Epidemiological study of hospital-acquired *Clostridium difficile* infection in Kuwait teaching hospitals and investigation of their virulence characteristics

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ABSTRACT

Clostridium difficile infection (CDI) is the most common type of infectious nosocomial diarrhoea. Overwhelming evidence indicate that the most important risk factors are prior antibiotic use and elderly patients. The severity of the disease varies from asymptomatic carrier to mild diarrhoea to colitis (AAC) and life threatening pseudomembranous colitis (PMC). Because little is known about *C. difficile* and CDI in Kuwait, this study was undertaken to determine the nosocomial acquisition of *C. difficile* by new patients admitted to the intensive care units (ICU) of 4 teaching hospitals in Kuwait between February 2001 and January 2002 (first part) and January 2003 to December 2005 (second part) and evaluate cytotoxin (toxin B) production by clinical isolates upon exposure to minimum inhibitory concentrations (MICs) and sub-MICs of certain antibiotics. The first part of the study was accomplished by serially culturing the stool specimens of 922 newly admitted patients to the ICUs; screening their stools for toxins A/B and screening their immediate environment for *C. difficile*. The isolates were typed by the PCR ribotyping technique developed in the Anaerobe Reference Unit, Cardiff. The effects of various concentrations of antibiotics that could predispose to CDI and those used for its therapy on the production of cell-bound and cell-free toxin B produced by *C. difficile* was investigated by experiments using cell cultures of the Vero cell line. Prevalence, epidemiology and risk factors of CDI in Kuwait hospitals was investigated during second part of the study by culturing patients' stool specimens, ribotyping the isolates and detection of toxin A/B in stool samples. The susceptibility of all isolates was assessed by MIC determination to 16 antibiotics using the E test method. During the first part of the study, 95 (10.3%) out of 922 patients with negative cultures initially on the day of admission acquired *C. difficile* during their hospitalisation at various time intervals. Of these, 65 (68%) remained symptom-free while 30 (32%) were symptomatic; 2 patients had PMC, 4 AAC and 24 AAD. *C. difficile* toxin A/B was present in 28 (93%) of 30

symptomatic patients but in only 7 (10.8%) of 65 symptom-free patients. The hospital environments occupied by symptomatic patients as well as those occupied by asymptomatic patients were contaminated by *C. difficile*. The 95 isolates from patients belonged to a total of 32 different ribotypes. Ribotypes 097 and 078 were responsible for >40% of *C. difficile* infections in Kuwait ICUs. There was a heterogeneous relationship between antibiotic exposure and intra- and extra-cellular toxin production by the toxigenic strains. Clinical strains of *C. difficile* when exposed to MIC and sub-inhibitory concentrations of certain antibiotics produced high level of cytotoxin. Ampicillin and clindamycin were the most potent inducers of cytotoxin followed by metronidazole and vancomycin. Cefotaxime induced the least amount of the cytotoxin activity.

During the second part of the study, 73 (10.5%) out of 697 met the diagnosis of CDI. Out of these 73, 56 (76.7%) were hospital-acquired and 17 (23.3%) were from outpatient clinics. Thus, the prevalence of hospital-acquired CDI was 8% over the study period. The prevalence of hospital-acquired CDI in 2003, 2004 and 2005 were, 9.7%, 7.8% and 7.2%, respectively. Our data showed that 42.9% of the CDI patients were above 60 years out of which over 79% were aged 71 years and above. Patients with CDI were more likely than the controls to have been exposed to immunosuppressive drugs and feeding via naso-gastric tube. The most common ribotypes isolated during the second part of the study were 002 and 001. The later was isolated only from one environmental sample in the first part of the study. PCR-ribotype 027 was not isolated during 2003-2005 study. None of our 151 *C. difficile* isolates were resistant to amoxicillin-clavulanic acid, ampicillin, linezolid, metronidazole, piperacillin-tazobactam, teicoplanin or vancomycin. Resistance to penicillin and meropenem among the clinical isolates increased from 2.4 to 16.4% and 4.8 to 21.4%, respectively while resistance to imipenem (another carbapenem) was extremely high in both studies.

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Publications

Published Manuscripts

1) **Jamal WY**, Mokaddas EM, Verghese TL, VO Rotimi. In vitro activity of 15 antimicrobial agents against clinical isolates of *Clostridium difficile* in Kuwait. Int J Antimicrob Agents 2002; 20: 270-274.

2) Rotimi VO, **Jamal WY**, Mokaddas EM, Brazier JS, Johny M, Duerden BI. Prevalent PCR-ribotypes of clinical and environmental strains of *Clostridium difficile* isolated from intensive–therapy unit patients in Kuwait. J Med Microbiol 2003; 52: 705-709.

