การวิเคราะห์ toxin genes และ *tcdC* genotypes ของเชื้อ
คลอสตริเดียม ดิฟฟิไซล์ ที่แยกได้ในโรงพยาบาลจุฬาลงกรณ์

นางสาวประเสริฐศรี ตั้งเลิศสัมพันธ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต

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MOLECULAR ANALYSIS OF TOXIN GENES AND *tcdC* GENOTYPES AMONG *CLOSTRIDIUM DIFFICILE* ISOLATES IN KING CHULALONGKORN MEMORIAL HOSPITAL

Miss Prasertsri Tunglertsumphan

A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science Program in Medical Microbiology

(Interdisciplinary Program)

Graduate School

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ประเสริฐศรี ตั้งเลิศสัมพันธ์ : การวิเคราะห์ toxin genes และ *tcdC* genotypes ของเชื้อคลอสตริเดียม ดิฟฟิไซล์ ที่แยกได้ในโรงพยาบาลจุฬาลงกรณ์ (Molecular analysis of toxin genes and *tcdC* genotypes among *Clostridium difficile* isolates in King Chulalongkorn Memorial Hospital) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : รศ.ดร.สมหญิง ธัมวาสร , 66 หน้า.

คลอสตริเดียม ดิฟฟิไซล์ เป็นแบคทีเรียแอนแอโรบ ชนิดแกรมบวก รูปแท่ง สามารถสร้างสปอร์ได้ เชื้อนี้เป็นสาเหตุทำให้เกิดโรคท้องเสียและลำไส้อักเสบซึ่งถูกเรียกรวมว่าโรคจาก คลอสตริเดียม ดิฟฟิไซล์ ปัจจัยก่อโรคของเชื้อที่สำคัญคือ ท็อกซิน เอ (TcdA) และ ท็อกซินบี (TcdB) ซึ่งสร้างใน เชื้อที่เป็ นสายพันธุ์ที่สร้างท็อกซิน (toxigenic strain) เนื่องจากมีรายงานอัตราการเกิดโรคสูงขึ้น และ มีความรุนแรงมากขึ้น การศึกษาครั้งนี้ จึงมุ่งศึกษาคุณลักษณะของเชื้อคลอสตริเดียม ดิฟฟิไซล์ ที่แยกได้ในโรงพยาบาลจุฬาลงกรณ์ โดยศึกษายีนที่สร้างท็อกซินเอ (toxinA) ท็อกซินบี (toxin B) ไบนาริท็อกซิน (binary toxin) และการกลายพันธุ์ของ *tcdC* ทำการศึกษาในช่วงเดือน สิงหาคม 2554 ถึงเดือนกันยายน 2555 โดยนำตัวอย่างอุจจาระจำนวน 1,114 ตัวอย่างจากผู้ป่วยที่สงสัยว่าเป็นโรคจาก คลอสตริเดียม ดิฟฟิไซล์ มาทำการเพาะเชื้อในสภาวะไร้อากาศ เลือกเชื้อที่สงสัยว่าเป็น *C. difficile* โดยใช้คุณสมบัติของเซลล์ กลิ่น และความสามารถสร้างสารเรืองแสงได้เมื่อสัมผัสกับรังสีอุลตราไวโอเลตที่ ความยาวคลื่น 365nm ผลการเพาะเชื้อพบเชื้อที่สงสัยว่าเป็นคลอสตริเดียม ดิฟฟิไซล์ ในอุจจาระจำนวน 242 ตัวอย่าง การทดสอบด้วยวิธี multiplex polymerase chain reaction เพื่อพิสูจน์เชื้อและหายีนที่สร้างท็อกซินเอ และท็อกซินบี โดยมีเป้าหมายที่ยีน triose phosphate isomerase (*tpi*), *tcdA* (toxin A) และ *tcdB* (toxin B) พบว่า เป็นเชื้อ คลอสตริเดียม ดิฟฟิไซ ล์ จำนวน 235ตัวอย่าง ซึ่งในจำนวนนี้เป็นสายพันธุ์ที่สร้างท็อกซินจำนวน 149 (63.40%) ตัวอย่าง และ สายพันธุ์ที่ไม่สร้างท็อกซิน จำนวน 86 (36.60%) ตัวอย่าง สายพันธุ์ที่สร้างท็อกซินจำนวน 149 ตัวอย่างเป็นสายพันธุ์ที่มียีนที่สร้างท็อกซิน เอ และ ท็อกซินบี (A^+B^+) จำนวน 84 (56.38%) ตัวอย่าง และเป็นสายพันธุ์ที่ไม่สร้างท็อกซิน เอ แต่มียีนที่สร้างท็อกซินบี (A^-B^+) จำนวน 65 (43.63%) ตัวอย่าง และไม่พบสายพันธุ์ที่มียีนที่สร้างท็อกซิน เอ แต่ไม่สร้างท็อกซินบี (A^+B^-) ในการศึกษาครั้งนี้ ผลการตรวจวิเคราะห์ ไบนาริท็อกซิน ด้วยวิธี multiplex polymerase chain reaction โดยหายีน *cdtA* และ *cdtB* พบคลอสตริเดียม ดิฟฟิไซล์ ที่มี ไบนาริท็อกซิน จำนวน 1 ตัวอย่าง คือ คลอสตริเดียม ดิฟฟิไซล์ No.38 ซึ่งตัวอย่างนี้พบทั้งยีนที่สร้างท็อกซินเอ และ ท็อกซินบี ด้วยเมื่อนำตัวอย่างนี้ทดสอบยีน *tcdC* ด้วยวิธีการหาลำดับเบส พบว่ายีน *tcdC* มีการขาดหายไป 18 bp และเป็น in-frame deletion ซึ่งมีผลต่อการขาดหายกรดอะมิโนจำนวน 6 โมเลกุลโดยไม่มีผลต่อตำแหน่งอื่น จาก ข้อมูลอายุที่สามารถหาได้ในผู้ป่วยจำนวน 176 ราย พบว่า ตรวจพบคลอสตริเดียม ดิฟฟิไซล์ ได้จากผู้ป่วยกลุ่มอายุมากกว่า 60 ปีมากที่สุด (69.81%) ซึ่งสอดคล้องกับที่พบว่า กลุ่มผู้สูงอายุเป็นกลุ่มมีความเสี่ยงสูง โดยสรุปการศึกษานี้พบความชุกของคลอสตริเดียม ดิฟฟิไซล์ สายพันธุ์ที่มียีนที่สร้างท็อกซินใน กลุ่มผู้ป่วยที่สงสัยว่าเป็นโรคจาก คลอสตริเดียม ดิฟฟิ ไซล์ คือ 13.37% และไม่พบคลอสตริเดียม ดิฟฟิไซล์ สายพันธุ์ที่มีความรุนแรงสูง

สาขาวิชา จุลชีววิทยาทางการแพทย์ ลายมือชื่อนิสิต...

ปีการศึกษา 2555 ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์หลัก.................................

#5387341020 : MAJOR MEDICAL MICROBIOLOGY
KEYWORD *: CLOSTRIDIUM DIFFICILE* / DIARRHEA / TOXIN A / TOXIN B / BINARY TOXIN

PRASERTSRI TUNGLERTSUMPHAN : MOLECULAR ANALYSIS OF TOXIN GENES AND *tcdC* GENOTYPES AMONG *CLOSTRIDIUM DIFFICILE* ISOLATES IN KING CHULALONGKORN MEMORIAL HOSPITAL.ADVISOR ASSOC. PROF. SOMYING TUMWASORN, Ph.D., 66 pp.

Clostridium difficile, a Gram-positive, anaerobic spore-forming bacterium, is a major cause of antibiotic-associated diarrhea and pseudomembranous colitis known as *C. difficile*-associated disease (CDAD). Major virulence factors contributing to the diseases are toxins A (TcdA) and B (TcdB) produced by toxigenic strain. With the increased frequency and severity of CDAD, this study aimed to characterize *Clostrdium difficile* isolates in King Chulalongkorn Memorial hospital for the presence of genes encoding toxins A and B, binary toxin and mutation in TcdC gene. From August 2011 to September 2012, 1,114 stool samples from suspected CDAD patients were cultured anaerobically. Suspected *C. difficile* colonies characterized by cell morphology, odor, and fluorescence under 365-nm UV illumination were isolated from 242 samples. Toxigenic *C. difficile* cultures were confirmed by multiplex polymerase chain reaction (PCR) targeting a species-specific internal fragment of triose phosphate isomerase (*tpi*) gene, toxin A gene (*tcdA*) and toxin B gene (*tcdB*). Of 242 *C. difficile* culture, 235 were confirmed to be *C. difficile* which 149(63.40%) were toxigenic and 86(36.60%) were non-toxigenic. Of 149 toxigenic *C. difficile*; toxins A and B-positive (A^+B^+) *C. difficile* were found in 84 (56.38%) samples and toxin A-negative, toxin B-positive (A^-B^+) *C. difficile* were found in 65 (43.63%) samples. Toxin A- positive, toxin B- negative (A^+B^-) *C. diffcile* was not found. Binary toxin investigated by multiplex PCR targeting *cdtA* and *cdtB* revealed that binary toxin-positive *C. difficile* (No.38) was found in one sample. *C. diffcille* No.38 which also harbor TcdA and TcdB genes was subjected to *tcdC* sequencing and the result showed 18 bp in frame deletion resulting in the truncate peptide of 232 amino acids. Clinical data available from 176 patients revealed that toxigenic *C. difficile* isolates were recovered from patients aged > 60 years (69.81%), corresponding with the finding that advanced age is the risk factor of toxigenic *C. difficile* infection. In conclusion, the prevalence of toxigenic *C. difficile* in suspected CDAD patients was 13.37% and the hypervirulent strain was not found in this study.

Field of study : Medical Microbiology Student's Signature..

Academic Year : 2012 Advisor's Signature.......................................

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LIST OF ABBREVIATIONS

ATCC	American type culture collection
bp	Base pair
°C	Degree celsius
DNA	Deoxyribonucleic acid
DW	Distilled water
et al.	et alii
g	Gram
h	Hour
l	Liter
M	Molar
mg	Milligram
mg/l	Milligram per liter
min	Minite(s)
ml	Milliliter
mm	Millimeter
PCR	Polymerase chain reaction
pmol	Picomol

rpm	Round per minute
sec	Second
TBE	Tris-boric acid
Tris	Tris-(hydroxymethayl)-aminoethane
µg	Microgram
µl	Microliter
µM	Micromolar

CHAPTER I

INTRODUCTION

Clostridium difficile is gram positive, anaerobic, spore-forming bacterium which was first isolated in 1935 from human newborn stool with named *Bacillus diciffilis* [1]. Commonly, *C. difficile* was found in humans as asymptomatic carrier [2-4], feces of animals such as horses, pigs, dogs and cats, and the environment such as soil [5-8]. In addition *C. difficile* was found in food ; retailed meat [9, 10], retailed pork [11], salad [12] and vegetable [13] but still unproven for foodborne pathogen [14]. Some strains of *C. difficile*, toxigenic strains, can produce toxins which are the important virulence factors of *C. difficile*. There are three known toxins; toxin A, toxin B and binary toxin which were found variably in toxigenic *C. difficile* strains. *C. difficile* plays important role in nosocomial diarrhea and colitis called *Clostridium difficile*-associated diseases (CDAD) which have wide-range of clinical symptoms from asymptomatic carrier, mild self limted diarrhea, severe diarrhea, colitis [15], ileus, pseudomembranus colitis [16], toxic megacolon and death [17-19]. The risk factors are antibiotic exposure which disturbs normal flora, leading to more number of toxigenic *C. difficile* which can produce and release toxins to damage intestinal epithelial cells. All antibiotics can cause CDAD, especially clindamycin, cephalosporins, quinolones and penicillin [20] ; long-term hospitalization offers more opportunity to be exposed with *C. difficile* spores which contaminate in hospital worker, environment and facility [21]; advanced age with changing of intestinal microbiota [22, 23] and patients with underlying diseases such as cancer, organ transplantation [24] and HIV [25]. The main virulence factors of *C. difficile* are toxins A and B which cause cell death and disruption of epithelium tight junction resulting in diarrhea and colitis [26]. The third toxin called binary toxin which is found in 6-12.5% of C. *difficile* isolates [14]. The other factor contributing to *C. difficile* virulence is its spore which helps *C. difficile* to resist heat, acid, harsh environment and disinfectants [27-29]. Moreover, CDAD are the burden in health system for the budget to cure the patients, stay in hospital longer [30] , more effective antibiotic , postsurgical and recurrent of CDAD [31].

Since 2000, there has been a changing of the incidence of CDAD with more severity [32] and mortality rate [33, 34] ; in non- risk group, non- antibiotic exposure, young age, children [35] [36], pregnant women [37], non-hospitalized patients [38, 39] and community-

acquired cases [40, 41]. *C. diffcile* PCR-Ribotype 027/NAP1was well known as *C. difficille* hypervirulent strain which was responsible for *C. difficile* epidemic in Canada [42, 43], England [44], Europe [45] with more patient number, more severity and more mortality rate. There were many reports of this strain being found around the world [46], North America [42, 47], Europe [48-51] and Asia [52-54]. The hypervirulent strain had these properties; production of higher amount of both of toxins A and B *in vitro* 16-23 times than reference strain (VPI 10463), presence of binary toxin and 18 bp deletion in *tcdC* which is known as negative regulator for toxins A and B production and single base deletion at position 11. Another *C. difficile* hypervirulent strain is *C. difficile* PCR-Ribotype 078 which had properties similar to *C. difficile* PCR-Ribotype 027 for production of both toxins A and B, presence of binary toxin and *tcdC* mutation with C184T transition that introduces a stop codon leading to a presumptive truncated protein of 61 residues, and a 39-bp deletion located downstream of the alternative stop codon. This strain was associated with community acquired cases more than *C. difficile* PCR-Ribotype 027 [55]. With the increase in frequency and severity of CDAD, this study aimed to search for the hypervirulent *C. difficile* strain by cultivation of *C. difficile* from stool samples of suspected CDAD patients and characterize for the presence of genes encoding toxin A, B and binary toxin and mutation in TcdC gene encoding anti-sigma factor which is negative regulator for toxin A and B production.

