium sulfate anhydrous ($MgSO_4$), 5% solution	0.1	ml
glucose	1	g
agar	12	g
distilled water	1000	ml
Supplement : egg yolk	1	

Media preparation

All of ingredients were dissolved in 1000 ml of distilled water and heat to boiling to dissolved completely. The medium steriled by autoclaving at 12°C, 15 pounds/inch^2 pressure, for 15 minutes. The sterile medium was cooled to 45°C to 50°C.

For sterile medium, supplement was added aseptically. The medium was mix and then dispensed into sterile petri dishes.

Solution

1. Neutral red

neutral red	1	g

This solution was prepared by dissolved 1 g of neutral red in 100 ml of 50% ethanal.

2. Resazurin solution

resazurin	25	mg

To prepared this solution, 25 mg of resazurin was dissolved in 100 ml of distilled water.

3. Vitamin K I stock solution

vitamin K	0.15	ml

This solution was prepared by dissolved 0.15 ml of vitamin K in 130 ml of 130% ethanal.

4. Vitamin K- hemin solution

1N NaOH	1	ml
hemoglobin	0.1	g

All of ingredients were dissolved in 199 ml distilled water. The solution steriled by autoclaving at 121°C, 15 pounds/inch^2 pressure, for 15 minutes. The sterile solution was cooled to 45°C to 50°C.

vitamin K	1	ml (10 mg /ml)

This sterile solution, 1 ml of vitamin K was added aseptically. The solution was mix.

Identification procedures

Gram stain

Four different kinds of the reagents , primary stain , mordant , decolorizer and counter stain were prepared

primary stain

- Alkaline crystal violet stain

solution A :	crystal violet	10	g
	distilled water	1000	ml
solution B :	$NaHCO_3$	50	g
	distilled water	1000	ml

mordant

- Gram iodine solution

iodine	20	g
NaOH	4	g
potassium iodide	1	g
distilled water	1000	ml

decolorizer

acetone	300	ml
95% ethyl alcohol	700	ml

safranin counter stain

slock solution : safranin	20.0 g melt in 95 % ethyl alcohol	
distilled water	1000	ml

APPENDIX 2

Reagent for molecular analysis

Stock reagents and buffer for PCR

1. 2M Tris HCl (pH 7.5 and pH 8.0)

Tris base	12.11	g
deionized water	50	ml

This stock reagent was prepared by dissolved 12.11 g of Tris base in 35 ml of deionized water, then the pH was adjusted to 7.5 or to 8.0 with HCl (conc.). The final volume was bought up to 50 ml with deionized water. The stock reagent steriled by autoclaving at 121°C, 15 pounds/inch^2 pressure, for 15 minutes. The stock reagent was stored at room temperature.

2. 0.5M EDTA

ethylene diaminetetraacetic acid	93.05	g
deionized water	500	ml

This stock reagent was prepared by dissolved 93.05 g of ethylene diaminetetraacetic acid in 400 ml of deionized water, then the pH was adjusted to 8.0 with NaOH (pellets). The final volume was bought up to 500 ml with deionized water. The stock reagent steriled by autoclaving at 121°C, 15 pounds/inch^2 pressure, for 15 minutes. The stock reagent was stored at room temperature.

3. 2M NaCl

NaCl	2.922	g
deionized water	500	ml

To prepared this stock reagent, 2.922 g of NaCl was dissolved in 50 ml of deionized water. The stock reagent steriled by autoclaving at 121°C, 15 pounds/inch^2 pressure, for 15 minutes. The stock reagent was stored at room temperature.

4. 5X TBE

Tris base	54.0	g
boric acid	27.5	g
0.5M EDTA pH 8.0	20	ml
deionized water	1000	ml

This stock reagent was prepared by dissolved all of ingredients in 1000 ml of deionized water. The stock reagent steriled by autoclaving at 121°C, 15 pounds/inch^2 pressure, for 15 minutes. The stock reagent was stored at room temperature.