3) **Jamal WY**, Rotimi VO, Grubesic A, Rupnik M, Brazier J, Duerden BI. Correlation of multidrug resistance, toxinotypes and PCR ribotypes in *Clostridium difficile* isolates from Kuwait. Accepted in Journal of Chemotherapy 2009; 21:

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1. Jamal WY, Rotimi VO, Suzcs G, Duerden BI. Influence of antibiotic exposure on cell-bound and cell-free *Clostridium difficile* cytotoxin. The 8th Biennal Congress of Anaerobe Society of the Americas 'Anaerobe 2006', Boise, ID., USA. July 25-28, 2006.

2. **Jamal WY**, Rotimi VO, Grubesic A, Rupnik M, Brazier J, Duerden BI. Correlation of multidrug resistance, toxinotypes and PCR ribotypes in *Clostridium difficile* isolates from Kuwait. Health Science Poster Day, Kuwait University, Kuwait. April 2005.

LIST OF ABBREVIATIONS

AAC	Antibiotic-associated colitis
$AACD_{50}$	Toxin lethal to 50% of animals
AAD	Antibiotic-associated diarrhea
Amox/clav	Amoxicillin-clavulanic acid
AMP	Ampicillin
AST	Antibiotic susceptibility testing
BHI	Brain Heart Infusion
CCEYA	Cycloserine-cefoxitin Egg-Yolk Agar
CCFA	Cycloserine-cefoxitin Fructose Agar
CDAD	*Clostridium difficile*-associated diarrhea
CDC	Centers for Disease Control and Prevention
CDI	*Clostridium difficile* Infection
CFU	Colony Forming Unit
Clin	Clindamycin
CNS	Central Nervous System
CNSIP	Canadian Nosocomial Infection Surveillance Program
COPD	Chronic Obstructive Pulmonary Disease
CT	Computerized Tomography
CTX	Cefotaxime
CU	Cytotoxic Unit
CVA	Cerebrovascular Accident
D-MEM	Minimal Essential Media
Eh	Redox potential

EIA	Enzyme immunoassay
ELISA	Enzyme linked immunoassay
FAA	Fastidious anaerobe agar
FAB	Fastidious anaerobe broth
FBS	Foetal Bovine Serum
FDA	Food and Drug Administration
Fox	Cefoxitin
GLC	Gas liquid chromatography
GDH	Glutamate dehydrogenase
HCW	Healthcare Worker
HPA	Health Protection Agency
ICU	Intensive Care Unit
IDSA	Infectious Disease Society of America
IL-8	Interleukin-8
Imip	Imipenem
ITU	Intensive Therapy Unit
K	Kuwaiti
KCCC	Kuwait Cancer Control Centre
MDR	Multi-drug resistant
MIC	Minimum inhibitory concentration
MIC_{50}	Concentration that kills 50% of the isolates
MIC_{90}	Concentration that kills 90% of the isolates
MTZ	Metronidazole
NAP	North American pulsed-field
NCCLS	National Committee for Clinical Laboratory Standards

NGT	Nasogastric tube
NK	Non-Kuwaiti
NT	Non-toxigenic
PaLoc	Pathogenicity locus
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
Pip/taz	Piperacillin-tazobactam
PMC	Pseudomembranous colitis
RCM	Robertson Cooked Meat
RFT	Renal Function Test
RR	Relative risk
SHEA	Society for Health Epidemiology of America
S-layer	Surface-protein layer
SLE	Systemic lupus erythromatosis
SS	Salmonella-Shigella agar
T	Toxigenic
Tox+	Toxin-positive
TcdA	Toxin A gene
TcdB	Toxin B gene
TcdA-,TcdB+	*tcd*A-negative and *tcd*B-positive.
TNFα	Tumor necrosis factor α
Trov	Trovafloxacin
TSI slant	Triple Sugar Iron slant
UK	United Kingdom
USA	United States of America

+ve	Positive
VRE	Vancomycin-resistant enterococci
WBC	White Blood Cells
XLD	Xylose Lysine Deoxycholate

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CHAPTER 1

GENERAL INTRODUCTION

1.1 General background

Clostridium difficile was first described in 1935 by American workers, Hall and O'Toole, who were studying the microbial flora of the meconium and faeces of newborn infants (Hall and O'Toole, 1935). They observed that this organism produced a toxin highly lethal to mice. During the Second World War, Hambre *et al.* (1943), while investigating the role of penicillin in the treatment of *Clostridium perfringens*-induced gas gangrene, demonstrated that penicillin caused typhlitis in rodent model which was more lethal to the rodent models than *C. perfringens* gas gangrene. This reaction in rodents was thought to be due to a direct action of penicillin on the gut mucosa. Latter, Green in 1974, who was studying penicillin-induced death in guinea pigs, found that stool specimens contained cytopathic changes, which the author related to the activity of a latent virus (Green, 1974). But this, in retrospect, may appear to be the first identification of *C. difficile* cytotoxin.

Pseudomembranous colitis (PMC) was first reported in 1893 (Finney, 1893) who reported pseudomembranous changes in the intestinal tract of a young 22-year-old patient who was being treated by William Osler. PMC, then became a commonly recognized complication of antibiotic use in the early 1950's especially among surgeons (Altemeier, 1963). Early work in 1960's implicated *Staphylococcus aureus* as a causative agent (Hall and Khan, 1966) and oral vancomycin was the standard treatment for PMC (Khan and Hall, 1966).

