การตรวจหา *Clostridium difficile* toxin A และ B genes ในอุจจาระผู้ป่วยซึ่งเป็นโรคอุจจาระร่วง โดยใช้เทคนิคปฏิกิริยาลูกโซ่

นางสาวศิริพร รักดีแข

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาจุลชีววิทยาทางการแพทย์ สหสาขาวิชาจุลชีววิทยาทางการแพทย์
บัณฑิตวิทยาลัย จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2544

ISBN 974-03-1629-8

DETECTION OF *CLOSTRIDIUM DIFFICILE* TOXIN A AND B GENES FROM STOOL SAMPLES OF DIARRHEAL PATIENTS BY POLYMERASE CHAIN REACTION (PCR) TECHNIQUE

Miss Siriporn Rugdeekha

A Thesis Submitted in Partial Fulfillment of the Requirements
For the Degree of Master of Science in Medical Microbiology
Inter-Departmental Medical Microbiology
Graduate School
Chulalongkorn University
Academic Year 2001

ISBN 974-03-1629-8

Thesis Title	Detection of *Clostridium difficile* toxin A and B genes from stool samples of diarrheal patients by polymerase chain reaction (PCR) technique
By	Siriporn Rugdeekha
Field of study	Medical Microbiology
Thesis Advisor	Associate Professor Pintip Pongpech, Ph.D.
Thesis Co-advisor	Siripan Wongwanich, M.Sc.

Accepted by the Graduate School, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master 's Degree

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

THESIS COMMITTEE

..... Chairman
(Associate Professor Somying Tumwasorn, Ph.D.)

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

..... Thesis Co-advisor
(Siripan Wongwanich, M.Sc.)

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

ศิริพร รักดีแข : การตรวจหา *Clostridium difficile* toxin A และ B genes ในอุจจาระผู้ป่วยซึ่งเป็นโรคอุจจาระร่วง โดยใช้เทคนิคปฏิกิริยาลูกโซ่ (Detection of *Clostridium difficile* toxin A and B genes from stool samples of diarrheal patients by polymerase chain reaction [PCR] technique) อ. ที่ปรึกษา : รศ.ดร.พิณทิพย์ พงษ์เพ็ชร, อ. ที่ปรึกษาร่วม : นางศิริพรรณ วงศ์วานิช 136 หน้า. ISBN 974-03-1629-8

อัตราการพบเชื้อ *Clostridium difficile* ในตัวอย่างอุจจาระจากผู้ป่วยคนไทยซึ่งเป็นโรคอุจจาระร่วงหรือสงสัยว่ามีการติดเชื้อนี้หลังการได้รับยาต้านจุลชีพ ในระยะเวลาการศึกษา 17 เดือน (ม.ค. 2543 ถึง พ.ค. 2544) เป็น 18.64% สามารถเพาะแยกเชื้อ *C. difficile* 107 สายพันธุ์จากอุจจาระทั้งหมด 574 ตัวอย่าง การตรวจหา toxin A และ B genes ของเชื้อ *C. difficile* จากอุจจาระโดยตรงโดยวิธี polymerase chain reaction (PCR) พบ toxin A ใน 47 ตัวอย่าง และพบ toxin B ใน 48 ตัวอย่าง เมื่อเปรียบเทียบผลจากการตรวจหา toxin A และ B genes โดยตรงจากอุจจาระและจากเชื้อ *C.difficile* ที่แยกได้โดยใช้วิธี PCR พบว่า สามารถตรวจพบทั้ง toxin A และ B genes ใน 33 ตัวอย่าง และตรวจไม่พบทั้ง toxin A และ B genes ใน 41 ตัวอย่าง ผลการตรวจไม่สอดคล้องเมื่อตรวจพบ toxin A และ B genes เมื่อตรวจโดยตรงจากอุจจาระ 14 ตัวอย่างแต่ตรวจไม่พบ genes ทั้งคู่เมื่อตรวจจากเชื้อ *C. difficile* และตรวจไม่พบ toxin A และ B genes เมื่อตรวจในตัวอย่างอุจจาระโดยตรง 18 ตัวอย่าง แต่ตรวจพบเมื่อตรวจจากเชื้อ *C. difficile* ที่แยกได้ ตรวจพบ toxin A และ B genes ในเชื้อ *C. difficile* ที่แยกได้จากอุจจาระ 1 ตัวอย่างซึ่งตรวจพบเพียง toxin B genes เมื่อตรวจโดยตรงจากอุจจาระ

การตรวจหา *C. difficile* สายพันธุ์ที่สร้าง toxin genes จากตัวอย่างอุจจาระโดยตรงโดยวิธี PCR พบว่าให้ผลสอดคล้องกับการตรวจหาการสร้าง toxins A และ B โดยวิธี EIA ใน 98 ตัวอย่าง นอกจากนี้วิธี PCR สามารถตรวจพบ toxin A และ B genes ในอุจจาระ 1 ตัวอย่างจาก 50 ตัวอย่างที่ตรวจไม่พบเชื้อ *C. difficile* และให้ผลการตรวจหา toxins ด้วย EIA เป็นลบ

โดยสรุปพบว่าการตรวจหา toxin A และ B genes โดยตรงจากอุจจาระ การตรวจจากเชื้อที่แยกได้และการตรวจหา toxin โดยตรงโดยวิธี EIA ให้ผลสอดคล้องกัน 69 ตัวอย่าง (64.49%) แต่เมื่อเปรียบเทียบผลที่ได้จากการตรวจหา toxin genes จากอุจจาระโดยตรงกับการตรวจจากเชื้อโดยวิธี PCR พบว่ามีความสอดคล้อง 69.16% ดังนั้นการศึกษาครั้งนี้ชี้ให้เห็นว่า การตรวจหา toxin A และ B genes ของ *C.difficile* ในตัวอย่างอุจจาระผู้ป่วยโดยตรงโดยใช้เทคนิค PCR เป็นวิธีที่เหมาะสมสำหรับการตรวจหาการติดเชื้อ *C. difficile* แต่ยังคงต้องมีการพัฒนาวิธีการนี้เพิ่มเติมเช่น ความไวของเทคนิค PCR หรือการกำจัด inhibitory substances เพื่อให้ได้ผลการตรวจที่ถูกต้องแม่นยำขึ้น

ภาควิชาสหสาขาจุลชีววิทยาทางการแพทย์ ลายมือชื่อนิสิต.....

สาขาวิชา จุลชีววิทยาทางการแพทย์ ลายมือชื่ออาจารย์ที่ปรึกษา.....

ปีการศึกษา 2544 ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

4175257630 : MAJOR MEDICAL MICROBIOLOGY

KEYWORD: *Clostridium difficile* / toxin A and B genes / diarrhea / PCR / stool samples

Siriporn Rugdeekha : detection of *Clostridium difficile* toxin A and B genes from stool samples of diarrhea patients by polymerase chain reaction (PCR) technique. (THESIS) THESIS ADVISOR : Assoc. Prof. Pintip Pongpech, Ph.D., THESIS COADVISOR : Siripan Wongwanich, M.Sc., 136 pp. ISBN 974-03-1629-8

The prevalence of *Clostridium difficile* isolated from stools of diarrheal Thai adults patients with suspected antibiotic-associated diarrhea during the 17 months of study (January 2000 to May 2001) were 18.64%. The *C. difficile* strains were found in 107 of 574 stool samples. Detection of *C. difficile* toxin A and B genes directly from stool samples by PCR were performed. It was found that 47 (43.93%) out of 107 stools with culture positive were positive for toxin A gene and 48 (44.86%) out of 107 stools with culture positive were positive for toxin B gene. Comparative results between the detection of toxin A and B genes directly from stools and *C. difficile* isolates by PCR technique were done. It was shown that 33 out of 107 stools with culture positive were positive and 41 samples were negative for both toxin A and B genes. The results were not concordance in 14 samples with positive genes in stool samples but negative in *C. difficile* isolates. Eighteen samples were toxin genes negative in stool samples but positive in *C. difficile* isolates. One of the stools was positive in *C. difficile* isolate but that stool was only toxin B gene positive. When the results from PCR technique were compared to those from EIA, 98 out of 107 stool samples gave the same results. One out of 50 stools with negative culture and negative EIA was both toxin A and B genes positive by PCR.

In conclusion, sixty-nine out of 107 samples (64.49%) gave same results in all three methods, PCR detection in stool specimens directly, PCR detection in *C. difficile* isolates and EIA in stool specimens directly. However, the direct detection of toxin A and B genes from the stool samples provided similar results with the detection from *C. difficile* isolates in 74 stool samples (69.16%). Thus, this study indicated that PCR detection of *C. difficile* toxin A and B genes in stool samples directly seems to be appropriate method for the detection of *C. difficile* infection even though technique development such as the sensitivity of PCR assays or the elimination of inhibitory substances is needed in order to increase the sensitivity of the technique.

Department Medical Microbiology	Student's signature……..
Field of study Medical Microbiology	Advisor's signature……..
Academic year 2001	Co-advisor's signature…….

ACKNOWLEDGEMENTS

I wish to express my deep gratitude to Associate Professor Dr. Pintip Pongpech, Department of Microbiology, Faculty of Pharmaceutical Science, Chulalongkorn University, my advisor, and Mrs. Siripan Wongwanich, Anaerobic bacteriology Section, National Institute of Health, Department of Medical Sciences, Ministry of Publish Health, my co-advisor, for their valuable advices, indispensable help, encouraging guidance, initiating ideas and construction criticisms through out my study.

My sincere gratitude is also given to the member of my advisory committee, Associate Professor Dr. Somying Tumwasorn, Department of Microbiology, Faculty of Medicine, Chulalongkorn University, and especially to Associate Professor Dr. Chertsak Dhiraputra, Department of Microbiology, Faculty of Medicine, Siriraj Hospital, Mahidol University, for their kindness, valuable comments helpful suggestion for the completeness of this thesis.

I also wish to thanks all staff members of the Siriraj Hospital and National Institute of Health, Department of Medical Sciences, Ministry of Publish Health, for their co-operation, encouragement and kindness in every way.

Finally, I wish to express all my deepest gratitude to my beloved parents for their eternal love, encouragement, and kindness will always be remembered.

CONTENTS

CONTENTS (cont.)

page

CONTENTS(cont.)

page

LIST OF TABLES

LIST OF TABLES (cont.)

LIST OF FIGURES

CHAPTER I

Introduction

Clostridium difficile is a causative agent of pseudomembranous colitis and a principle pathogen causing antibiotic–associated diarrhea (AAD) and antibiotic–associated colitis (AAC). Although many infants and hospitalized patients can be asymptomatically colonized with *C.difficile*, the disease can develop rapidly in patients who are taking antibiotics which suppress the normal intestinal flora, allowing *C.difficile* to increase in numbers and to produce its toxin (Lyverly et al., 1988). *C.difficile* can be detected in the stools of 5% or more of healthy adults and even more freguently in the stools of healthy infants (30-50%) and patients without diarrhea in some hospitals and nursing homes (up to 30%) (Fekety et al., 1997). Infants who carry the organism rarely develop *C.difficile* colitis. The reason for this protection might be the immaturily of the toxin binding site (Kelly et al., 1994, Mitty et al., 1994). In addition, *C.difficile* is also widely distributed in the soil, water and hospital environment. In some hospitals and nursing homes, as many as 20-30% or more of patients who have received antibiotics are asymptomatic carriers and shedders of the organism into the environment. Transmission of *C.difficile* is often via the hands of hospital personnel (Mcfarland et al., 1989). The organism is able to survive for long period of time in the hospital enveironment in the form of heat-resistant spores. Spores of *C.difficile* can survive for months on the floors of hospital rooms long after symptomatic patients have been discharged. The organism was also cultured from the fingers and medical apparatus of asymptomatic hospital personnel (Fekety et al., 1981).

Two toxins are known to be produced by *C.difficile*, toxin A and toxin B, which are involved in the pathogenicity of this organism. These toxins are thought to play a major role in diarrhea and colitis. Toxin A is a 308 kDa enterotoxin capable of causing mucosal damage in experimental animal. It is cytotoxic for certain cell lines in culture. It is also a chemoattractant for neutrophils and an activator of macrophages and mast cell, thus, causing them to produce various inflammatory mediators (Bartlett et al., 1994). Toxin A causes actin disaggregation and intracellular calcium release, and damage neurons (Kelly et al., 1994). Toxin B is a 270 kDa cytotoxin that causes depolymerization of filamentous actin, disrupts the actin cytoskeleton and is also a necrotizing enterotoxin 10 times more potent than toxin A in causing damage to human colonic mucosa in cell cutures (Riegler et al., 1995). In general toxin-producing strains produce both toxins and nontoxigenic strains of *C.difficile* lack the genes for both toxin A and toxin B (Fluit et al., 1991). However, a strain that produce only one toxin, toxin B, has also been reported. A strain named *C.difficile* 8864 has been reported to be toxin A-negative and toxin B-positive (Borriello et al.1992, Lyverly et al., 1992). The strain failed to produce toxin A but shows enterotoxic activity in a rabbit ileal loop test. The activity may be due to the fact that toxin B has a potent enterotoxin activity (Lyverly et al., 1992). In addition, a case of severe pseudomembranous colitis due to a toxin A^- B^+ strain of *C.difficile* in the immunosuppressed patient who developed the *C.difficile*-associated diarrhea at a Canadian tertiary care hospital has been reported recently (Limaye et al., 2000, al-Barrak et al., 1999). In both reports, a presumptive case was defined when stool studies were negative for toxin A by a toxin A enzyme immunoassay (EIA) but positive by either cytotoxicity assay or a combination of toxin A/B EIA. Limaye et al. used the PCR technique to amplify the repeating units of the toxin A gene in the

C.difficile isolates from such patients and could detect the toxin A gene with the deletion of 1.7 kb (Limaye et al., 2000). Thus, the detection of both toxin A and toxin B for the diagnosis of *C.difficile* associated diarrhea and colitis is recommended.

The laboratory diagnosis of *C.difficile* infection depends on the demonstration of *C.difficile* toxins in stool. The stool cytotoxin test is a tissue-culture assay based on the detection of cytotoxic activity in stool specimens by the induction of cell rounding by *C.difficil*e by toxin B in stool filtrate and neutralization of the activity of *C.difficile* by *C.sordellii* antitoxin. This assay has been the gold standard for a long time because many researchers indicated that the assay had high sensitivity (94 to 100 persent) and specificity (99 percent) and it was capable to detect as low as 10 pg of cytotoxin (toxin B) in stool samples (Pothoulakis et al., 1993, Kelly et al., 1994). Later on, the disadvantages of the technique include the assay time and expense have been shown such that the experted tissue culture assays required specialized equipment and personnel, making the assay expensive (Karasawa et al., 1999, Lyverly et al., 1988). In addition, the assay can produce up to 30% false-negative results in low producer strains, after toxin degradation by free proteases in stools and in diluted stool specimens in the laboratory (Cleary et al., 1998, Alonso et al., 1999). There are variations in the dilutions of stool specimens tested, the antiserum used, and the time of recording the results. Many hospital laboratories have to send stool samples for *C.difficile* toxin assay to the outside testing laboratories, further adding to the delay and expense.

In the past several years, rapid immunoassays have been developed to replace the tissue culture assay. The latex agglutination test was introduced in 1986 to provide a simple and rapid test for *C.difficile*. Its major advantage is that it is easy to

perform and gives results within 30 minutes (Bartlett et al., 1994). Its major disadvantage is that it does not work very well. This test was originally designed to detect toxin A, but subsequent work showed that it also detected a nontoxic enzyme, glutamate dehydrogenase, which was produced by both toxigenic and nontoxigenic strains of *C.difficile* and other nonpathogenic clostridia (Kelly et al., 1994). The sensitivity and specificity of the available latex tests for the glutamate dehydrogenase antigen have varied widely. When compared to the other tests in patients with clinical criteria for *C.difficile* associated diarrhea, this assay has sensitivity of 58% to 68% and specificity of 94% to 96% (Shanholtzer et al., 1992, Peterson et al., 1988, DiPersio et al., 1991). The latex test for *C.difficile*-associated antigen is not sufficiently sensitive for the routine laboratory detection of *C.difficile*, eventhough it is rapid, simple, relatively inexpensive, and specific.

Several new commercial enzymeimmuno assays or EIA have been developed for the detection of the toxin A and toxin B of *C.difficile* in stool samples (Laughon et al., 1984, Lyerly et al., 1983). These tests are based on the detection of either toxin A or B with specific toxin antibody and capable of detecting 100 to 1000 pg of either toxin. A clinical EIA which detect either toxin A or toxin B or both could be suitable since both toxins are present in the stool of patients. The EIA tests are more specific than they are sensitive. The sensitivity of the commercial EIA kits used for toxin A and B detection has varied widely when evaluated in different laboratories using the same kits but differing criteria for a positive endpoint. Whereas sensitivities have ranged from as low as 70% to as high as 95%, specificity was generally very good (Barbut et al., 1993, Staneck et al., 1996). Although not as sensitive as the tissue

culture assay, EIA are quicker, do not require specialized training of laboratory personnel, and provide reasonable sensitivity and specificity at a lower cost.

The isolation of toxigenic *C.difficile* from the stool has been used for the presumptive diagnosis *C.difficile* infection because patients with the disease contain high number (10^7 or greater) of *C.difficile* in their stool (Bartlett et al., 1980). The most widely use medium for the isolation of *C.difficile* is the cycloserine-fructose-egg yolk medium (George et al., 1989). This medium is a selective and differential medium for *C.difficile* and can detect as few as 2000 organisms in a total count of 6 x 10^{10} bacteria per g (wet weight) of stools. A stool culture of *C.difficile* is highly sensitive if properly done, however, the time required is at least 2-3 days and does not differentiate between toxigenic and nontoxigenic *C.difficile* strains (Kelly et al., 1994).

New tests based on detection of the toxin A and B genes offer the potential for increased speed, specificity and sensitivity. Polymerase chain reaction (PCR) has been used for detection of toxigenic *C.difficile*. Amplification of a portion of either the toxin A gene (Kato et al., 1991, Wren et al., 1990), the toxin B gene (Gumerlock et al., 1993) or both toxin A and B genes has been performed (Kato et al., 1998). The first study on the use of PCR to detect *C.difficile* toxin gene was performed by Wren et al. who amplified a fragment of repeating sequence of *C.difficile* toxin A gene and distinguished toxigenic *C.difficile* strains from nontoxigenic strains (Wren et al., 1990). Later on, Kato et al. amplified the segments of nonrepeating and repeating sequences of the *C.difficile* toxin A gene. They showed that there was no reaction when demonstrated in DNA of toxigenic *C.sordellii* and other *Clostridium spp* (Kato

et al., 1991). The method used by Gumerlock et al. for the detection of toxin B gene of *C.difficile* directly in stool specimens was even more sensitive than detection of cytotoxin by tissue culture cytotoxicity assay (Gumerlock et al., 1993). In a series of 12 patients using this PCR approach, Kuhl et al. detected toxigenic *C.difficile* directly from stool of four patients before relapse when toxin B was undetectable by the tissue culture assay and results of anaerobic culture were negative (Kuhl et al., 1993). The recent study in Thailand has been done by Wongwanich et al. who used the PCR technique to detect toxin A gene from *C.difficile* isolated from feces of premature infant, full-term infant, children with anticancer drug and adult with diarrhea. It was shown that PCR technique was a sensitive and useful tool in the detection of toxin A gene from *C.difficile*(Wongwanich et al.,2001). It is very interesting to perform the study using PCR technique to detect both toxin genes directly from stools of Thai adult patients with suspected antibiotic-associated diarrhea because there has been no such study reported before. Along with this study, the toxin detection by EIA should be performed as the comparative study. The results obtained would indicate whether or not the application of PCR has the potential for adaptation in clinical microbiology laboratory as the effective tool for such fast diagnosis of ADD caused by toxigenic *C.difficile*. Furthermore, conclusive evidence could be made on the usefulness of both PCR and EIA techniques as the tools for detection of toxigenic *C.difficile* directly from stool specimens.

CHAPTER II

Objectives

The purpose of this study are :

1. To detect toxin A and B genes directly from the *C.difficile* positive stools and from isolated *C.difficile* by PCR technique.
2. To detect toxin A and toxin B directly from the same *C. difficile* positive stools using enzyme immunoassay (EIA).
3. To compare the results between PCR and EIA in the detection of toxigenic *C.difficile* directly from stool.

CHAPTER III

Literature Review

1. Historical Review

The disease of pseudomembranous colitis (PMC) has been firstly reported in 1893 by Finney (Finney et al., 1893), who noted diphtheritic pseudomembranous and hemorrhagic diarrhea in young woman following surgery. The number of reported cases of PMC increased dramatically, particularly following the widespread use of broad-spectrum antibiotics. In 1960s and 1970s, antibiotic-associated pseudomembranous colitis became a major clinical problem. The use of the broad-spectrum antimicrobial agents included clindamycin and lincomycin has been shown to be the cause of diarrhea and pseudomembranous colitis in approximately 10% and 1 % of the patients,respectively. (Gurwith et al., 1977, Kabin et al., 1975, Swartzberg et al., 1977). The incidence of PMC varied widely between different hospitals and even between different wards in the same hospital. The higher incidence in some studies probably resulted from transmission within the hospital prior to the recognition that this was a nosocomial infection.

The organism that is a causative agent of PMC, *C.difficile* was initially isolated from stools of healthy newborn infants by Hall and O'Toole in 1935 (Hall et al., 1935). These investigators referred to the organism as *Bacillus difficilis* because of the difficulty they encountered in the isolation of the organism and they were also the first investigators who showed that the organism was toxigenic. This observation was based on the finding that broth cultures and culture filtrates of the organism

caused lesions, respiratory arrest, and dealth when injected into rabbits and guinea pigs. They proposed the interesting idea that the toxin was a neurotoxin because it caused convulsions in animals.

C.difficile was not known to be a pathogen so the toxin of the organism was not studied in detail until the late 1970s, when the association of *C.difficile* with PMC became apparent. At this time, it was shown that stools from patients with PMC contained high levels of cytotoxic activity. Initially, it was suspected that the activity was due to viral infection or perhaps mycoplasma, but the tests for these agents were consistently negative. Larson and his colleagues reported in 1977 (Larson et al., 1977) that stool specimens from affected patients contained a toxin that produced cytopathic changes in tissue-culture cells. When the individual antisera were tested, only the antiserum against *C.sordellii* neutralized the cytotoxicity. This led investigators to believe that *C.sordellii* might be causing PMC. This organism, unfortunately, was almost never isolated from patients with the disease. Later on, it was demonstrated that *C.difficile* was present in high numbers in patients with PMC and that the organism produced a cytotoxin which was neutralized by *C.sorsellii* antiserum (Bartlett et al., 1979. George et al., 1978). It was suggested that *C.difficile* and *C.sordellii* produced toxins that were almost identical. Once these findings were reported, the researchers had developed a suitable animal model for studying the role of *C.difficile* in the pathogenesis of PMC. It had been shown by Small in 1968 that hamsters injected with lincomycin developed severe enterocolitis and died (Small et al., 1968). In the late 1970s, it was shown that hamsters that died from cecitis after treatment with antibiotics contained high numbers of toxigenis *C.difficile* in their stools and that the toxin in the stools of

these animals was very similar to the toxin found in stools of the PMC patients (Bartlett et al., 1977, Rifkin et al., 1978).

Up until 1980, it had been believed that the cytotoxic factor was the only toxin produced by *C.difficile*. There were several reports deseribing enterotoxic activity in fecal extracts from hamsters with antibiotic-associated cecitis (Humphrey et al., 1979, Rifkin et al., 1978), but there was no indication that this activity and the potent cytotoxic activity represented two different toxins. Finally, Banno et al. successfully demonstrated that the cytotoxic activity could be separated from the enterotoxic activity by anion-exchange chromatography. These findings provided conclusive evidence that the organism produces at least two distinct toxins (Banno et al., 1981, Banno et al., 1984). The cytotoxin is referred to as toxin B. The enterotoxin is referred to as toxin A. The designations A and B refer to the elution pattern of the toxins on anion-exchange resins. Toxin A binds less tightly to the resin than toxin B and elutes before toxin B. Over the past decade, a great deal more has been learned about this interesting pathogen and its toxin. Although *C.difficile* is no longer difficult to culture, it occasionally provides difficult diagnostic and therapeutic challenges for the clinician and is now the major identifiable infectious cause of nosocomial diarrhea in many hospitals (Samore et al., 1994).