5. Proteinase K (20 mg/ml)

proteinase K	100	mg
deionized water	5	ml

To prepared this stock reagent, 100 mg of proteinase K was dissolved in 5 ml of sterile deionized water. The stock reagent was stored at -20°C.

proteinase K (5 mg/ml)

This stock reagent was prepared by mixed 0.25 ml of proteinase K (20 mg/ml) in 0.75 ml of sterile deionized water. The stock reagent was stored at -20°C.

6. 10% Sarkosyl

sarkosyl	0.5	g
deionized water	5	ml

Buffer

1. TEN

2M Tris HCl (pH 7.5)	0.25	ml
0.5M EDTA (pH 8.0)	0.1	ml
2M NaCl	0.25	ml
sterile deionized water up to	50	ml

2. TES

2M Tris HCl (pH 8.0)	1.25	ml
0.5M EDTA (pH 8.0)	0.5	ml
2M NaCl	1.25	ml
sterile deionized water up to	50	ml

3. Lysis buffer I

sucrose	20	g

To prepared this buffer, 20 g of sucrose was dissolved in 80 ml of TES and autoclaving at 115°C, 10 pounds/inch^2 pressure, for 10 minutes to dissolved completely. The buffer was cooled to 45°C to 50°C.

lysozyme	400	mg

This buffer, 400 mg of lysozyme was added aseptically and then mixed well. The buffer was stored at -20°C.

4. Lysis buffer II (200 µl)

10% sarkosyl	16	µl
5 mg/ml proteinase K	4	µl
TEN	180	µl

This buffer was freshly prepared before use.

5. 5% polyacrylamide gel

sterile deionized water	7.3	ml

30% acrylamide (29:1 acrylamide/bisacrylamide)	1.7	ml
5X TBE	1	ml
ammonium persulfate	5-10	partical
TEMED	50	µl

This polyacrylamide gel was prepared by mixing sterile deionized water, 30% acrylamide and 5X TBE. Ammonium persulfate was dissolved in gel mixture and the last added TEMED then mix well.

Stock reagents and buffer for PFGE

1. 2500 units Mutanolysin

mutanolysin (Sigma, USA)	10000	units
sterile deionized water	4	ml

This stock reagent was prepared by dissolved 10000 units of mutanolysin in 4 ml of sterile deionized water. The stock reagent was stored at –20°C.

2. 20 mg/ml Protease

protease (Sigma, USA)	100	mg
sterile deionized water	5	ml

This stock reagent was prepared by dissolved 100 mg of proteinase in 5 ml of sterile deionized water. The stock reagent was stored at -20°C.

3. Lysis buffer A

10mM Tris-HCl pH 7.6, 10mM EDTA	5.8	ml
2500 units mutanolysis	0.2	ml

This buffer was freshly prepared before use.

4. Lysis solution I

SDS	0.06	g
0.5M EDTA	6	ml
25 mg/ml RNase	4.8	µl

This buffer was freshly prepared before use.

5. Lysis solution II

sarkocyl	0.06	g
0.5M EDTA	5.4	ml
20 mg/ml protease	0.6	ml

6. Phenyle thylsulfonyl fluoride (PMSF)

phenyle thylsulfonyl fluoride	0.087	g
1X TE	50	ml

This stock reagent was prepared by dissolved 0.087 g of PMSF in 50 ml of 1X TE. The stock reagent was stored at -20°C.

Working PMSF

This working was prepared by mixing 2 ml of PMSF with 18 ml of 1X TE. This buffer was freshly prepared before use.

7. 1X TE

2M Tris-HCl pH 8.0	5	ml
0.5M EDTA	2	ml
sterile deionized water up to	1000	ml

This buffer was prepared by mixing these stock solution in 1000 ml of sterile deionized water

8. 10mM Tris-HCl + 10mM EDTA

2M Tris-HCl pH 7.6	5	ml
0.5M EDTA	20	ml
sterile deionized water up to	1000	ml

This buffer was prepared by mixing these stock solution in 1000 ml of sterile deionized water

9. 1.6% Low melting point agarose

low melting point agarose	0.16	g
TES	10	ml

This low melting point agarose was prepared by suspending 0.16 g of low melting point agarose in 10 ml of TES. The agarose was melted by microwave or placing the flask into a beaker of boiling water.