The "*C. difficile* era" started in 1974, when Tedesco and colleagues reported high rate of PMC among their patients who received clindamycin at the Barnes Hospital (St Louis, MO, USA)

(Tedesco *et al.*, 1974). Diarrhoea developed in 42 (21%) out of 200 patients who were given clindamycin. Twenty (10%) of the clindamycin recipients had PMC which was diagnosed by endoscopy (Tedesco *et al.*, 1974).

The initial work on *C. difficile* detection involving clinical specimens and hamster models were performed by several investigators in the 1970's. They were primarily Keighley *et al.* (1978) in Birmingham, UK, Bartlett *et al.*, (1977) in Boston, USA and Lusk *et al.* (1977) in Michigan, USA. In these early studies, the main method of detection of *C. difficile* infection was the hamster model as well as the cell cytotoxin assays (Bartlett *et al.*, 1978).

The work on cell cytotoxicity assays began with Te-Wen Chang, who showed that the stool samples from hamsters with antibiotic-associated typhlitis and from patients with PMC contained a powerful cytotoxin that could be neutralized with *C. sordellii* antitoxin (Chang *et al.*, 1978). In spite of this finding, they did not demonstrate the presence of *C. sordellii* from hamsters stool cultures. However, Bartlett and his colleagues found that *C. difficile* was the only *Clostridium* species that could be recovered from the stool samples of the hamsters and produced a cytopathic effect which was neutralized by *C. sordellii* antitoxin (Bartlett *et al.*, 1977). Since *C. difficile* antitoxin was not available at that time, the standard way of detection of *C. difficile* infection was to detect the presence of cytotoxin neutralized by *C. sordellii* antitoxin. In 1978, *C. difficile* was reported as the definitive causative agent of antibiotic-associated pseudomembranous colitis (Bartlett *et al.*, 1978).

1.2 Genome of *Clostridium difficile*

1.2.1 General features of the genome

The complete gemome sequence of *C. difficile* strain 630 (epidemic type X) has been determined recently by Sebaihia *et al.*, 2006. It consists of a circular chromosome of 4,290,252 bp and a plasmid, pCD630, of 7,881 bp (Table 1.1). The chromosome encodes 3,776 predicted coding sequences (CDSs); in common with other low G+C content Gram-positive bacteia. The chromosome has a coding bias, with 82.1% of the CDSs encoded on the leading strand. The plasmid carries 11 CDSs, none of which has any function. The CDSs of *C. difficile* encode many accessory functions and mobile elements (Figure 1.1).

Table 1.1. General features of *C. difficile* genome (Sabaihia *et al.*, 2006).

	Chromosome	Plasmid
Size (bp)	4,290,252	7,881
G + C content (%)	29.06	27.9
Coding sequences	3,776	11
Coding density	0.87	1.39
Average gene size (bp)	943	563
Pseudogenes	32	0
rRNA operons	11	0
tRNA	87	0
Stable RNA	54	0