2. Bacteriology

C.difficile is a gram-positive spore-forming anaerobic bacillus that can be cultured on selective medium containing cycloserine and cefoxitin. The organism produces two toxins: an enterotoxin (toxin A) and a cytotoxin (Toxin B). Nontoxin-producing strains have also been isolated from human carriers, domestic and wild

animals. *C.difficile* colonized the human intestinal tract when the normal flora is altered by antibiotic therapy. The normal human colonic flora, at least in adults and in children over 12 months old, quite effectively prevents colonization by *C.difficile*. A similar microbial barrier exists in experimental and domestic animals, who are likewise resistant to *C.difficile* colonization except when the colonic microflora is altered by antibiotics. Virtually, all antibiotic agents have been associated with *C.difficile* colitis, but the main offenders are ampicillin, clindamycin, cephalosporins and aminoglycosides, as well as combinations of these agents (Bartlett et al., 1979). The precise mechanism of how antibiotics disturb the colonic barrier is not known, but indirect evidence points to elimination of *Bacteroides* species as an requirement for colonization by *C.difficile*. *Bacteroides* species reappear as *C.difficile* disappears during recovery, and treatment with lyophilized *Bacteroides* species eliminates *C.difficile* from the stool flora in patients with recurrent disease (Tvede et al., 1989). Antibiotics, the primary predisposing agent of PMC, act in this fashion by upsetting the normal flora of the bowel. Most cases of PMC result from nosocomial infections. This is apparent from the increasing number of reports describing outbreaks of the disease and from the fact that *C.difficile* is part of the normal flora in only a low percentage of healthy adults (Kim et al., 1981, Malamou-Ladas et al., 1983, Mulligan et al., 1984). The clustering of cases in hospitals and even within hospital wards is the reason why such a wide range of incidence of the disease (0.01 to 10%) has been reported (Gurwith et al., 1977). PMC cases in hospitals can be especially difficult to control because these patients have diarrhea. This mode of transmission increases the release of the organism into the environment, and the organism can be isolated from the clothing and room fixtures of the patient (Kim et al., 1981). Compromised elderly patients undergoing antibiotic therapy are

especially at risk. The organism is present in hospitals not only in the patients but also as “normal” flora of the infants in many hospital nurseries. Most infants carry high numbers of the organism and high levels of toxins A and B in their stool but only few develop the disease indicating the is quite effective protection in the gut (Al-Jumaili et al., 1984, Holst et al., 1981, Larson et al., 1982).

3. Pathogenesis

The two major toxins referred to as toxin A and B are thought to be primarily responsible for the virulence of the bacterium and the major contributors to the pathogenesis of antibiotic-associated gastrointestinal disease. The molecular organization and control of expression of toxin A and B is now starting to be understood. At the same time, the cellular mechanism of action of both toxins, glucosylation of Rho family protein, has also been discovered. Other factors, such as production of proteolytic and hydrolytic enzymes, expression of fimbriae and flagella, chemotaxis and adhesion to gut receptors, and production of capsule, may all play parts in pathogenesis by facilitation colonization of by directly contributing to tissue damage, or both. The details of important virulence factor were as followed:

3.1 Toxins

3.1.1 Pathogenicity locus in *C.difficile*

Futher research established the toxins as the major virulence factors in *C.difficile* disease. Toxin A (Tcd A), an enterotoxin, causes tissue damage in the intestinal mucosa, and induces hemorrhagic fluid secretion in the intestine. Toxin B (Tcd B) lacks overt enterotoxicity but is a potent cytotoxin (Lyerly et al., 1988, Hatheway, 1990). Tcd B exceeds Tcd A in its cytotoxicity by a factor of 100-1000

(Bettle et al., 1991). *In vitro* both toxins show cytotoxic effects on cultured cells, toxin B however is about three orders of magnitude more potent than toxin A. Characteristic cytotoxic effects are rounding up and arborization cells, accompanied by disruption and dramatic redistribution of the microfilament network in the cells (Ottlinger and lin, 1988).

The sequence of the genes of Tcd A and Tcd B have been completely determined from strain VPT 10463. (Barroso et al., 1990, Dove et al., 1990, Eichel-Streiber et al., 1990, Sauerborn and Eichel-Streiber, 1990). These genes are located in close proximity on the chromosome and are transcribed in the same orientation. An additional ORF (*tcdE*) with so far undefined function has also been identified. Apart from toxigenic strains, non-toxigenic strains lacking both toxin genes *tcdA* and *tcdB* are known (Toma et sl.,1988). These strains do not induce any of the symptoms which are specific for *C.difficile*- associated diseases.

From the year 1995, many researchers have successfully determined the nucleotide sequence 3.8 kb upstream and 5.2 kb downstream of the toxin genes A and B of *C.difficile*, as shown in figure 1. They found that toxin genes *tcdA* and tcdB were located together with the three accessory genes *tcdC-E*, the toxin genes from a genetic unit of 19.6 kb that was designated toxigenic element (Hammond and Johnson, 1995) or the pathogenicity locus (Paloc) of *C.difficile* (Braun et al., 1996). The latter designation points to the fact that the existence of this genetic unit was a prerequisite for the pathogenicity of a strain. Comparative analysis of five toxigenic versus five nontoxigenic isolates showed that the integration site of the Paloc was unique on the genome of *C.difficile* and that integration occurs in one direction only,

The stretch of 115 bp found in nontoxigenic strains was replaced by the 19.6 kb locus in toxigenic strains and the locus was obviously not a mobile genetic element by itself. The 115 bp of nontoxigenic strains replaced by the locus in toxigenic strains carry the putative transcription terminator of the cdu1, the ORFs cdu1 upstream of the PaLoc displayed a repressor protein of gram-positive bacteria. A possible polar effect of the loss of this terminator of cdu1 in toxigenic strains could have important consequences for the regulation of the genes of the PaLoc. Such an effect would explain the unidirectional insertion of the PaLoc at a single site of the *C.difficile* genome and might have a rationale for the development of the disease which was induces after antibiotic treatment (Braun et al., 1996).

Toxigenic *C.difficile* isolates from stool specimens of patients suffering from *C.difficile*-associated diseases differ in their capacity to cause disease. This is most probably due to the amount of toxins produced (Wren et al., 1987). Some strains were reported to be deficient in TcdA production while still produciong TcdB (Torres, 1991). This phenomenon could in part be explained by the observation of the deletions within their tcdA genes (Lyerly et al., 1992, Depitre et al., 1993). However, some of the deficient strains were inactive in the hamster model (Delmee / and Avesani, 1990) while others were as potent as the laboratory strain *C.difficile* VPI 10463 (Torres, 1991). Apart from the obvious defects in the *tcdA* genes, differences in regulation of toxin transcription yet to be identified could account for this phenomenon. These toxin genes have their own promotors and ribosome binding sites. Transcriptional analysis of *tcdA-E* genes of the PaLoc of *C.difficile* showed that the five genes are transcribes in a coordinate fashion according to phase of growth (Hammond et al., 1997, Hundsberge et al., 1997). Transcription of *tcdC* is

high in early exponential growth phase, whereas transcription of *tcdA-E* is high is late stationary phase. Sequence analysis of the accessory genes *tcdC, E, D* gave some hints for the function of these genes in *C.difficile.* Due to its basic nature and similarity to BcnA of *Clostridium perfringens* and to Orf-22 of *Clostridium botulinum*, TcdD is most probably a regulatory protein with DNA-binding properties. On this basis, a model for the growth-phase-related, coordinate regulation of toxin expression wherein *tcdC* has a negative and *tcdD* a positive regulatory function on transcription of the *tcdD,B,E* and *tcdA* genes have been discussed (Hundsberger et al., 1997). Analysis of the PaLoc in *C.difficile* strains suggests that the PaLoc is highly stable and conserved genetic unit in toxigenic strains and nontoxigenic isolates lack this genetic unit (Cohen et al., 2000).

3.1.2 Sequencing and expression of the toxin A and B genes.

In 1990, Dove et al. sequenced the entire toxin A gene and confirmed that the toxin was indeed, as indicated by protein chemistry studies, an unusually large protein. The structural gene is 8130 nucleotides long and encodes a protein of 2710 amino acids with a deduced Mr of 30800. The toxin A gene contains a 26.9% G+C content, consistent with the overall G+C content of 28 mol% of the *C.difficile* chromosome (Dove et al., 1990). The sequence of the toxin A gene has been shown in figure 2. Previous findings showed that toxin A agglutinated rabbit erythrocytes and was precipitated by monoclonal antibody PCG-4, demonstrated the multivalent properties and the possibility that the toxin contained the repeats. This was also confirmed by the sequencing of the toxin A gene. Nearly one-third of the gene at the 3′ end encodes a series of 38 contiguous repeating units composed of 855 amino acids. The repeating units form the domain responsible for the binding and

agglutination properties of toxin A. The deduced amino acid sequence of toxin A does not contain any sequence consistent with a signal peptide at its amino- terminal end. This is in agreement with lack of post-translational processing of the N-terminal end of the protein. Toxin A contains a small hydrophobic C-terminal sequence following the repeating units. Therefore, if toxin A is secreted, it may be by an atypical pathway, such as those mediated by a C-terminal secretion signal (Saverborn and Von Eichel-Streiber, 1990). C-terminal signals are known to mediate the export of a number of bacterial proteins (Koronakis et al., 1989).

The toxin B gene has been sequenced by Barroso et al. in the same year as toxin A gene, as shown in figure 3 (Barroso et al.,1990). The structural gene is 7098 nucleotides long and has a deduced amino acid sequence of 2366 (Mr 270 000). Once again, the gene sequence confirmed earlier findings that toxin B, like toxin A, is an extremely large protein expressed as a single polypeptide. The 3′ end of the toxin B gene is located 1350 bp upstream of the toxin A initiation codon. Comparison of the deduced amino acid sequences of the toxin A and B genes confirmed their relatedness at the structural level. A comparative sequence analysis has been reported (Eichel-Streiber et al., 1992). Alignment of the amino acid sequences showed extensive sequence identity ; the toxin share 44.8% identical amino acids and are 63.1% similar if conservative subsitutions are considered. Toxin A and B both contain a series of repeating units at the carboxy terminus that comprise nearly one-third of the molecule. The repeating units of the toxins share homology with the carbohydrate binding region of streptococcal glycosyl transferases (Eichel-Streiber and Saverborn, 1990). The repeating units of toxins A and B are rich in aromatic amino acids and, with rare exceptions, each unit contains

three consecutive aromatic amino acids, YYE. It has been proposed that the repeats have a modular design in which a stretch of aromatic amino acids function in primary protein-carbohydrate interaction (Eichel-Streiber et al., 1992). In this manner, the proteins have evolved an ability to bind different carbohydrate structures based on a similar fundamental unit.

3.1.3 Toxin production

Numerous media have been used for the growth of *C.difficile* and the production of the toxins. Taylor and Bartlett purified toxin B from cultures grown anaerobically in brain-heart infusion broth (Taylor and Bartlett, 1979). The organism grows well in a complex medium such as brain heart infusion broth and produces high levels of both toxin A and toxin B (Sullivan et al., 1982). Under these conditions, the production of the toxins appears to be coregulated. Highly virulent isolates produce high levels of both toxins and weakly virulent isolates produce low levels of the toxins under these conditions. Avirulent strains do not produce either of the toxins. Under straved condition, however, the coproduction of the toxins does not appear to be as tightly regulated. For example, it has been reported that some isolates produce only one of the toxins when grown in media deficient in certain amino acid (Haslam et al., 1986). The level at which this inhibition occurs in the cell is not known. It is unlikely that this type of inhibition of toxin A or B production occurs in the intestine of PMC patients since the organism is in an environment rich in nutrients and relatively free of competing organisms. In many researches, it was found that the toxins were produced and released post-exponentially, near the beginning of the decline phase of the bacterial growth cycle. Although the toxins are produced during stationary phase, there is no correlation of toxin production with

sporulation as occurs with *C.perfringens* enterotoxin (Ketly et al., 1984, Ketly et al., 1986).

3.1.4 Purification and physicochemical properties

Toxin A and B are present in the supernatant fluids from the culture of *C.difficile* and can be purified from the culture filtrates. A number of methods have been developed for the purification of toxins A and B. Most of the purification procedures which have been described utilize an initial concentration step such as ammonium sulfate precipitation or ultrafiltration, followed by gel filtration and ion-exchange chromatography. Ion-exchange chromatography has been used extensively because it separates toxins A and B very effectively. The simplest and most gentle procedure developed thus far for the purification of toxin A is the thermal affinity chromatography based on its ability to bind to immobilized bovine thyroglobulin (Krivan and Wilkins, 1987). Bovine thyroglobulin contains large amounts of the toxin A carbohydrate receptor Gal∝1-3 Galβ1-4 GlcNAc. Toxin A binds the carbohydrate tightly at 4°C, but binding is markedly decreased at 37°C. An affinity matrix can be prepared by covalently coupling thyroglobulin to agarose. Toxin A binds tightly to the affinity column at 4°C. Contaminating proteins are removed by washing at 4°C. Toxin A is then eluted at 37°C. Elution is gentle and yields highly purfied toxin A with full biological activity.

Toxin A purified by Banno et al., Sullivan et al. and Lyerly et al. (Banno et al., 1981, Sullivan et al., 1982 and Lyerly et al., 1986) exhibited similar physicochemical properties. All these researchers reported the toxin to be unusually large. Under denaturing conditions, the toxin had an Mr > 250 000 and did not

associate into subunits. Cloning and sequencing of the toxin A gene, however, has established that the toxin is extremely large with an Mr of 308 000 calculated from the deduced amino acid sequence (Dove et al., 1990).

Toxin B purified by Banno et al., Taylor et al., Sullivan et al. and Lyerly et al. was also found to be an extremely large toxin. Like toxin A, under denaturing conditions the toxin did not dissociate and has an Mr of at least 250 000 (Banno et al., 1981, Taylor et al., 1979, Sullivan et al., 1982 and Lyerly et al., 1986). The large size of toxin B has been established by cloning and sequencing of the toxin B gene (Barroso et al., 1990). The calculated Mr from the deduced amino acid sequence of toxin B is 270 000.

Toxin B is more susceptible to extremes of pH and proteases than toxin A. Both toxins are inactivated by oxidizing agents and can be protected by reducing agent (Sullivan et al., 1982 and Lyerly et al., 1986). The toxins contain few cysteine residues, and the fact that they are not inactivated by reducing agents indicates that disulfide bonds are probably not involved in toxin conformation. Site-directed mutagenesis of the toxin B gene, however, indicates that conserved cysteine residues of the toxins are important for cytotoxicity activity. It has been determined that both toxins contain zinc and iron. Both toxins contain high amounts of the amino acids Asp, Glu, and Gly, and low amounts of His and sulfur-containing amino acids.

3.1.5 Biological properties

Toxin A is an extremely potent enterotoxin (Lyerly et al., 1982 and Lima et al., 1988). On a molar basis, it is as active as cholera toxin in the rabbit ileal loop assay for enterotoxic activity. Its mechanism of action, however, is quite different.

Unlike cholera toxin, toxin A causes extensive damage to the intestine. The villus tips of the epithelium are initially disrupted, followed by damage to the brush border membrane. The mucosa eventually becomes eroded. Damage to the intestinal mucosa is accompanied by extensive infiltration with inflammatory neutrophils, and this probably plays an important role in the extensive damage caused. The fluid responsed presumably results from the tissue damage.

Toxin B is a much more potent cytotoxin than toxin A, it does not cause a fluid response in animal models. The lack of enterotoxicity of toxin B may be due to an inability to bind tightly to a receptor on the brush border membrane cells that line the intestine. Thus, toxin A, which is able to bind to specific carbohydrate receptors on the surface of intestinal cells, initiates damage to the intestine. Toxin B then gains access to the underlying tissues and contributes to the extensive damage during the course of disease (Lyerly et al., 1982 and Lima et al., 1988).

Both toxins cause a similar type of rounding of tissue culture cells, and are active against every cell line which has been tested. Toxin A is far less cytotoxic than toxin B on most cell lines, but when tested on certain cell lines, particularly those of intestinal origin, its activity approaches that of toxin B (Tucker et al., 1990). This is presumably due to an increased density of toxin A carbohydrate receptors on these cell lines. Rounding of intoxicated cell is acconpanied by disassembly of the action microfilaments, whereas microtubules and intermediate filaments are only affected secondarily (Fiorentini et al., 1990). Although the microfilament cytoskeleton is preferentially affected, actin itself does not appear to be the target (Mitchell et al., 1987).

Both toxins are lethal when injected intraperitoneally or subcutaneously in small amounts (Lyerly et al., 1982). Contrary to the cytotoxic activity of the toxins, the lethal activity of toxin A is at least as potent as toxin B, with both toxins having a minimun lethal dose of 50 ng. The mechanism by which the toxins cause death when administered systemically is unknown.

3.1.6 Mechanism of action of *C.difficile* toxins A and B

Because of the similarity of toxins A and B, both structurally and in their effect on cells, they probably have a similar mechanism of action. Although toxin B is significantly more potent as a cytotoxin, the effects of each toxin on tissue culture cells is qualitatively very similar. Intoxicated cells exhibit a retraction of cell processes and rounding of the cell body (Henrigues et al., 1987). The cytopathic effect results from disassembly of filamentous actin, accompanied by a decrease in F-actin and an increase in G-actin prior to the onset of cell rounding. The fact that the toxins are active in very small amounts supports the idea that the mechanism is dependent on enzymatic activity within the target cell. Since the toxins cause cell rounding by disruption of the actin cytoskeleton, proteins regulating this process are likely targets for the toxins.

The Rho/Rac family of low molecular weight GTP-binding proteins are therefore ideal candidates for modification by the toxins. This Ras-related family of proteins, which includes RhoA, RhoB, RhoC, Rac1, Rac2 and CDC42, has recently been implicated in the regulation of actin microfilament assembly. These regions appear to be involved in GTP-binding and in coupling with effector proteins (Bourne

et al., 1991). Like other GTP-binding proteins, Rho proteins are active in the GTP bound form and inactive when GDP is bound.

Two research groups have now independently reported that toxins A and B modified Rho proteins (Just et al., 1994, Just et al., 1995 and Dillon et al., 1995). The finding that Rho from cells pretreated with toxin B was resistant to modification by C3 provided the first evidence that the toxins modify Rho. Just et al. first demonstrated that treatment of cells with toxin B resulted in a time-and concentration-dependent inhibition of C3-catalysed ADP-ribosylation of Rho in cell lysates. Furthermore, toxin B caused an inhibition of C3 activity on Rho, not only in intact cells, but also in cell lysates. The investigators concluded that toxin B acts directly or indirectly by modification of Rho, and that the cytopathic effect involves dysfunction of Rho (Just et al., 1994).

A similar study by Just et al. revealed that toxin A also inhibits C3 modification of Rho. This provided the first evidence that, as suspected, the toxins have a similar molecular mode of action. They also demonstrated that the action of toxin A is not due to proteolytic degradation of Rho or an intrinsic ADP-ribosyl transferase activity. As with toxin B, the activity occurs in cell lysates and also in intact cells treated with toxin A. The modification of Rho by the toxins was determined to be dependent on heat-stable low molecular mass cytosolic factor (Just et al., 1995). Dillon et al. later confirmed that modification of Rho was involved in the mechanism of action of toxins A and B. They extended these findings to show that Rho is covalently modifies in cell lysates treated with the toxins and that the covalent modification prevents ADP-ribosylation of Rho by the C3 exoenzyme

(Dillon et al., 1995). To study whether glucosylation of Rho proteins is the basis of *C.difficile* toxin-induced cytotoxic effects, recombinant toxin-glucosylated RhoA protein was microinjected into cells after toxin removal by membrane filtration. Microinjection of modified RhoA caused morphological alterations, accompanied by depolymerization of actin filaments, which resemble *C.difficile* toxin-induced effects (Just et al., 1995).

3.1.7 Characterization of a Toxin A-negative, Toxin B-positive strain of *C.difficile*

Toxigenic strains of *C.difficile* have been reported to produce both toxin A and B nearly always, and nontoxigenic strains have been reported to produce neither of these toxins. Current studies indicate that it is not always true. Recently, a strain named *C.difficile* 8864 has been reported to be toxin A-negative and toxin B-positive (toxin A^-, toxin B^+)(Borriello et al., 1992, Lyerly et al., 1992 and Torres, 1991). In their study, Borriello et al. demonstrated the absence of toxin A in filtrates by enzyme-linked immunosorbent assay (ELISA) with PCG–4 monoclonal antibody, an antibody that reacts with the series of repeating units located at the COOH terminus of the toxin A molecule. These repeating units represent the portion of the molecule thought to be necessary for the binding of toxin A to intestinal cells. In addition, these investigators reported that the portion of the toxin A gene encoding the repeating units was absent, substantiating their immunoassay results. However, these investigators noted that this strain still caused diarrhea and death in hamsters treated with clindamycin, even though toxin A was absent (Borriello et al., 1992).

Studies by Torres (Torres, 1991) also demonstrated the absence of toxin A in strain 8864 filtrates and showed that the cytotoxic activity produced by strain 8864 was neutralized by antiserum against toxin B from VPI strain 10463, a strain that has been studied extensively because of the high levels of toxins A and B that it produces. Torres noted that toxin B from strain 8864 showed slight differences in its structure from VPI strain 10463 toxin B. This researcher along with Lyverly et al. suggested that it was a modified toxin B. Their results confirmed that although toxin B from strain 8864 is highly related to toxin B from VPI strain 10463, it was more toxic and possesses small amounts of enterotoxic activity. Thus, the ability of this strain to cause disease in hamsters may result from the production of a modified toxin B molecule that is more active than toxin B from VPI strain 10463 (Lyerly et al., 1992).

In 1998, Kato et al., successful used a PCR technique targeted to the repeating sequences of the toxin A gene to distinguish the strains which were toxin A-negative, toxin B-positive strains from both toxin-positive strains and both toxin-negative strains (Kato et al., 1998). By the PCR with NK11-NK9 primers, it was shown that toxin A+, toxin B+ strains generated an approximately 1200-bp product, while a shorter segment of ca. 700 bp was amplifies from toxin A-, toxin B+ strains, suggesting a constitutive difference of the repetitive region between toxin A-, toxin B+ strains and toxin A+, toxin B+ strains (Kato et al., 1998). These researchers also determined the whole repeating sequences of the toxin A from toxin A-negative, toxin B-positive strains of *C.difficile* by PCR and examined the transcription of the nonrepeating regions of the toxin A gene in toxin A-, toxin B+ strains by RT-PCR (Kato et al., 1999). This results showed that toxin A-, toxin B+ strains had identical

repeating sequences with two deletions of the toxin A gene, which encodes the epitopes fully responsible for the reaction with the polyclonal antiserum (Kato et al., 1999).

Although the clinical significance and pathogenicity of toxin A-, toxin B+ strains of *C.difficile* are incompletely understood (Kato et al., 1998). An outbreak of toxin A-, toxin B+ *C.difficile*-associated diarrhea at a Canadian tertiary care hospital has recently been reported (al-Barrak et al., 1999). In this report, a presumptive case was defined when stool studies were negative for toxin A by a toxin A EIA, but positive by either cytotoxicity assay or a combination toxin A-toxin B EIA. However, the molecular characterization of the *C.difficile* strains from these patients was not reported. In a recent report from United States, Limaye and colleagues reported a case of severe pseudomembranous colitis due to a toxin A^-,B^+ strain of *C.difficile* in an immunosuppressed patient (Limaye et al.,2000). *C.difficile* was cultured from stool of patient and analyzed by ELISA, tissue culture and PCR assay. They found that *C.difficile* were negative for toxin A by a toxin A EIA but positive by either tissue culture assay or a combination toxin A/B EIA. PCR results indicated that the *C.difficile* strain from their patient contained a deletion of approximately 1.7 kb in the toxin A gene. This is the portion of the gene that encodes the epitope that reacts with the monoclonal antibody sued in the diagnostic EIA kits for detection of toxin A (PCR-4 epitope).

3.2 Adhesion

Adhesion to host tissue is important for full expression of virulence for many pathogens. The first indication that *C.cifficile* adhered to human gut was obtained in

1979 following its recovery from a washed biopsy specimen from a patient with PMC (Borriello et al., 1979). In a hamster model of the disease, a high virulent toxigenic strain adhered better than a low virulent strain, and both strains adhered better than an avirulent non-toxigenic strain (Borriello et al., 1988). In all cases, adherence was most pronounced in the terminal ileum and caecum, in keeping with the pathology of ileocaecitis. A further observation from the same study was that co-administration of toxin A with the non-toxigenic strain raised adhesion by the latter strain to the same level as that seen for the high virulent toxigenic strain. This implies either that adhesion is facilitated by toxin A mediated damage or that toxin A is directly involved in binding *C.difficile* to the gut, and could contribute to other adhesion mechanisms. 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 (Borriello et al., 1988).However, their role in colonization is unclear, especially as no correlation could be found between presence of fimbriae and the relative ability of different strains of *C.difficile* to adhere 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 adhesins.