10. 1% Ultrapure high melting temperature agarose

ultrapure high melting temperature agarose	1	g
0.5X TBE	100	ml

This ultrapure high melting temperature agarose was prepared by suspending 1 g of ultrapure high melting temperature agarose in 100 ml of 0.5X TBE. The agarose was melted by microwave or placing the flask into a beaker of boiling water.

APPENDIX 3

Chemical agents, Materials, Instruments

Chemical agents

Tris base (Sigma, USA)

EDTA (Sigma, USA)

NaCl (Merck, Germany)

Boric acid (Merck, Germany)

Sucrose (Sigma, USA)

Lysozyme (Sigma, USA)

HCl (Merck, Germany)

Sarkocyl (Sigma, USA)

Proteinase K (Sigma, USA)

Deoxynucleoside triphosphates (Bio Rad, USA)

$MgCl_2$ (Bio Rad, USA)

KCl (Bio Rad, USA)

Taq polymerase (Promega, USA)

Mineral oil (Sigma, USA)

Phl x 174 DNA/*Hae* III (Promega, USA)

Ethidium bromide (Bio Rad, USA)

Arcylamide (Bio Rad, USA)

Ammonium persufate (Bio Rad, USA)

TEMED (Bio Rad, USA)

Mc Farland No.4 (bio Merieux)

Low melting point agarose (Bio Rad, USA)

Ultrapure high melting temperature agarose (Bio Rad, USA)

Rnase (Bio Rad, USA)

SDS (Bio Rad, USA)

Protease (Sigma, USA)

Phenylethylsulfonyl fluoride (Sigma, USA)

Mutanolysin (Sigma, USA)

Restriction enzyme *Sma* I (Bio Rad, USA)

Restriction enzyme *Sma* I buffer (Bio Rad, USA)

Lambda ladder (Bio Rad, USA)

Meterials

Eppendorf microcentrifuge

Micropippet

Tip

Cotton swab

Cylinder

Test tube

X-ray film cassettes

Plug mold

Pasture pipet

Gel block

Gas Pak (Oxoid, England)

Palidium

Instruments

Heat block (Scientific, USA)

Centrifuge (Tomy Seiko, Japan)

pH meter (Orion, USA)

Microcentrifuge (Tomy Seiko, Japan)

Magnetic stirrer (VELP Scientifica, Italy)

Automated thermal cycle (Perkin-Elmer Cetus, USA)

Electrophoresis chamber (Bio Rad, USA)

Power supply (Bio Rad, USA)

UV transilluminator (Spectroline, USA)

Water bath shaker (Yamato, Japan)

Polaroid camera (Polaroid, USA)

Colling water bath

Biological safety cabinet (Yamato, Japan)

Automatic pipette (Brand, Germany)

Rotary shaker (Bellco Glass, USA)

Incubator (Sanyo, Japan)

Refrigerator (Sharp, Japan)

Freezer -20°C (Sanyo, Japan)

Balance (Mettler, Japan)

Autoclave (Yamato, Japan)

Spectrophotometer (Bio-Tek instruments, USA)

Anaerobic jar (Oxoid, England)

Microwave (Sanyo, Japan)

Vortex mixer (Scientific, USA)

Contour-clamped homogenous electric field apparatus (Bio Rad, USA)

BIOGRAPHY

Miss Sriwanna Huttayananont was born on June 7, 1965 in Bangkok, Thailand. I graduated with the Bachalor degree of Science in Biology from the Faculty of Science, Ramkhamhaeng University in 1989. I am medical scientist rank 5 at National Institute of Health, Nonthaburi, Thailand.

Publication

Krongkaew Supawat, **Sriwanna Huttayananont**, Mayura Kusum, Thareerat Kalambaheti, and Wanpen Chaicumpa. 1994. A monoclonal antibody-based dot-blot ELISA diagnostic kit for the detection of *Vibrio cholerae* O1 in stool of diarrheic patients and household contacts. **Asian Pacific Journal of Allergy and Immunology**. 12 : 155-159.

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