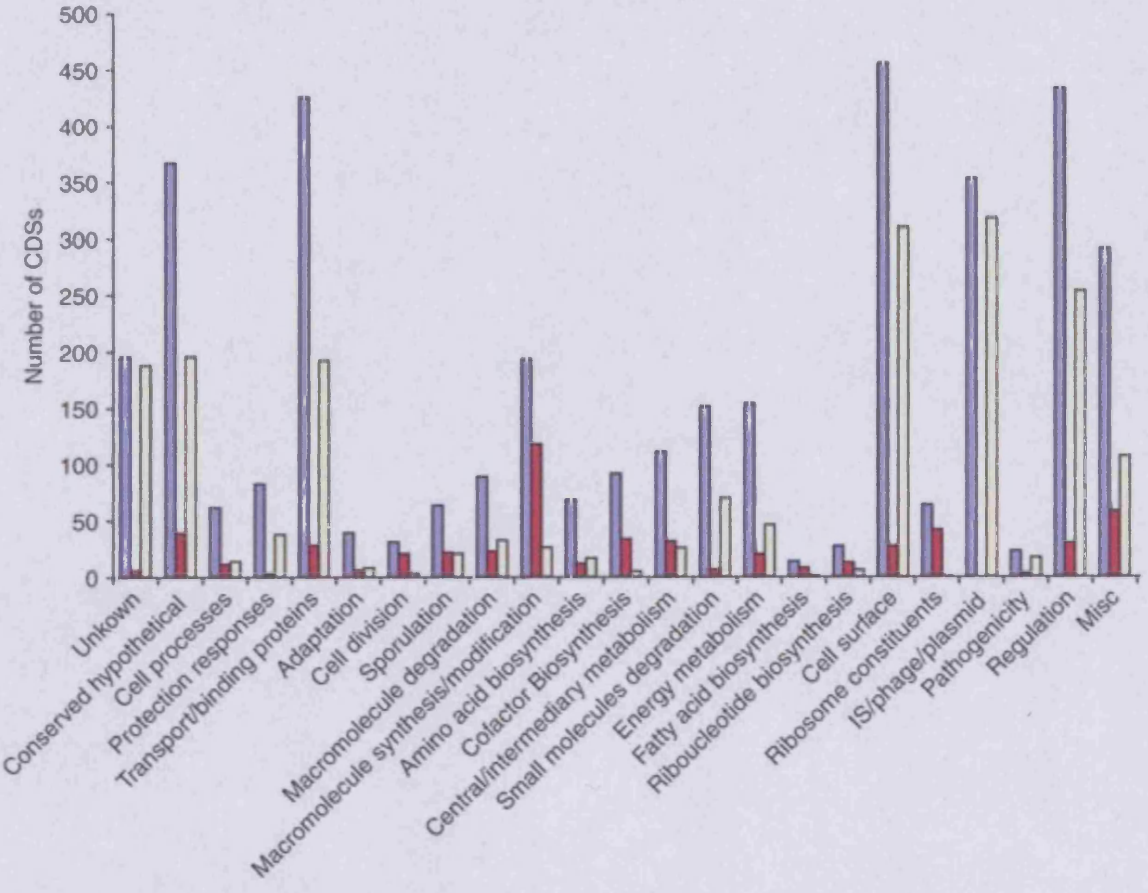


Figure 1.1. Representation of the distribution, by functional categories, of the shared and unique *C. difficile* CDSs relative to the sequences clostridial genomes. Blue: *C. difficile* CDSs, purple: *C. difficile* CDSs shared with the sequenced clostridia, yellow: CSDs unique to *C. difficile* relative to the sequenced clostridia (from Sabaihia *et al.*, 2006).

1.2.2 Mobile genetic elements

The G+C content of the chromosome is highly variable, with numerous high G+C regions and some anomalies in the GC bias (Sebaihia *et al.*, 2006). Many of these anomalous regions are consisted of mobile elements. Eleven percent of *C. difficile* genome consists of these mobile

genetic elements. The identified mobile elements include the following: 7 putative conjugative transposons (CTn), one element that is likely to be a mobilizable transposon (Tn5398) (Farrow *et al.*, 2001), two prophages, a skin element (Haraldsen and Sonenshein, 2003), IStrons (Braun *et al.*, 2000) and several other unknown mobile elements. On conjugative transposon, Tn5397, mediates tetracycline resistance and has broad host range (Sebaihia *et al.*, 2006). Conjugative transposons, known as integrative and conjugative elements (ICE), are genetic elements which are able of integrate into and excise from the host genome and thus transfer themselves into other unrelated bacteria (Burrus *et al.*, 2002). One of the conjugative transposon from *C. difficile*, Tn5397, mediates tetracycline resistance and has a wide host range.

The genome of *C. difficile* carries a prophage-like element, *skin* (*sigK* intervening sequence), inserted within the gene *sigK* encoding a sporulation-specific sigma factor. *Skin* is required for effective sporulation of *C. difficile* after its excision at the onset of sporulation (Farrow *et al.*, 2003).

1.2.3 The genome of hypervirulent hyperendemic *C. difficile*

Recently, Stabler and his colleagues compared the genomic and phenotypic character of 3 *C. difficile* strains: an epidemic, hyperepidemic 027 strain, a non-epidemic ribotype 027 strain and a previously sequenced PCR-ribotype 012 strain (Stabler *et al.*, 2009). They found that these 3 strains share 3,247 core genes, including those encoding determinants for pathogenesis like antibiotic resistance. In addition there are 234 genes unique to both 027 ribotypes which include motility, drug resistance genes and toxicity. However, their sequence data showed that there are at least 5 genetic regions unique to the epidemic ribotype 027 strain compared to the non epidemic 027. These genetic elements include a unique phage island of high G + C DNA content

inserted into a 027 unique conjugative transposoon, transcriptional regulators and a two-component response regulator, i.e. a putative lantibiotic ABC transporter and a putative cell surface protein along with a number. The phage island also encodes a toxin/antitoxin system which is important to maintain the stability of mobile elements (Stabler *et al.*, 2009).

1.2.4 Virulence factors and surface proteins

As shown in Figure 1.2, *C. difficile* genome consists of one complete locus, termed the pathogenicity locus (PaLoc), which has 5 genes (*tcdABCDE*) responsible for the synthesis, and regulation of toxin A and B (Voth and Balard, 2006). Some *C. difficle* strains produce a third toxin, binary toxin, a two subunit actin-specific ADP-ribosyltransferase. Its genes found only in about 6-15% of isolates. This suggests that it is not a major virulence factor. However, whether it contributes to disease formation is still a debate.