Physicochemical 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 (Krishna et al., 1996). This charge is evenly distributed and resides predominantly in the cell wall. Charge interactions with negatively charged host cells may contribute to gut colonization.

3.3 Chemotaxis

The ability of an enteric bacterial pathogen to move from the lumen to the gut mucus would enhance its chances of adhering to gut receptors. The gut mucus of different animals and humans serves as a chemoattractant for *C.difficile* (Borriello, et al., 1998). The toxin (attractant) is heat-stable and resistant to proteolysis. The degree of chemotaxis correlated positively with the relative virulence of the strains examined in a hamster model. Chemotaxis is impossible without motility. For the vast majority of bacteria, motility is mediated by flagella. Little was known about flagella of *C.difficile* until very recently, the researchers purified the flagellin of different strains of *C.difficile*. The flagellin has a molecular mass of about 39 kDa, and the same group has now cloned and sequenced the gene encoding this flagellin and shown it to have 60% similarity to the hag flagellin gene of *Baillus subtilis* (Tasteyre et al., 1997).

3.4 Capsule

C.difficile requires opsonization for significant phagocytosis, suggesting that there might be an anti-phagocytic factor on its cell surface (Dailey et al., 1987). Removal of the cell-surface carbohydrates did not affect the degree of phagocytosis, suggestion that *C.difficile* may have a polysaccharide capsule. This was subsequently shown to be the case. The accumulation of polymorphonuclear cells in gut tissue in PMC may in part be due to toxin A induced recruitment and, although they appear to have little effect on *C.difficile*, they may contribute to the tissue damage characteristic of this infection (Davies and Borriello, 1990; Triadafilo poulous et al., 1987).

3.5 Hydrolytic enzymes

There has been sporadic work on the production of hydrolytic enzymes by *C.difficile*. Hafiz and Oakley found that all of 21 isolates examined were positive for hyaluronidase activity, though the amount produced was variable (Hafiz and Oakley, 1985). Steffen and Hentges examined one isolate of *C.difficile* in a study of hydrolytic enzyme production by anaerobes from human infections. This isolate was positive for hyaluronidase, chondroitin-4-sulphatase, gelatinase and collagenaws, but negative for heparinase, fibrinolysin, lecithinase and lipase (Steffen and Hontges, 1981). It is possible that some of these tissue degradative enzymes contribute to the observed pathology and help to compromise further gut integrity and subsequent fluid accumulation. It is also likely that *C.difficile* derives nutritional benefit from such activity. For example, it is known that *C.difficile* utilizes N-acetylglucosamine, but cannot derive this saccharide by cleavage of mucus glycoproteins (Wilson et al., 1988).

4. *C.difficile* diarrhea and colitis

4.1 Epidemiology

C.difficile is a unique enteric pathogen in that the effected hosts have nearly always been exposed to antimicrobials. The role of antimicrobials in the pathogenesis of *C.difficile* disease appears to be in the alteration of the normal intestinal flora that normally provides protection or resistance to infection with *C.difficile* (Aronsson et al., 1985 and Bartlett et al., 1981). Specifically, *C.difficile* was thought to be part of the normal human intestinal flora, present in low numbers but proliferating under the influence of antimicrobial pressure on the other indigenous flora. In addition, most cases of *C.difficile* diarrhea and colitis are

recognized in patients who are, or recently have been hospitalized (McFarland et al., 1990). Despite this association, recognition that *C.difficile* is a true nosocomial infection was slow. Pseudomembranous colitis, a distinct clinical manifestation of *C.difficile* infection, was originally considered a complication of antimicrobials (partcularly clindamycin) by most hospital infection control practitioners and was not recognized as an infection transmitted between patients in the hospital (Samore et al., 1994). Although *C.difficile* diarrhea has traditionally been discussed separately from other enteric pathogens and other nosocomial pathogens, it is now clear that *C.difficile*, like other enteric pathogens, is exogenously acquired and that hospitalized patients exposed to antimicrobials are uniquely susceptible. *C.difficile* can be detected in stool specimens of many healthy children (Viscidi et al., 1981) and some adults (Aronsson et al., 1985). In both infants and adults, *C.difficile* infection is generally acquired in the hospital. Neonatal colonization is common but almost invariably asymptomatic despite stool cytotoxin levels that may be similar to those in adults with severe colitis. Surveys in neonatal nurseries and outpatient clinics indicate that over 50 percent of healthy infants have transient colonies of toxigenic *C.difficile* (Larson et al., 1982). A possible explanation for the resistance of infants to *C.difficile* toxins is the immaturity of their enterocyte membrane toxin recepters (Eglow et al., 1992). However, children become susceptible to *C.difficile* colitis after the first year of life.

4.2 Risk factor

The administration of antibiotics is the most significant and most frequently reported predisposing factor for *C.difficile*-associated disease (Kelly et al., 1994). One of the main defences against *C.difficile* colonization is the maintenance of a

normal bowel ecosystem. Even when it is present in the colon, the organism is suppressed by other components of the intestinal flora and usually produces no symptoms (Borriello et al., 1990). The use of antibiotics can distrupt this normal ecosystem and allow *C.difficile* to become established and colonize the empty bowel. Although most antibiotics have been associated with predisposition to *C.difficile* infection, the most commonly implicated have been clindamycin, cephalosporins and ampicillin (Aronsson et al., 1984, George et al., 1982). One of these three agents, or groups of agents, can be implicated as the precipitating cause of the disease in approximately 90% of cases.

Parenteral aminoglycosides have never been associated with *C.difficile* infection and other antibiotics with relatively low risk include trimethoprim, rifampicin and quinolones (Bartoett et al., 1992). This probably reflects their lack of effect on endogenous anaerobic gut flora. The use of multiple antibiotics increases the risk of infection. Patients with *C.difficile* infection were more likely to have received multiple courses of therapeutic antibiotics when compared with matched controls (Gerding et al., 1986). It is difficult to extrapolate from frequency of association to relative risk of infection as the former will be very dependent on the amount of antibiotic used (George et al., 1982). For instance, clindamycin is no longer the most commonly implicated antibiotic, reflecting its decreased use rather than a change in its ability to predispore patients to *C.difficile* infection .

As stated previously, nearly all antibacterial agents, given either orally or parenterally, have been associated with *C.difficile* diarrhea. However, susceptibility to any particular antibiotic does not predict the likelihood of *C.difficile*-associated

disease following exposure to that agent, For instance, aminopenicillins can precipitate *C.difficile* infection even though most strains of *C.difficile* are susceptible to them *in vitro,* a phenomenon also seen with salmonella infection (Bartlett et al., 1992).

Antibiotics might influence the risk of *C.difficile*-associated disease by affecting adhesion, toxin production and the microflora of the large bowel (Starr et al., 1997). No data are avaiable to determine whether antibiotics alter adhesion properties of *C.difficile*. Little is known about the effects of antibiotics on toxic production, although the published data suggest that this is not an important mechanism for *C.difficile* diarrhea associated with antibiotic used (Borc et al., 1992). Thus, the most likely pathway by which antibiotics promote *C.difficile*-associated disease is by altering the colonic microflora and ecosystem (Spencer, 1998).

An understanding of the microecology of *C.difficile* provides a better understanding of the disease that this organism causes. *C.difficile* is not a significant component of the microflora in the colon of healthy adult humans or animals. However, it can establish large populations in antibiotic-treated of gnotobiotic animals and in infants before they acquire a complete flora (Onderdonk et al., 1980, Borriello et al., 1986). Major factors that determine whether or not disease develops are the size of the *C.difficile* population, the toxigenicity of the colonizing strain, the presence of other organisms that affect toxin expression or activity, susceptibility of the host and possiby a strain's adhesion to colonic epithelium (Wilson, 1993). The rest of the colonic flora determines the size of the *C.difficile* population, at least in part by limiting available nutrients (Wilson and Perini, 1988). In outbreaks, most

C.difficile disease is caused by nosocomial strains. Environmental comtamination with spores and spread via the hands of health care workers have been implicated in trasmission (McFarland et al., 1989, Simpson et al., 1995). Information with regard to this orgainsm's microecology suggests alternative approaches to the control of disease.

There are a number of risks and host-related factors which should alert both the clinician and the laboratory to the likelihood of *C.difficile* infection in a given hospitalized patient, with old age as a major risk factor. *C.difficile* associated diarrhea(CDAD) is generally more of a problem in patients over 65 years of age. In the first quarter of 1997 the PHLS Communicable Disease Surveillance Centre (CDSC) reported that stools from patients in this age group accounted for approximately 80% of all positive laboratory reports in England and Wales (Brazier, 1998). By contrast, carriage rates in neonates are high, but disease is low. Many asymptomatic neonates and infants colonized with *C.difficile* have detecable toxin B in facces often at very high titres (Cooperstock et al., 1983). It remains unclear why neonates, who are in the process of producing a complex gut bacterial ecosystem, remain unaffected, even though *C.difficile* and its toxins are frequently present in their feces (Libby et al., 1983). The absence of such symptoms could be due to the immature nature of the intestinal flora and lack of development of the toxin receptors in the intestine (Lyerly et al., 1988). Prevalence studies in infants have shown that 15-63% healthy newborns frequently become colonized with *C.difficile* during the first 2 weeks of life(Tabaqchali et al., 1984). After that, the colonization rates remain constant until about 1-2 years of age. In this age range prevalence has been reported at between 7% and 60% (Brettle et al., 1982, Larson et al., 1982). The

reported carrier rates in children > 2 years of age, like those in adults, are < 4% (Stark et al., 1982).

Reported carriage rates in healthy adult have varied from 0.3% in Europe to 15% in Japan (Mulligan, 1988). These difference probably reflect differences in the sensitivity of the culture methods and in selecting subjects who have not previously received antibiotics. Risk factors associated with acquisition of the organism include the advanced age, the severe underlying disease and the length of hospital stay (McFarland et al., 1990). By the fourth week of hospital stay, over half the patients are likely to be culture-positive (McFarland et al., 1990, Clabots et al., 1992). Other risk factors for carriage include the use of antacids and use of stool softeners (McFarland et al., 1990).

4.3 Transmission

The two major potential reservoirs of *C.difficile* in hospitals are both infected human (symptomatic or asymptomatic) and inanimate objects. Patients with symptomatic intestinal infection probably are the major reservoir. Careful studies have indicated that asymptomatic colonization is remarkably common in hospitals with a high prevalence of symptomatic disease (Gerding et al., 1995). Admission to another hospital in which *C.difficile* was endemic resulted in nosocomial acquisition for 20% of patients who initially were culture negative, of those patients, two thirds were asymptomatic (McFarland et al., 1989). Many of these asymptomatic individuals could be implicated as the source of strain recovered from other patients who developed symptomatic disease (Johnson et al., 1990, Clabots et al., 1992). Intestinal infection of healthcare workers also could provide a reservior of *C.difficile*,

but there has been little evidence to indicate that this is an important concern (Gerding et al., 1986). It is for more likely that healthcare workers contribute to transmission because of transient hand carriage (Fekety et al., 1981). Johnson et al. showed the association between the reduction in CDAD rates and the use of gloves. These researcher concluded that personnel hand carriage probably accounted for the majority of hospital transmission of *C.difficile* (Johnson et al., 1990).

Contamination of environment surfaces in the hospital has been well documented (Fekety et al., 1981). Environmental contamination is due to the persistence of spores, which can be highly resistant to cleaning and disinfection measures. Whether environmental contamination has a direct role in transmission is not clear, although transfer to hands could occur when contaminated surfaces are touched. In this way, the hands of the patients or of their healthcare workers could become transiently colonized. In one study, the same strains were found on environmental surfaces in hospital rooms and in cultures of patients in those rooms who subsequently became infected or colonized (McFarland et al., 1989). Direct exposure of patients to certain contaminated items in hospitals may be important in transmission. Contaminated commodes, bathing tubs for neonates, telephones, and rectal thermometers have been implicated as potential sources of *C.difficile* (Brooks et al., 1990). Other potential sources of *C.difficile* have been identified but have not been implicated in transmission to humans.

4.4 Clinical manifestations

The clinical presentation of CDAD is variable and includes diarrhea, colitis without pseudomembranes, pseudomembranous colitis, and fulminant colitis. Some

individuals with toxigenic strains in stool remain totally asymptomatic (Shim et al., 1998). Mild to moderate CDAD is usually accompanied by lower abdominal cramping pain but no systemic symptoms or physical findings (Kelly et al., 1994). Moderate or severe colitis usually presents with profuse diarrhea, abdominal distention with pain, and, in some cases, occult colonic bleeding. Also, systemic symptoms such as fever, nausea, anorexia, and malaise are usually present (Kelly et al., 1994). A minority of patients have disease primarily in the cecum and right colon, presenting with marked leukocytosis and abdominal pain but little or no diarrhea. Fulminant colitis develops in approximalely 1% to 3% of patients, with ileus, toxic megacolon, perforation, and death (Kelly and LaMont, 1998). Clinicians should be aware that the development of these life-threatening complications may be accompanied by a decrease in diarrhea due to loss of colonic muscular tone and ileus. Other complications of *C.difficile* infection include hyperpyrexia, chronic diarrhea, and hypoalbuminemia with anasarca (Bartlett, 1992). *C.difficile* infection may occasionally complicate idiopathic inflammatory bowel disease. Also, a reactive arthritis occurring 1 to 4 weeks after *C.difficile* colitis develops in some patients, resembling that seen after other enteric infections such as *Yersinia*, *Shigella* and *Salmonella* disease (Putlerman and Rubinow, 1993).

4.5 Treatment

The inciting antibiotic therapy should be discontinued if possible, and supportive therapy should be given with fluids and electrolytes as needed (Mylonakis et al., 2001). Antiperistaltic and opiate drugs should be avoided in patients with CDAD, because they mask symptoms and may worsen the course of the disease (Fekety, 1997). Diarrhea will resolve without specific antimicrobial

therapy in up to one fourth of patients (Johnson and Gerding, 1998). Antimicrobial therapy for CDAD is indicated for patients with moderate or severe disease or significant underlying conditions. There is no evidence that treatment of asymptomatic carriers of *C.difficile* provides any clinical benefit, and such therapy has been associated with a prolongation of the carrier state (Kelly et al., 1998, Johnson et al., 1992). In the appropriate clinical setting, antimicrobial therapy should be instituted even before the laboratory results for *C.difficile* are available.

Oral metronidazole and vancomycin hydrochloride are the antibiotics most commonly used, whereas bacitracin methylene disalicylate, teicoplanin, and fusidic acid also have some clinical efficacy. *C.difficile* is uniformly susceptible to vancomycin, while occasional strains have been reported to be resistant to metronidazole (Fekety and Shah, 1993). In many series of patients, therapy with metronidazole or vancomycin was effective in resolving symptoms in more than 95% of patients, although 10% to 20% of patients subsequently relapse (Kelly et al., 1994, Fekety and Shah, 1993).

4.6 Prevention and control

Education of hospital personnel and infection control issues are of paramount importance of handwashing between patients and the need for glove use for the handling of bodily substances of all patients. Education should reinforce the disinfect objects contaminated with *C.difficile* and should be familiar with the disease and its epidemiological features (Johnson and Gerding, 1998). Enteric isolation precautions are recommended for patients with CDAD, and patients should be moved to a private room if possible (Fekety, 1997).

Advances in the prevention and management of antibiotic-associated diarrhea and colitis may depend, in part, on future research efforts on the host defense mechanisms against the organism and toxin and the examination of the virulent properties of the organism, including adherence factors and tissue-degrading enzymes. Further insight in managing relapses may depend on unraveling the conditions under which the normal fecal flora confers colonization resistance, whether it is competition for nutrients or the regulation of the oxidation-reduction potential, pH, or concentrations of short-chain fatty acids (Fekety, 1997). Further elucidation of the inflammatory cytokines involved in the colonic submucosa or in the liver may allow therapeutic interventions directed at minimizing their systemic toxic effects. The development of vaccines against Toxin A in an effort to immunize high-risk patients is under consideration at this time (Tabaqchali and Jumaa, 1995). Better ways to use control strategies and epidemiologic modeling methods to evaluate and manage the contribution of the contaminated hospital environment in cases of *C.difficile* disease is also warranted (Cleary, 1998).

5. Laboratory diagnosis

Since the original observations that *C.difficile* toxins are responsible for antibiotic-associated colitis, several diagnostic tests have been developed that detect the cytotoxin (toxin B) and/or enterotoxin(toxin A) produced by *C.difficile* (Lyverly et al.,1988). Although the issue remains controversial, a combination of *C.difficile* culture and one nonculture-based test (for toxin) has been recommended particularly stool toxin testing (Gerding et al., 1993, Barbut et al.,1993, Gumerlock et al., 1993, Kuhl et al., 1993.).

The specimen

The proper laboratory specimen for the diagnosis of *C.difficile* associated diarrhea is a watery or loose stool (Gerding et al.,1993). Except in rare instances when patients have ileus without diarrhea, swabs are unacceptable specimens because toxin testing cannot be done reliably on these specimens. Specimens should be submitted in a clean, watertight container. For optimal laboratory investigation, a freshy taken faecal specimen should be submitted immediately to the laboratory. Specimens should be kept either refrigerated or frozen. *C.difficile* will survive in faeces for many months at 4^{o}C and for over a decade frozen at -70^{o}C (Brazier,1998).

Method for detecting *C.difficile*

5.1 Culture methods

Culture has been a mainstay in the laboratory diagnosis of CDAD and is essential for the epidemiologic study of nosocomial isolates. The description of an egg yolk agar base medium containing cycloserine, cefoxitin, and fructose (CCFA) by George et al provided laboratories with a selective culture system for recovery of *C.difficile* (George et al.,1979). The two selective agents, cycloserine and cefoxitin, were used at concentrations of 500 mg/L and 16 mg/L, respectively, and the medium also incorporated added carbohydrate, in the form of fructose, and neutral red as a pH indicator in an egg yolk agar base. The successful combination of these antibiotics paved the way for detailed study of *C.difficile* in many centers, and several modifications in both agar and broth formulae were also published (George et al., 1979). Formulations incorporating an egg yolk agar base have a theoretical

advantage over blood agar based media, in that *C.difficile* produces neither lecithinase nor lipase, while other clostridia commomly present in the gut, such as *C.perfringens*, *C.sordellii*, and *C.bifermantans* (which occasionally grow on the selective medium), produce lecithinase and can therefore be easily recognized (Brazier, 1998).

As *C.difficile* is a spore-forming bacillus, procedures aimed at selection for bacterial spores can be applied to specimens before culture. Various groups have reported in the efficacy of alcohol-shock treatment of stool specimens. The selection of spores by alcohol shock greatly diminishes competing flora which enhances both the isolation and easier recognition of *C.difficile* (Bartley and Dowell,1991, Brazier, 1998). One important point regarding the survival of *C.difficile* colonies on solid media is that the organism does not readily sporulate on agar containing the selective agents. Colonies will therefore become non-viable if such agar plates are left in air for prolonged periods. In contrast, on non-selective agars, colonies will usually sporulate heavily after incubation for 72 hours and hence survive prolonged exposure to air.

5.2 Latex agglutination test

Originally thought to detect toxin A, this simple, rapid and inexpensive immunologic test actually detects the presence of glutamate dehydrogenase produced by *C.difficile* , not toxin A (Kelly et al., 1994,Fekety, 1997). This enzyme appears to play no role in the pathogenesis of *C.difficile* diarrhea, but many clinicians and laboratory personnel still incorrectly believe the latex agglutination test detects toxin A. Nontoxigenic strains of *C.difficile* (which do not cause diarrhea) are also positive

with the latex test. In addition, the latex agglutination test is nonspecific, because several other organisms commonly found in stools can produce an antigen that cross-reacts with the antibody directed against glutamate dehydrogenase produced by *C.difficile*. Overall, the latex agglutination test is about as sensitive as but not as specific as the EIA tests for the toxins (Barbut et al., 1993,Lyerly et al., 1988).

Methods for detecting *C.difficile* toxins

5.3 Toxin B (cytotoxin) assay by tissue culture

One of the two exotoxins produces by toxigenic strains of *C.difficile*, toxin B, has a potent effect on the cytoskeletal or microfilament structure of mammalian tissue culture cell lines. This manifests itself either as a rounding-up or as an actinomorphic-like cytopathic effect (CPE) depending on the cell lines, and forms the principle of the cytotoxin assay for toxin B in stools. The detection of cytotoxin in faeces by tissue culture is regarded by many as the " gold standard" with which other diagnostic tests are compared in evaluations of sensitivity and specificity. The cell lines used in these evaluations is important, however, and often overlooked: if Vero cells are used, this may make the alternative method appear less sensitive than a comparison with another, less sensitive cell line. The standard procedure for cytotoxin assay is to make a suspension of the faecal sample in phosphate-buffered saline (PBS), centrifuge it to remove large debris and pass the supernatant through a 0.2 μm membrane filter. The filtrate is then inoculated on to the cell monolayer which is examined after overnight incubation and again after 48 h. To comfirm its specificity , any CPE should ideally be compared with a negative PBS control and be neutralizable with either *C.diffcile* or *C.sordellii* antitoxin. Non-specific CPEs may result from viruses or enterotoxins of other organisms such as *C.perfringens*. The

disadvantages are having to maintain tissue culture cell lines and the slowness of the test (24-48 h) compared with EIA (1-2 h)(Brazier, 1998).

5.4 Enzyme immunoassay for *C.difficile* toxin

In 1981, Yolken et al., evaluated an EIA for detecting *C.diffcile* toxin in faecal samples (Yolken et al., 1981). Of 277 specimens examined, the 84 positives by EIA were also positive for cytotoxin. The authors reported discrepant results in stools from three patients who had a history of treatment of CDAD, which were EIA-positive but cytotoxin-negative, concluding that the method did not give false-positive results. The excellent results of 100% sensitivity and 98.4% specificity paved the way forward for other studies using this methodology. Whereas, Loziewski et al. (2001) reported the sensitivities and specificities of the EIA in comparison with the cytotoxic assay were 74.1% and 100%, respectively. They suggested that EIA was not sensitive enough to be relied on as the sole laboratory test. There have been numerous publications regarding the efficacy of commercial EIA kits. Most of the kits are designed to detect toxin A (the enterotoxin), but a few are designed to detect both toxins. Until recently, it was not clear whether this offered a diagnostic advantage, since it was believed that virtually all toxin-producing strains produced both toxins. However, recent analysis of the strains received by the PHLS Anaerobe reference Unit for typing has revealed that a toxin A-negative, toxin B-positive strain in present in several hospitals in England and accounts for 3% of the strains referred for typing. This strain, termed PCR ribotype 17, corresponds to Delmee's serogroup F and, because of the practice of testing stool samples for toxin A only, may be significantly underreported (Brazier, 1998).

Molecular methods

The advent of methods based on the polymerase chain reaction (PCR) for detecting nucleic acid specific for a given gene or organism directly in clinical specimens heralds a revolution in diagnostic microbiology. For the diagnostic of CDAD, research has been aimed mainly at direct amplification in specimens of the genes for toxin production, but initially other approaches were tried. Wren et al. successfully amplified a repeat sequence of the toxin A gene and used this method to distinguish between 58 toxigenic and 17 non-toxigenic strains. This probe also gave a positive result with a known toxigenic strain of *C.sordellii* but was negative for 17 other clostridial isolates (Wren et al.1990,). Kato et al. also used PCR methodology to detect both non-repeating and repeating segments of the toxin A gene in *C.difficile* isolates. They differentiated 26 non-toxigenic from 35 toxigenic strains and demonstrated no adverse cross-reactions with other clostridia (Kato et al.,1991). Alonso et al. reported the use of a nested PCR method to detect 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 (Alonso et al.,1999).