HYPOTHESIS

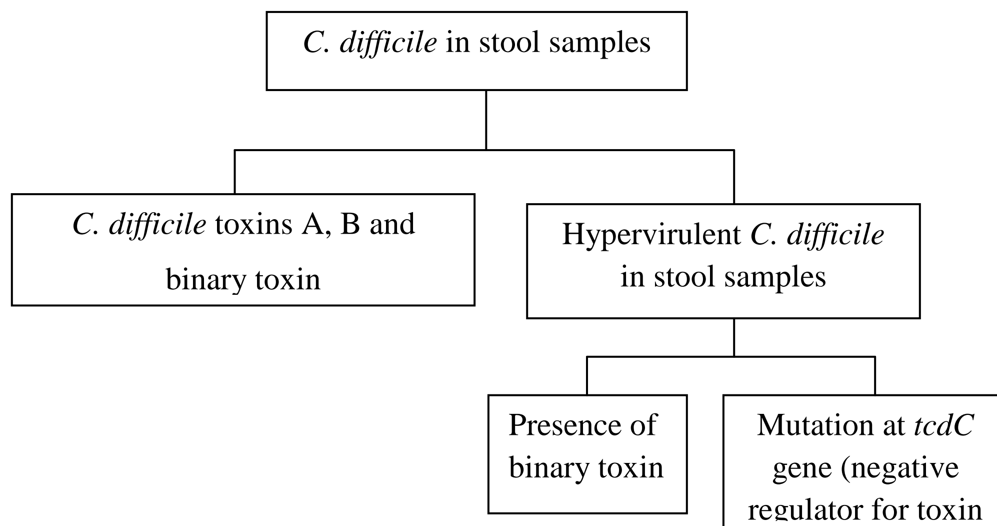
1. Some *C. difficile* Thai isolates have binary toxin gene together with toxin A (*tcdA*) and or toxin B (*tcdB*).

2. *C. difficile* PCR-Ribotype 027 may be found in King Chulalongkorn Memorial hospital.

OBJECTIVE

Characterize *Clostrdium difficile* isolates in King Chulalongkorn Memorial Hospital for the presence of genes encoding toxins A and B, and binary toxin and mutation in TcdC gene.

Conceptual framework



CHAPTER II

LITERATURE REVIEWS

Clostridium difficile

Clostridium difficile is gram positive, anaerobic, spore-forming bacterium which is major cause of nosocomial diarrhea and colitis called *Closridium diffcile*–associated disease (CDAD). Risk factors of CDAD are antibiotic exposure which eliminates normal flora in colon resulting in *C. difficile* colonization and overgrowth[56], advanced age by changing of gut microbiota[57], reduced immune status, and more frequent hospitalization [22]. Long term hospitalization has more chance for exposure to contaminated *C. difficile* spores in hospital environment such as air, facilities and from heath care workers whose hands transmitted *C. difficile* spores from person to person [58].

Virulence factors of *C. difficile*

1. Toxins A and B

Toxin A (TcdA) 308 kDa and toxin B (TcdB) 270 kDa encoded by *tcdA* and *tcdB* which located on Pathogenecity locus (PaLoc) in *C. difficile* chromosome Figure1 has size 19.6 kb, integrated with five genes encoded different proteins; *tcdA* encoded TcdA (toxin A), *tcdB* encode TcdB (toxin B), *tcdC* encodes TcdC; anti sigma factor which is negative regulator for toxin A and B, *tcdE* encode TcdE, *tcdR* encode TcdR, In non toxigenic strain ($TcdA^-$, $TcdB^-$), PaLoc was replaced with short nucleotides of 115 bp.

Both toxins A and B belong to the group of large clostridial cytotoxins (LCT) and are characterized by a high molecular mass (more than 250 kDa). These toxins share common domain structure, approximately 66% sequence similarity and similar enzymatic activities. TcdA has an extended C-terminus, different cell tropism and is generally less potent in cell cytotoxicity than TcdB. The structure of *C. difficile* toxins A and B were shown in Figure 2. Toxins A and B are composed of three domains; enzymatic domain in N-terminal region, translocation domain in middle and binding domain in C-terminal region. These toxins share a common domain structure, similarity and similar enzymatic activities. These toxins are glycosyltransferase to inactivate small proteins, Rho, Rac and

Cdc42, which have important role in signaling pathway involved in morphological changes, tight junction disruption and cell apoptosis[59] TcdA and TcdB covalently modify and inactivate the Rho family GTPases, Rho, Rac and Cdc42 [59], which play key roles in regulating signaling pathways. Glucosylation of Rho GTPases results in disaggregation of the actin cytoskeleton, cell rounding, cell death and loss of intestinal epithelium barrier function. [60]

2. Binary toxin (*Closridium difficile* Transferase ,CDT)

Binary toxin or called *Clostridium difficile* transferase (CDT) is encoded by *cdtR*, *cdtA* and *cdtB* which located on CDT locus (CDTLoc) of *C. difficile* chromosome (Figure1) which encoded CdtR, CdtA and CdtB [Figure3]. In non binary toxin strains, CDTLoc is replaced with conserved sequence of 68 bp [61]. Binary toxin alone may not be the cause of disease [62] but Schwan *et al*. [63]found that *C. difficile* VPI 10463 with toxin A but no binary toxin, when manipulated to express binary toxin, can increase the attachment with cell.

CDT is one of Clostridial binary toxins family such as *C. perfringens* iota toxin, *C. spiroforme* toxin which destroys filamentous actin in epithelial cells[64] which also impair the structure of actin cytoskeleton. Recent study reported that binary toxin not only affects the actin cytoskeleton but also induces the formation of microtubule-based protrusions on the surface of epithelial cells, leading to increased adherence.

3. Spore-production

Spore-production property helps *C. difficile* to resist heat, acid, harsh environment and disinfectants [27-29] and to transmit *C. difficile* [65]. Highly purified spores permitted the study of their biology and infectivity. Cholate, taurocholate, or glycocholate supplementation of brain heart infusion (BHI) increased germination rates 100- to 1000-fold more than BHI agar plates alone. This observation has implications for recovery of *C. difficile* from clinical and environmental samples. Purified spores demonstrated resistance to high temperatures and 70% ethanol but were inactivated by sporicidal agents. Environmental spores infect mice in a dose-dependent manner; the dose required to infect 50% of the mice (ICD50) is~7 spores per cm.

4. Other virulence factors

There are many factors involved in the initiation of *C. difficile* pathogenesis by facilitating *C. difficile* to colonize host cell. These are surface layer protein, adhesin, capsule and flagella.

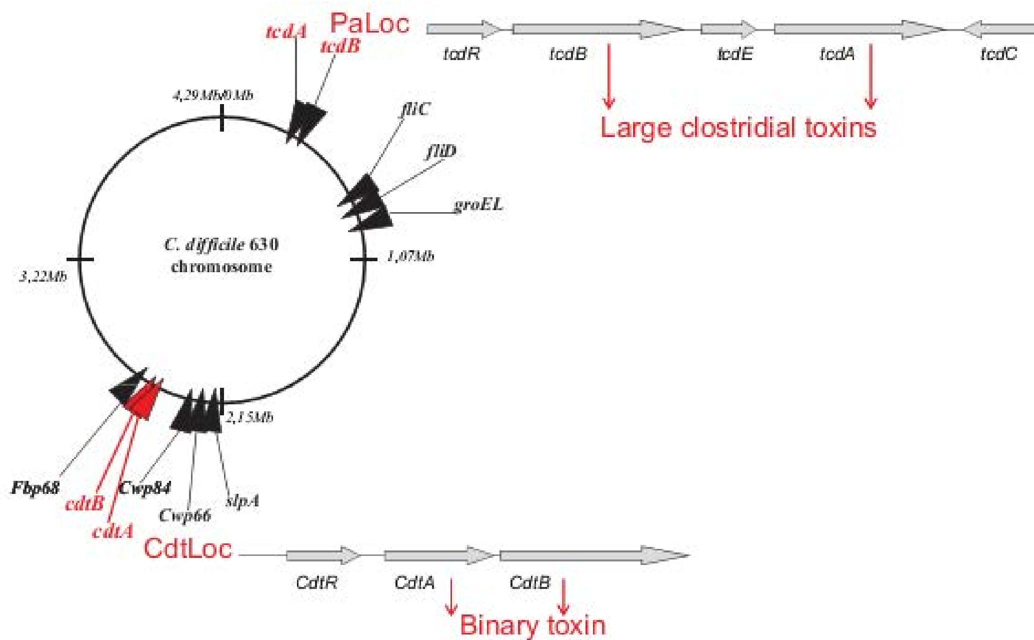


Figure 1.*C. difficile* toxin A, B and binary toxin locate on *C. difficile* 630 chromosome [66]

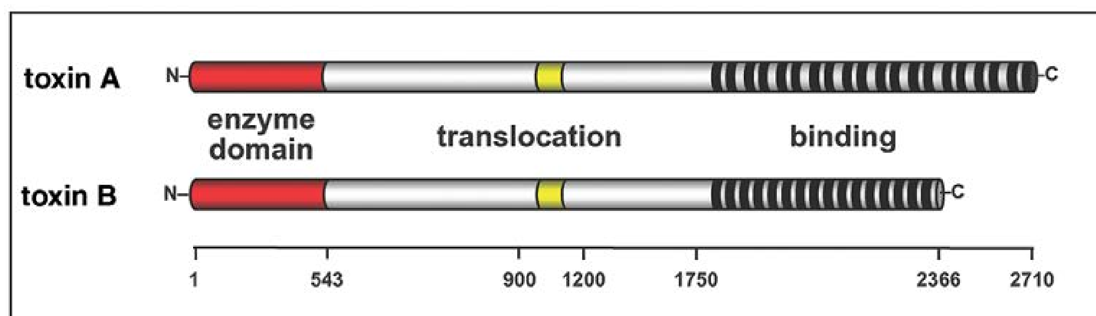


Figure 2. Structure of *C. difficile* toxins A and B. Toxins A and B composed of three domains; enzymatic domain in N-terminal region, translocation domain in middle and binding domain in C-terminal region .[67]

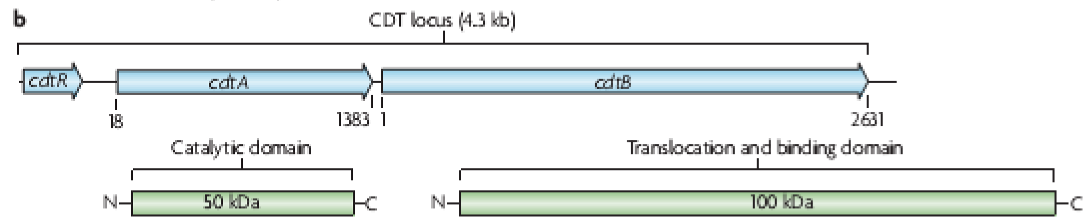


Figure 3. Structure of binary toxin called *Clostridium difficile* transferase (CDT) in CDT locus [23]

Clinical Symptoms

Colon manifestations

1. Asymptomatic carrier

Host contacted with *C. difficile* spores will colonized with *C. difficile* toxingenic strains or non toxigenic strain (Figure4). In toxigenic strains can develop to CDI when host exposed with risk factor such as antibiotics, changing of intestinal microbiota.

2. Diarrhea

C. difficile was cause of antibiotic-associated diarrhea. Water diarrhea, unformed stool but not blood (rare) during use antibiotics, diarrhea will stops when stop use antibiotics [68].

3. Severe diarrhea

Patients had diarrhea with/without blood associated with hypovolemia or hypoalbuminemia, high fever and leukocytosis.

4. Colitis without Pseudomembrane formation [15, 68]

Patients had symptoms high white blood count, abdominal pain, nausea, anorexia, and watery diarrhea

5. Pseudomembranous colitis (PMC)

Patients had symptoms with high white blood count, hypoalbuminemia, ascites, abdominal tenderness [16, 69-71], *C. difficile* is considered the only cause of antibiotic-associated pseudomembranous colitis [72]. The endoscopic presence of many white-yellowish plaques with diameters 2–10 mm in all of the colon (Figure5) [68] and in histopathology pseudomembranous colitis were showed volcanic eruption.

6. Fulminant colitis [18, 73]

Found about 3-8 % of patients. Fulminant colitis was the most severe complication cause of *C. difficile* infection. Patients have symptom with severe diarrhea, but in some patients may not have diarrhea from prolong ileus as a consequence of which secretions accumulate in the dilated [72] , toxic megacolon (defined by distension of colon diameters more than 6cm) [74] toxin shock and death

7. Recurrent *C. difficile*

A first episode of *C. difficile* infection (CDI) is followed by a symptomatic recurrence in approximately 19-20% of patients affected. The pathophysiology is not quite clear and may be due to persistently altered fecal flora.