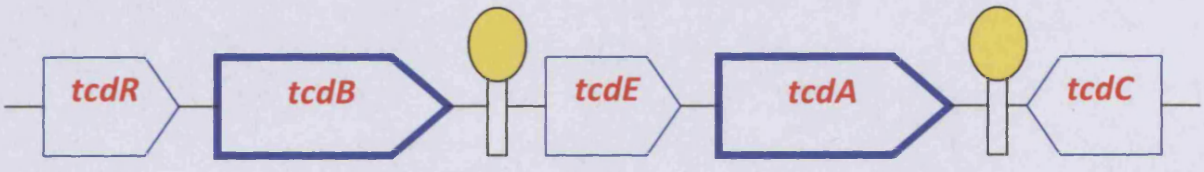


Figure 1.2. Pathogenicity locus of *C. difficile* (PaLoc); *tcdR*: an alternative sigma factor – positive regulator of toxin production; *tcdC*: negative regulator of toxin production; *tcdE*: encodes a holin like protein (with permission from Professor I Poxton).

1.2.5 Antimicrobial resistance

Tetracycline and erythromycin resistance genes in *C. difficile* are carried on the conjugative and putatively mobilizable transposons Tn5397 and Tn5398, respectively (Sebaihia *et al.*, 2006). In addition, Sebaihia and his colleagues described the genes that mediates bacitracin resisctance, β-lactam resistance i.e. β-lactamase, β-lactamase regulatory protein and β-lactamse repressor in *C. difficile.*

1.3 Virulence Factors

C. difficile exhibits four main types of virulence factors which can be divided into the following: adhesion, chemotaxis, hydrolytic enzymes and toxins. Some are true virulence factors that contribute directly to infection process e.g. toxin A and B while others help *C. difficile* to colonize the colon and produce the true virulence factors.

1.3.1 Adhesion

Adhesion to the target site of infection is an important initial process. In 1979, adherence of *C. difficile* to the human gut epithelial cells was indicated by the recovery of *C. difficile* from a washed biopsy specimen from a patient with PMC (Borriello, 1979). In 1988, Borriello and colleagues found that highly virulent toxigenic *C. difficile* strain adhered better to the terminal ileum and caecum than the poorly virulent strain and both toxigenic strains adhered better than the non-virulent non-toxigenic strains in hamster model. The same group observed that co-administration of toxin A with a non-toxigenic *C. difficile* strain enhanced its adhesion to the

same level as the virulent toxigenic strain (Borriello *et al.*, 1988a). There have been several attempts made to identify the adhesion factors. Polar fimbriae were detected in 1988 (Borriello *et al.*, 1988a). But there was no correlation between the presence of these fimbriae and the ability to adhere to hamster gut mucus (Borriello *et al.*, 1998b). Some *C. difficile* have flagella but it is not known if they are involved in the adhesion of the organism to the gut epithelial cells. Some investigators have identified and characterized heat stimulated 27 kDa and 40 kDa proteins of *C. difficile* which may be involved in adhesion to human colonic enterocyte-like Caco-2 and mucus secreting HT29 cells *in vitro* (Eveillard *et al.*, 1993). Type IV pillus biosynthesis locus is present in the C. difficile genome (CD3503-CD3513). This locus encodes one pillin subunit, two type IV pillin leader peptidases and several proteins (Sebaihia *et al.*, 2006).

1.3.2 Chemotaxis

Chemotaxis is the ability of a microorganism to move to its target site along a chemical gradient, mostly by the activity of flagella. It has been shown that gut mucosa of animals as well as human acts as chemoattractant for *C. difficile* (Borriello and Bhatt, 1995). The degree of chemotaxis is correlated positively with the virulence of the strains in hamster model (Borriello, 1998). Flagellin has been demonstrated in *C. difficile* which is about 39kDa and appears to be an important chemotactic factor; the gene of this flagellin has been cloned and sequenced (Borriello, 1998).

1.3.3 Capsule

Some investigators have detected a polysaccharide capsule in *C. difficile* but the removal of this capsule did not appear to affect the degree of pathogenicity of the organism (Davies and Borriello, 1990). In addition, it has been demonstrated that *C. difficile* has an outer coat called Surface layer (S-layer) protein. It is encoded by a single gene, *splA*. This S-layer protein consists of two polypeptides that form a regular crystalline array over the whole surface of the bacterium which may have a yet-to-be identified role in the virulence of *C. difficile* (McCoubrey and Poxton, 2001). However, the same group in Edinburgh has shown some degree of correlation between S-layer type, ribotype and serotype of *C. difficile*.

1.3.4 Hydrolytic enzymes

Various hydrolytic enzymes have been detected in *C. difficile* strains, e.g., hyaluronidase, chondroitin-4-sulphatase, heparinase and collagenase (Hafiz and Oakey, 1976; Seddon *et al.*, 1990). Highly virulent strains seem to produce higher level of enzymes than the less virulent strains (Seddon *et al.*, 1990). It is possible that some of these enzymes are involved in tissue destruction and help to compromise gut integrity leading to further fluid accumulation (Borriello, 1998). Conceivably, *C. difficile* acquire nutritional benefit from such enzymatic activity.