The next logical step was to bypass the need for culture and to apply molecular methodology directly to stool samples in order to effect rapid diagnosis. A 33 bp oligonucleotide probe designed to detect a sequence of the toxin B gene was tried by Green et al. using both radiolabelling and digoxigenin-labelling. Of 196 stools examined, a 96% correlation with stool cytotoxicity was obtained, with a sensitivity and specificity of 83% and 100%, respectively. This compared very closely with a parallel evaluation of a commercial EIA kit which gave respective

values of 80% and 98% (Green et al.,1994). The approach of Gumerlock et al. was to use a PCR method with a pair of primers to detect a 270 bp fragment of the 16S rRNA gene of *C.difficile*. The authors claimed this method could detect as few as ten cells of *C.difficile* in a stool containing 10^{11} organisms/g. It also could discriminate between *C.difficile* and related organisms such as *C.bifermentans/C.sordellii*, but was unable to differentiate between toxigenic and non-toxigenic strains (Gumerlock et al.,1991). Kato et al. applied their earlier experience to amplify a segment of the toxin A gene in stool specimens, but experience difficulty with PCR inhibitory substances which required prior treatment by an ion-exchange column. In the 39 stool specimens examined in this study, PCR results agreed with both culture and cytotoxin results, and the authors suggested that PCR amplification may be an effective method for the laboratory diagnosis of CDAD. However, the necessity of an ion-exchange process for each specimen proved a barrier to the routine application of this method (Kato et al.,1993). Gumerlock et al. designed a primer which amplified a 399 bp sequence of the toxin B gene and applied it to stool samples. In a small study of 18 cytotoxin-positive specimens they reported 100 % agreement by PCR, and claimed an increased sensitivity of between 10- and 100-fold over tissue culture methodology, detecting as little as 1 pg of DNA. In two 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; the two patients concerned had symptoms typical of CDAD. Equally, one could construe such results as false-positives, and the problem of resolving discrepancies between PCR and other diagnostic methods results needs to be addressed (Gumerlock et al.1993,). Boondeekhun et al. also developed a PCR method to apply directly to stools amplifying 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 (Boondeekhun et al.,1993). Arzese et al. used a PCR method to detect toxin A gene fragments in faeces from patients in a long-term care ward, and claimed improved detection of toxigenic strains (Arzese et al.,1995).

A different molecular approach was adopted by Wolfhagen et al. who used a magnetic immuno-PCR assay (MIPA). This involved separation of *C.difficile* in a faecal sample by monoclonal antibody coated in magnetic material, followed by DNA extraction and PCR amplification with primers aimed at the toxin B gene. Compared with isolation of a cytotoxin-producing strain, this method had a reported sensitivity of 96.7%, a specificity of 100%, and a negative predictive value of 94.1%. Perhaps the major benefit of direct PCR methods could be in elucidating the epidemiology of CDAD, allowing detection of very low numbers of bacteria in specimens and the environment (Wolfhagen et al.,1994).

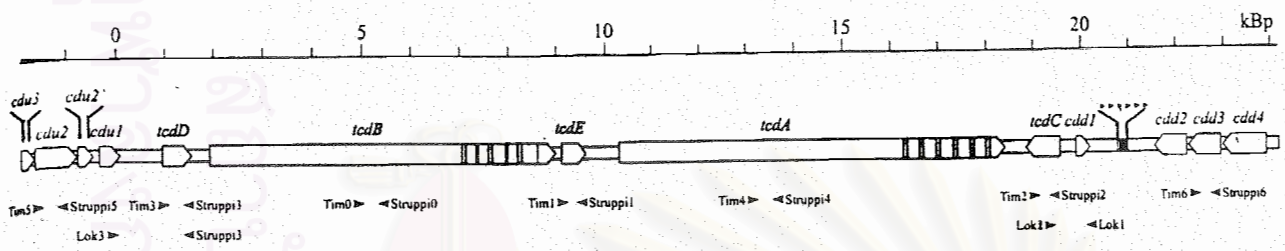


Figure 1. The pathogenicity locus of *C. difficile*

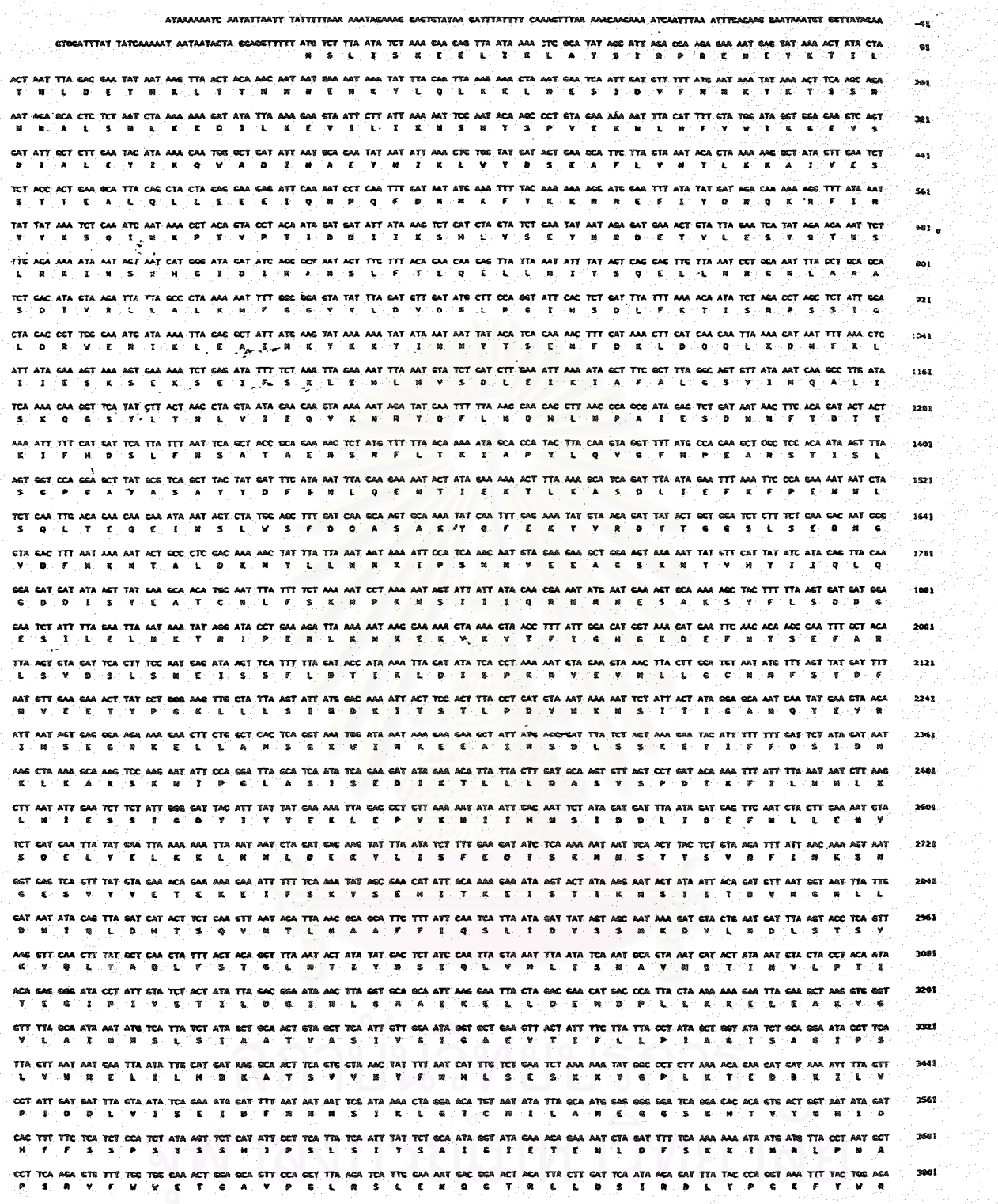


Figure 2. Nucleotide sequences of *C. difficile* toxin A gene (Dove et al., 1990)

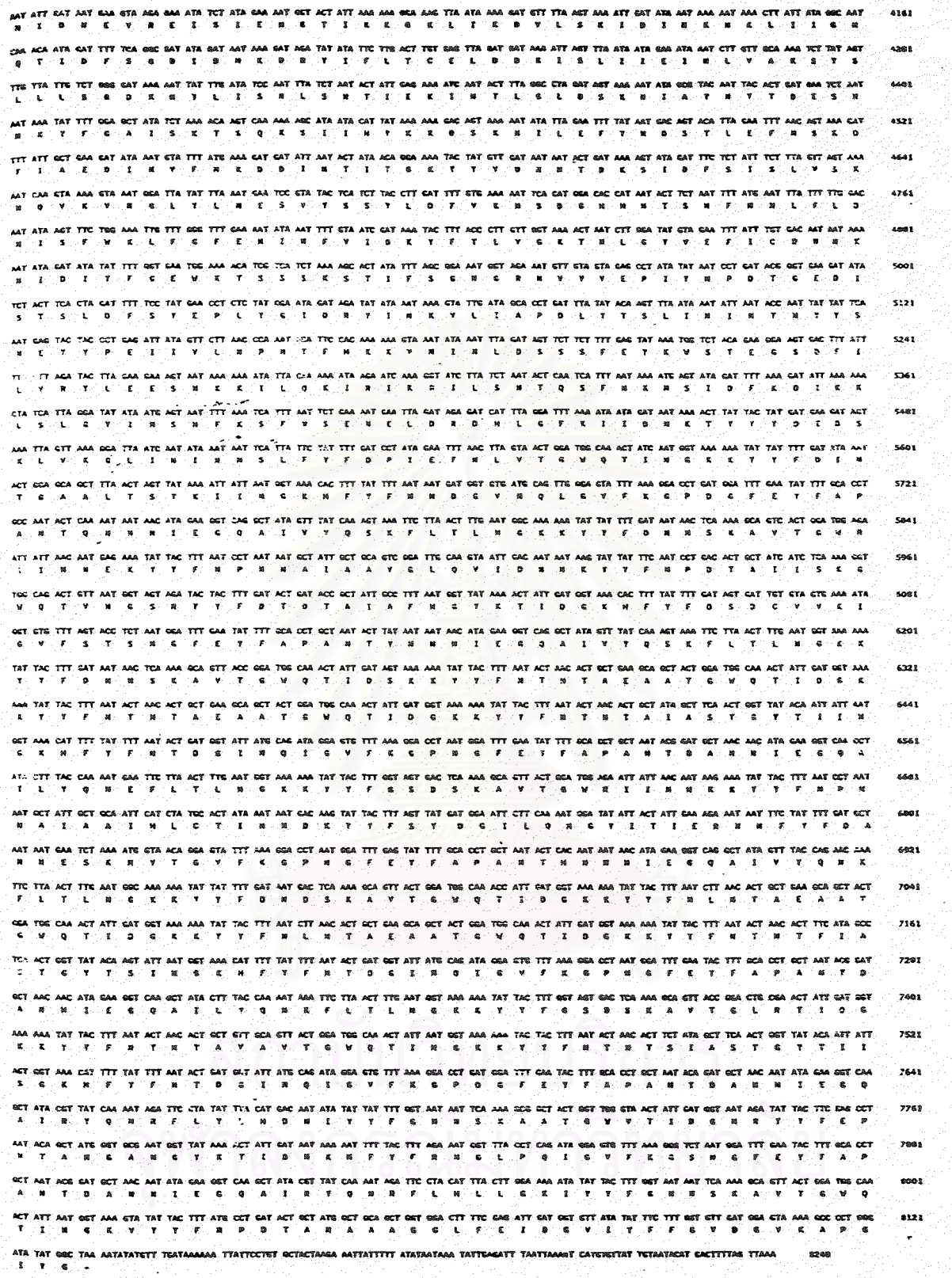


Figure 2 –*Cont.*

1 tctagacaag ctgttaataa ggctaaaaat agagcattta aaaaaataaa aaaagactat
61 gaaaattatt ttaacttgta attaatgagc ttaaagagat atttataata gaaatcaaat
121 tttagaatta actttattgt aaaatcaata acttaatcta agaatatctt aatttttata
181 ttttatatag aacaaagttt acatatttat ttcagacaac gtctttattc aatcgaagag
241 caaattaatc aactgagtgt cttaaattta aaatgttagg aagtgaatgt atatgaaaac
301 ctaagtagat attagtatat tttataaata gaaaggagga tatataaaag agttttagca
361 tttagatgta aaaatattat agtaaaggag aaaattttat gagtttagtt aatagaaaac
421 agttagaaaa aatggcaaat gtaagatttc gtactcaaga agatgaatat gttgcaatat
481 tggatgcttt agaagaatat cataatatgt cagagaatac tgtagtcgaa aaatatttaa
541 aattaaaaga tataaatagt ttaacagata tttatataga tacatataaa aaatctggta
601 gaaataaagc cttaaaaaaa tttaaggaat atctagttac agaagtatta gagctaaaga
661 ataataattt aactccagtt gagaaaaatt tacattttgt ttggattgga ggtcaaataa
721 atgacactgc tattaattat ataaatcaat ggaaagatgt aaatagtgat tataatgtta
781 atgtttttta tgatagtaat gcatttttga taaacacatt gaaaaaaact gtagtagaat
841 cagcaataaa tgatacactt gaatcattta gagaaaactt aaatgaccct agatttgact
901 ataataaatt cttcagaaaa cgtatggaaa taatttatga taaacagaaa aatttcataa
961 actactataa agctcaaaga gaagaaaatc ctgaacttat aattgatgat attgtaaaga
1021 catatctttc aaatgagtat tcaaaggaga tagatgaact taatacctat attgaagaat
1081 ccttaaataa aattacacag aatagtggaa atgatgttag aaactttgaa gaatttaaaa
1141 atggagagtc attcaactta tatgaacaag agttggtaga aaggtggaat ttagctgctg
1201 cttctgacat attaagaata tctgcattaa aagaaattgg tggtatgtat ttagatgttg
1261 atatgttacc aggaatacaa ccagacttat ttgagtctat agagaaacct agttcagtaa
1321 cagtggattt ttgggaaatg acaaagttag aagctataat gaaatacaaa gaatatatac
1381 cagaatatac ctcagaacat tttgacatgt tagacgaaga agttcaaagt agttttgaat
1441 ctgttctagc ttctaagtca gataaatcag aaatattctc atcacttggt gatatggagg
1501 catcaccact agaagttaaa attgcattta atagtaaggg tattataaat caagggctaa
1561 tttctgtgaa agactcatat tgtagcaatt taatagtaaa acaaatcgag aatagatata
1621 aaatattgaa taatagttta aatccagcta ttagcgagga taatgatttt aatactacaa
1681 cgaatacctt tattgatagt ataatggctg aagctaatgc agataatggt agatttatga
1741 tggaactagg aaagtattta agagttggtt tcttcccaga tgttaaaact actattaact
1801 taagtggccc tgaagcatat gcggcagctt atcaagattt attaatgttt aaagaaggca
1861 gtatgaatat ccatttgata gaagctgatt taagaaactt tgaaatctct aaaactaata
1921 tttctcaatc aactgaacaa gaaatggcta gcttatggtc atttgacgat gcaagagcta
1981 aagctcaatt tgaagaatat aaaaggaatt attttgaagg ttctcttggt gaagatgata
2041 atcttgattt ttctcaaaat atagtagttg acaaggagta tcttttagaa aaaatatctt
2101 cattagcaag aagttcagag agaggatata tacactatat tgttcagtta caaggagata
2161 aaattagtta tgaagcagca tgtaacttat ttgcaaagac tccttatgat agtgtactgt
2221 ttcagaaaaa tatagaagat tcagaaattg catattatta taatcctgga gatggtgaaa
2281 tacaagaaat agacaagtat aaaattccaa gtataatttc tgatagacct aagattaaat
2341 taacatttat tggtcatggt aaagatgaat ttaatactga tatatttgca ggttttgatg
2401 tagattcatt atccacagaa atagaagcag caatagattt agctaaagag gatatttctc
2461 ctaagtcaat agaaataaat ttattaggat gtaatatgtt tagctactct atcaacgtag
2521 aggagactta tcctggaaaa ttattactta aagttaaaga taaaatatca gaattaatgc
2581 catctataag tcaagactct attatagtaa gtgcaaatca atatgaagtt agaataaata
2641 gtgaaggaag aagagaatta ttggatcatt ctggtgaatg gataaataaa gaagaaagta
2701 ttataaagga tatttcatca aaagaatata tatcatttaa tcctaaagaa aataaaatta
2761 cagtaaaatc taaaaattta cctgagctat ctacattatt acaagaaatt agaaataatt
2821 ctaattcaag tgatattgaa ctagaagaaa aagtaatgtt aacagaatgt gagataaatg
2881 ttatttcaaa tatagatacg caaattgttg aggaaaggat tgaagaagct aagaatttaa
2941 cttctgactc tattaattat ataaaagatg aatttaaact aatagaatct atttctgatg
3001 cactatgtga cttaaaacaa cagaatgaat tagaagattc tcattttata tcttttgagg
3061 acatatcaga gactgatgag ggatttagta taagatttat taataaagaa actggagaat
3121 ctatatttgt agaaactgaa aaaacaatat tctctgaata tgctaatcat ataactgaag
3181 agatttctaa gataaaaggt actatatttg atactgtaaa tggtaagtta gtaaaaaaag
3241 taaatttaga tactacacac gaagtaaata ctttaaatgc tgcatttttt atacaatcat
3301 taatagaata taatagttct aaagaatctc ttagtaattt aagtgtagca atgaaagtcc
3361 aagtttacgc tcaattattt agtactggtt taaatactat tacagatgca gccaaagttg
3421 ttgaattagt atcaactgca ttagatgaaa ctatagactt acttcctaca ttatctgaag
3481 gattacctat aattgcaact attatagatg gtgtaagttt aggtgcagca atcaaagagc
3541 taagtgaaac gagtgaccca ttattaagac aagaaataga agctaagata ggtataatgg
3601 cagtaaattt aacaacagct acaactgcaa tcattacttc atctttgggg atagctagtg
3661 gatttagtat acttttagtt cctttagcag gaatttcagc aggtatacca agcttagtaa
3721 acaatgaact tgtacttcga gataaggcaa caaaggttgt agattatttt aaacatgttt
3781 cattagttga aactgaagga gtatttactt tattagatga taaaataatg atgccacaag
3841 atgatttagt gatatcagaa atagatttta ataataattc aatagtttta ggtaaatgtg
3901 aaatctggag aatggaaggt ggttcaggtc atactgtaac tgatgatata gatcacttct

Figure 3. Nucleotide sequences of *C. difficile* toxin B gene (Barroso et al., 1990)

3961 tttcagcacc atcaataaca tatagagagc cacacttatc tatatatgac gtattggaag
4021 tacaaaaaga agaacttgat ttgtcaaaag atttaatggt attacctaat gctccaaata
4081 gagtatttgc ttgggaaaca ggatggacac caggtttaag aagcttagaa aatgatggca
4141 caaaactgtt agaccgtata agagataact atgaaggtga gttttattgg agatattttg
4201 cttttatagc tgatgcttta ataacaacat taaaaccaag atatgaagat actaatataa
4261 gaataaattt agatagtaat actagaagtt ttatagttcc aataataact acagaatata
4321 taagagaaaa attatcatat tctttctatg gttcaggagg aacttatgca ttgtctcttt
4381 ctcaatataa tatgggtata aatatagaat taagtgaaag tgatgtttgg attatagatg
4441 ttgataatgt tgtgagagat gtaactatag aatctgataa aattaaaaaa ggtgatttaa
4501 tagaaggtat tttatctaca ctaagtattg aagagaataa aattatctta aatagccatg
4561 agattaattt ttctggtgag gtaaatggaa gtaatggatt tgtttcttta acattttcaa
4621 ttttagaagg aataaatgca attatagaag ttgatttatt atctaaatca tataaattac
4681 ttattttctgg cgaattaaaa atattgatgt taaattcaaa tcatattcaa cagaaaatag
4741 attatatagg attcaatagc gaattacaga aaaatatacc atatagcttt gtagatagtg
4801 aaggaaaaga gaatggtttt attaatggtt caacaaaaga aggtttattt gtatctgaat
4861 tacctgatgt agttcttata agtaaggttt atatggatga tagtaagcct tcatttggat
4921 attatagtaa taatttgaaa gatgtcaaag ttataactaa agataatgtt aatatattaa
4981 caggttatta tcttaaggat gatataaaaa tctctctttc tttgactcta caagatgaaa
5041 aaactataaa gttaaatagt gtgcatttag atgaaagtgg agtagctgag attttgaagt
5101 tcatgaatag aaaaggtaat acaaatactt cagattcttt aatgagcttt ttagaaagta
5161 tgaatataaa aagtattttc gttaatttct tacaatctaa tattaagttt atattagatg
5221 ctaatttttat aataagtggt actacttcta ttggccaatt tgagtttatt tgtgatgaaa
5281 atgataatat acaaccatat ttcattaagt ttaatacact agaaactaat tatactttat
5341 atgtaggaaa tagacaaaat atgatagtgg aaccaaatta tgatttagat gattctggag
5401 atatatcttc aactgttatc aatttctctc aaaagtatct ttatggaata gacagttgtg
5461 ttaataaagt tgtaatttca ccaaatattt atacagatga aataaatata acgcctgtat
5521 atgaaacaaa taatacttat ccagaagtta ttgtattaga tgcaaattat ataaatgaaa
5581 aaataaatgt taatatcaat gatctatcta tacgatatgt atggagtaat gatggtaatg
5641 attttattct tatgtcaact agtgaagaaa ataaggtgtc acaagttaaa ataagattcg
5701 ttaatgtttt taaagataag actttggcaa ataagctatc ttttaacttt agtgataaac
5761 aagatgtacc tgtaagtgaa ataatcttat catttacacc ttcatattat gaggatggat
5821 tgattggcta tgatttgggt ctagtttctt tatataatga gaaattttat attaataact
5881 ttggaatgat ggtatctgga ttaatatata ttaatgattc attatattat tttaaaccac
5941 cagtaaataa tttgataact ggatttgtga ctgtaggcga tgataaatac tactttaatc
6001 caattaatgg tggagctgct tcaattggag agacaataat tgatgacaaa aattattatt
6061 tcaaccaaag tggagtgtta caaacaggtg tatttagtac agaagatgga tttaaatatt
6121 ttgccccagc taatacactt gatgaaaacc tagaaggaga agcaattgat tttactggaa
6181 aattaattat tgacgaaaat atttattatt ttgatgataa ttatagagga gctgtagaat
6241 ggaaagaatt agatggtgaa atgcactatt ttagcccaga aacaggtaaa gcttttaaag
6301 gtctaaatca aataggtgat tataaatact atttcaattc tgatggagtt atgcaaaaag
6361 gatttgttag tataaatgat aataaacact attttgatga ttctggtgtt atgaaagtag
6421 gttacactga aatagatggc aagcatttct actttgctga aaacggagaa atgcaaatag
6481 gagtatttaa tacagaagat ggatttaaat attttgctca tcataatgaa gatttaggaa
6541 atgaagaagg tgaagaaatc tcatattctg gtatattaaa tttcaataat aaaatttact
6601 attttgatga ttcatttaca gctgtagttg gatggaaaga tttagaggat ggttcaaagt
6661 attattttga tgaagataca gcagaagcat atataggttt gtcattaata aatgatggtc
6721 aatattattt taatgatgat ggaattatgc aagttggatt tgtcactata aatgataaag
6781 tcttctactt ctctgactct ggaattatag aatctggagt acaaaacata gatgacaatt
6841 atttctatat agatgataat ggtatagttc aaattggtgt atttgatact tcagatggat
6901 ataaatattt tgcacctgct aatactgtaa atgataatat ttacggacaa gcagttgaat
6961 atagtggttt agttagagtt ggggaagatg tatattattt tggagaaaca tatacaattg
7021 agactggatg gatatatgat atggaaaatg aaagtgataa atattatttc aatccagaaa
7081 ctaaaaaagc atgcaaaggt attaatttaa ttgatgatat aaaatattat tttgatgaga
7141 agggcataat gagaacgggt cttatatcat ttgaaaataa taattattac tttaatgaga
7201 atggtgaaat gcaatttggt tatataaata tagaagataa gatgttctat tttggtgaag
7261 atggtgtcat gcagattgga gtatttaata caccagatgg atttaaatac tttgcacatc
7321 aaaatacttt ggatgagaat tttgagggag aatcaataaa ctatactggt tggttagatt
7381 tagatgaaaa gagatattat tttacagatg aatatattgc agcaactggt tcagttatta
7441 ttgatggtga ggagtattat tttgatcctg atacagctca attagtgatt agtgaataga
7501 taaaaatatg ttaaatatat cctcttatac ttaaatatat aaaaataaac aaaatgatac
7561 actacataaa gtgttctatc taatatgaag atttaccaat aaaaaggtgg actatgatga
7621 atgcacagta gttcaccttt ttatattact aatggtaaca aaatattttt ttatataaac
7681 ctaggaggcg tt