Extracolonic manifestations

Commonly, *C. difficile* infection due to *C. difficile* outside colon have infrequent found, such as bacteriamia [75-77] , reactive arthritis [78-80]

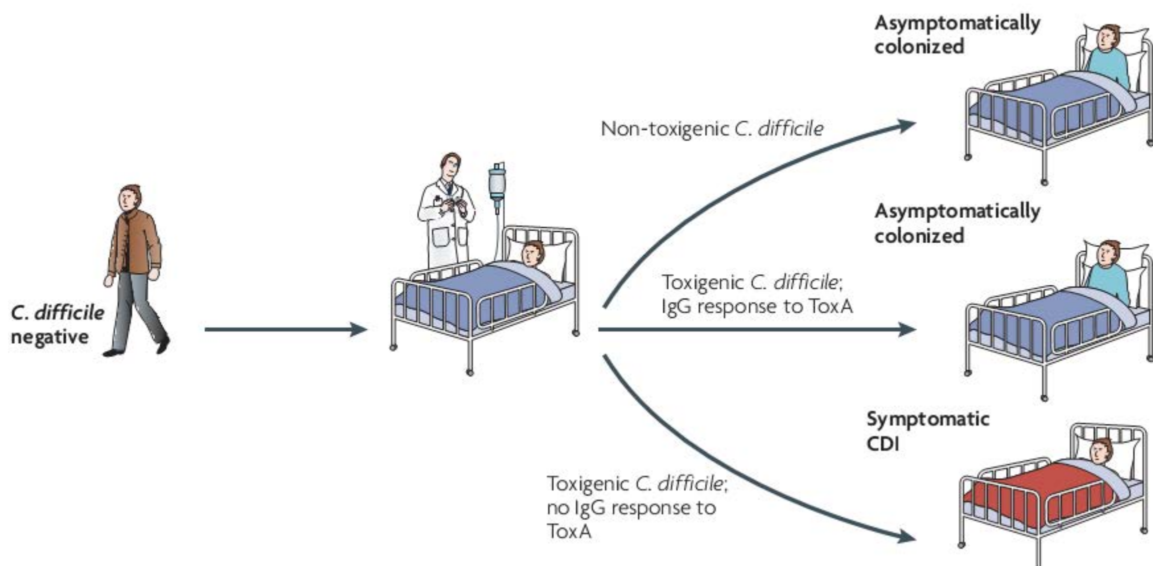


Figure 4. Model of CDI transmission [23]

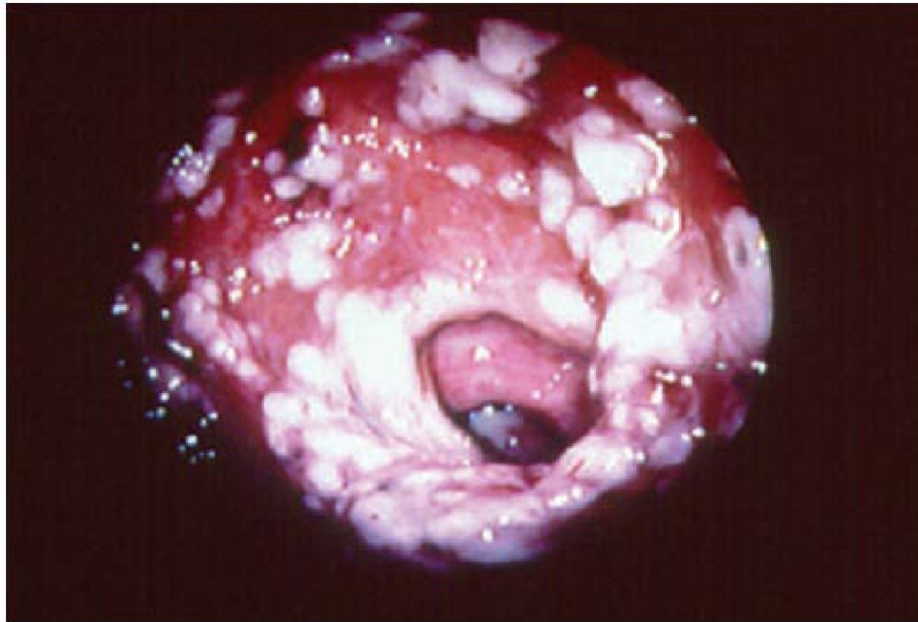


Figure 5. Endoscopic of pseudomembranous colitis in CDAD patient [81]

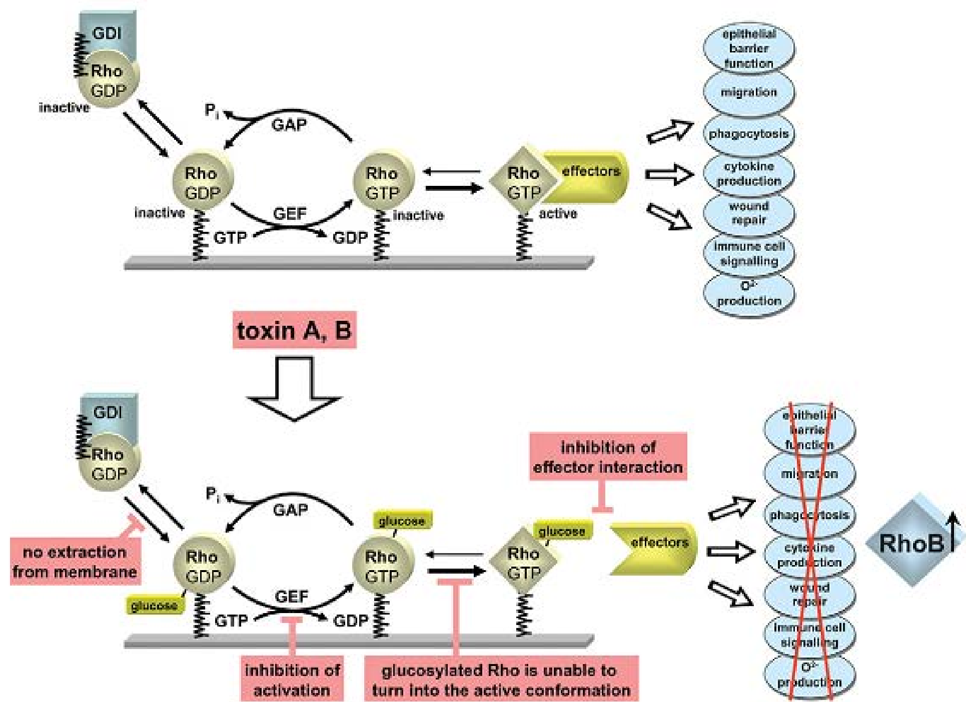


Figure 6. Regulation of Rho GTPases and inhibition by toxin A and B [67]

Pathogenesis

Host exposed with *C. difficile* spores from CDI patients by fecal-oral route, healthcare environment or facility. Normally vegetative cells and spores of *C. difficile* transmitted into gut but vegetative cells killed by acid in gastric juice, only spores survived to small intestine and germinated by bile salt to vegetative cells which colonized in colon. When hosts were susceptible for *C. difficile* from many factors (advanced age, antibiotic use, impaired immunity) [82]. *C. difficile* colonized in colon epithelium cell by use colonizing factors such as; adhesion, surface layer protein, then *C. difficile* will secrete toxins A and or B to destroy epithelium cell and epithelium tight junction by toxin A and B will inactive Ras superfamily of small GTPases due to morphology change and apoptosis (Figure6).

Epidemiology of *Clostridium difficile*

C. difficile is gram positive spore forming bacterium. In past decade, there are many reports about the changing epidemiology of *C. difficile*. Pepin et al. [32] reported that in Quebec, Canada between 1991-2003, there was dramatically increase in number of CDAD patients from 35.6 per 100,000 population in 1991 to 156.3 per 100,000 population in 2003. Especially in age group over 65 years, the rate increased from 102.0 to 866.5 per 100,000 population and, in 2005 Warny *et al*. [42] reported the outbreak of *C. difficile* in Sherbrook, Canada between June 2004 -April 2005. Cause of this of outbreak is *C. difficile* NAP1/PCR-Ribotype 027/Toxinotype III strain. This strain had the following properties: produced toxin A and toxin B in higher amount than reference strain (*C.difficile* VPI 10463 /Toxinotype 0) 16 and 23 times, along with binary toxin and *tcdC* 18 bp deletion. Furthermore, analysis of *C. difficile* NAP1/PCR-Ribotype 027/Toxinotype III strain which isolated from this study compared with *C. difficile* isolates of the same molecular type from UK, USA (Maine, Georgia, Pennsylvania, New Jersey) and France showed 94-100% similarity. In 2006, MacCannell *et al.* studied *C. difficile* PCR Ribotype 027 from Eastern and Western Canada by sequencing *tcdc* gene and found single-base-pair deletion at position 117 and deletion 18 bp at positions 330 to 347 of the gene due to premature truncation of TcdC protein (Figure7) [83].

```
                     *        20         *        40         *        60         *        80         *       100
PQ_027_F    : MFSKKNEGNEFSNERKGSSKKIIKFFKSTKDIALLAFILVCFLA---------------------IYPHQLVLKTMRRS-------FLIKHQL------- :  65
BC_027_A    : MFSKKNEGNEFSNERKGSSKKIIKFFKSTKDIALLAFILVCFLA---------------------IYPHQLVLKTMRRS-------FLIKHQL------- :  65
BC_027_C    : MFSKKNEGNEFSNERKGSSKKIIKFFKSTKDIALLAFILVCFLA---------------------IYPHQLVLKTMRRS-------FLIKHQL------- :  65
PQ_027_A    : MFSKKNEGNEFSNERKGSSKKIIKFFKSTKDIALLAFILVCFLA---------------------IYPHQLVLKTMRRS-------FLIKHQL------- :  65
AB_027_A    : MFSKKNEGNEFSNERKGSSKKIIKFFKSTKDIALLAFILVCFLA---------------------IYPHQLVLKTMRRS-------FLIKHQL------- :  65
AB_027_G    : MFSKKNEGNEFSNERKGSSKKIIKFFKSTKDIALLAFILVCFLA---------------------IYPHQLVLKTMRRS-------FLIKHQL------- :  65
UK_027_REF  : MFSKKNEGNEFSNERKGSSKKIIKFFKSTKDIALLAFILVCFLA---------------------IYPHQLVLKTMRRS-------FLIKHQL------- :  65
ATCC43255   : MFSKKNEGNEFSNEGKGSSKKIIKFFKSTKGIALLAFILGVFFGNISSPSCSEDHEEVISNQTSVIDSQKTEIETLNSKLSDAEPWFKMKDDEKKAIEAE : 100
              MFSKKNEGNEFSNErKGSSKKIIKFFKSTKdIALLAFILvcFla                     Iyphqlv6kT6rrs       Fl6Khql
```

Figure 7. Comparison of *tcdC* sequence of *C. difficile* PCR-Ribotype 027 with *C. difficile* ATCC 43255 reference strain [83].

Clostridium difficile in Thailand

The reports of *C. diffcile* in Thailand were very few. In, 2003 Wongwanich *et.al* [84] reported that the prevalence of *C. difficile* isolated from the stools of Thai adult patients with suspected CDAD was 18.64% by using PCR and (of toxin genes (*tcdA* and *tcdB*) by polymerase chain reaction (PCR) from stool samples yielded almost the same compared to the recovery rate of the toxin detection by enzyme immunoassay (EIA). In 2011, Thipmontree *et al.* [85] reported that the prevalence of CDAD in suspected *C.* difficile-associated hospital-acquired diarrhea was 12.3% (95% CI 8.5% to 17.6%) by detection of *C.* difficile toxins A and B in stool samples.

PCR-Ribotyping

PCR-ribotyping is the standard typing method in Europe by uses specific primers to amplify the variable-length intergenic spacer region (ITS) between 16S and 23S rDNA. *C. difficile* genome and copies also differ in the length of ITS and a single primer pair can result in a pattern of bands ranging from 200 to 700 bp. DNA band patterns are referred to as ribotypes. PCR-ribotyping is the molecular typing method with a high discriminatory power, type-ability, and reproducibility [86]. This method use for investigate epidemiology of *C. difficile* which found around the world [87]; such as PCR Ribotype 027 in Canada, Europe [50, 88-94] ,PCR Ribotype 078 in Europe [93] and PCR-Ribotype 027 in Asia [54, 95, 96] .

CHAPTER III

MATERIALS AND METHODS

Stool samples and cultivation of *C.difficile*

A total of 1,114 stool samples of suspected CDAD patients in King Chulalongkorn Memorial Hospital from August 2011 to September 2012 were included in this study. Fresh, loose, watery, unbound stool was sent to anaerobic bacteriology laboratory for toxin detection as routine service and the leftover sample was cultured for *C. difficile* with the method described by UK Standards for Microbiology Investigations [97].

Processing of Faeces for *Clostridium difficile* [97] with modification by using phenyl ethyl alcohol agar supplemented with 5% sheep blood instead of cefoxitin-cycloserine egg yolk agar. Alcohol shock was first performed to eliminate viable microbial cells except spores by mixing stool sample with absolute ethanol in a ratio of 1:1 and incubated at room temperature for 1 hr. The sample was then plated on phenyl ethyl alcohol agar (PEA) supplemented with 5% sheep blood and incubated in an anaerobic condition (mixture of 80% N2, 10% CO2 and 10% H2) at 35±2 °C for 48 hrs. *C. difficile* was presumptively identified with colony appearance as white-yellow and flat, gram positive rod and fluorescence under UV 365 nm. All up to 10 colonies of presumptively identified *C. difficile* were pooled and subcultured onto 2 plates of Brucella agar (BBL, Becton-Dickison) supplemented with 5% sheep blood and incubated in an anaerobic condition at 35±2 °C for 48 hrs. *C. difficile* culture from one plate was used for DNA extraction and the other was kept as stock culture in 20% skim milk and stored at -80 °C.

DNA extraction

All colonies from 48 h culture on Brucella agar were suspended in 200 µl sterile distilled water in 1.5 ml sterile microcentrifuge tube and centrifuged for 15 s. Cell pellet was washed twice with and resuspended with in180 µl sterile distilled water . Cell suspension was added with 20 µl digestion buffer and incubated in a water bath at 60°C for 1 hr. After incubation in heat box at 100°C for 15 min, the suspension was centrifuged at 15,280.6 x g for 5 min and the supernatant was collected in 1.5 ml sterile microcentrifuge tube and stored at -20 °C.

Molecular analysis of *Clostridium difficile*

1. Multiplex PCR for *C.difficile* , toxins A and B

Multiplex PCR was performed as described by Lemee *et al.* [98] to detect *C. difficile* , toxins A, and B by targeting species-specific internal fragment of the *tpi* (triose phosphate isomerase), internal fragment of the *tcdA* (toxin A) and internal fragment of the *tcdB* (toxin B) as shown in Figure 8. PCR mixture in a volume of 25 µl contains PCR buffer (50 mM Tris-HCl, 10 mM KCl, 5 mM (NH4)2SO4 pH 8.3), 2.6 mM MgCl2 , 1.0 U of *Taq* DNA polymerase, 200 µM each dATP, dCTP , dGTP and dTTP, each primer [Table 1]; *tpi-F* and *tpi-R* 1.0 µM, *tcdA-F* and *tcdA-R* 2.0 µM, *tcdB-F* and *tcdB-R* 2.5 µM . PCR products were electrophoresed in 2 % agarose gel at 100 V for 45 min and stained with ethidium bromide. The stained gel was recorded by gel documentation system. Reference strains of *C. difficile* were used as controls: *C. diffcile* ATCC 9689 as positive control for toxigenic *C. difficile* (A+B+) and *C. difficile* ATCC 700057 as negative control for non- toxigrnic *C. difficile*.