1.3.5 Toxins

C. difficile produces two high molecular weight toxins, toxin A and toxin B, which are responsible for the disease manifestations. Both toxin A (TcdA) and B (TcdB) are large single-

chain polypeptides with molecular masses of 308 kDa for enterotoxin A and 269kDa for cytotoxin B. These toxins are encoded by two genes, *tcdA* and *tcdB*, which have been mapped to a 19.6kb chromosomal pathogenicity locus (PaLoc) (Rupnik *et al.*, 1998). Beside these genes, 3 regulatory genes (*tcdC*, *tcdD* and *tcdE*) are located within the PaLoc (Braun *et al.*, 1996; Hammond and Johnson, 1995; Cohen *et al.*, 2000a). Both toxins A and B are 50% identical at the amino acid level and have similar primary structures (Poxton *et al.*, 2001). The enzymatic and the cytotoxic activity of toxin B is found at the N-terminal of the toxin, which also holds the enzyme and cytotoxic activity of toxin A (Hofmann *et al.*, 1997). The C-terminal of the toxin includes a receptor-binding domain and is made up of repetitive peptide elements (Poxton *et al.*, 2001).

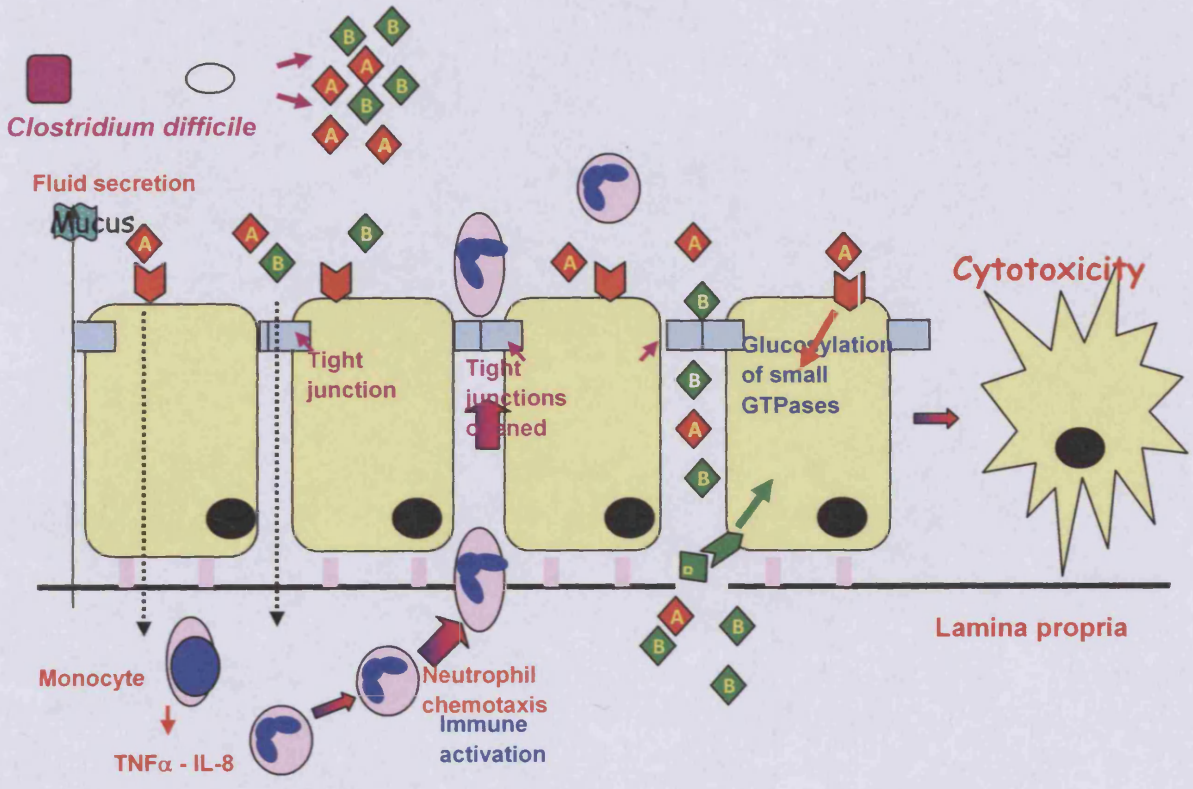


Figure 1.3. Mechanism of action of *C. difficile* toxin A and B on intestinal epithelial cells (Thelestam and Chaves-Olarte, 2000)

The carboxyl terminal of toxin A forms binding domains for the carbohydrate structure that occurs on the surface of the epithelial cells. Toxin B binds to the cells which are not covered by a thick carbohydrate matrix. They, then enter the epithelial cells via receptor-mediated endocytosis (Thelestam and Chaves-Olarte, 2000). Both toxins have to pass through an acidic intracellular compartment so that they can intoxicate the cells. Once the cells are intoxicated by the toxins A and B, both toxins inactivate Rho proteins, a group of low molecular weight GTP-binding

proteins that regulate actin cytoskeletal filaments in the cells and various signal transduction process. Once Rho proteins are inactivated by *C. difficile* toxins, the cytoskeleton of the cells is disrupted, actin filaments disintegrate, roundening of the cells takes place and the resultant damaged cells cannot function (Salyers and Whitt, 2002). In addition, both toxins cause disruption of the barrier function by opening the tight junctions between the colonic epithelial cells and break down the actin filaments thus increasing the colonic permeability leading to watery diarrhoea (Poxton *et al.*, 2001). This condition leads to increased permeability and peristalsis (Riegler *et al.*, 1995; Lamont 2002). The inflammation of the colonic mucosa may result in toxic megacolon and perforation in fulminant cases (Longo *et al.*, 2004).