Figure 3 –*Cont.*

Figure 4. Colony characteristics of *C. difficile* on blood agar

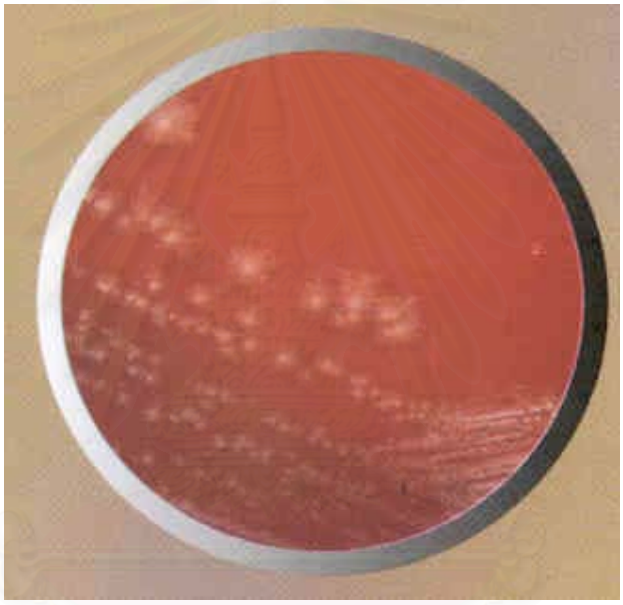


Figure 5. Colony characteristics of *C. difficile* on cycloserine cefoxitin fructose agar (CCFA)

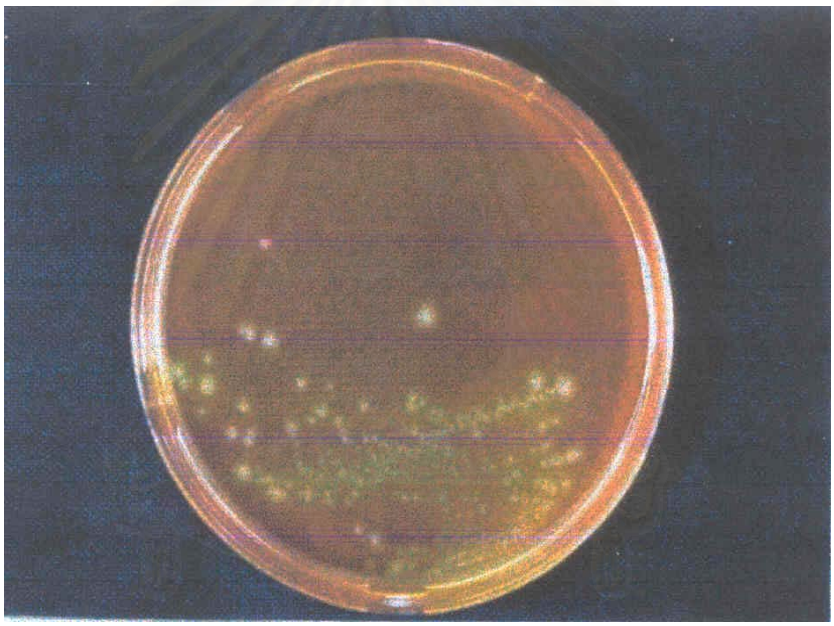
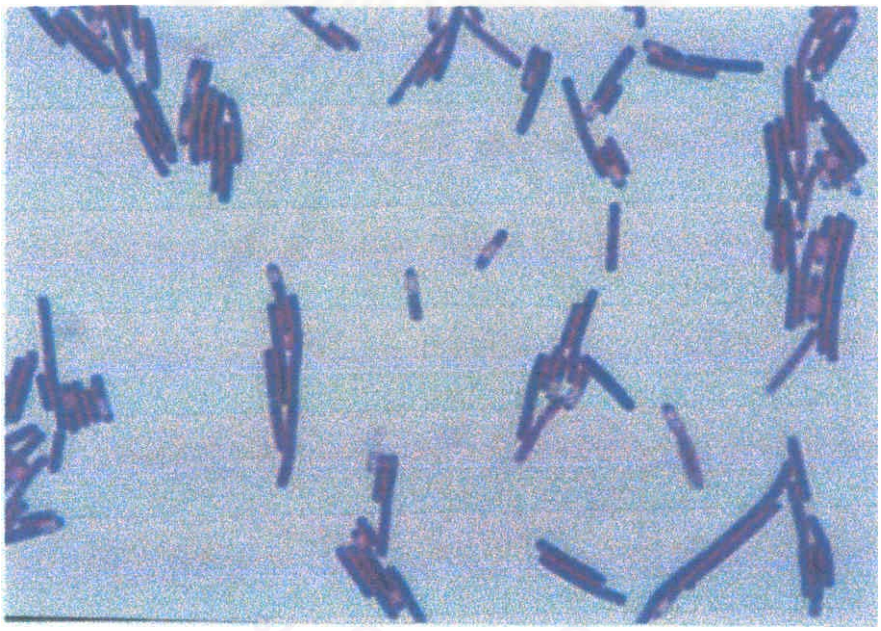


Figure 6. Microscopic characteristic of *C. difficile*



CHAPTER IV

Materials and Methods

1. Specimens

Two hundred and eighty-four stool specimens from inpatients with suspected *C.difficile* antibiotic-associated diarrhea in Siriraj hospital and 290 stool specimens from inpatients with suspected *C.difficile* antibiotic-associated diarrhea admitted at the hospitals all over the country which were sent to National Institute of Health (NIH) for laboratory identification were included in the study.

2. Bacterial control strains

C.difficile GAI 10029 (toxigenic strain) and *C.difficile* A4897 (nontoxigenic strain) were used in this study as the control strains.

3. Bacterial isolation and identification

C.difficile from stool specimens were isolated and identified using the standard procedure as recommended in Wadworth anaerobic bacteriology manual, fifth edition, 1997. Briefly, the stool specimens were inoculated on blood agar (BA) and cycloserine cefoxitin fructose agar (CCFA). The inoculated media were incubated at 35°C for 48 hours under anaerobic condition. Colony morphology of *C.difficile* on BA was white, grey, opaque, round, flat, 2-4 mm. in diameter, spread of edge looked like root, nonhemolytic, specific odor like horse manure, and yellow-green fluorescense. Colony morphology on CCFA was yellow, round, spread of edge looked like root, 5-8 mm.in diameter, specific odor like horse manure, and yellow-green fluorescense. Cell

morphology of *C.difficile* after gram staining were gram positive bacillus with oval subterminal spore. Identification of *C.difficile* was based on the carbohydrate fermentation by the organism. The substrates were arabinose, dextose, fructose, lactose, maltose, mannose, manital, salicin, sucrose and xylose. The fermentation reaction was determined by measuring the pH of the fermentation media as followed:

Carbohydrate substrate	Result	Carbohydrate substrate	Result
Arabinose	-	Mannitol	w^a
Dextrose	a^w	Salicin	$-^w$
Fructose	a^w	Sucrose	$-^w$
Lactose	-	Xylose	$-^w$
Maltose	-	Esculin	+
Mannose	w^-	Starch	-

+	=	positive
-	=	negative
a^w	=	most strains acid, some weakly positive
w^-	=	most strains weakly positive, some negative
w^a	=	most strains weakly positive, some acid
$-^w$	=	most strains negative, some weakly positive

The other biochemical characteristics of *C.difficile* were enzyme lecithinase positive, lipase and catalase negative, no growth under aerobic condition, and gelatin hydrolysis.

4. DNA extraction from *C.difficile*

C. difficile were extracted as described previously by Wongwanich et al.; all *C.difficile* isolates were inoculated on brain heart infusion (BHI) agar and incubated at 35°C for 48 hours under anaerobic condition. One colony of each isolate was transferred with sterile cotton swab into a 1.5 ml microcentrifuge tube containing 500 µl of lysis buffer 1 (formular was shown in appendix II) which was then incubated at 37°C for 15 minutes. After incubation, the culture suspension was centrifuged at 14000 rpm for 2 minutes. The supernatant was then removed using transfer pipett. The pellet was resuspended in 200 µl of lysis buffer 2 (formular was shown in appendix II). The suspension was further incubated for 60 minutes at 56°C. After the incubation, the sample was centrifuged at 14000 rpm for 2 minutes. The supernatant containing the DNA was used in the amplification reactions.

5. DNA extraction from stool specimens

DNA was extracted from stool specimens using QIAamp DNA stool mini kit (QIAGEN, USA). In brief, approximately 180-220 mg stool were weighed in a 2 ml microcentrifuge tube which was then placed on ice. The 1.4 ml of lysis buffer 1 (buffer ASL,QIAGEN,USA) was added to each stool sample which was then vortexed for 1 minute or until the stool sample was throughly homogenized. The suspension was then heated for 5 min at 70°C to lyse the bacteria and then vortexed for 15 seconds and centrifuged at 14000 rpm for 1 min to pellet the stool particles. The 1.2 ml of supernatant was pipetted into a new 2 ml microcentrifuge tube. The one inhibitor tablet (Inhibit EX tablet ,QIAGEN,USA) was added to each sample tube which was then vortexed immediately and continuously for 1 minute or until the tablet was completely suspended. The suspension was incubated for 1 minute at room temperature to allow

the inhibitors to adsorb to the Inhibit EX matrix. The sample was centrifuged at 14000 rpm for 3 minutes to pellet all the inhibitors which bound to Inhibit EX. The supernatant was then pipetted into a new 1.5 ml microcentrifuge tube. The sample was again centrifuged at 14000 rpm for 3 min. After that, 200 μl supernatant of sample was pipetted into the 1.5 ml micrecentrifuge tube containing 15 μl proteinase K. Two hundread microlite of lysis buffer 2 (buffer AL,QIAGEN,USA) was added. The mixture was vortexed for 15 seconds then incubated at 70°C for 10 minutes. After incubation, 200 μl of ethanol (96-100%) was added to the lysate and the sample was mixed by vortexing. The complete lysate was carefully applied to the QIAamp spin column (QIAGEN,USA) which was sitting in a 2 ml collection tube without moistening the rim. The cap was closed and the tube was centrifuged at 14000 rpm for 1 minute. The QIAamp spin column was removed to a new 2 ml collection tube. Then, 500 μl of wash buffer1 (buffer AW1,QIAGEN,USA) was added to the column which was then centrifuged at 14000 rpm for 1 minute. The QIAamp spin column was placed in a new 2 ml collection tube and 500 μl of wash buffer2 (buffer AW2,QIAGEN,USA) was added into the column and centrifuged at 14000 rpm for 3 minutes. The QIAamp spin column was transferred to a new collection tube. The 200 μl elution buffer (buffer AE,QIAGEN,USA) was pipetted directly onto the QIAamp membrane. The tube was incubated for 1 minute at room temperature and then centrifuged at 14000 rpm for 1 minute to elute DNA. The eluted DNA was used for the amplification reaction.

6. Amplification of toxin A and B genes from bacteria and stool samples.

Toxin A and B genes were amplified as described previously by Wongwanich et al.; PCR assay was done in a total volumn of 30 μl containing 0.3 μl of the bacterial DNA preparation, 25 mM $MgCl_2$, $MgCl_2$ free buffer, distilled water, the four deoxynucleoside triphosphates (100 mM each), 45 mg of each primer and 5 U / μl Taq DNA polymerase (Promega, USA). A segment of the toxin A gene was amplified by using primers NK2 and NK3 which were derived from the nonrepeating portion of the *C.difficile* toxin A gene (Kato et al., 1991) and primers YT17 and YT18 was used to detect toxin B gene (Gumerlock et al., 1993). The sequences of the oligonucleotide primers for PCR amplification used in this study were listed in table 1. The reaction mixture was overlaid with 50 μl of mineral oil to prevent evaporation. The thermal profile for the 35 cycles of PCR was used in these experiments included a denaturing step at 95°C for 20 seconds followed by annealing of the primers at 55°C for 30 seconds with an DNA extension at 60°C for 2 minutes. As a quality control for the amplification of toxin A and B genes of *C.difficile* from stool samples, DNA extracted from a negative stool sample spiked with 10^8 cells of *C.difficile* ATCC GAI 10029 were used as positive control. In all cases, a negative control containing all PCR reagents but no DNA was used to monitor the contamination.

Table 1. Sequences of the PCR primers

Gene	Primer	Sequence	Product
TcdA	NK2	CCC AAT AGA AGA TTC AAT ATT AGG CTT	252
	NK3	GGA AGA AAA GAA CTT CTG GCT CAC TCA GGT	
TcdB	YT17	GGT GGA GCT TCA ATT GGA GAG	399
	YT18	GTG TAA CCT ACT TTC ATA ACA CCA G	

7. Quantitative determination of the amount of *C.difficile* in the DNA extraction from stool

The limitation for DNA extraction from stool samples was determined by spiking 10^4 to 10^8 cells of *C.difficile* GAI 10029 to the negative stool culture. The DNA extraction and toxin A and B genes amplification were performed from all five spiked stools. The lowest number of *C.difficile* in stool that showed the positive PCR results would be used as a positive control for the DNA extraction step.

8. Detection of amplified product

Amplification products were analyzed by running 10 μl of the product in a 5% polyacrylamide gel in TBE buffer. DNA markers included in all gels were 100 bp DNA ladder. Gels were run in TBE buffer at a constant 125 volts for 30 minutes. Gels were

stained in an ethidium bromide solution(10 mg/ml) for 30 minutes destained for 15 minutes, and photograghed under UV light.

9. Detection of *C.difficile* toxin A and B in stool samples by EIA

The detection of *C.difficile* toxin A and toxin B in stool samples were performed in 107 stool samples with culture positive and 50 stool samples with negative culture using commercial enzyme immunoassay kit (Premier Cytoclone A+B EIA, Meridian, USA). The basic principle and detection method of the kit were as followed: breakaway microwells were coated with toxin specific monoclonal antibodies. If either toxin was present in the diluted stool samples, toxin monoclonal antibody and biotinylated goat polyclonal conjugate (specific for both toxins) complexes would be formed and remained in the microwells after washing. A Streptavidin-horseradis peroxidase conjugate was then added into the wells to bind to the biotinylated conjugate-toxin complex. After a final washing step, a substrate (urea peroxide) and chromogen (tetramethylbenzidine) mixture was added to the wells. Any bound conjugated would convert the substrate/chromogen to a blue color. Addition of acid (stop solution) converted the blue to a yellow color. The intensity of the yellow was the proportional to the amount of toxins bound in the wells.

In this study, 100 µl of throughly mixed stool samples in 400 µl sample diluent were vortexed until homogenous mixed. One hundread µl of the sample were pipetted into a microtiter will while 100 µl of positive control and sample diluent (negative control) were also added into the other separate microtiter wells. One hundread µl of step 1 conjugate (Meridian, USA) were added to the microtiter wells containing samples and controls. The plate was covered with the plate sealer and incubated at 37° C for 60

minutes. Each well was aspirated into a biohazard receptacle and refilled with the 300 μl wash buffer (Meridian, USA). The aspiration of the wells was repeated. The fill and aspiration cycle were repeated for 5 times. After the final wash, 100 μl of diluted 1x step 2 conjugated (Meridian, USA) was pipetted into each well. The plate was covered with the plate sealer and incubated for 15 minutes at room temperature (20-25°C). After that the plate was washed for 5 times with wash buffer. One hundread μl of the mixed substrate solution were added covered with the plate sealer and incubated for 15 minutes at room temperature. One drop (50 μl) of the stop solution (Meridian, USA) was added to each well. The plate was swirled on the lab bench in order to mix the solution in each well. The absorbance of each solution at 450 nm and 450/630 nm were read within 30 of minutes after the adding of the stop solution.

CHAPTER V

Results

C.difficile in diarrheal patients

In total, 284 fecal specimens were collected from diarrheal patients at Siriraj hospital, Bangkok, Thailand, from January 2000 to May 2001. In addition, 290 fecal specimens which were delivered from government and individual hospitals to the Anaerobe Bacteria Laboratory at the National Institute of Health (NIH), Department of Medical Science, Ministry of Public Health, Nontaburi, Thailand for *C.difficile* identification during March 2000 to May 2001 were also included. *C.difficile* were isolated from 52 out of 284 diarrheal patients from Siriraj hospital (18.31%), and 55 out of 290 diarrheal patients from NIH (18.97%). The total of 107 *C.difficile* isolates were obtained from 574 diarrheal patients (18.64%), as shown in table 3.

The lowest number of *C.difficile* GAI 10029 cells in stool samples detected by PCR technique.

Only the DNA extracted from negative stool spiked with 10^8 CFU/g of *C.difficile* GAI 10029 could be amplified both toxin A and B genes by PCR technique while DNA extracts from stool with the lower amount of bacteria (10^4 – 10^7 CFU/g) could not be detected by PCR. The sensitivity of the PCR technique in detecting toxin A and B genes of *C. difficile* GAI 10029 DNA contained in 10^8-10^4 cells which spiked in the negative stool were showed in figures 7 and 8.

Amplification of toxin A and B genes from *C.difficile* isolates and directly from stool specimens by PCR

A pair of primers directed at the toxin A and B genes was used to specifically ampified toxin A and B sequences from strains of *C.difficile* and amplified directly from stool specimens by the PCR. With these primers, a 252-bp amplified product from toxin A gene and a 399-bp amplified product from toxin B gene was obtained, as shown in figures 9 and 10.

Toxigenic *C.difficile* were found in 52 out of 107 *C.difficile* isolates from diarrheal patients (48.6%). They were positive for both toxin A and B genes. On the other hand, 47 out of 107 stool specimens were toxin A gene positive and 48 specimens were toxin B gene positive which were 43.9% and 44.9% of the total stool specimens with positive culture, respectively. When the DNA extraction and amplification were done with 50 *C.difficile* culture negative stools, it was found that there was 1 specimen which was toxin A and B genes positive (2.0%) All these results were also summarized in table 4. The correlation between the PCR detection of toxin A and B genes from *C.difficile* isolates and directly from stools were shown in table 5. It was shown that the same results were obtained in 74 isolates / specimens. Among these, 33 isolates / specimens were toxin A and B genes positive and 41 isolates / specimens were negative. Positive results were obtained from 18 isolates from which the stools were negative and positive results were obtained from 14 stools while the isolates were negative.

Additional results were shown in table 6. Toxin A gene could not be detected in one stool specimen while toxin A and B genes were positive in *C.difficile* isolated from the same specimen. The details of each individual isolate and specimen were shown in table 1 in the appendix I.

EIA for toxin A and B detection in clinical specimens

Fifty out of 107 (46.73%) stool samples with *C.difficile* positive were positive for toxin A and B when using EIA to detect both toxins directly from stool specimens while all 50 *C.difficile* culture negative stool samples were negative for toxin A and B production, as shown in table 7.

Comparative results between PCR and EIA

The summary results of toxin A and B genes detection from *C.difficile* isolates, directly from stool samples with *C.difficile* positive and negative by PCR and toxin A and B detection by EIA (A+B) directly from stool samples were compared as shown in table 8. Fifty- two out of 107 (48.6%) of *C.difficle* isolates were positive for both toxin A and B genes while 47 out of 107(43.93%) of stool samples with *C.difficile* positive were positive for toxin A gene and 48 out of 107(44.86%) of stool samples with *C.difficile* were positive for toxin B gene. On the other hand, the detection of toxin A and B production in stool samples with *C.difficile* positive by EIA showed that 50 out of 107 stools (46.73%) were positive for toxin A and B.

The results from the detection of toxin A and B genes directly from stools by PCR technique and the detection of toxin A and B from stools by EIA (A+B) were compared in details as shown in tables 9 and 10. In table 9, both methods showed similar results in 97 out of 107 specimens that 44 stools were positive and 53 stools were negative by both techniques. However, 6 stools were negative for genes but positive for toxins and 3 stools were positive for genes but negative for toxins.

In table 10, the detections of individual toxin A and B genes were compared to the detection of toxins by EIA (A+B). Fourty-four out of 107 stool samples were toxin A and B genes positive and positive for toxin production and 53 out of 107 stool

samples were both negative for toxin A and B genes and toxin production. Whereas, 9 out of 107 stool samples with organism positive were not concordance between PCR and EIA results from the direct detection in stool specimens. Three out of 107 stool samples were positive for toxin A and B genes but negative for toxin A and B production detected by EIA, while 6 out of 107 stool samples were negative for toxin A and B genes but positive for toxin production (sensitivity of the PCR detected of *C.difficile* toxin A and B genes in stool specimens directly *versus* EIA was 93.6% and specificity 89.8%).

The comparative results between PCR and EIA was shown in table 11. Thirty-one out of 107(28.97%) samples were positive by all techniques used while 38 out of 107 (35.51%) were negative by all techniques. The results from the other 38 isolates/specimens were not concordance between the techniques used. Three out of 107 (2.8%) were negative both toxin A and B genes when detected in *C.difficile* isolates or stool specimens by PCR but positive results were shown when using EIA (A+B). The other three (2.8%) were positive for toxin A and B genes when detected in *C.difficile* isolates by PCR and by EIA but toxin A and B genes negative in stool samples directly detected by PCR. Thirteen out of 107(12.15%) were negative for toxin A and B genes when detected in *C.difficile* isolates by PCR but positive in stool samples directly and also toxins positive when detected by EIA. Two out of 107(1.87%) were positive for toxin A and B genes when detected in *C.difficile* isolates and stool samples directly but negative when detected by EIA. Fifteen out of 107(14.02%) were positive for toxin A and B genes only when detected in *C.difficile* isolates by PCR and 1 out of 107(0.93%) were positive for toxin A and B genes only when detected in stool samples directly. In 1 out of 107(0.93%), the results could not be discrepanted between PCR and EIA.

The detected results from each individual samples were also shown in table 2 and 3 of the appendix I. The summary on the correlation between the results from PCR and EIA in 50 stool samples with *C.difficile* negative. It was found that one out of 50 stool samples was positive for toxin A and B genes while negative toxins with EIA, as shown in the table 12 and 13. The detailed results from each samples were also shown in table 4 of the appendix I.