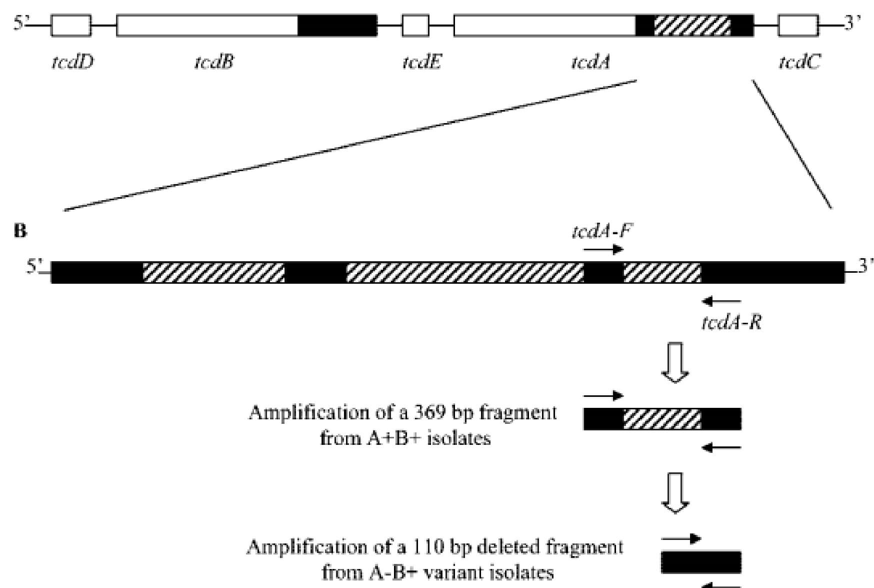


Figure 8. Positions of tcdA primers allowing differentiation between A+B+ and AB+ isolates[98]. (A) Partial map of the pathogenicity locus with *tcdA* and *tcdB* genes and their adjacent accessory genes *tcdD*, *tcdE*, and *tcdC*. Black regions represent the 3' repetitive sequences characteristic of *C. difficile tcdA* and *tcdB* genes. (B) Details of the 1.8-kb deletion, resulting in combination of three small deletions (hatched regions). The *tcdA* primers flank the smallest deletion (in the 3' end of the gene) and generate a 369-bp amplified fragment from A+B+ strains but a 110-bp amplified fragment from A-B+ variant strains

Table 1. Primers for multiplex PCR detect *C. difficile* , toxins A, and B

Target genes	Primer	Sequence (5'–3')	Reference
tpi	*tpi-F*	AAAGAAGCTACTAAGGGTACAAA	[98]
	tpi-R	CATAATATTGGGTCTATTCCTAC	[98]
tcdA	*tcdA-F*	AGATTCCTATATTTACATGACAATAT	[98]
	tcdA-R	GTATCAGGCATAAAGTAATATACTTT	[98]
tcdB	*tcdB-F*	GGAAAAGAGAATGGTTTTATTAA	[98]
	tcdB-R	ATCTTTAGTTATAACTTTGACATCTTT	[98]

2. Multiplex PCR for binary toxin

Multiplex PCR was performed to detect binary toxin target to *cdtA* and *cdtB* in Table 2 performed by Persson *.et al* [99] in PCR mixture volume 25 µl contain PCR buffer (50 mM Tris-HCl, 10 mM KCl, 5 mM (NH4)2SO4 pH 8.3) 2.6 mM MgCl2, 1.0 U of *Taq* DNA polymerase, 200 µM each dATP, dCTP, dGTP and dTTP, each primer from [Table2] 1.0 µM . PCR condition, start from denatured at 94 °C for 10 min, follow with 94 °C for 50 s 54 °C 40 s 72 °C 50 s for 35 cycles and final extension at 72 °C for 3 s . PCR products were electrophoresed in 2% agarose gel at 100 V for 45 min and stained with ethidium bromide. The stained gel was recorded by gel documentation system. Reference strains of *C. difficile* were used as controls: *C. diffcile* ATCC BAA-1870 as positive control (*ctdA+,cdtB+*) and *C. difficile* ATCC 9689 as negative control (*cdtA-,cdtB-*).

Table 2. Primers for multiplex PCR to detect binary toxin

Target genes	Primer	Sequence (5'–3')	Reference
cdtA	cdtA-F739A	GGGAAGCACTATATTAAAGCAGAAGC	[99]
	cdtA-F739B	GGGAAACATTATATTAAAGCAGAAGC	[99]
	cdtA-R958	CTGGGTTAGGATTATTTACTGGACCA	[99]
cdtB	cdtB-F617	TTGACCCAAAGTTGATGTCTGATTG	[99]
	cdtB-R878	CGGATCTCTTGCTTCAGTCTTTATAG	[99]

3. PCR screening for *tcdC* deletion

Screening for *tcdC* gene deletion by PCR was performed as described by Persson *et al.* [100] using primers as in Table 3. PCR mixture in a volume of 25 µl contains PCR buffer (50 mM Tris-HCl, 10 mM KCl, 5 mM (NH4)2SO4 pH 8.3), 2.6 mM MgCl2 , 1.0 U of *Taq* DNA polymerase, 200 µM each dATP, dCTP, dGTP and dTTP, each primer from [Table3] 1.0 µM . PCR products were electrophoresed in 2.5 % agarose gel at 100 V for 45 min and stained with ethidium bromide. The stained gel was recorded by gel documentation system. *C. difficile* ATCC 9689 was used as a control with intact *tcdC*. Strain with tcdC deletion had smaller band size compared with that from C. *difficile* ATCC 9689.

Table 3. PCR primers for screening *tcdC* deletion

Target gene	Primer	Sequence (5'–3')	Reference
tcdC	tcdC-F252	CATGGTTCAAAATGAAAGACGAC	[100]
	tcdC-R415	GGTCATAAGTAATACCAGTATCATATCCTTTC	[100]

4. Sequencing of *tcdC*

Amplifation of *tcdC* was performed with primers as described by Spigaglia *et al.* [101] as shown in table 4. PCR condition was as described by Cohen .et al [102] as follow. PCR mixture in a volume of 100 µl contains PCR buffer (50 mM Tris-HCl, 10 mM KCl, 5 mM (NH4)2SO4 pH 8.3), 2.6 mM MgCl2 , 5.0 U of *Ta*q DNA polymerase, 200 µM each dATP, dCTP, dGTP and dTTP, 1.0 µM each primer from [Table4]. PCR condition was 95 °C for 1 min, 52 °C for 1 min and 72 °C 1 min in a total of 35 cycles and final extension at 72 °C for 10 min. PCR products were electrophoresed in 2 % agarose gel at 100 V for 45 min and stained with ethidium bromide. The stained gel was recorded by gel documentation system. PCR products were purified by High Pure PCR Template Preparation Kit (Roche, Germany) and sent to sequencing by automated sequencer at 1st BASE DNA Sequencing Services, Singapore.

Table 4. Primers for *tcdC* sequencing

Target gene	Primer	Sequence (5'–3')	Reference
tcdC	tcdC-C1	TTAATTAATTTTCTCTACA GCTATCC	[101]
	tcdC-C2	TCTAATAAAAGGGAGATT GTATTATG	[101]

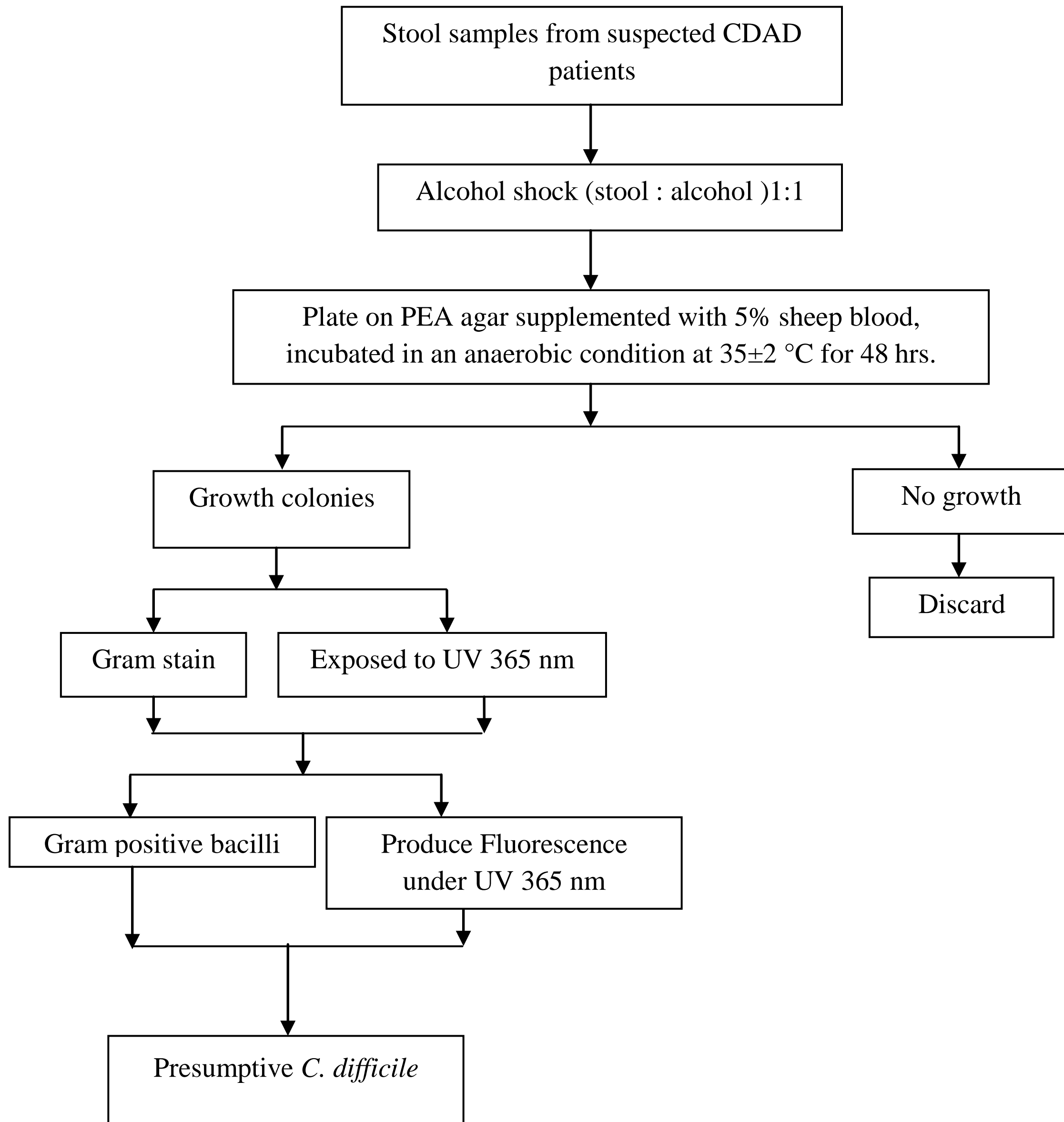
5. PCR ribotyping for *C.difficile* ribotype 027 (hypervirulent strain)

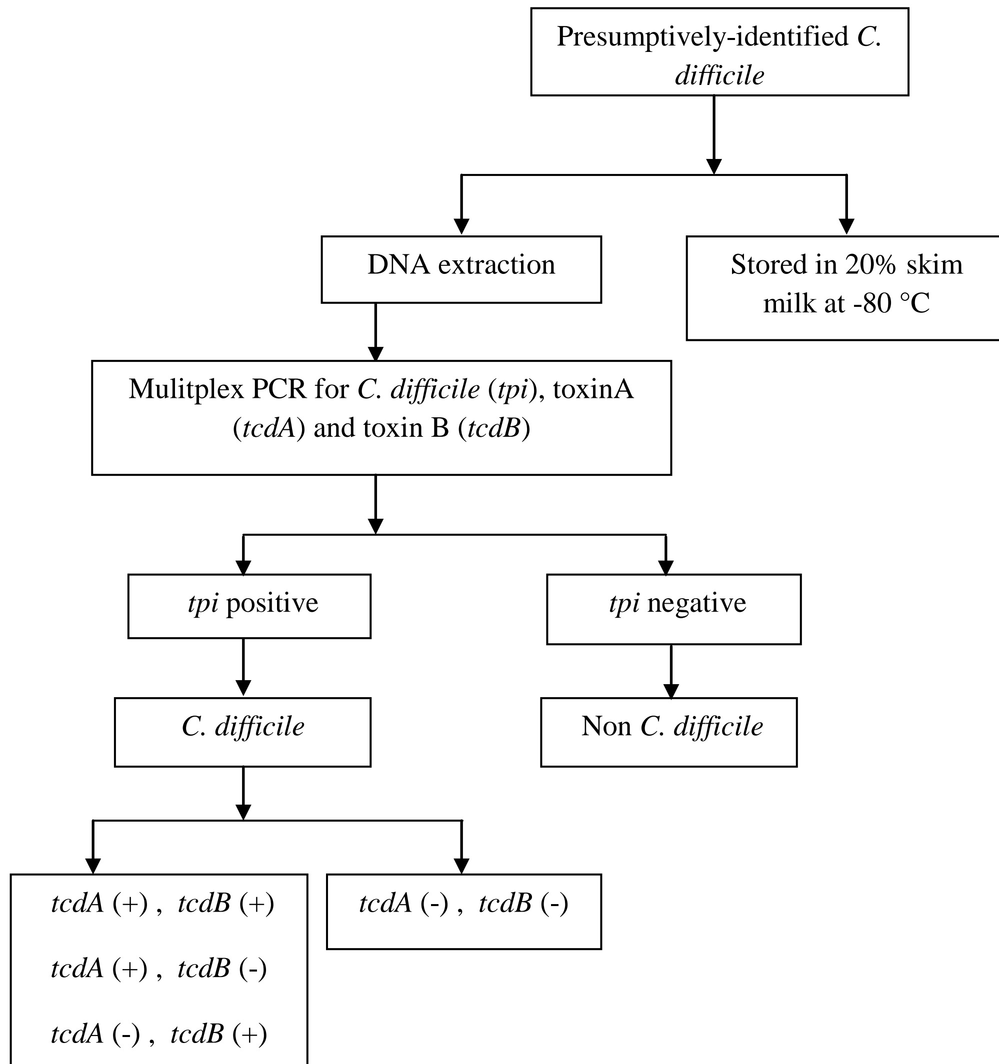
Suspected *C. difficile* with properties similar with hypervirulent strain *C. difficile* which has toxin A, toxin B , binary toxin and deletion at *tcdC* gene. PCR reaction was performed with the method described by Bidet, *et al*. [103]. PCR mixture in a volume of 100 µl contain PCR buffer 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 1.5 mM MgCl , 2.5 U of *Taq* DNA polymerase, 200 µM each dATP, dCTP, dGTP and dTTP, 50 pmol of each primer from (Table5). PCR started from 1 cycle of 6 min at 94°C for denaturation; 35 cycles of 1 min at 94°C, 1 min at 57°C, and 1 min at 72°C; and a final extension cycle of 7 min at 72°C. Amplification products were fractionated by electrophoresis through 3% agarose gel for 6 h at 85 V in TBE buffer with a distance of 24 cm between electrodes (3.5 V/cm) and stained with ethidium bromide. The stained gel was recorded by gel documentation system.