Toxin production varies among different toxigenic strains of *C. difficile* and is influenced by environmental growth condition (Hundesberger *et al.*, 1997), availablility of glucose (Dupuy and Sonenhein, 1998), temperature (Karlsson *et al.*, 2003), various amino acids in the growth media (Yamakawa *et al.*, 1994; Karlsson *et al.*, 1999), butyric acid and butanol (Karlsson *et al.*, 2000) and biotin (Yamakawa *et al.*, 1998). Sub-inhibitory concentration of vancomycin and penicillin can induce increased toxin A production (Honda *et al.*, 1983) and clindamycin and cephradine increase toxin A production (Onderdonk *et al.*, 1979).

1.4 Pathophysiology

The pathogenesis of *C. difficile* diarrhoea starts with the disruption of the gut normal flora by various antimicrobial agents, followed by colonization of the gut with *C. difficile* and the production of toxins A and B that mediate the cytoskeletal derangement of the colon epithelial cells and finally by inflammation and mucosal injury.

The indigenous microflora of the gut is one of the host defense mechanism against infection. About 10^{12} organisms are present in a gram of stool and disruption of microbial ecosystem by antibiotics is required before the development of *C. difficile* infection (CDI) (Thielman, 2000). Once the normal gut flora has been altered by an antibiotic, *C. difficile* colonization occurs after fecal-oral transmission. Most of the vegetative cells are killed by the gastric acid but the spores resist the acidity of the stomach. Thereafter, the spores are converted into vegetative cells in the small bowel after exposure to the bile acid (Thielman, 2000). Then, *C. difficile* multiplies in the colon reaching colony counts of $> 10^8$ Colony Forming Units (CFU)/gram of stool. Once the pathogenic *C. difficile* organisms reach the logarithmic and early stationary phases of growth, they release the cyto- and enterotoxins that mediate the disease (Thielman, 2000).

Systemic symptoms are often related to toxin-induced inflammatory mediators released into the colon, such as the tumour necrosis factor α (TNFα), interleukin-8 (IL-8), macrophage inflammatory protein-2 and substance P (Lamontagne *et al.*, 2007; Flegel *et al.*, 1991; Castagliuolo *et al.*, 1997; Castagliulo *et al.*, 1998). Neutrophils, present in the pseudomembranes and within the intestinal mucosal layer underneath the pseudomembranes, are said to play an important role in the pathogenesis of the disease. In the study of Thielman (2000), toxin A was observed to activate human neutrophils which promote in vitro chemotaxis and chemokinesis.

It is not known why only a fraction of those exposed to certain antibiotics become colonized or why only a small proportion of those colonized with *C. difficile* develop CDI. It has been suggested that serum IgG to toxin A may play a role (Kyne *et al.*, 2000). However, a number of of *C. difficile* have been reported with increasing frequency that have deletions, insertions or polymorphic restriction sites in one or more of the genes within the PaLoc. Until recently, it was believed that all *C. difficile* strains responsible for disease produce both TcdA and TcdB.

However, several studies have shown that TcdA negative and TcdB positive ($TcdA^-$ $TcdB^+$) *C. difficile* strains can be involved in CDI (Al-Barrak *et al.*, 1999; Brazier *et al.*, 1999). The prevalence rate of these $TcdA^-$ $TcdB^+$ strains ranged from 0.2 to 56% in different studies in Europe, Asia, and North America (Al-Barrak *et al.*, 1999; Barbut *et al.*, 2002; Rupnik *et al.*, 2003).

1.5 Isolation and identification

C. difficile is an obligate Gram-positive, spore-forming anaerobic bacillus. The designated name is related to the difficulty faced by the original investigators in finding it in culture. The vegetative cells are characterized by formation of spores, which allow the organism to survive the harsh environments by its resistance to oxygen, heat, drying and chemical agents. Its spores are usually subterminal but occasionally may be terminally located (Figure 1.4).