Table 3. Recovery of *C.difficile* from diarrheal patients

Patient source	No. of stool sample	No. of *C.difficile* positive (%)
Siriraj hospital	284	52(18.31)
NIH	290	55(18.97)
Total	574	107(18.64)

Table 4. Toxin A and B genes from *C.difficile* isolates and stool samples as detected by PCR

PCR results	*C.difficile* isolates (107 isolates)*	Stool with positive isolates (107 specimens)	Stool with negative isolates (50 specimens)
Toxin A gene positive	52(48.6)**	47(43.9)	1(2.0)
Toxin B gene positive	52(48.6)	48***(44.9)	1(2.0)

* Total number

** Percent positive

*** One specimen was only toxin B gene positive

Table 5. Correlation between the toxin A and B genes detection by PCR from *C.difficile* isolates and directly from stools

Results from PCR	Number of isolates or stools / Total number (%)
Isolates + Stool +	33/107 (30.84)
Isolates - Stool +	14/107 (13.08)
Isolates + Stool -	18/107 (16.82)
Isolates - Stool -	41/107 (38.32)

Table 6. Comparison of the presence of toxin A and B genes from *C.difficile* isolates and from stool samples as detected by PCR

C.difficile isolates / Stool	ToxA + ToxB +	Tox A-Tox B-	ToxA - ToxB +	Total
ToxA + ToxB +	33	14	0	47
Tox A- ToxB -	18	41	0	59
Tox A- ToxB +	1	0	0	1
Total	52	55	0	107

Table 7. Toxins A and B from stool samples as detected by EIA (A+B)

Source	Total	EIA
Positive stools	107	50(46.73)
Negative stools	50	0

Table 8. Toxin A and B genes from *C.difficile* isolates and stool samples as detected by PCR as compared to toxins A and B detection from stool samples by EIA (A+B)

Sample	No. of samples	PCR		EIA(A+B) Positive(%)
		Toxin A gene Positive(%)	Toxin B gene Positive(%)	
C.difficile isolates	107	52(48.6)	52(48.6)	-
Stool				
- with organism positive	107	47(43.93)	48(44.86)	50(46.73)
- with organism negative	50	1(2.0)	1(2.0)	0

Table 9. Correlation between the toxin A and toxin B genes detection by PCR in stools and toxin A and B detection by EIA (A+B)

Result from PCR and EIA	Number of stool/ Total number (%)
PCR + EIA +	44/107 (41.12)
PCR - EIA +	6/107 (5.61)
PCR + EIA -	3/107 (2.8)
PCR - EIA -	53/107 (49.53)

Table 10. Comparison of the presence of toxin A and B genes and toxin A and B in stool samples

Toxin A+B / Toxin genes	Positive	Negative	Total
A+B+	44	3	47
A-B-	6	53	59
A-B+	-	1	1
Total	50	57	107

Table 11. Comparative results from toxin A and B genes detection from *C.difficile* isolates and stool samples by PCR and toxin A and B detection by EIA (A+B) in 107 stool samples with organism positive

PCR *C.difficile* isolates		PCR stool samples		EIA	No. of sample with indicated characteristics (%)
Toxin A	Toxin B	Toxin A	Toxin B	(A+B)	
+	+	+	+	+	31(28.97)
-	-	-	-	-	38(35.51)
+	+	+	+	-	2(1.87)
+	+	-	-	+	3(2.8)
+	+	-	-	-	15(14.02)
+	+	-	+	-	1(0.93)
-	-	+	+	-	1(0.93)
-	-	+	+	+	13(12.15)
-	-	-	-	+	3(2.8)
				Total	107

Table 12. Correlation between the toxin A and B genes detection by PCR directly from stool samples with negative culture and EIA for toxins A and B production

Result from PCR and EIA	Number of stool/total number
Stool + EIA +	0
Stool + EIA -	1
Stool - EIA +	0
Stool - EIA -	49

Table 13. Comparison between the presence of toxin A and B genes from 50 stool samples with negative culture to EIA for toxin A and B production

EIA / PCR stool	Positive	Negative	Total
A+B+	-	1	1
A-B-	-	49	49
Total	-	50	50

Figure 7. Sensitivity of the PCR technique in detecting toxin A gene of *C. difficile* GAI 10029 DNA contained in 10^8-10^4 cells which spiked in the negative stools. Lane 1: 100 bp DNA marker ladder ; Lane 2,3,4,5, and 6 : 10^8, 10^7,10^6,10^5 and 10^4 cells of *C. difficile* GAI 10029 ; Lane 7 : DNA of *C. difficile* GAI 10029 used as positive ; Lane 8 : negative control

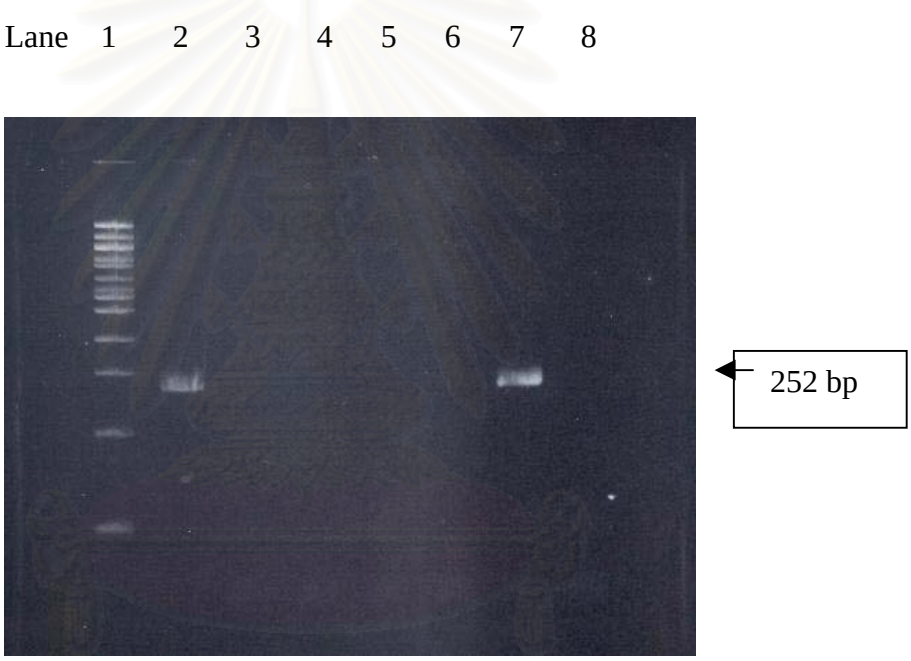


Figure 8. Sensitivity of the PCR technique in detecting toxin B gene of *C. difficile* GAI 10029 DNA contained in 10^8-10^4 cells which spiked in the negative stools. Lane 1: 100 bp DNA marker ladder ; Lane 2,3,4,5, and 6 : 10^8, 10^7,10^6,10^5 and 10^4 cells of *C. difficile* GAI 10029 ; Lane 7 : DNA of *C. difficile* GAI 10029 used as positive ; Lane 8 : negative control

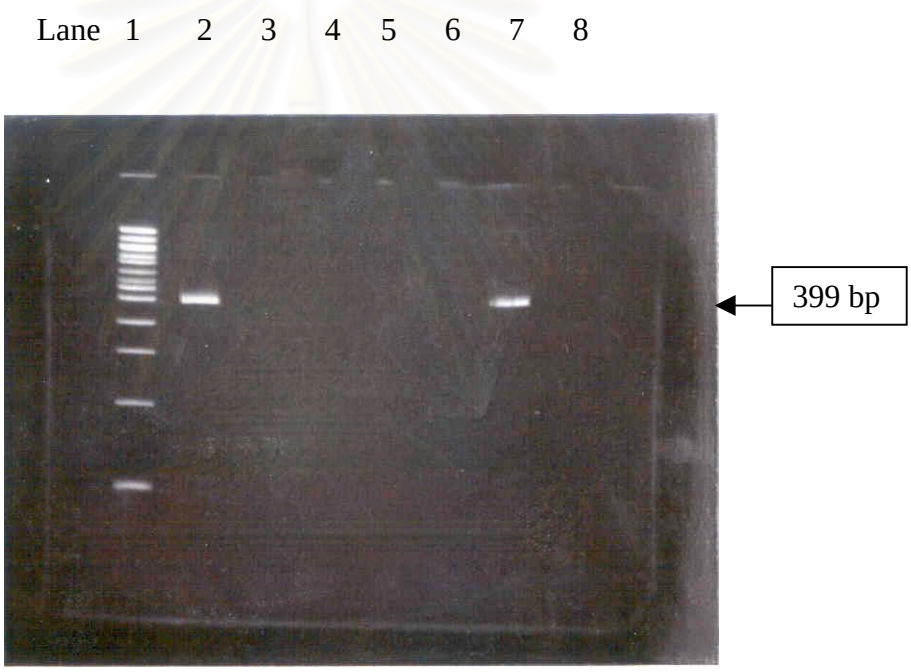


Figure 9. Polyacrylamide gel electrophoresis of PCR products from *Clostridium difficile* GAI 10029 with two primers, NK3-NK2 (toxin A gene), and YT 18-YT17 (toxin B gene). Lane 1: 100 bp DNA marker ladder ; Lane 2 : reference strain of toxigenic *C.difficile* (GAI 10029) with primers NK3-NK2 ; Lane 4 : reference strain of toxigenic *C.difficile* (GAI 10029) with primers YT18-YT17 ; Lane 3 and 5 : negative control

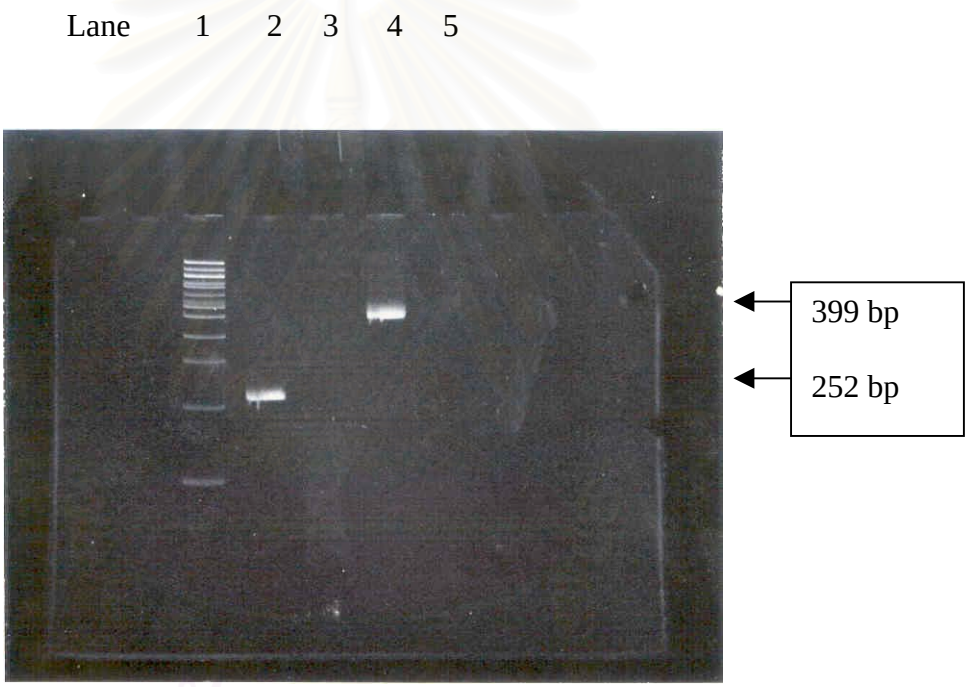
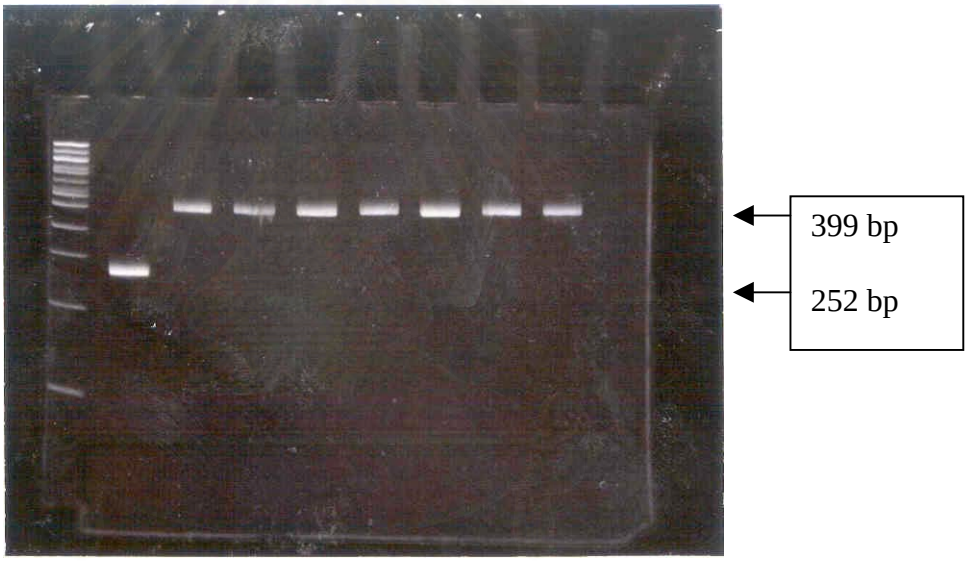


Figure 10. Polyacrylamide gel electrophoresis of PCR products from DNA from *Clostridium difficile* isolates from patients with two primers, NK3-NK2 (toxin A gene), and YT 18-YT17 (toxin B gene). Lane 1: 100 bp DNA marker ladder ; Lane 2: 252 bp amplified product of toxin A gene ; Lane 3 though 8 : 399 bp amplified product of toxin B gene ; Lane 9 : DNA of *C. difficile* GAI 10029 used as positive ; Lane 10 : negative control



CHAPTER VI

Discussion

The prevalence of *C.difficile* in the diarrheal patients in this study was determined. It was shown that 18.64% of Thai patients with diarrhea had *C.difficile* in their stools. This results agreed with the recovery rates in previous reports. Samore et al.(1994) showed that 18% of the diarrheal patients who were admitted at New England Deaconess hospital(Boston, USA) were culture positive for *C.difficile*. Another study done by Wongwanich et al. (2000) showed that 25% of adult patients had *C.difficile* in their stools. McFarland et al. (1990) found that the incidence of *C.difficile*-associated diarrhea was 7.8% in the admitted patient. Jobe et al. (1995) showed that the incidence of *C.difficile* colitis appeared to be sharply increased during the 10 years of study associated with increasing use of antibiotics. This result correlated with the study which performed by Wilcox and Smyth (1998). They found that *C.difficile* infection had increased markedly from 5% in 1993 to 16% in 1996. Gerding et al. (1986) reported an incidence of 20% and Talbot et al. (1986) reported an incidence of 38% after administration of prophylactic antibiotics. McFarland et al. (1989) found that 7% of 428 patients had the positive cultures at admission, and 21% became culture positive during the course of their hospitalizations. The review by Bartlett (1994) showed that toxigenic *C.difficile* was implicated in 10-25% of cases of antibiotic-associated diarrhea, and in 50-75% of cases of antibiotic-associated colitis. The increase of the incidence of *C.difficile*-associated diarrhea or colitis is most probably caused in part by the development and liberal use of broad-spectrum antibiotics (Jobe et al., 1995).

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). Increased age with more severe underlying illness were associated with increased risk of *C.difficile* carriage and diarrhea (McFarland et al., 1990). Factors important in the development of diarrheal disease are exposure to antibiotics. It is due to increasing of population of *C.difficile* in the patient's indigenous flora or acquisition of the organism from an environment source with strains producing of toxin A and B. Many authors have already admitted that nosocomial infection of *C.difficile* is facilitated by the persistance of its spores in environment. It transmitted through personnel or patient-to-patient by contact, and also by intensive antibiotic therapy (Johnson et al., 1990, Kristiansson et al., 1994, Simor et al., 1993).

In this study, PCR technique was applied in the direct detection of *C.difficile* toxin A and B genes in stool specimens. The primer sets used were the NK3 – NK2 and YT18 – YT17, respectively. The NK3 – NK2 primers (Kato et al.,1991) were selected because of its relatively high G+C contents that ranged from 27 to 61% and its sensitivity that could differentiate between toxigenic *C.difficile*, nontoxigenic *C.difficile* and other *Clostridium* spp. The primers set YT18 – YT17 (Gumerlock et al., 1993) were able to ampify the target 399-bp fragment. The sensitivity of their study allowed the detection of toxin B sequences when only 1 pg of toxigenic bacterial DNA was present and differentiate toxigenic *C.diffcile* from nontoxigenic *C.diffcile* (Gumerlock, et al., 1993).

The results between PCR detection of toxin A and B gene from *C.difficile* isolates and PCR detection of stool samples directly were correlated in 74 samples. Fourteen strains of *C.difficile* isolates were toxin A and B genes negative by when they were detected from the isolates but stool samples gave positive PCR results. This may be explained by the fact that there were mixed population of the nontoxigenic *C.diffcile*

strains and toxigenic strains in the same stool specimens. The nontoxigenic isolates might be picked for further toxin genes detection instead of toxigenic ones. This speculation could be supported by various previous studies. Borrello and Honour had shown the toxigenic and nontoxigenic isolates were cultured concurrently from the stools of individual patients, indicating that more than one strains of *C.difficile* could be harbored simultaneously (Borriello and Honour,1983). Limitation of culture method was that toxigenic strains could not be distinguished from nontoxigenic strains (Kelly et al., 1994).

The major problem of direct detection of toxigenic *C.difficile* in stool specimens by PCR in this study was the presence of PCR inhibitory substances in the clinical specimens and the sensitivity of PCR technique for direct detection of toxigenic *C.difficile* in stool specimens. Blood and stool specimens were known to contain potent PCR inhibitors. Stools constituted the complex biological samples which caused problem when PCR was used as a diagnostic method, not only because of the presence of numerous types of bacteria but also because of the different kinds of food degradation products presented (Greenfield et al., 1993). Monteiro et al. (1997) had developed the model for the study of the inhibitors presented in the feces which was later modified as QIAamp tissue method (QIAGEN, USA). The results showed that the inhibitors in the feces were the complex polysaccharides possibly originating from vegetable material in the diet. Processing of specimens is the most critical step in PCR assays. Although it is not essential to isolate DNA in great purify, it is necessary to remove or inactivate substances that inhibit PCR assays. Alonso et al. (1999) described the PCR sample preparation before using a commercial extraction system (QIAamp Tissue Kit, QIAGEN, USA). The samples were diluted ten-fold in distilled water, homogenized, filtered and centrifuged to obtain pellet and DNA was extracted from the

pellet. They showed that only 2 out of 102 stool samples were inhibited in PCR assays. Lou et al. (1997) developed a method for the preparation of fecal specimens for PCR assays. They purified bacterial DNA by Sepharose CL-6B spin column chromatography. The results showed that toxigenic *C.difficile* were found to be positive by both the toxin A and B genes and the negative *C.difficile* PCR results were not due to the presence of the inhibitors in the specimens.

Even though, eighteen stool specimens in this study still gave negative results in the PCR detection of *C.difficile* toxin A and B genes in stool directly while they gave positive results for both toxin genes when detected in *C.diffcile* isolated from such stools. This part of the results might be due to the PCR inhibitory substances in stool specimens were not completely removed by the technique used. The QIAamp DNA stool mini kit (QIAGEN, USA) was used in the extraction of the DNA from stool samples. This method was based on the lysis of the bacterial cells, adsorption of the DNA damaging substances and PCR inhibitors, proteins digestion and binding of DNA to a silica gel membrane. Nucleic acids were eluted under low-salt conditions.

Another reason for the limitation of the direct detection of toxigenic *C.difficile* in stool specimens might be due to the sensitivity of PCR assays. In this study, the sensitivity of PCR assays for detection of toxin A and B genes was performed. It showed that as much as 10^8 CFU/g of *C. difficile* GAI 10029 with spiked in negative stool samples were needed in the assay. In contrast, Gumerlock et al. (1991) showed that the sensitivity of the PCR technique allowed the detection of as few as 10 cells of *C.difficile* among a total of 10^{10} - 10^{11} bacterial cells present in 1 g. in stools.

There was one stool sample that was toxin A and B genes positive when detected in *C.difficile* isolates by PCR but was positive only toxin B gene when detected in stool sample directly. The test was repeated twice but the same results was obtained.

The speculations that could be made were either the limitation of the sensitivity of primers used for toxin A gene detection or the lower amount of toxin A gene than toxin B gene obtained in DNA perparation.

The PCR amplification of the toxin A and B genes of *C.difficile* directly in stool with culture negative were also performed. Surprisingly, the result showed that one of 50 stool samples were positive by both toxin A and B genes. This might be explained by the fact that such stool specimen was not handled properly for the cultivation of anaerobic bacteria such as *C.difficile*.

Boondeekhun et al. (1993) compared three methods for direct examination of *C.difficile* infection in stool samples; cytotoxin assays, culture method and PCR method. They showed that the PCR method for detection of a segment of the *C.difficile* enterotoxin gene directly from stool samples was more rapid than the culture method, and of similar sensitivity to the cytotoxin assay. It agreed with the results obtained by Kato et al.(1993) who showed that PCR results for the detection of toxigenic *C.difficile* directly in stool samples were completely agreed with the cell culture assay results. Kuhl et al. (1993) developed the PCR assay for the rapid direct detection of toxigenic *C.difficile* in stool samples and compared the assay with the cytotoxin assay and culture method. They claimed that the PCR assay is 100 fold more sensitive than anaerobic culture methods. *C.difficile* have not been cultured from stool samples. It was due to the death of organisms between delay in specimen transport to the laboratory or may be explained by sampling problems inherrent to the uneven distribution of *C.difficile* in the fecal samples (Fekety, 1997).

The detection of toxin A and B directly in stool specimens by using Premier Cytoclone A+B enzyme immunoassay (Meridian diagnostic, Inc.) which was a rapid EIA that utilizes microwells coated with toxin A- and B- specific monoclonal antibodies

were also applied in this study. One hundred and fifty seven stool samples which were either positive or negative cultured were determined by EIA. The results showed that the results from PCR detection in stool directly and EIA results were not concordance in 9 stool samples.

Several studies have evaluated the Cytoclone A+B kit (Cambridge, USA). This test has the potential advantage of being able to detect both toxin A and B. Most strains of *C.difficile* produce both toxins, but the relative amounts of toxin produced may vary between strains. When chart reviews were performed to establish diagnoses of *C.difficile*-associated diarrhea and colitis, the previously reported sensitivities and specificities of the Cytoclone A+B EIA ranged from 75.5 to 84.5%, and from 97.8 to 100%, respectively (Barbut et al., 1993, and Doern et al., 1992). They concluded that EIA was not sensitive enough to be relied on as the sole laboratory test, although it had excellent specificity but lacked of the sensitivity and the high rate of indeterminate results (6.7%). Whereas, Loziewski et al. (2001) reported the sensitivities and specificities of the Cytoclone A+B EIA in comparison with the cytotoxic assay to be 74.1% and 100%, respectively. They suggested that the laboratories should combined a rapid toxin A+B EIA direct toxin detection in stool with other method in case that there is a negative stool toxin assay. Doern et al. (1992) compared Cytoclone A+B EIA kit with a cytotoxicity assay and recorded that the technique provided both high sensitivity (94.9%) and specificity (98.9%) while Arrow et al. (1994) found a slightly higher sensitivity (96.2%), and specificity was slightly lower (93.5%). The study by O' Connor et al. (2001) showed that the sensitivities and specificities of the Premier A+B EIA (Meridian, USA) in comparison with the cytotoxic assay were 80 and 99%, respectively. The superior performance of the Premier assays might be in part be related

to their ability to detect *C.diffcile* toxin $A^- B^+$ strains that were nondetectable with toxin A- specific assays (Al-Barrak et al., 1999, and Alfa et al., 2000).

In determining *C.difficile* disease, the relationship between the laboratory results and the clinical diagnosis is not always clear-out. Culture for toxigenic *C.difficile* and cytotoxin assays may be positive for patients without enteric disease. In the first months of life, up to 50% of infants may be colonized with highly toxigenic strains and do not express any clinical symptoms (Cooperstock et al., 1983, and Eglow et al., 1992.).

In this study, EIA showed both the false-negative and false-positive in comparison with the PCR results which were obtained from *C.difficile* isolates and in stool samples directly. In case of false negative, it may be explained by low toxin titers in stool specimens, toxin degradation by free proteases in feces or delayed in specimen transport to laboratory (Knoop et al., 1993, and Merze et al.,1994). In case of false-positive, it might be due to cross reaction from toxin that produced by *C.sordellii*. This organism produces two toxins which are very similar to toxins A and B of *C.difficile* (Lyerly et al., 1988, and Lyerly et al.,1998).

Rapid diagnosis of *C.difficile* in patients with pseudomembranous colitis and antibiotic-associated diarrrhea is very important and guides both the treatment and the control of nosocomial spread of infection. The need for rapid, sensitive, and specific detection and diagnosis of *C.difficile* diarrhea is demonstrated by the wide array of new tests developed recently for the detection of toxin gene of *C.difficile* by molecular methods based on the polymerase chain reaction (Brazier, 1998).

This study was one of the very first studies using PCR technique in the detection of *C.difficile* toxin A and B genes directly from stool specimens of adult patients with suspected antibiotic-associated diarrhea in Thailand. The comparative study on PCR detection of *C.difficile* isolates and toxin detection by EIA was also performed. The

major problem in this study was the incomplete removal of the PCR inhibitory substances in stool specimens which decreased the sensitivity of PCR technique. However, if the modified methods for removal or inactivation of PCR inhibitors in fecal specimens could be obtained, PCR should be the powerful molecular biology technique for the detection of target DNA in fecal specimens. This method has been successfully shown to be rapid, and simple to perform for diagnostic of *C.difficile* infection even though the sensitivity and specificity of the technique should be improved.

CHAPTER VII

Conclusions

Stool specimens collecting from Thai adult patients with suspected antibiotic-associated diarrhea in this study were 574 samples. The 107 isolates of *C.difficile* (18.64%) were obtained. Detection of toxin A and B genes from of *C.difficile* isolates and from stool specimens by PCR were performed. The 33 samples with culture positive were positive for both toxin genes while 41 samples with culture positive were negative. The results were not concordance in 14 samples which toxin genes were positive in stool samples but negative when detected in *C.difficile* isolates and 18 samples which were toxin genes negative in stool samples but positive when detected in *C.difficile* isolates. One of the *C.difficile* isolate was toxin genes positive while the stool was positive only toxin B gene.

Detection of toxin A and B directly in stool specimens by EIA were also performed. Nine out of 107 stool samples gave different results from those PCR detection in stools directly. In 50 stools with negative culture and negative EIA, one sample was positive for both toxin A and B genes by PCR.

The comparison results among the three methods; PCR detection in *C.difficile* isolates, PCR detection in stool specimens directly, and EIA in stool specimens directly were summarized. Sixty-nine out of 107 samples (64.49%) gave same results in all three methods. However, the direct detection of toxin A and B genes from the stool samples provided similar results with the detection from *C. difficile* isolates in 74 stool samples (69.16%).

PCR detection of *C.difficile* toxin A and B gene in stool specimens directly seems to be the appropriate method for detection of *C.difficile* infection. Although PCR

inhibitory substances were still recognized in DNA prepared from stool specimens, suggesting that the inhibitory substance should be removed by the modified method in the future study. EIA has an advantage that it is the test for the toxin detection but the limitation of EIA such as the maintenance of the quality of the specimens is still a serious problem. It could be concluded that the PCR detection technique for toxigenic *C.difficile* was more valuable than the culture method. The method has the potential for the adoption in routine laboratory practice.