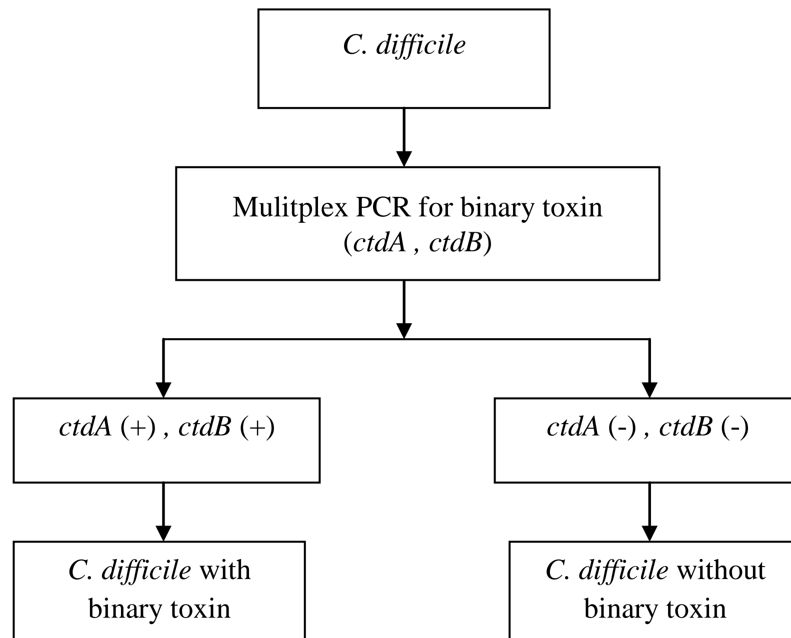
Table 5. Primers for *C.difficile* PCR ribotyping

Target genes	Primer	Sequence (5'–3')	Reference
16S	16S	GTGCGGCTGGATCACCTCCT	[103]
23S	23S	CCCTGACCCTTAATAACTTGACC	[103]

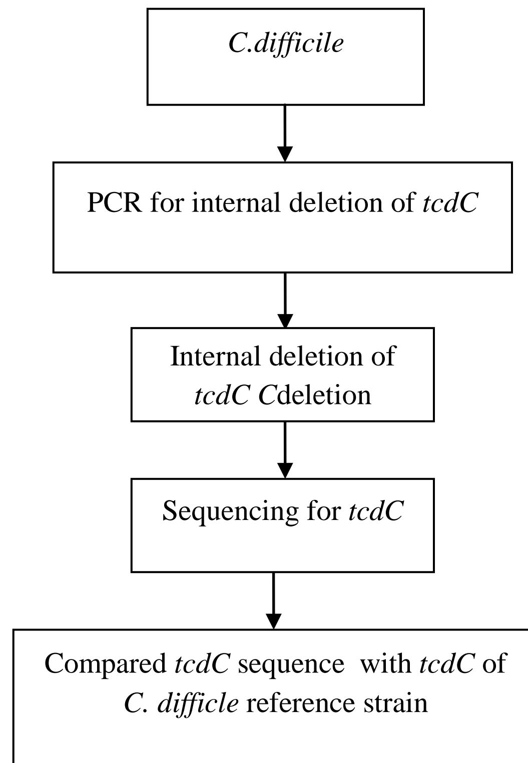
Flowcharts

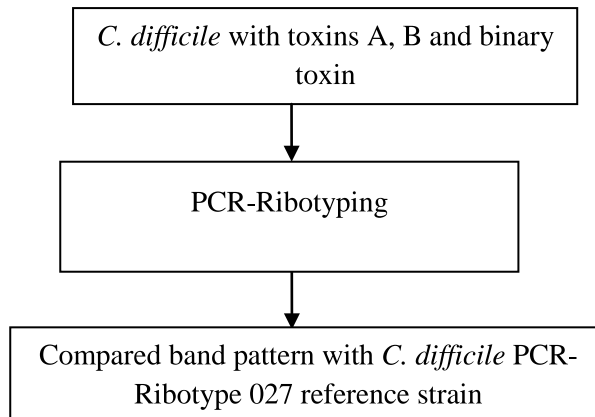



Presumptively-identified *C. difficile*
DNA extraction
Stored in 20% skim milk at -80 °C
Mulitplex PCR for *C. difficile* (*tpi*), toxinA (*tcdA*) and toxin B (*tcdB*)
tpi positive
tpi negative
C. difficile
Non *C. difficile*
tcdA (+) , *tcdB* (+)
tcdA (+) , *tcdB* (-)
tcdA (-) , *tcdB* (+)
tcdA (-) , *tcdB* (-)



C. difficile
Mulitplex PCR for binary toxin
(ctdA , ctdB)
ctdA (+) , ctdB (+)
ctdA (-) , ctdB (-)
C. difficile with
binary toxin
C. difficile without
binary toxin


C.difficile
PCR for internal deletion of *tcdC*
Internal deletion of *tcdC* *C*deletion
Sequencing for *tcdC*
Compared *tcdC* sequence with *tcdC* of *C. difficile* reference strain


C. difficile with toxins A, B and binary toxin
PCR-Ribotyping
Compared band pattern with *C. difficile* PCR-Ribotype 027 reference strain

CHAPTER IV

RESULTS

Cultivation of *C. difficile*

A total of 1,114 stool samples were cultured anaerobically on PEA supplemented with 5% sheep blood. Suspected *C. difficile* colonies with white-gray and flat, horse manure odor were shown in (Figure 9) and fluorescence under 365-nm UV shown in (Figure 10). They were Gram stained and subcultured onto Brucella agar supplemented with 5% sheep blood for promoting the growth of *C. difficile*. Colonies of *C. difficile* on Brucella agar were shown in (Figure 11).

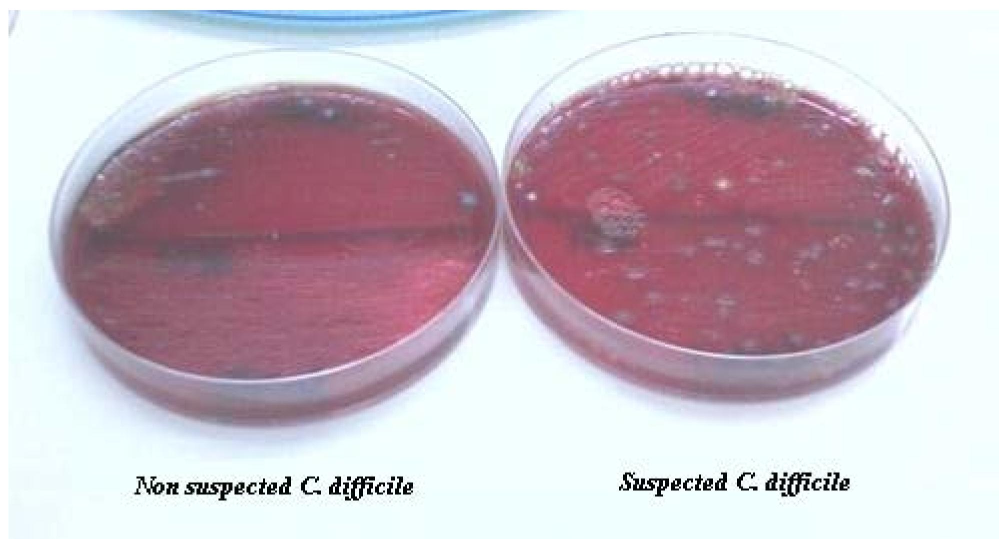


Figure 9. Suspected *C. difficile* colonies on phenyl ethyl alcohol agar supplemented with 5% sheep blood in anaerobic condition at 35±2 C° for 48 h.

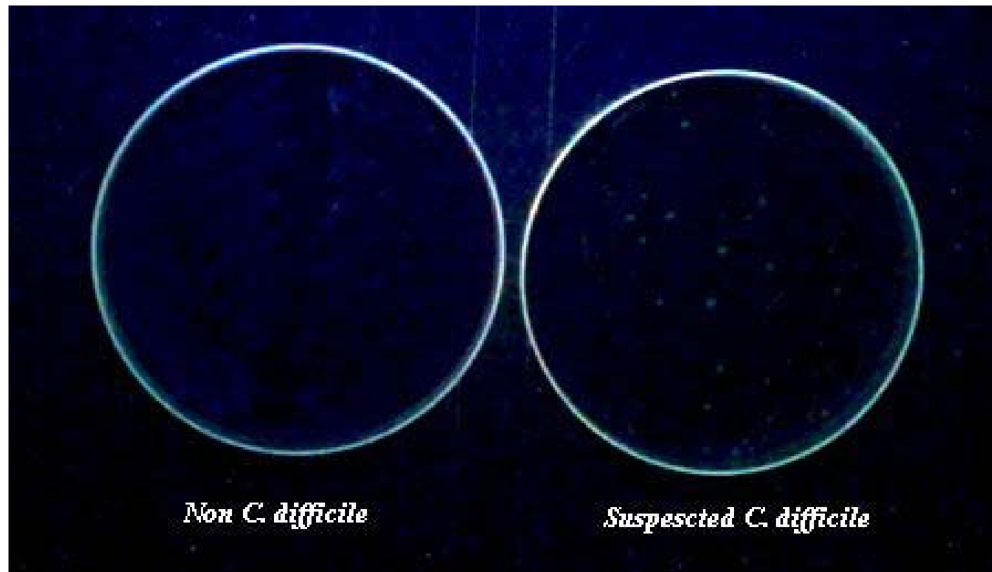


Figure 10. Suspected *C. difficile* colonies (right hand) on phenyl ethyl alcohol agar supplemented with 5% sheep blood produce fluorescence under UV 365 nm from this study.

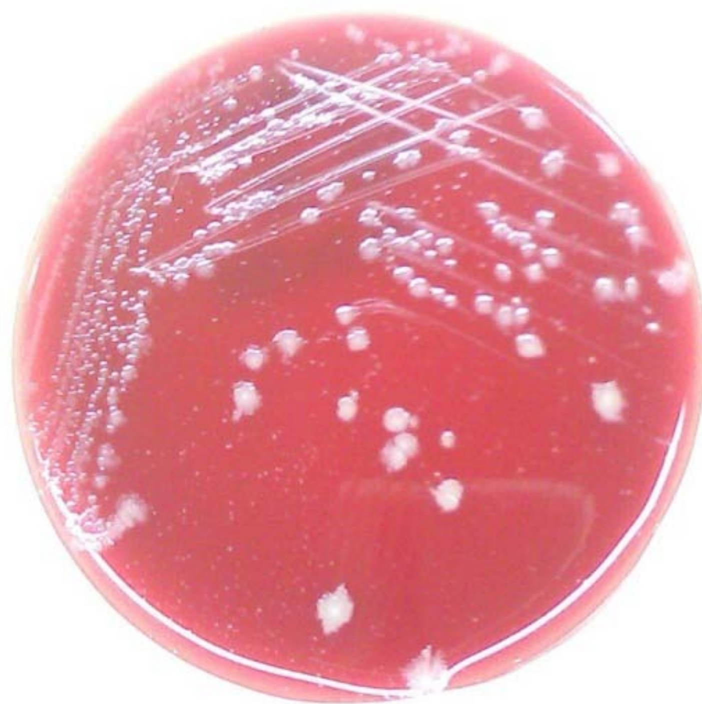


Figure 11. *Clostridium difficile* colonies on Brucella agar supplemented with 5% sheep blood at 48 h in anaerobic condition.

Molecular analysis of suspected *C. difficile*

Multiplex PCR for *C. difficile* , toxin A and toxin B

Of 1,114 stool samples, suspected *C. difficile* colonies, which may be toxigenic (A^+B^+), (A^-B^+) or non-toxigenic (A^-,B^-) were found in 242 samples. Identification by multiplex PCR revealed that there were toxigenic *C. difficile* in 149 (61.57%) samples. Out of these 149 samples, toxins A and B-positive (A^+B^+) *C. difficile* were found in 84 (56.38%) samples, whereas toxin A-negative, toxin B-positive (A^-B^+) *C. difficile* were found in 65 (43.62%) samples. Non-toxigenic (A^-,B^-) *C. diffi*cile were found in 86 (35.54%) samples and non *C. difficile* (*tpi* negative) were found in 7(2.89%) samples. Representative result of multiplex PCR was shown in (Figure 12) and details of *C. difficile* isolates were shown in (Table 6).

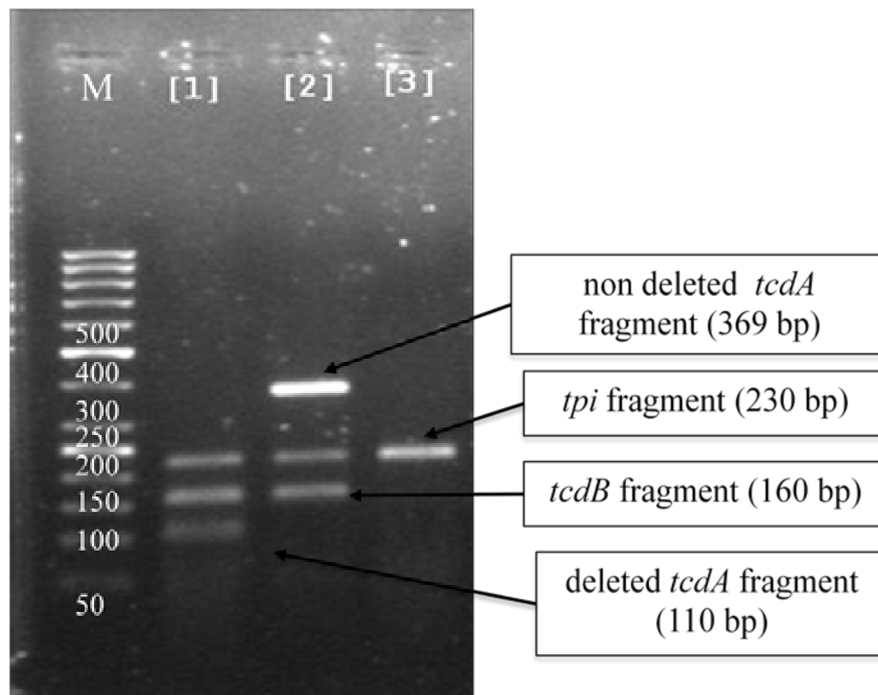


Figure12. Multipex PCR for detection of *C. difficile*, *tcdA* and *tcdB* targeted to internal fragment of *tpi*, internall fragment of *tcdA* and internal fragment of *tcdB*. Lane M: 50 bp molecular weight marker , lane 1 : toxin A-negative, toxinB-positive *C. difficile* , lane 2 : toxin A-positive, toxin B-positive *C. difficile*, lane 3 : toxin A-negative, toxin B-negative (non-toxigenic) *C. difficile.*

Table 6 Molecular analysis for *C. difficile* and toxins A and B genes

Number of *C. difficile* cultures	PCR target for			Interpretation
	tpi	*tcdA*	*tcdB*	
84	+	+	+	*C. diffcile* A^+B^+
0	+	+	-	*C. diffcile* A^+B^-
65	+	-	+	*C. diffcile* A^-B^+
86	+	-	-	*C. diffcile* A^-B^-
7	-	-	-	Non *C. diffcile*
Total 242				

Multiplex PCR for Binary toxin

Of 1,114 samples, suspected *C. difficile* colonies which may be *C. difficile* were found in 242 samples. After identification by multiplex PCR as described above, it revealed that there were *C. difficile* (*tpi* positive) in 235 samples. These samples were then identified for the presence of binary toxin genes (*cdtA* and *cdtB*). Binary toxin genes were found in *C. difficile* culture No.38 from 1 sample (0.43%) which was also positive for toxins A and B genes (A^+B^+)(Table 7). The result of multiplex PCR was shown in (Figure13).