REFERENCES

Al-Barrak, A., Embil, J., Dyck, B., et al. 1999. An outbreak of toxin A negative, toxin B positive *Clostridium difficile*-associated diarrhea in a Canadian tertiary-care hospital. Can Commun Dis Rep 25 : 65-69.

Alfa, M. J., Kabani, A., Lyerly, D., et al. 2000. Characterization of a toxin A-negative, toxin B-positive strain of *Clostridium difficile* responsible for a nosocomial outbreak of *C. difficile*-associated diarrhea. J Clin Microbiol 31: 963-967.

Al-Jumaili, I. J., Shibley, M., Lishmann, A. H., and Record, C. O. 1984. Incidence and origin of *Clostridium dificile* in neonates. J Clin Microbiol 19 : 77-78.

Alonso, R., Munoz, C., Gros, S., et al. 1999. Rapid detection of toxigenic *Clostridium difficile* from stool samples by a nested PDR of toxin B gene. J Hosp Infect 41 : 145-149.

Aronsson, B., Mollby, and Nord C-E. 1984. Diagnosis and epidemiology of *Clostridium difficile* enterocolitis in Sweden. J Antimicrob Chemother 14(suppl D) : 85-95.

Aronsson, B., Mollby, and Nord C-E. 1985. Antimicrobial agents and *Clostridium difficile* in acute enteric disease: epidemiologic data from Sweden, 1980-1982. J Infect Dis 151 : 476-481.

Arrow, S. A., Croese, L., Bowman, R. A., and Riley, T. V. 1994. Evaluation of three commercial enzyme immunoassay kits for detecting faecal *Clostridium difficile* toxins. J Clin Pathol 47: 954-956.

Arzese, A., Trani, G., Riul, L., and Botta. G.A. 1995. Rapid PCR method for specific detection of toxigenic *Clostridium difficile*. Eur J Clin Microbial and Infect Dis 14 : 716-719.

Banno, Y., Kobayashi, T., Kono, H., et al. 1984. Biochemical characterization and biologic actions of two toxins (D-1 and D-2) from *Clostridium difficile*. Rev Infect Dis 6(Suppl 1) : s11-20.

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

Barbut, F., Kajzer, C., Planas, N., and petit, J-C. 1993. Comparison of three enzyme immunoassays, a cytotoxicity assay, and toxigenic culture for diagnosis of *Clostridium difficile*-associated diarrhea. J Clin Microbiol 31(4) : 963-967.

Barc, M. C., Depitre, C., Cortimer, G., Collignon, A., Su, W. J., and Bourlioux, P. 1992. Effects of antibiotics and other drugs on toxin production in *Clostridium difficile in vitro* and *in vivo*. Antimicrobial Agents Chemother 36 : 1332-1335.

Barroso, L. A., Wang, S. Z., Phelps, C. J., Johnson, J. L., and Wilkins, T. D. 1990. Nucleotide sequence of *Clostridium difficile* toxin B gene. Nucleic Acids Research 18 (13) : 4004.

Bartlett, J. G. 1981. Antimicrobial agents implicated in *Clostridium difficile* toxin associated diarrhea or colitis. Johns Hopkins Med J 149 : 6-8.

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

Bartlett, J. G. 1994. *Clostridium difficile* : History of its role as an enteric pathogen and the current state of knowledge about the organism. Clin Infect Dis 18(suppl 4) : 265-272.

Bartlett, J. G., Chang, T. W., Taylor, N. S., and Onderdonk, A. B. 1979. Colitis induced by *Clostridium difficile*. Rev Infect Dis 1 : 370-378.

Bartlett, J. G., Onderdonk, A. B., Cisneros, R. L., and Kasper, D. L. 1977. Clindamycin-associated colitis due to a toxin-producing species of *Clostridium* in hamsters. J Infect Dis 136 : 701-705.

Bartlett, J. G., Taylor, N. S., Chang, T., and Dzink, J. 1980. Clinical and laboratory observations in *Clostridium difficile* colitis. Am J Clin Nutr 33 : 2521-2526.

Bartley, S. L., and Dowell, V. R. 1991. Comparison of media for the isolation of *Clostridium difficile* from faecal specimens. Laboratory Medicine 22 : 335-338.

Bette, P., Oksche, A., Mauler, F., et al. 1991. A comparative biochemical, pharmacological, and immunological study of *Clostridium novyi* α- toxin, *C. difficile* toxin B and *C. sordellii* lethal toxin. Toxicon 29: 877-887.

Boondeekhun, H. S., Gurtler, V., Odd, M. L., Wilson, V. A., and Mayall, B. C. 1993. Detection of *Clostridium difficile* enterotoxin gene in clinical specimens by the PCR. J Med Microbiol 38 : 384-387.

Borriello, S. P. 1979. *Clostridium difficile* and its toxin in the gastrointestinal tract in health and disease. Res Clin Forums 1 : 33.

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

Borriello, S. P. 1990. Pathogenesis of *Clostridium difficile* infection of the gut. J Med Microbiol 33 : 207-215.

Borriello, S. P., and Barclay, F. E. 1986. An in-vitro model of colonization resistance to *Clostridium difficile* infection. J med Microbiol 21 : 299-309.

Borriello, S. P., Davies, H. A., and Barclay, F. E. 1988b. Detection of fimbriae among strains of *Clostridium difficile*. FEMS microbiol letters 49 : 65-67.

Borriello, S. P., and Honour, P. 1983. Concomitance of cytotoxigenic and noncytotoxigenic *Clostridium difficile* in stool specimens. J Clin Microbiol 18: 1006.

Borriello, S. P., Welch, A. R., Barclay, F. E., and Davies, H. E. 1988a. Mucosal association by *Clostridium difficile* in the hamster gastrointestinal tract. J Med Microbiol 25 : 191-196.

Borriello, S. P., Wren, B. W., Hyde, S., et al. 1992. Molecular, Immunological, and biological characterization of a toxin A-negative, toxin B-positive strain of *Clostridium diffcile.* Infect Immun 60 : 4192-4199.

Bourne, H. R., Sanders, D. A., and McCormick, F. 1991. The GTPase superfamily : conserved structure and molecular mechanism. Nature 349 : 117-127.

Braun, V., Hundsberger, T., Leukel, P., Sauerborn, M., and Eicher-Streiber, C. v. 1996. Definition of the single integration site of the pathogenicity locus in *Clostridium difficile*. Gene 181 : 29-38.

Brazier, J. S. 1998a. The diagnosis of *Clostridium difficile*-associated diarrhea. J Antimicrobial Chemother 41(Suppl C) : 29-40.

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

Brettle, R. P., and Wallace, E. 1982. *Clostridium difficile* from stools of normal children. Lancet i, 1983.

Brooks, S., Real, R., Kramer, M., and Adachi, M. 1990. *Clostridium difficile*-associated diarrhea. Infect Control Hosp Epidemiol 11 : 574.

Cartmill, T. D. I., Panigrahi, H., Noraley, H. A., Mc Cann, D., Nice, C. N., and Keith, E. 1994. Management and control of a large outbreak of diarrhea due to *Clostridium difficile*. J Hosp Infect 27 : 1-15.

Clabots, C. R., Johnson, S., Olson, M.M., Peterson, L. R., and Gerding, D.N. 1992. Acquisition of *Clostridium difficile* by hospitalized patients : evidence for colonized new admissions as a source of infection. J Infect Dis 166 : 5561-567.

Cleary, R. K. 1998. *Clostridium difficile*-associated diarrhea and colitis. Dis Colon Rectum 41 : 1435-1449.

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

Cooperstock, M., Riegle, L., Woodruff, C. W., and Onderdonk, A. 1983. Influence of age, sex, and diet on asymptomatic colonization of infants with *Clostridium difficile*. J Clin Microbiol 17 : 830-833.

Dailey, D. C., Kaiser, A., and Schloemer, R. H. 1987. Factors influencing the phagocytosis of *Clostridium difficile* by human polymorphonuclear leukocytes. Infect Immun 55 : 1541-1546.

Davies, H. A., and Borriello, S. P. 1990. Detection of capsule in strains of *Clostridium difficile* of varying virulence and toxigenicity. Microb Patho 9 : 141-146.

Delmee', M., and Avesani, V. 1990. Virulence of ten serogroups of *Clostridium difficile* in hamster. J Med Microbiol 33 : 85-90.

Depitre, C., Delmee, M., Avesani, V., et al. 1993. Serogroup F strains of *Clostridium difficile* produce toxin B but not toxin A. J Med Microbiol 38: 434-441.

Dillon, S. T., Rubin, E. J., Yakubovich, M., et al. 1995. Involvement of Ras-related Rho proteins in the mechanism of action of *Clostridium difficile* toxin A and toxin B. Infect Immun 63 : 1421-1426.

DiPersio, J. R., Varga, F. J., Conwell, D. L., Kraft, J. A., Kozak, K. J., and Willis, D. H. 1991. Development of a rapid enzyme immunoassay for *Clostridium difficile*

toxin A and its use in the diagnosis of *C. difficile*-associated disease. J Clin Microbiol 29 : 2724-2730.

Doern, G. V., Coughlin, R. T., and Wu, L. 1992. Laboratory diagnosis of *Clostridium difficile*-associated gastrointestinal disease: comparison of a monoclonal antibody enzyme immunoassay for toxins A and B with a monoclonal antibody enzyme immunoassay for toxin A only and two cytotoxicity assays. J Clin Microbiol 30: 2042-2046.

Dove, C. H., Wang, S. Z., Price, S. B., et al. 1990. Molecular characterization of the *Clostridium difficile* toxin A gene. Infect Immun 58 : 480-488.

Eglow, R., Pothoulakis, C., Itzkowitz, S., et al. 1992. Diminished *Clostridium difficile* toxin A sensitivity in newborn rabbit ileum is associated with decreased toxin A receptor. J Clin Invest 90 : 822-829.

Eicher-Streiber, C. v., Laufenberg-Feldmann, R., Sartingen, S., Schulze, J., and Sauerborn, M. 1990. Cloning of *Clostridium difficile* toxin B gene and demonstration of high N-terminal homology between toxin A and B. Med Microbiol Immunol. 179 : 271-279.

Eicher-Streiber, C. v., Laufenberg-Feldmann, R., Sartingen, S., Schulze, J., and Sauerborn, M. 1992. Comparative sequence analysis of the *Clostridium difficile* toxins A and B. Mol Gen Genet 230 : 260-268.

Eicher-Streiber, C. v., and Sauerborn, M. 1990. *Clostridium difficile* toxin A carries a C-terminal repetetive structure homologous to the carbohydrate binding region of streptococcal glycosyltransferases. Gene 96 : 107-113.

Fekety, R. 1997. Guidelines for the diagnosis and management of *Clostridium difficile*-associated diarrhea and colitis. Am J Gastroenterol 92(5) : 739-750.

Fekety, R., McFarland, L. V., Surawicz, C. M., et al. 1981. Epidemiology of antibiotic-associated colitis : isolation of *Clostridium difficile* from the hospital environment. Am J med 70 : 906.

Fekety, R., and Shah, A. B. 1993. Diagnosis and treatment of *Clostridium difficile* colitis. JAMA 269 : 71-75.

Finney, J. M. T. 1893. Gastroenterostomy for cicatrizing ulcer of the pyloris. Bull Johns Hopkins Hosp 4 : 53-55.

Fiorentini, C., Malorni, W., Paradisi, S., et al. 1990. Interaction of *Clostridium difficile* toxin A with cultured cells: cytoskeletal changes and nuclear polarization. Infect Immun 58 ; 2329-2336.

Fluit, A. C., Wolfhagen, M. J., Verdonk, G. P., et al. 1991. Nontoxigenic strains of *Clostridium difficile* lack the genes for both toxin A and B. J Clin Microbiol 29 (11) : 2666-2667.

George, R. H., Goldstein, E. J. C., Sutter, V. L., Ludwig, S. L., and Finegold, S. M. 1978. Etiology of antimicrobial agent associated colitis. Lancet i : 802-803.

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

George, W. L., Rolfe, R. D., and Finegold, S. M. 1982. *Clostridium difficile* and its cytotoxin in feces of patients with antimicrobial agent-associated gartroenteritis. Diag Microbiol Infect Dis 4(suppl 3) : s87-s91.

Gerding, D. N., Olson, M. M., Peterson, L. R., et al. 1986. *Clostridium difficile*-associated diarrhea and colitis in adults : a prospective case-controlled epidemiologic study. Arch Intern Med 146 : 95-100.

Gerding, D. N., and Brazier, J. S. 1993. Optimal methods for identifying *Clostridium difficile* infections. Clin Infect Dis 16(suppl 4) : s439-442.

Gerding, D. N., Johnson, S., Peterson, L. R., et al. 1995. *Clostridium difficile*-associated diarrhea and colitis. Infect Control Hosp Epidemiol 16 : 459-477.

Green, G. A., Riot, B., and Monteil, H. 1994. Evaluation of an oligonucletide probe and an immunological test for direct detection of toxigenic *Clostridium difficile* in stool samples. Eur J Clin Microbiol Infect Dis 13 : 576-581.

Greenfield, L., and White, T. J. 1993. Sample preparation methods, p. 122-137. *In* D. H. Persing, T. F. Smith, F. C. Tenover, and T. J. White (ed). Diagnostis molecular microbiology: principles and application. American society for Microbiology, Washington, D.C.

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

Gumerlock, P. H., Tang, Y. T., Weiss, J. B., and Silva, J. 1993. Specific detection of toxigenic strains of *Clostridium difficile* in stool specimens. J Clin Microbiol 31 : 507-511.

Gurwith, M. J., Rabin, H. R., and Love, K. 1977. Diarrhea associated with clindamycin and ampicillin therapy : preliminary results of a cooperative study. J Infect Dis 135 : s104.

Hafiz, S., and Oakley, C. L. 1976. *Clostridum difficile*: Isolation and characteristics. J Med Microbiol 9: 129-136.

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

Hammond, G. A., and Johnson, J. L. 1995. The toxigenic element of *Clostridium difficile* strain VPI 10463. Microbiol Pathol 19 : 203-213.

Hammond, G. A., Lyerly, D. M., and Johnson, J. L. 1997. Transcriptional analysis of the toxigenic element of *Clostridium difficile*. Microbiol Pathol 22 : 143-154.

Haslam, S. C., Ketley, J. M., Mitchell, T. J., et al. 1986. Growth of *Clostridium difficile* and production of toxins A and B in complex and defined media. J Med Microbiol 21 : 293-297.

Hatheway, C. L. 1990. Toxigenic Clostridia. Clin Microbiol Rev 3(1) : 66-98.

Henriques, B., Florin, I., and Thelestam, M. 1987. Cellular internalization of *Clostridium difficile* toxin A. Microb Pathog 2: 455-463.

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

Humphrey, C. D., Condon, C. W., Cantey, J. R., and Pittman, F.E. 1979. Partial purification of a toxin found in hamsters with antibiotic-associated colitis. Gastroenterol 76 : 478-486.

Hundsberger, T., Braun, V., Weidmann, M., et al. 1997. Transcription analysis of the genes tsdA-tcdE of the pathogenicity locus in *Clostridium difficile*. Eur J Biochem 244 : 735-742.

Jobe, B. A., Grasley, A., Deveney, K. E., Deveney, C. W., and sheppard, B. C. 1995. *Clostridum difficile*: an increasing hospital-acquired illness. American J Surg 169: 480-483.

Johnson, S., Clabots, C. R., Linn, F. V., et al. 1990. Nosocomial *Clostridium difficile* colonization and disease. Lancet 336 : 97-100.

Johnson, S., Gerding, D. N., and Janoff, E. N. 1992. Systemic and mucosal antibody responses to toxin A in patients infected with *Clostridium difficile*. J Infect Dis 166 : 1287-1294.

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

Just, I., Fritz, G., Aktories, K., et al. 1994. *Clostridium difficile* toxin B acts on the GTP-binding protein Rho. J Biol Chem 269 : 10706-10712.

Just, I., Seizer, J., Eicher-Streiber, C. v., and Aktories, K. 1995. The low molecular mass GTP-binding protein Rho is affected by toxin A from *Clostridium difficile*. J Clin Invest 95 : 1026-1031.

Kabins, S. A., and Spira, T. J. 1975. Outbreak of Clindamycin-associated colitis. Ann Tern Med 83 : 830-831.

Karasawa, T., Nojiri, T., Hayashi, Y., Megawa, T., et al. 1999. Laboratory diagnosis of toxigenic *Clostridium difficile* by PCR : Presence of toxin genes and their stable expression in toxigenic isolates from Japanese individuals. J Gastroenterol 34 : 41-45.

Kato, H., Kato, N., Watanabe, K., Iwai, N., et al. 1998. Identification of toxin A-negative, Toxin B-positive *Clostridium difficile* by PCR. J Clin Microbiol 36(8) : 2178-2182.

Kato, H., Kato, N., Katow, S., Maegawa, T., et al. 1999. Deletion in the repeating sequences of the toxin A gene of toxin A-negative, toxin B-positive *Clostridium difficile* strains. FEMS Microbiol letters 175 : 197-203.

Kato, N., Ou, C. Y., Kato, H., Bartley, S. L., et al. 1991. Identification of toxigenic *Clostridium difficile* by polymerase chain reaction. J Clin Microbiol 29 : 33-37.

Kato, N., Ou, C. Y., Kato, H., Bartley, S. L., et al. 1993. Detection of toxigenic *Clostridium difficile* in stool specimens by the PCR. J Infect Dis 167 : 455-458.

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

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

Ketly, J. M., Haslam, S. C., Mitchell, T. J., et al. 1984. Production and release of toxins A and B by *Clostridium difficile*. J Med Microbiol 18 : 385-391.

Ketly, J. M., Mitchell, T. J., Haslam, S. C., et al. 1986. Sporogenesis and toxin A production by *Clostridium difficile*. J Med Microbiol 22 : 33-38.

Kim, K. H., Fekety, R., Batts, D. H., et al. 1981. Isolation of *Clostridium difficile* from the environment and contacts of patients with antibiotic-associated colitis. J Infect Dis 143 : 42-50.

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

Koronakis, V., Koronakis, E., and Hughes, C. 1989. Isolation and analysis of the *C*-terminal signal directing export of the *Escherichia coli* hemolysin protein across both bacterial membranes. EMBO J 8 : 595-605.

Krishna, M. M., Powell, N. B. L., and Borriello, S. P. 1996. Cell surface properties of *Clostridium difficile*: haemmagglutination, relative hydrophobicity and charge. J Med Microbiol 44 : 115-126-3.

Kristiansson, M., Samore, M. H., Gerding, D. N., et al. 1994. Comparison of restriction endonuclease analysis, ribotyping, and pulse-field gel electrophoresis for molecular differentiation of *Clostridium difficile* strains. J Clin Microbiol 32 (8): 1963-1969.

Krivan, H. C., and Wilkins, T. D. 1987. Purification of *Clostridium difficile* toxin A by affinity chromatography on immobilized thyroglobulin. Infect Immun 55 : 1873-1877.

Kuhl, S. J., Tang, Y. T., Navarro, L., Gumerlock, P. H., and Silva, J. Jr. 1993. Diagnosis and monitoring of *Clostridium difficile* infections with the polymerase chain reaction. Clin Infect Dis 16(suppl 4) : s234-238.

Larson, H. E., Barclay, F. E., Honuor, P., and Hill, I. D. 1982. Epidemiology of *Clostridium difficile* in infants. J Infect Dis 149 : 727-733.

Larson, H. E., Parry, J. V., Price, A. B., et al. 1977. Undescribed toxin in pseudomembranous colitis. Brit Med J I : 1246-1248.

Laughon, B. E., Viscidi, R. P., Gdovin, S. L., Yolken, R. H., and Bartlett, J. G. 1984. Enzyme immunoassays for detection of *Clostridium difficile* toxins A and B in fecal specimens. J Infect Dis 149 : 781-788.

Libby, J. M., Donta, S. T., and Wilkins, T. D. 1983. *Clostridium difficile* toxin A in infants. J Infect Dis 148 : 606.

Lima, A. A., Lyerly, D. M., Wilkins, T. D., et al. 1988. Effects of *Clostridium difficile* toxins A and B in rabbit small and large intestine *in vivo* and on cultured cells *in vitro*. Infect Immun 56(3) : 582-588.

Limaye, A. P., Turgeon, D. K., Cookson, B. T., and Fritsche, T. R. 2000. Pseudomembranous colitis caused by a toxin A- B+ strain of *Clostridium difficile*. J Clin Microbiol 38 (4) : 1696-1697.

Lou, Q., Chong, S. K. F., Fitzgerald, J. F., et al. 1997. Rapid and effective methods for preparation of fecal specimens for PCR assays. J Clin Microbiol 35(1): 281-283.

Lozniewski, A., Rabaud, C., Dotto, E., Weber, M., and Mory, F. 2001. Laboratory diagnosis of *Clostridium difficile*-associated diarrhea and colitis: usefulness of Premier Cytoclone A+B enzyme immunoassay for combined detection of stool toxins and toxigenic *C. difficile* strains. J Clin Microbiol 39(5): 1996-1998.

Lyerly, D. M., Barroso, L. A., Wilkins, T. D., Depitre, C., and Corthier, G. 1992. Characterization of a toxin A-negative, toxin B-positive strain of *Clostridium difficile*. Infect Immun 60(11) : 4633-4639.

Lyerly, D. M., Krican, H. C., and Wilkins, T. D. 1988. *Clostridum difficile* : Its disease and toxins. Clim Microbiol Rev 1 : 1-18.

Lyerly, D. M., Lockwood, D. E., Richardson, S. H., and Wilkins, T. D. 1982. Biological activities of toxins A and B of *Clostridium difficile*. Infect Immun 35 : 1147-1150.

Lyerly, D. M., Nevilie, L. M., Evans, D. T., et al. 1998. Muticenter evaluation of the *Clostridium difficile* TOX A/B test. J Clin Microbiol 36(1) : 184-190.

Lyerly, D. M., Phelps, C. J., Toth, J., and Wilkins, T.D. 1986a. Characterization of toxin A and B of *Clostridium difficile* with monoclonal antibodies. Infect immun 54 : 70-76.

Lyerly, D. M., Roberts, M. D., Phelps, C. J., and Wilkins, T.D. 1986b. Purification and properties of toxin A and b of *Clostridium difficile*. FEMS Microbiol Letters 33: 31-35.

Lyerly, D. M., Sullivan, N. M., and Wilkins, T. D. 1983. Enzyme-linked immunosorbent assay for *Clostridium difficile* toxin A. J Clin Microbiol 17 : 72-78.

Malamou-Ladas, H. S., O' Farrell, S., Nash, J. Q., and Tabaqchali, S. 1983. Isolation of *Clostridium difficile* from patients and the environment of hospital wards. J Clin Microbiol 36 : 88-92.

McFarland, L. V., Mulligan, M. E., Kwok, R. Y., and Stamm, W. E. 1989. Nosocomial acquisition of *Clostridium difficile* infection. N Engl J Med 320 : 204-210.

McFarland, L. V., Surawicz, C. M., and Stamm, W. E. 1990. Risk factors for *Clostridium difficile* carriage and *C. difficile*-associated diarrhea in a cohort of hospitalized patients. J Infect Dis 162 : 678-684.

Merz , C. S., Kramer, C., Forman, M., et al. 1994. Comparison of four commercially available rapid enzyme immunoassays with cytotoxin assay for detection of *Clostridium difficile* toxin(s) from stool specimens. J Clin Microbiol 32: 1142-1147.

Michell, J., Laughon, B. E., and Lin, S. 1987. Biochemical studies on the effect of *Clostridium difficile* toxin B on actin *in vivo* and *in vitro*. Infect Immun 55 : 1910-1615.

Mitty, R. D., and LaMont, T. 1994. *Clostridium difficile* diarrhea: Pathogenesis, epidemiology, and treatment. Gastroenterologist 2: 61-69.

Monteiro, L., Bonnemaison, D., Vekris, A., et al. 1997. Complex polysaccharides as PCR inhibitors in Feces: *Helicobactor pylori* model. J Clin Microbiol 35(4) : 995-998.

Mulligan, M. E. 1984. Epidemiology of *Clostridium difficile* induced intestinal disease. Rev Infect Dis 6 : s222-228.

Mulligan, M. E. 1988. General epidemiology, potential reservoirs and typing procedures. In *Clostridium difficile*-Its role in intestinal disease (Rolfe, R. D., and Finegold, S. M., Eds), pp. 129-156. Academic Press, London.

Mylonakis, E., Ryan, E. T., and Calderwood, S. B. 2001. *Clostridium difficile*-associated diarrhea. Arch Intern Med. 161(26) : 525-533.