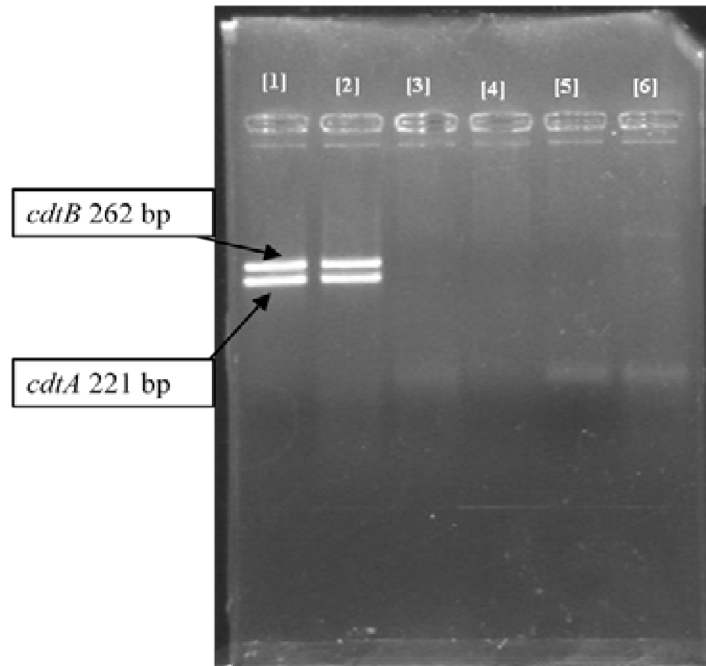


Figure 13. Multipex PCR for detection of *C. difficile* binary toxin gene. Lane 1 : *C. difficile* ATCC BAA-1870 with binary toxin, lane 2 : No. 38 ; *C. difficile* clinical isolate with binary toxin, lane 3 : *C. difficile* ATCC 9689 ; without binary toxin, lanes 4-6 : *C. difficile* clinical isolates without binary toxin.

Table 7. Molecular detection of *C. difficle* binary toxin gene

C. difficile culture	Number	
	Positive	Negative
Toxin $A^{+}B^{+}$ (n= 84)	1	83
Toxin $A^{-}B^{+}$ (n= 65)	0	65
Toxin $A^{-}B^{-}$ (n= 86)	0	86
Total (n=235)		

PCR screening for internal deletion of *tcdC* of *C. difficle*

C. difficile culture from 235 samples which include both toxigenic and non-toxigenic strains were screened for *tcdC* deletion. The result was shown in Table 8 and representative result was shown in (Figure14). *C. difficile* ATCC 9689 strain with complete *tcdC* was used for comparison. *C. diffcile* culture No.38 in this study had internal deletion of *tcdC* .

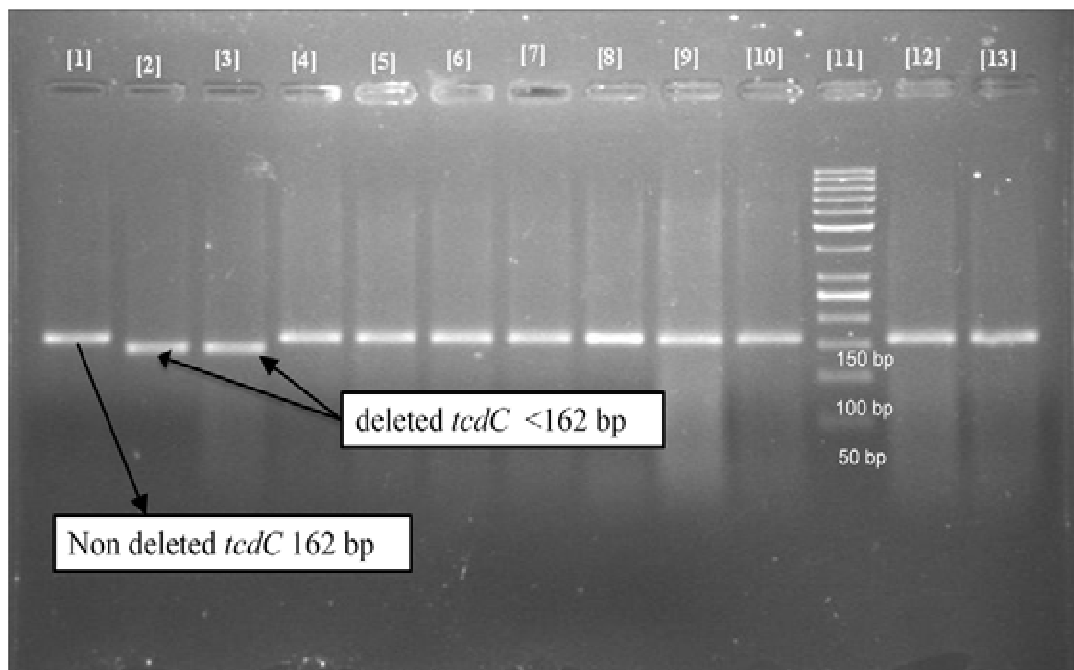


Figure 14. PCR results for screening internal deletion of *tcdC* of *C. difficle*. Lane 1 : *C. difficile* ATCC 9689, lane 2 : *C. difficile* ATCC BAA-1870, lane 3 : No. 38 showed internal in frame deletion of *tcdC*, lanes 4-10,12-13 : *C. difficile* clinical isolates, lane 11 : 50 bp molecular weight marker.

Table 8. PCR screening for internal deletion of *tcdC*

C. difficile culture	Number of isolates with	
	Intact gene	Deleted gene
Toxin A^+B^+ (n= 84)	83	1
Toxin A^-B^+ (n= 65)	65	0
Toxin A-B- (n= 86)	0	0
Total (n= 235)		

tcdC sequencing by automated sequencer

The sequence of amplified product of *tcdC* from *C. difficile* culture No.38 was aligned and compared with *tcdC* sequence from *C. difficile* VPI 10463 reference strain in GenBank with Multalin program as shown in (Figure15). The sequence showed 18 bp deletion at position 373-390, base substitution from thymine to cytosine at position 115, from cytosine to thymine at position 322, from guanine to adenine at position 337, from cytosine to thymine at position 363, from guanine to adenine at position 516 and 517, from guanine to adenine at position 580, from cytosine to thymine at position 608 and base insertion thymine at positions 244, 253 and 258. The result showed *tcdC* mutation pattern is different from *tcdC* of *C. difficile* PCR Ribotype 027 from previous study [83] which had single base deletion at position 117 and the 18 bp deletion at position 330 to 347.

The *tcdC* sequences of *C. difficile* culture No.38 was translated to protein by ExPASy translate, and compared with TcdC protein from *C. difficile* VPI 10463 reference strain in GenBank by Multalin program as shown in (Figure16). TcdC from *C. difficile* No.38 had 6 amino acid residues in frame deletion at position 114-119. In addition, it has amino acid substitution from glycine to aspartic acid at position 31, leucine to phenylalanine at position 38, glutamic acid to lysine at position 107, serine to lysine at position 148, threonine to tyrosine at position 149, glycine to lysine at position 151, valine insertion at position 152 and one amino acid deletion at position 62.

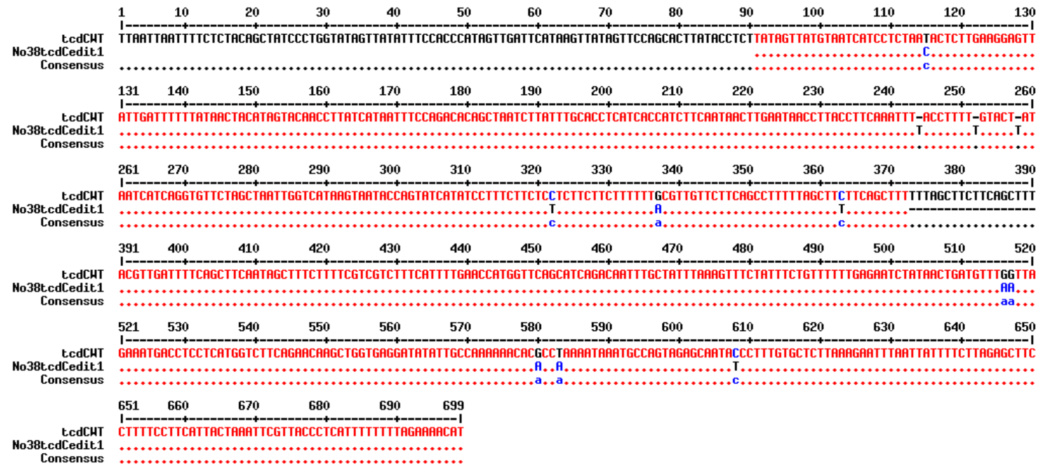


Figure 15. Comparison between *tcdC* from *C. difficile* VPI10463 reference strain and *tcdC* from *C. difficile* culture No.38 (large picture was showed in appendix C)

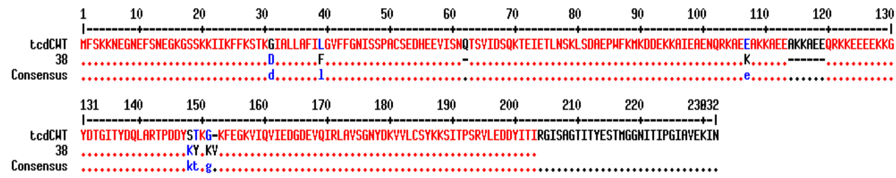


Figure 16. Comparison between TcdC from *C. difficile* VPI10463 reference strain and TcdC from *C. difficile* culture No.38 (large picture was showed in appendix C)

PCR ribotyping for *C. difficile* PCR ribotype 027

Since it has been reported that *C. difficile* PCR-Ribotype 027 harbors toxins A and B, binary toxin and has 18 bp *tcdC* deletion [42], *C. difficile* isolate No.38 which had these properties may be *C. difficile* PCR-Ribotype 027. PCR-ribotyping of No.38 was performed and the result in (Figure17) showed that it is not PCR –ribotype 027. We used *C. difficile* PCR-Ribotype 027 (ATCC BAA-1870) reference strain as positive control to compare with the suspected *C. difficile* PCR-Ribotype 027 isolated from this study.

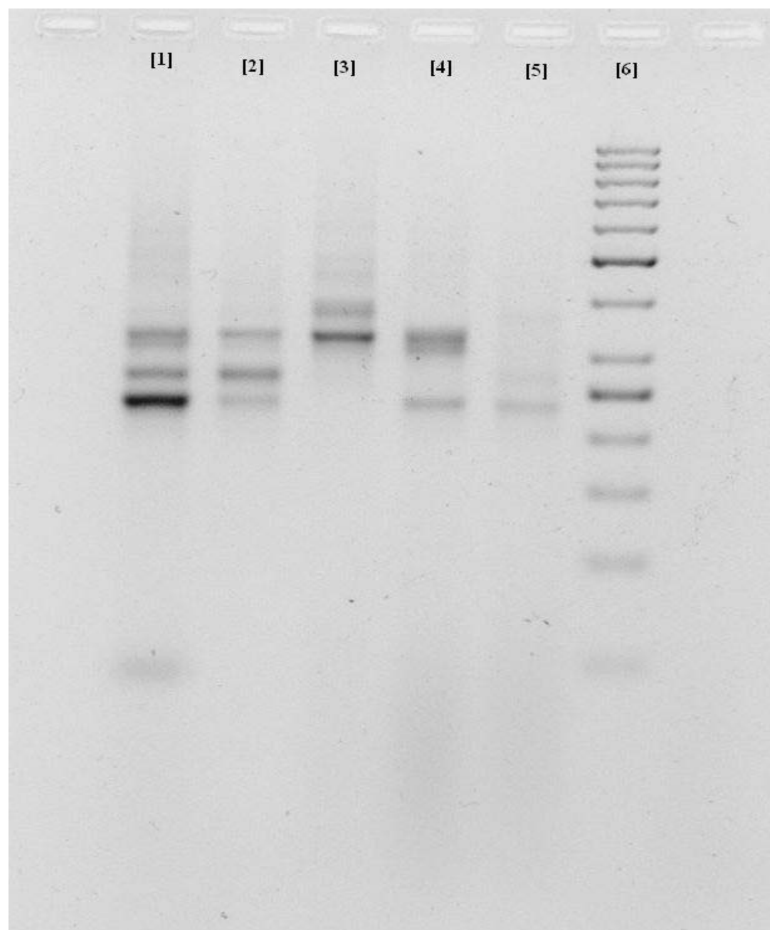


Figure 17. PCR ribotyping of *C.difficile* culture No.38 compare with ATCC strain; Lane 1 : *C. diffcile* ATCC 9689 (Ribotype 001), lane 2 : *C. diffcile* ATCC BAA 1870 (Ribotype 027), lane 3 : *C. difficile* culture No.38, lane 4-5 : other *C. diffcile* culture from this study No. 228 , No. 238 and lane 6 : 100 bp molecular marker.

Age group correlation with CDAD

The correlation between age and *C. difficile* isolates was determined. Of 176 *C. difficile* isolates had age data available (76 males and 100 females whose mean age is 63.56 years (range 1-102 years), 106 (60.23%) were toxigenic and 70 (39.77%) were non-toxigenic. Among toxigenic isolates, 1 (0.94%) were ($A^+B^+CDT^+$), 65 (61.32%) were ($A^+B^+CDT^-$) and 40 (37.74%) were ($A^-B^+CDT^-$). For age group distribution, toxigenic *C. difficile* was recovered from patients with different age group as follow: more than 60 yrs ; 74 (69.81%), 30-39 yrs ;11(10.38%), 40-49 yrs; 9 (8.49%), 50-59 yrs; 6 (5.66%), 0-9 yrs; 3 (2.83%), 10-19 yrs; 2 (1.89%) and 20-29 yrs; 1(0.94%) as showed on (Table 9) and (Figure 18).

Table 9. Age group distribution of CDAD in this study

Age	Toxigenic *C. difficile*
0-9	3
10-19	2
20-29	1
30-39	11
40-49	9
50-59	6
>60	74
Total	**106**

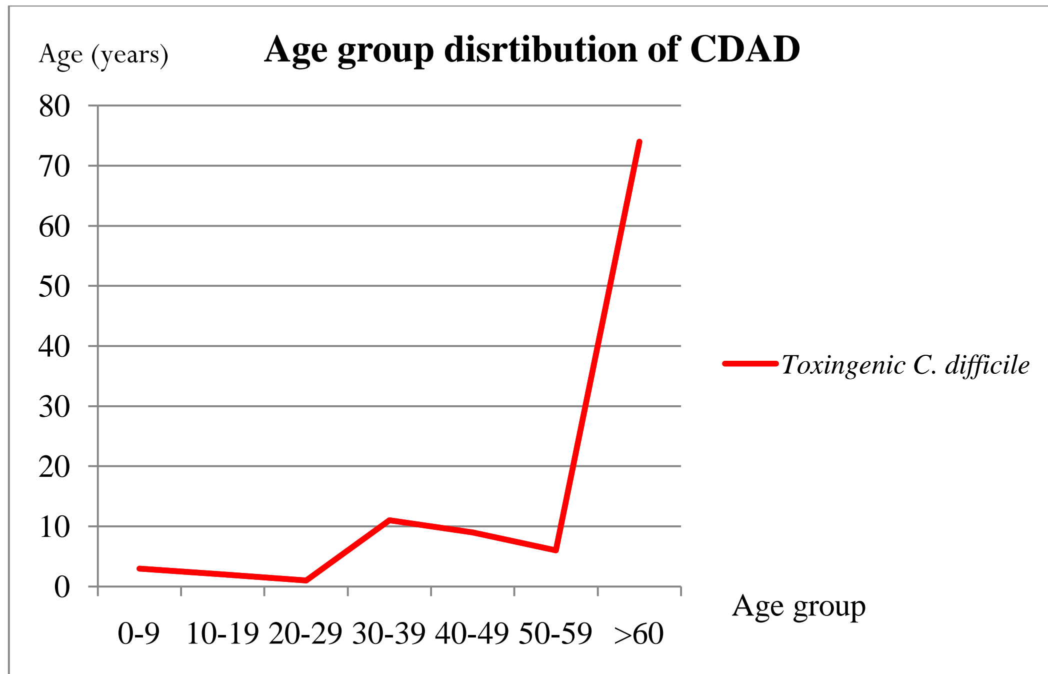


Figure 18. Graph displayed age group distribution for CDAD

CHAPTER V

DISCUSSION

Isolation and identification of toxigenic *C. difficile* from 149 of 1,114 stool samples revealed that the prevalence of toxigenic *C. difficile* isolated from diarrhea patients in this study was 13.37%. This prevalence rate was not much different from those reported in previous studies. Wongwanich *et al*.[84] reported in 2003 that the prevalence of *C. difficile* isolated from the stools of Thai adult patients with suspected CDAD was 18.64% and Thipmontree *et al*.[85] has recently reported the prevalence rate of 12.3% in 2011. The result of this study thus suggested that the frequency of CDAD in Thailand was not increasing, which is different from the reports of increasing CDAD in western countries. For examples, Pepin *et al.* [32] reported in 2004 that there was dramatically increase in number of CDAD patients from 35.6 per 100,000 population in 1991 to 156.3 per 100,000 population in 2003 in Canada, Lyytikainen *et al.* in Finland reported in 2009 that CDAD cases doubled from 810 (16/100,000 population) in 1996 to 1,787 (34/100,000 population) in 2004 [33].

Clinical data were available from 176 patients who are 76 males and 100 females whose mean age is 63.56 years (range 1-102 years). For age group distribution as shown in (Figure 17), toxigenic *C. difficil*e was recovered from patients with different age group as follow: more than 60 yrs ; 74 (69.81%), 30-39 yrs ; 11 (10.38%), 40-49 yrs; 9 (8.49%), 50-59 yrs; 6(5.66%), 0-9 yrs; 3(2.83%)10-19 yrs; 2(1.89%) and 20-29yrs; 1(0.94%). This result agreed with the previous reports of Pepin *et al.* [32] and Lyytikainen *et al.* [33] that CDAD cases were found in patients more than 65 and 64 years of age, respectively. Our study indicated that elder age is still the important risk factor for *C. difficile* infection. People with elder age have decreased abundance and diversity of protective gut microbiota which play important role in preventing toxigenic *C. difficile* to proliferate in the colon and producing toxins leading to CDAD [57].

For molecular characteristic of *C. difficile* toxins genes, out of 149 toxigenic *C. difficile* ; toxins A and B-positive (A+B+) *C. difficile* were found in 84 (56.38%) samples and toxin A-negative, toxin B-positive (A-B+) *C. difficile* were found in 65 (43.63%) samples. Toxin A- positive, toxin B- negative (A+B-) *C. difficile* was not found in this study which is in agreement with the previous report by Cohen, *et al.* in1998 that

toxin A- positive, toxin B- negative (A+B-) was extremely rare [104]. Toxin A-negative, toxin B-positive (A-B+) *C. difficile* in this study was found in 43.6% samples. Previous studies showed the presence of toxin A-negative, toxin B-positive (A-B+) *C. difficile*. For examples, Samra *et al.*[105] reported in 2002 that 58.5% of *C. difficile* isolates in Israel were toxin A-negative, toxin B-positive (A-B+) and Drudy *et al.*[106] reported in 2007 that that 95% of *C. difficile* isolates in Dublin, Ireland were toxin A–negative and toxin B–positive. This study found binary toxin–positive *C. difficile* (culture No.38) in only one stool sample. *C. difficile* culture (No.38) also had both toxins A and B as shown in (Table 7). In term of *tcdC* deletion, 235 *C. diffcile* cultures which include both toxigenic and non-toxigenic isolates were screened for *tcdC* deletion. Non-toxigenic *C. difficile* was also tested to confirm the result obtained by PCR for *C. difficile*, *tcdA* and *tcdB*. Amplification for screening of *tcdC* deletion did not show amplified product in non-toxigenic *C. difficile* (Table8). The *tcdC* deletion was found in *C. difficile* culture (No.38) and the *tcdC* sequence showed 18 bp deletion resulting in in- frame deletion of 6 amino acid residues .This deletion is different from that of *C. difficile* PCR-Ribotype 027 reported by MacCannell *et al.* in 2006 that a single-base-pair deletion at position 117 resulting in truncated TcdC of 65 amino acids in addition to 18 bp deletion. The predicted TcdC peptide of *C. difficile* culture (No.38) was found shorter than TcdC peptide of *C. difficile* VPI 10463 reference strain. The TcdC gene *of C. difficile* culture (No.38) was not like that of *C. difficile* PCR-Ribotype 078 which had properties similar to *C. difficile* PCR-Ribotype 027 for production of both toxins A and B, presence of binary toxin but different *tcdC* mutation [55]. *C. difficile* PCR-Ribotype 078 had C184T transition that introduces a stop codon leading to a presumptive truncated protein of 61 residues, and a 39-bp deletion located downstream of the alternative stop codon. This strain was associated with community acquired cases more than *C. difficile* PCR-Ribotype 027 [55]. PCR-Ribotyping of *C. difficile* culture (No.38) in this study showed that it was not Ribotype 027 from different band pattern. Clinical data from patient from whom *C. difficile* culture (No.38) was isolated revealed that the patient had only mild diarrhea and susceptible to vancomycin treatment. *C. difficile* culture (No.38) seems not to be hypervirulent strain as the truncate TcdC seems to be more intact than that of the reported hypervirulent strains of both Ribotypes 027 and 078.

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APPENDICES

APPENDIX A

MATERIALS AND EQUIPMENTS

Materials and reagents

- Agarose (Research organism, USA)

- Anaerobic indicator (Oxoid, Basingstroke, Hamps, UK)

- Boric acid (Sigma, USA)

- Brucellar agar (BBL, USA)

- Ethylene diamine tetraacetic acid (EDTA) (Sigma, USA)

- Ethidium bromide (Bio Rad, USA)

- Gaspak (AnaeroPack-Anaero, Mitsubishi, Japan)

- GeneRuler^{TM} 50bp DNA Ladder Plus (Fermentas, USA)

- GeneRuler^{TM} 100bp DNA Ladder Plus (Fermentas, USA)

- Phenylethyl Alcohol Agar (BBL, USA)

- Proteinase K (Sigma, USA)

- Skim milk (Difco, USA)

- Sodium chloride (NaCl) (Sigma, USA)

- *Taq* DNA polymerase (Invitrogen , USA)

- Tris base (Sigma, USA)

- Tween 20 (Merck, Germany)
- Tween 80 (Sigma, USA)

2. Equipments

- Anaerobic Chamber (Concept Plus, Ruskinn Technology, UK)
- Anaerobic Jar (BBL, USA)
- Autoclave (Hirayama, Japan)
- Autopipettes (Gilson, France)
- Deep Freezer (-20^{0}C) (Sanyo, Japan)
- Deep Freezer (-80^{0}C) (Sanyo, Japan)
- Electrophoresis chamber (BioRad, USA)
- Gel doc (BioRad, USA)
- Heat block (Scientific, USA)
- Hot air oven (Haraeus, Germany)
- Incubator (Forma Scientific, USA)
- Light Microscope (Nikon, Japan)
- Microcentrifuge (Eppendorf, USA)
- pH meter (Orion, USA)
- Thermal cycler (Eppendorf, Hamburg, Germany)
- Vortex mixer (Scientific, USA)

- Water bath (Memmert, USA)

3. Software and program

- GenBank DNA database search (http://www.ncbi.nlm.gov/BLAST).

- Multalin program (http://bioinfo.genotoul.fr/multalin)

- ExPASy translate http://web.expasy.org/translate/

APPENDIX B

PREPARATION OF MEDIA AND REAGENT

Media for *Clostridium difficile*

1. 5% Sheep blood Brucella agar

Brucella agar (BBL)	43	g
Sterile sheep blood	50	ml
Distilled water	1,000	ml

2. 5% Sheep blood PEA agar

PEA agar (BBL)	42.5	g
Sterile sheep blood	50	ml
Distilled water	1,000	ml

PEA agar using for *Clostridium difficile* isolated from stool sample.

3. 20% Skim milk

Skim milk (BBL)	37	g
Distilled water	1,000	ml

The pH was adjusted to 7.2 before autoclaving at 121^0C for 15 minutes.

Reagent for molecular analysis

1. 0.5M EDTA, pH 8.0

Ethylene diamine tetraacetic acid (EDTA)	93.05	g
Distilled water	500	ml

Dissolve 93.05 g of EDTA in 400 ml of distilled water, adjusted pH to 8.0 with NaOH (pellets) and final volume was bought up to 500 ml. The stock reagent sterile by

autoclaving at 121^0C at 15 pounds/inch^2 pressure for 15 minutes. The solution was stored at room temperature.

2. 5X TBE

Tris base	54	g
Boric acid	27.5	g
0.5M EDTA pH 8.0	20	ml
Distilled water	1,000	ml

Dissolve all of ingradients in 1,000 ml of distilled water. The stock reagent sterile by [107]autoclaving at 121^0C at 15 pounds/inch^2 pressure for 15 minutes. The solution was stored at room temperature.

3. 1M Tris-HCl, pH 8.0

Tris base	121.1	g
Distilled water	1,000	ml

Dissolve 121.1 g of Tris base in 800 ml of distilled water. Adjust the pH to the desired value by adding concentrated HCl 42 ml and allow the solution to cool to room temperature before making final adjustments to the pH 8.0. Adjust the volume of the solution to 1 liter with distilled water. Dispense in to aliquots and sterilize by autoclaving.

4. 10X Digestion buffer

The stock reagent 10X digestion buffer contained 5% tween 20 and 10 mg/ml proteinase K in 0.2 M Tris pH 8.3. For example prepare 4 ml of the stock reagent.

Tween 20	0.2	ml
Proteinase K	40	mg
1M Tris pH 8.3	0.8	ml
Distilled water	3.0	ml

Dissolve 40 mg of Proteinase K in 3 ml of distilled water adding Tween 20 and 1M Tris pH 8.3 making final volume to 4 ml. Mix well and store at 4^0C.