O'connor, D., Hynes, P., cormican, M., et al. 2001. Evaluation of methods for detection of toxins in specimens of feces submitted for diagnosis of *Clostridium difficile*-associated diarrhea. J Clin Microbiol 39(8): 2846-2849.

Onderdonk, A. B., Cisneros, R. L., and Bartlett, J. G. 1980. *Clostridium difficile* in gnotobiotic mice. Infect Immun 28 : 277-282

Ottlinger, M. E., and Lin, S. 1988. *Clostridium difficile* toxin B induces reorganization of actin, vinculin and talin in cultured cells. Exp Cell Res 174: 215-229.

Peterson, L. R., Olson, M. M., Shanholtzer, C. J., et al. 1988. Results of a prospective, 18-month clinical evaluation of culture, cytotoxin testing, and culturette brand (CDT) latex testing in the diagnosis of *Clostridium difficile*-associates diarrhea. Diagn Microbiol Infect Dis 10 : 85-91.

Pothoulakis, C., and LaMont, J. T. 1993. *Clostridium difficile* colitis and diarrhea. Gastroenterol Clin North Am 22 (3) : 623-637.

Pothoulakis, C., Karmeli, F., Kelly, C. P., et al. 1993. Ketotifen inhibits *Clostridium difficile* toxin A – induced enteritis in rat ileum. Gastroenterol 105: 701-707.

Putterman, C., and Rubinow, A. 1993. Reactive arthritis associated with *Clostridium difficile* pseudomembranous colitis. Semin Arthritis Rheum 22 : 420-426.

Riegler, M., Sedivey, R., Pothoulakis, C., et al. 1995. *Clostridium difficile* toxin B is more potent than toxin A in damaging human colonic mucosa *in vitro*. J Clin Invest 95 : 2004-2011.

Rifkin, G. D., Fekety, R., and Silva, J. 1987. Neutralization by *Clostridium sordellii* antitoxin of toxins implicated in clindamycin-induced cecitis in the hamster. Gastroenterology 75: 422-424.

Rifkin, G. D., Silva, J. Jr, and Fekety, F. R. 1987. Gastrointestinal and systemic toxicity of fecal extracts from hamsters with clindamycin-induced colitis. Gastroenterology 74: 52-57.

Samore, M. H., DeGirolami, P. C., Tlucko, A., et al. 1994. *Clostridium difficile* colonization and diarrhea at a tertiary care hospital. Clin Infect Dis 18 : 181-187.

Shanholtzer, C. J., Willard, K. E., Holter, J. J., et al. 1992. Comparison of VIDAS *Clostridium difficile* toxin A immunoassay (CDA) with *C. difficile* culture, cytotoxin, and latex test. J Clin Microbiol 30: 1837-1840.

Shim, J. K., Johnson, S., Samore, M. H., Bliss, D. Z., and Gerding D. N. 1998. Primary symptomless colonization by *Clostridium difficile* and decreased risk of subsequent diarrhea. Lancet 351: 633-636.

Simpson, A. J. H., Das, S. S., and Tabaqchali, S. 1996. Nosocomail empyema caused by *Clostridium difficile*. J Clin Pathol 49: 172-173.

Simor, A. E., Yake, S. L., and Tsimidis, K. 1993. Infection due to *Clostridium difficile* among elderly residents of a long-term-care facility. Clin Infect Dis 17: 672-678.

Small, J. D. 1968. Fatal enterocolitis in hamsters given lincomycin hydrochloride. Lab Anim care 18 : 411-420.

Spencer,R.C. 1998a. Clinical impact and associated costs of Clostridium difficile-associated disease. J Antimicrob Chemother 41 (Suppl C): 5-12.

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

Staneck, J. L., Weckbach, L. S., Allen, S. D., et al. 1996. Multicenter evaluation of four methods for *Clostridium difficile* detection: ImmunoCard *C. difficile*, cytotoxin assay, culture, and latex agglutination. J Clin Microbiol 34: 2718-2721.

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

Stark, L. P., and Lee, A. 1982. Clostridia isolated from the feces of infants during the first year of life. J Pediar 100(3): 362-365.

Stark, L. P., Lee, A., and parsonage, B. 1982. Colonization of the large bowel by *Clostridium difficile* in healthy infants: Quantitative study. Infect Immun 35: 895-899.

Sullivan, N. M., Pellet, S., and Wilkins, T. D. 1982. Purification and characterization of toxins A and B of *Clostridium difficile*. Infect Immun 35 : 1032-1040.

Swartzberg, J. E., Maresca, R. M., and Remington, J. S. 1977. Clinical study of gastrointestinal complications associated with clindamycin therapy. J Infect Dis 135: S99-S103.

Tabaqchali, S., and Jumaa, P. 1995. Daignosis and management of *Clostridium difficile* infection. BMJ 310: 1375-1380.

Tabaqchali, S., O'Farrell, S., Nash, J. Q., and Wilks, M. 1984. Vagianl carriage and neonatal acquisition of *Clostridium difficile*. J Med Microbiol 18: 47-53.

Talbot, R. W., walker, R. C.,and beart, R. W. 1986. Changing epidemiology, diagnosis, and treatment of *Clostridium difficile* toxin-associated colitis. British J Surg 73: 457-460.

Tasteyre, A., Barc, M.-C., Dodson, P., et al. 1997. Isolation of a genetic determinant coding for *Clostridium difficile* flagellin and its relation to different serogroups. Bio Microflora 16 suppl : 19.

Taylor, N. S., and Bartlett, J. G. 1979. Partial purification and characterization of a cytotoxin from *Clostridium difficile*. Rev Infect Dis 1: 379-385.

Toma, S., Lesniak, G., Magnus, M., Lo, H-L., and Delmee, M. 1988. Serotyping of *Clostridium difficile*. J Clin Microbiol 28: 426-428.

Torres, J. F. 1991. Purification and characterisation of toxin B from a strain of *Clostridium difficile* that does not produce toxin A. J Med Micrbiol 35: 40-44.

Triadafilopoulos, G., Pothoulakis, C., O'Brien, M. J., and LaMont, J. T. 1987. Differential effects of *Clostridium difficile* toxins A and B on rabbit ileum. Gastroenterology 93 : 273-279.

Tucker,K. D.,Carrig, P. E., and Wilkins, T. D. 1990. Toxin A of *Clostridium difficile* is a potent cytotoxin. J Clin Microbiol 28: 869-871.

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

Viscidi, R. Wiley, S., and Bartlett, J. G. 1981. Isolation rates and toxigenic potential of *Clostridium difficile* isolates from various patient populations. Gastroenterology 81: 5-9.

Wilcox, M. H., and Smyth, E. T. M. 1998. Incidence and impact of *Clostridium difficile* infection in the UK, 1993-1996. J Hosp Infect 39: 181-187.

Wilson, K. H. 1993. *Clostridium difficile* enteric disease. Clin Infect Dis 16 (suppl 4): S214-S218.

Wilson, K. H., and Perini, F. 1988. Role of competition for nutrients in suppression of *Clostridium difficile* by the colonic microflora. Infect Immun 56 : 2610-2614.

Wolfhagen,M. J., Fluit, A. C., Torensma, R. Poppelier, M. J., and Verhoel, J. 1994. Rapid detection of toxigenic *Clostridum difficile* in fecal samples by magnetic immuno-PCR assay. J Clin Microbiol 32: 1629-1633.

Wongwanich, S., Pongpech, P., Dhiraputra, C. Huttayananont, S., and Sawanpayalert, P. 2001. Characteristics of *Clostridium difficile* strains isolated from asymptomatic individuals and from diarrheal patients. Clin Infect Dis 7: 438-441.

Wren, B. W., Clayton, C. L., and Tabaqchali, S. 1990b. Rapid identification of toxigenic *Clostridium difficile* by polymerase chain reaction. Lancet 335: 423.

Wren, B. W., Clayton, C. L., Castledin, N. B., et al. 1990a. Identification of toxigenic *Clostridium difficile* strains by using a toxin A gene-specific probe. J Clin Microbiol 38: 109-113.

Yolken, R. H., Whitchomb, L. S., Marien, G., et al. 1981. Enzyme immunoassay for the detection of *Clostridium difficile* antigen. J Infect Dis 144: 378.

APPENDICES

APPENDIX I

Table 1 PCR detection of toxin A and B genes from *C.difficile* isolates and stool samples from 107 stool samples with organism positive

No. of isolates or specimens	PCR *C.difficile*		PCR stool	
	Toxin A	Toxin B	Toxin A	Toxin B
1	+	+	+	+
2	-	-	-	-
3	-	-	+	+
4	+	+	+	+
5	+	+	+	+
6	+	+	+	+
7	+	+	+	+
8	+	+	+	+
9	-	-	-	-
10	-	-	-	-
11	+	+	-	-
12	-	-	-	-
13	-	-	-	-
14	-	-	-	-
15	-	-	-	-
16	+	+	+	+
17	-	-	-	-
18	-	-	-	-
19	+	+	-	-
20	+	+	-	-
21	-	-	-	-
22	+	+	+	+
23	-	-	-	-

Table 1- *Continued*

No. of isolates or specimens	PCR *C.difficile*		PCR stool	
	Toxin A	Toxin B	Toxin A	Toxin B
24	+	+	-	-
25	-	-	-	-
26	-	-	-	-
27	-	-	-	-
28	-	-	+	+
29	-	-	+	+
30	-	-	-	-
31	-	-	+	+
32	-	-	+	+
33	+	+	-	-
34	+	+	+	+
35	-	-	+	+
36	-	-	-	-
37	-	-	-	-
38	-	-	-	-
39	+	+	+	+
40	-	-	-	-
41	-	-	-	-
42	-	-	+	+
43	+	+	+	+
44	-	-	+	+
45	-	-	+	+
46	-	-	+	+
47	-	-	-	-
48	-	-	+	+
49	-	-	+	+
50	+	+	+	+
51	-	-	-	-

Table 1- *Continued*

No. of isolates or specimens	PCR *C.difficile*		PCR stool	
	Toxin A	Toxin B	Toxin A	Toxin B
52	+	+	+	+
53	-	-	-	-
54	+	+	-	-
56	-	-	-	-
57	-	-	-	-
58	-	-	+	+
59	-	-	+	+
60	+	+	+	+
61	+	+	+	+
62	+	+	+	+
63	+	+	+	+
64	-	-	-	-
65	+	+	-	-
66	-	-	-	-
67	-	-	-	-
68	+	+	+	+
69	-	-	-	-
70	+	+	-	-
71	+	+	-	-
72	+	+	+	+
73	+	+	+	+
74	-	-	-	-
75	+	+	+	+
76	+	+	+	+
77	+	+	+	+
78	+	+	+	+
79	+	+	+	+
80	-	-	-	-

Table 1- *Continued*

No. of isolates or specimens	PCR *C.difficile*		PCR stool	
	Toxin A	Toxin B	Toxin A	Toxin B
81	+	+	+	+
82	+	+	+	+
83	+	+	+	+
84	+	+	-	-
85	+	+	+	+
87	-	-	-	-
88	-	-	-	-
89	+	+	-	-
90	+	+	-	+
91	+	+	-	-
92	-	-	-	-
93	+	+	-	-
94	+	+	+	+
95	+	+	-	-
96	+	+	+	+
97	-	-	-	-
98	-	-	-	-
99	+	+	+	+
100	+	+	-	-
101	+	+	-	-
102	-	-	-	-
103	-	-	-	-
104	-	-	-	-
105	+	+	-	-
106	-	-	-	-
107	+	+	-	-

Table 2 PCR detection of toxin A and B genes from stool samples and toxin A and B detection by EIA from 107 stool samples with organism positive

No. of isolates or specimens	PCR stool		EIA (A+B) stool
	Toxin A	Toxin B	
1	+	+	+
2	-	-	+
3	+	+	+
4	+	+	+
5	+	+	+
6	+	+	+
7	+	+	+
8	+	+	+
9	-	-	-
10	-	-	-
11	-	-	-
12	-	-	-
13	-	-	-
14	-	-	-
15	-	-	-
16	+	+	+
17	-	-	-
18	-	-	-
19	-	-	+
20	-	-	-
21	-	-	-
22	+	+	-
23	-	-	-
24	-	-	-
25	-	-	-
26	-	-	-

Table 2- *Continued*

No. of isolates or specimens	PCR stool		EIA (A+B) stool
	Toxin A	Toxin B	
27	-	-	-
28	+	+	+
29	+	+	+
30	-	-	-
31	+	+	+
32	+	+	-
33	-	-	-
34	+	+	+
35	+	+	+
36	-	-	-
37	-	-	-
38	-	-	-
39	+	+	+
40	-	-	-
41	-	-	-
42	+	+	+
43	+	+	+
44	+	+	+
45	+	+	+
46	+	+	+
47	-	-	-
48	+	+	+
49	+	+	+
50	+	+	-
51	-	-	-
52	+	+	+
53	-	-	-
54	-	-	+

Table 2- *Continued*

No. of isolates or specimens	PCR stool		EIA (A+B) stool
	Toxin A	Toxin B	
55	+	+	+
56	-	-	+
57	-	-	-
58	+	+	+
59	+	+	+
60	+	+	+
61	+	+	+
62	+	+	+
63	+	+	+
64	-	-	-
65	-	-	-
66	-	-	-
67	-	-	-
68	+	+	+
69	-	-	-
70	-	-	+
71	-	-	-
72	+	+	+
73	+	+	+
74	-	-	-
75	+	+	+
76	+	+	+
77	+	+	+
78	+	+	+
79	+	+	+
80	-	-	-
81	+	+	+
82	+	+	+

Table 2- *Continued*

No. of isolates or specimens	PCR stool		EIA (A+B) stool
	Toxin A	Toxin B	
83	+	+	+
84	-	-	-
85	+	+	+
86	-	-	-
87	-	-	-
88	-	-	-
89	-	-	-
90	-	+	-
91	-	-	-
92	-	-	+
93	-	-	-
94	+	+	+
95	-	-	-
96	+	+	+
97	-	-	-
98	-	-	-
99	+	+	+
100	-	-	-
101	-	-	-
102	-	-	-
103	-	-	-
104	-	-	-
105	-	-	-
106	-	-	-
107	-	-	-

Table 3 PCR detection of toxin A and B genes from *C.difficile* isolates and stool samples, and toxin A and B detection by EIA from 107 stool samples with organism positive

No. of isolates or specimens	PCR *C.difficile*		PCR stool		EIA (A+B)
	Toxin A	Toxin B	Toxin A	Toxin B	
1	+	+	+	+	+
2	-	-	-	-	+
3	-	-	+	+	+
4	+	+	+	+	+
5	+	+	+	+	+
6	+	+	+	+	+
7	+	+	+	+	+
8	+	+	+	+	+
9	-	-	-	-	-
10	-	-	-	-	-
11	+	+	-	-	-
12	-	-	-	-	-
13	-	-	-	-	-
14	-	-	-	-	-
15	-	-	-	-	-
16	+	+	+	+	+
17	-	-	-	-	-
18	-	-	-	-	-
19	+	+	-	-	+
20	+	+	-	-	-
21	-	-	-	-	-
22	+	+	+	+	-
23	-	-	-	-	-
24	+	+	-	-	-
25	-	-	-	-	-

Table 3-*Continued*

No. of isolates or specimens	PCR *C.difficile*		PCR stool		EIA (A+B)
	Toxin A	Toxin B	Toxin A	Toxin B	
26	-	-	-	-	-
27	-	-	-	-	-
28	-	-	+	+	+
29	-	-	+	+	+
30	-	-	-	-	-
31	-	-	+	+	+
32	-	-	+	+	-
33	+	+	-	-	-
34	+	+	+	+	+
35	-	-	+	+	+
36	-	-	-	-	-
37	-	-	-	-	-
38	-	-	-	-	-
39	+	+	+	+	+
40	-	-	-	-	-
41	-	-	-	-	-
42	-	-	+	+	+
43	+	+	+	+	+
44	-	-	+	+	+
45	-	-	+	+	+
46	-	-	+	+	+
47	-	-	-	-	-
48	-	-	+	+	+
49	-	-	+	+	+
50	+	+	+	+	-
51	-	-	-	-	-
52	+	+	+	+	+
53	-	-	-	-	-

Table 3- *Continued*

No. of isolates or specimens	PCR *C.difficile*		PCR stool		EIA (A+B)
	Toxin A	Toxin B	Toxin A	Toxin B	
54	+	+	-	-	+
55	+	+	+	+	+
56	-	-	-	-	+
57	-	-	-	-	-
58	-	-	+	+	+
59	-	-	+	+	+
60	+	+	+	+	+
61	+	+	+	+	+
62	+	+	+	+	+
63	+	+	+	+	+
64	-	-	-	-	-
65	+	+	-	-	-
66	-	-	-	-	-
67	-	-	-	-	-
68	+	+	+	+	+
69	-	-	-	-	-
70	+	+	-	-	+
71	+	+	-	-	-
72	+	+	+	+	+
73	+	+	+	+	+
74	-	-	-	-	-
75	+	+	+	+	+
76	+	+	+	+	+
77	+	+	+	+	+
78	+	+	+	+	+
79	+	+	+	+	+
80	-	-	-	-	-
81	+	+	+	+	+

Table 3- *Continued*

No. of isolated or specimens	PCR *C.difficile*		PCR stool		EIA (A+B)
	Toxin A	Toxin B	Toxin A	Toxin B	
82	+	+	+	+	+
83	+	+	+	+	+
84	+	+	-	-	-
85	+	+	+	+	+
86	-	-	-	-	-
87	-	-	-	-	-
88	-	-	-	-	-
89	+	+	-	-	-
90	+	+	-	+	-
91	+	+	-	-	-
92	-	-	-	-	+
93	+	+	-	-	-
94	+	+	+	+	+
95	+	+	-	-	-
96	+	+	+	+	+
97	-	-	-	-	-
98	-	-	-	-	-
99	+	+	+	+	+
100	+	+	-	-	-
101	+	+	-	-	-
102	-	-	-	-	-
103	-	-	-	-	-
104	-	-	-	-	-
105	+	+	-	-	-
106	-	-	-	-	-
107	+	+	-	-	-

Table 4 PCR detection of toxin A and B genes and toxin A and B detection by EIA (toxin A and B) from 50 stool samples with negative culture

No.	PCR		EIA
	Toxin A	Toxin B	(A+B)
1	-	-	-
2	+	+	-
3	-	-	-
4	-	-	-
5	-	-	-
6	-	-	-
7	-	-	-
8	-	-	-
9	-	-	-
10	-	-	-
11	-	-	-
12	-	-	-
13	-	-	-
14	-	-	-
15	-	-	-
16	-	-	-
17	-	-	-
18	-	-	-
19	-	-	-
20	-	-	-
21	-	-	-
22	-	-	-
23	-	-	-
24	-	-	-
25	-	-	-
26	-	-	-

Table 4- *Continued*

No.	PCR		EIA
	Toxin A	Toxin B	(A+B)
27	-	-	-
28	-	-	-
29	-	-	-
30	-	-	-
31	-	-	-
32	-	-	-
33	-	-	-
34	-	-	-
35	-	-	-
36	-	-	-
37	-	-	-
38	-	-	-
39	-	-	-
40	-	-	-
41	-	-	-
42	-	-	-
43	-	-	-
44	-	-	-
45	-	-	-
46	-	-	-
47	-	-	-
48	-	-	-
49	-	-	-
50	-	-	-

APPENDIX II

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
Human or sheep blood	50	ml

2. Brain heart infusion broth-supplement

Brain heart infusion broth (dehydrated) (Difco,USA)	37	g
Yeast extract	5	g
Resazurin solution	4	ml
Distilled water	1000	ml
Supplement : cysteine HCI-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	g
Disodium phosphate (Na_2HPO_2)	5	g
Monopotassium phosphate (KH_2PO_4)	1	g
Sodium chloride (NaCl)	2	g
Magnesium sulfate anhydrous ($MgSO_4$)	0.1	g
Fructose	6	g
Neutral red	3 ml or 0.03	g

Agar	20	g
Distilled water	1000	ml
Supplement : cycloserine	0.5	g
Cefoxitin	0.016	g

4. Egg yolk agar (modified)

Trypticase (Difco, USA)	20	g
Disodium phosphate (Na_2HPO_2)	2.5	g
Sodium chloride (NaCl)	1	g
Magnesium sulfate anhydrous ($MgSO_4$), 5% solution	0.1	ml
Glucose	1	g
Agar	12	g
Distilled water	1000	ml
Supplement : egg yolk		

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 121^0C, 15 pounds/inch^2 pressure, for 15 minutes. The sterile medium was cooled to 45^0C to 50^0C. 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% ethanol.

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 10% ethanol.

4. Vitamin K-hemin solution

1 N NaOH	1	ml
Hemoglobin	0.1	g

All of ingredients were dissolved in 199 ml distilled water. The solution steriled by autoclaving at 121^0C, 15 pounds/inch^2 pressure, for 15 minutes. The sterile solution was cooled to 45^0C to 50^0C.

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 strain

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

Primary strain

- 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

stock solution : safranin	20 g melt in 95% ethyl alcohol	
distilled water	1000	ml

APPENDIX III

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 conc. HCl. The final volume was bought up to 50 ml with deionized water. The stock reagent steriled by autoclaving at 121^0C, 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 121^0C, 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 50 ml of deionized water. The stock reagent steriled by autoclaving at 121^0C, 15 pounds/inch^2 pressure, for 15 minutes. The stock reagent was stored at room temperature.

4. 5X TBE

Tris base	54	g
Boric acid	27.5	g
0.5 M 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 121^0C, 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	ml
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 ^{0}C.

Proteinase K (5mg/ml)

This stock reagent was prepared by mixed 0.25 ml of proteinase K (20mg/ml) in 0.75 ml of sterile deionized water. The stock reagent was stored at –20 ^{0}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 (pH8.0)	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
0.5 EDTA (pH 8.0)	0.5	ml
2M NaCl	1.25	ml
sterile deionized water up to	50	ml

3. Lysis buffer 1

Sucrose	20	g

To prepared this buffer, 20g of sucrose was dissolved 80 ml of TES and autoclaving at 115^0C, 10 pounds/inch^2 pressure, for 10 minutes to dissolved completely. The buffer was cooled to 45^0C to 50^0C.

Lysozyme

This buffer, 400 mg of lysozyme was added aseptically and then mixed well. This buffer was stored at -20^0C.

4. Lysis buffer 2 (200 µl)

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

This buffer was freshly prepared before use.

5. 5% polyacrylamide gel

sterile deionized water	13.3	ml
30% acrylamide (29:1 acrylamide/bisacrylamide)	4.6	ml
5X TBE	2	ml
ammonium persulfate	10	particles
TEMED	50	μl

This polyacrylamide gel was perpared 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.

APPENDIX IV

Chemical agents, Materials, and Instruments

1. Chemical agents

Tris base (Sigma, USA)

EDTA (Sigma, USA)

NaCl (Merck, Germany)

Sucrose (Sigma, USA)

Lysozyme (Sigma, USA)

HCl (Merck, Germany)

Sarkocyl (Sigma, USA)

Proteinase K (Sigma, USA)

Deoxynucleotide triphosphates (Bio Rad, USA)

$MgCl_2$ (Bio Rad, USA)

KCl (Bio Rad, USA)

Taq polymerase (Promega, USA)

Mineral oil (Sigma, USA)

100 bp DNA marker ladder (Promega, USA)

Ethidium bromide (Bio Rad, USA)

Arcylamide (Bio Rad, USA)

Ammonium persulfate (Bio Rad, USA)

TEMED (Bio Rad, USA)

Mc Farland No.2 (bio Merieux)

2. Meterials

Eppendorf microcentrifuge

Micropipette

Tip

Cotton swab

Cylinder

Test tube

X-ray film cassettes

Pasture pipette

Gas pak (Oxiod, England)

3. 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)

Polaroid camera (Polaroid, USA)

Biological safety cabinet (Yamato, Japan)

Automatic pipette (Brand, Germany)

Rotary shaker (Bellco Glass, USA)

Incubator (Sanyo, Japan)

Refrigerator (Sharp, Japan)

Freezer –20^{0}C (Sanyo, Japan)

Autoclave (Yamato, Japan)

Spectrophotometer (Bio-Tek instruments, USA)

Anaerobic jar (Oxoid, England)

Vortex mixer (Scientific, USA

BIOGRAPHY

Miss Siriporn Rugdeekha was born on April 24th,1976 in Prachuapkhirikhun, Thailand. I graduated with the Bachalor degree of Science in Microbiology from the Faculty of Science ,Chulalongkorn University in 1997.