APPENDIX C

Detail of PCR and Multiplex PCR results from *C. difficile* clinical isolates in this study

No.	Age	Sex	*C. difficile (tpi)*	*tcdA*	*tcdB*	Binary	*tcdC*
1	ND	M	+	0	+	0	N
2	34	F	+	0	+	0	N
3	54	F	+	+	+	0	N
4	87	M	+	0	0	0	-
5	ND	M	+	0	0	0	-
6	ND	F	+	0	+	0	N
7	ND	M	-	-	-	-	-
8	35	F	+	+	+	0	N
9	ND	F	+	0	0	0	-
10	37	F	+	0	+	0	N
11	7	F	+	0	0	0	-
12	54	F	+	0	0	0	-
13	89	F	+	0	+	0	N
14	25	F	+	0	+	0	N
15	ND	F	+	0	+	0	N
16	68	F	+	0	+	0	N
17	76	F	+	0	+	0	N
18	63	M	+	0	+	0	N
19	ND	F	+	0	+	0	N
20	ND	M	+	0	+	0	N
21	ND	M	+	0	+	0	N
22	95	M	+	+	+	0	N

No.	Age	Sex	*C. difficile (tpi)*	*tcdA*	*tcdB*	Binary	*tcdC*
23	ND	F	+	0	+	0	N
24	ND	M	+	0	+	0	N
25	ND	F	+	+	+	0	-
26	80	M	+	0	+	0	N
27	ND	F	+	+	+	0	N
28	ND	F	0	0	0	0	0
29	65	M	0	0	0	0	0
30	ND	M	0	0	0	0	0
31	ND	F	+	+	+	0	N
32	ND	F	+	+	+	0	N
33	ND	F	+	0	+	0	N
34	76	F	+	0	+	0	N
35	ND	F	+	0	0	0	-
36	ND	F	+	0	+	0	N
37	ND	M	+	0	+	0	N
38	77	M	+	+	+	+	D
39	51	M	+	+	+	0	N
40	48	F	+	+	+	0	N
41	87	F	+	0	+	0	N
42	81	M	+	+	+	0	N
43	39	F	+	0	+	0	N
44	ND	M	+	+	+	0	N
45	87	F	+	+	+	0	N
46	ND	F	+	+	+	0	N
47	ND	F	+	0	±	0	N

No.	Age	Sex	*C. difficile (tpi)*	*tcdA*	*tcdB*	Binary	*tcdC*
48	ND	M	+	0	0	0	-
49	ND	F	+	0	0	0	-
50	44	F	+	0	0	0	-
51	ND	M	+	+	+	0	N
52	27	F	+	0	0	0	-
53	33	F	+	0	0	0	-
54	ND	F	+	0	+	0	N
55	ND	M	+	0	+	0	N
56	67	F	+	+	+	0	N
57	99	F	+	0	0	0	-
58	ND	F	+	0	0	0	-
59	ND	-	+	0	0	0	-
60	32	F	+	0	0	0	-
61	32	F	+	0	0	0	-
62	ND	F	+	0	+	0	N
63	58	M	+	0	0	0	-
64	32	F	+	0	0	0	-
65	ND	M	+	0	0	0	-
66	102	F	+	+	+	0	N
67	87	M	+	0	0	0	-
68	95	M	+	0	0	0	-
69	62	F	+	+	+	0	N
70	14	M	+	0	+	0	N
71	ND	M	+	0	0	0	-
72	ND	F	+	+	+	0	N

No.	Age	Sex	*C. difficile (tpi)*	*tcdA*	*tcdB*	Binary	*tcdC*
73	44	F	+	+	+	0	N
74	44	F	+	+	+	0	N
75	75	F	+	0	0	0	-
76	62	F	+	+	+	0	N
77	86	F	+	+	+	0	N
78	68	M	+	+	+	0	N
79	68	M	+	+	+	0	N
80	2	M	+	0	+	0	N
81	91	F	+	0	0	0	-
82	73	M	+	0	0	0	-
83	ND	M	+	0	+	0	N
84	1	F	+	0	0	0	-
85	87	M	+	0	0	0	-
86	84	M	+	+	+	0	N
87	81	M	+	0	0	0	-
88	49	F	+	0	+	0	N
89	ND	F	+	+	+	0	N
90	73	F	+	0	+	0	N
91	40	F	+	+	+	0	N
92	47	F	+	0	0	0	-
93	ND	M	+	0	0	0	-
94	95	M	+	0	+	0	N
95	7	F	+	0	0	0	-
96	7	F	+	0	0	0	-
97	84	M	+	0	+	0	N

No.	Age	Sex	*C. difficile (tpi)*	*tcdA*	*tcdB*	Binary	*tcdC*
98	32	F	+	0	0	0	-
99	ND	M	+	0	0	0	-
100	ND	M	+	+	+	0	N
101	43	F	+	+	+	0	N
102	60	M	+	+	+	0	N
103	33	F	+	0	0	0	-
104	32	F	+	0	0	0	-
105	16	F	+	0	0	0	-
106	ND	M	–	–	–	0	-
107	ND	F	+	0	+	0	N
108	76	F	+	0	0	0	-
109	60	M	+	+	+	0	N
110	11	M	+	0	0	0	-
111	78	F	+	+	+	0	N
112	39	F	+	0	0	0	-
113	85	M	+	0	0	0	-
114	ND	M	+	0	0	0	-
115	60	M	+	+	+	0	N
116	82	M	+	0	+	0	N
117	82	M	+	0	+	0	N
118	60	M	+	0	0	0	-
119	40	M	+	0	+	0	N
120	74	M	+	+	+	0	N
121	ND	F	+	+	+	0	N
122	59	M	+	0	+	0	N

No.	Age	Sex	*C. difficile (tpi)*	*tcdA*	*tcdB*	Binary	*tcdC*
123	39	F	+	0	+	0	N
124	82	F	+	+	+	0	N
125	95	M	+	+	+	0	N
126	81	F	+	0	0	0	-
127	ND	M	+	0	0	0	-
128	58	M	+	0	+	0	N
129	88	F	+	0	0	0	-
130	ND	F	+	0	0	0	-
131	76	F	+	0	0	0	-
132	33	M	+	+	+	0	N
133	60	M	+	0	0	0	-
134	88	F	+	+	+	0	N
135	95	M	+	0	+	0	N
136	63	F	+	0	+	0	N
137	9	M	+	0	0	0	-
138	74	F	+	0	+	0	N
139	83	F	+	+	+	0	N
140	44	M	+	0	0	0	-
141	ND	M	+	+	+	0	N
142	65	F	+	+	+	0	N
143	85	M	+	+	+	0	N
144	85	M	+	+	+	0	N
145	33	M	+	+	+	0	N
146	74	F	+	+	+	0	N
147	26	F	+	0	0	0	-

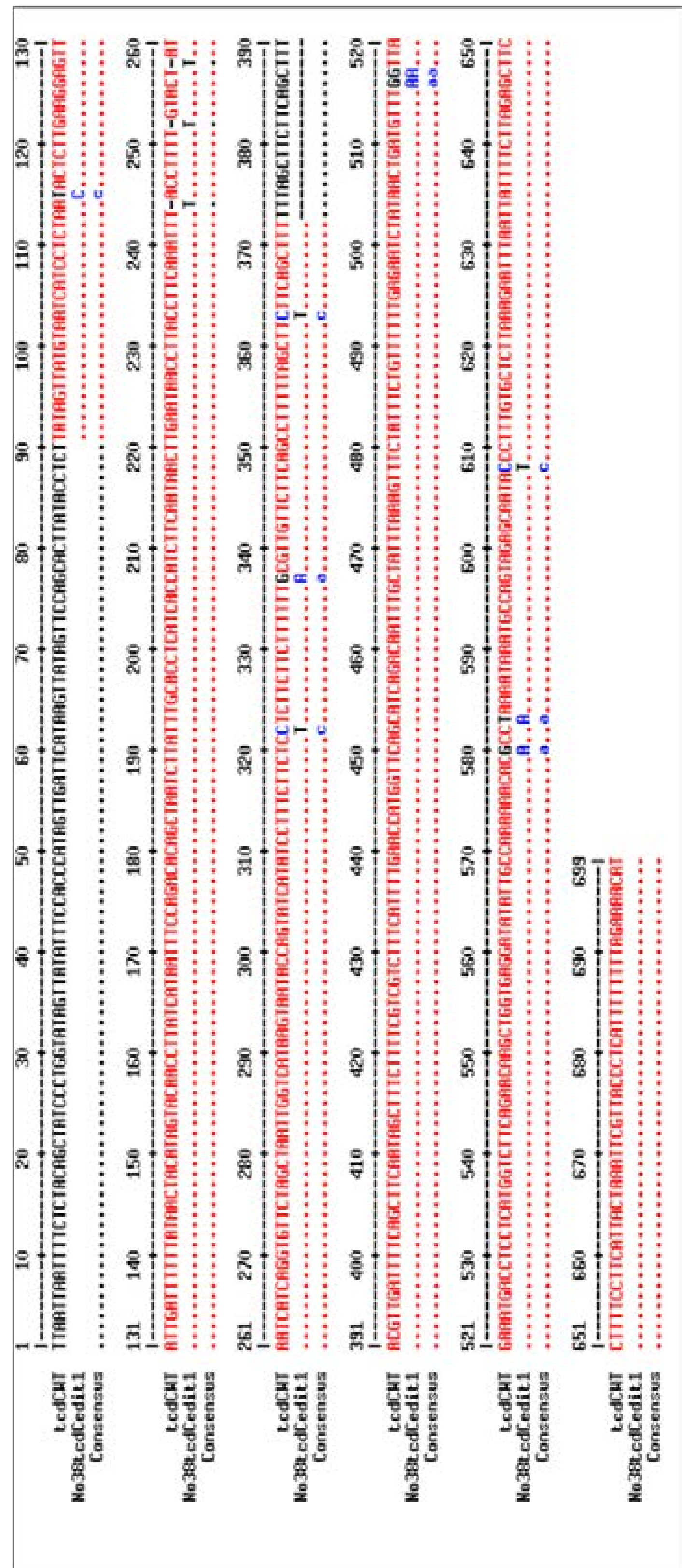
No.	Age	Sex	*C. difficile (tpi)*	*tcdA*	*tcdB*	Binary	*tcdC*
148	77	F	+	+	+	0	N
149	2	M	+	0	0	0	-
150	ND	F	+	0	0	0	-
151	85	M	+	+	+	0	N
152	82	F	+	0	0	0	-
153	42	M	+	0	0	0	-
154	42	M	+	0	0	0	-
155	49	M	+	0	0	0	-
156	49	M	+	0	0	0	-
157	95	M	+	0	+	0	N
158	93	F	+	+	+	0	N
159	96	F	+	0	0	0	-
160	ND	F	+	+	+	0	N
161	62	M	+	0	+	0	N
162	81	M	+	0	+	0	N
163	84	M	+	+	+	0	N
164	ND	F	+	+	+	0	N
165	78	F	+	+	+	0	N
166	ND	M	+	0	0	0	-
167	75	M	+	+	+	0	N
168	89	M	0	0	0	0	0
169	85	F	+	+	+	0	N
170	ND	F	+	0	+	0	N
171	69	F	+	0	0	0	-
172	1	M	+	+	+	0	N

No.	Age	Sex	*C. difficile (tpi)*	*tcdA*	*tcdB*	Binary	*tcdC*
173	ND	M	+	0	0	0	-
174	78	F	+	0	0	0	-
175	48	F	+	0	0	0	-
176	53	M	+	0	0	0	-
177	70	M	+	0	0	0	-
178	ND	M	+	0	0	0	-
179	96	F	+	0	0	0	-
180	96	F	+	0	0	0	-
181	96	F	+	0	0	0	-
182	46	M	+	0	0	0	-
183	61	M	+	0	0	0	-
184	58	F	+	0	0	0	-
185	95	F	+	0	0	0	-
186	79	F	+	0	0	0	-
187	71	M	+	+	+	0	N
188	75	M	+	+	+	0	N
189	35	F	+	0	+	0	N
190	17	M	+	0	+	0	N
191	93	F	+	+	+	0	N
192	96	F	+	0	0	0	N
193	82	F	+	0	0	0	N
194	74	M	0	0	0	0	-
195	96	F	+	0	0	0	N
196	96	F	+	0	0	0	N
197	84	M	+	0	+	0	N

No.	Age	Sex	*C. difficile (tpi)*	*tcdA*	*tcdB*	Binary	*tcdC*
198	87	M	+	0	0	0	-
199	88	M	+	+	+	0	N
200	ND	F	+	+	+	0	N
201	93	F	+	+	+	0	N
202	84	M	+	+	+	0	N
203	57	F	+	+	+	0	N
204	86	M	+	+	+	0	N
205	72	M	+	0	0	0	-
206	72	M	+	+	+	0	N
207	89	F	+	+	+	0	N
208	89	F	+	+	+	0	N
209	80	F	+	+	+	0	N
210	96	F	+	+	+	0	N
211	ND	F	+	+	+	0	N
212	80	F	+	+	+	0	N
213	35	F	+	+	+	0	N
214	ND	F	+	0	+	0	_
215	84	F	+	+	+	0	N
216	1	M	+	+	+	0	N
217	ND	M	+	0	+	0	N
218	ND	F	+	0	+	0	N
219	51	F	+	+	+	0	N
220	65	F	+	+	+	0	N
221	90	F	+	+	+	0	N
222	ND	M	+	+	+	0	N

No.	Age	Sex	*C. difficile (tpi)*	*tcdA*	*tcdB*	Binary	*tcdC*
223	ND	F	+	0	+	0	N
224	86	M	+	0	+	0	N
225	71	F	+	+	+	0	N
226	ND	M	+	+	+	0	N
227	82	M	+	0	+	0	N
228	47	F	+	0	0	0	-
229	80	F	+	0	0	0	-
230	37	F	+	0	+	0	N
231	84	F	+	+	+	0	N
232	ND	M	+	0	+	0	N
233	86	M	+	0	+	0	N
234	45	M	+	0	+	0	N
235	45	M	+	0	+	0	N
236	76	M	+	+	+	0	N
237	30	F	+	0	0	0	N
238	35	F	+	0	+	0	N
239	ND	M	+	0	+	0	N
240	ND	F	+	0	+	0	N
241	ND	F	+	0	0	0	-
242	84	M	+	0	+	0	N

M: Male, F: Female, ND : no data, N : normal, D : deleted, (+) : positive, (-) negative



Comparison between *tcdC* from *C. difficile* VPI10463 reference strain and *tcdC* from *C. difficile* culture No.38

```
          1        10        20        30        40        50        60        70        80        90        100       110       120       130
          |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
   tcdCWT MFSKKNEGNEFSNEGKGSSKKIIKFFKSTKGIALLAFILGVFFGNISSPACSEDHEEVISNQTSVIDSQKTEIETLNSKLSDAEPWFKMKDDEKKAIEAENQRKAEEAKKAEEAKKAEEQRKKEEEEKKG
       38 ..............................D.......F......................-.............................................K......------..........
Consensus ..............................d.......l.......................................................................e...................

          131      140       150       160       170       180       190       200       210       220      23032
          |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+-|
   tcdCWT YDTGITYDQLARTPDDYSTKG-KFEGKVIQVIEDGDEVQIRLAVSGNYDKVVLCSYKKSITPSRVLEDDYITIRGISAGTITYESTMGGNITIPGIAVEKIN
       38 .................KY.KV.................................................
Consensus .................kt.g.....................................................................................
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Comparison between TcdC from *C. difficile* VPI10463 reference strain and TcdC from *C. difficile* culture No.38 (large picture)

BIOGRAPHY

Miss Prasertsri Tunglertsumphan was born on April 23, 1981 in Bangkok, Thailand. She graduated with Bachelor degree of Science in Medical Technology from the Faculty of Allied Health Sciences at Chulalongkorn University in 2004.