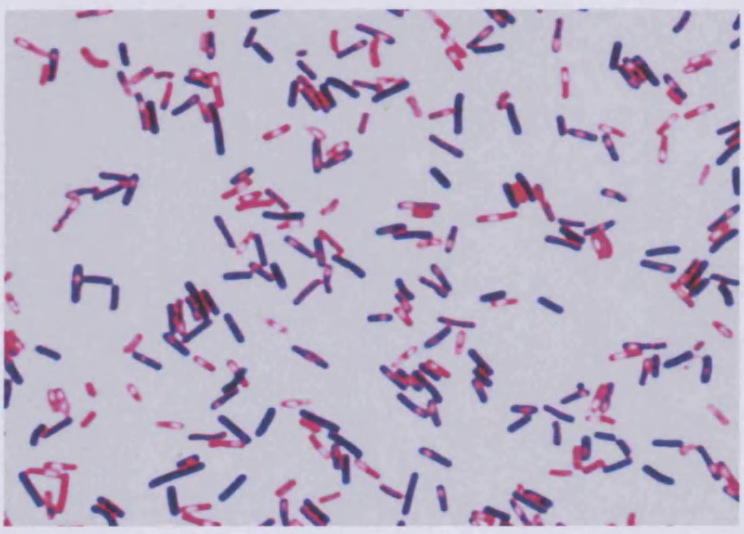


Figure 1.4. Gram stain of *C. difficile* bacilli showing spores (with permission from Dr J Brazier)

It can be isolated after alcohol shock or heat shock procedure that allows the survival of the spores and eliminate other vegetative non-spore forming faecal organisms. Then, the deposit of the spirit treated specimen or the broth is inoculated onto selective *C. difficile* media which could be either *C. difficile* Cefoxitin Cycloserine Egg Yolk Agar base (CCEY; Oxoid LtD, Basinstoke, Hampshire, England) or *C. difficile* Cefoxitin Cycloserine Agar Base containing cefoxitin 8mg/L and cycloserine 250mg/L (Oxoid LtD). The inoculated agar plates are incubated anaerobically in the presence of 10% CO_2, 80% H_2, 10% N_2 for 48-72 h. The colonies usually appear pleomorphic, grey, opaque and non-haemolytic (Figure 1.5; Figure 1.6). However, some strains may produce an alpha-type of haemolysis (Brazier, 1993). It produces a characteristic odour, often described as "elephant or horse manure" due to the production of *p*-cresol from parahydroxyphenylacetic acid. Under long-wave ultraviolet light, *C. difficile* colonies fluoresce a greenish yellow colour (George *et al.*, 1979). However, this is not a unique characteristic of *C. difficile*, as other *Clostridium* species, like *C. innocum* and *C. novyi*, also fluoresce the same way (Brazier, 1993). It can be identified by agglutination with *C. difficile* somatic antigen latex agglutination (however, false positive may occur with *C. glycolicum* and C. *bifermentans/sordellii*) or biochemical reactions like API20AN. Confirmation is usually by analysis of the metabolic end product of volatile fatty acid on gas liquid chromatography (GLC).

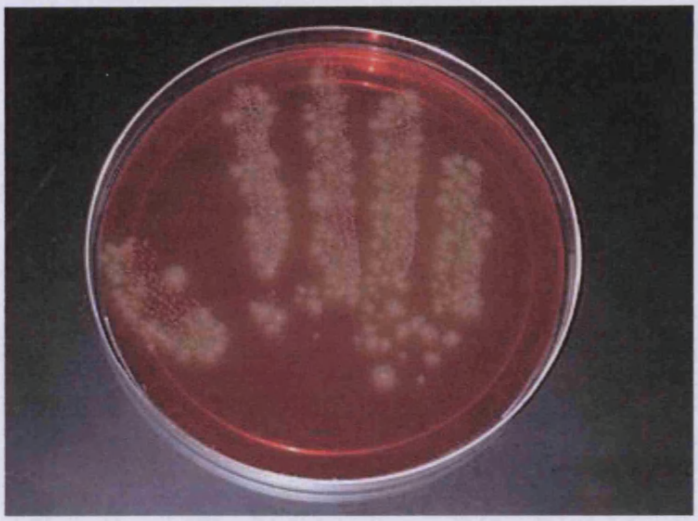


Figure 1.5. Culture of hand for *C. difficile* after 48h incubation.

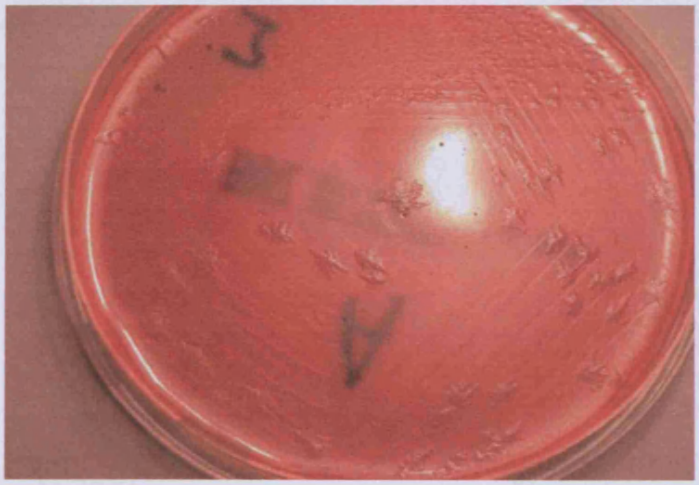


Figure 1.6. Colonial morphology of *C. difficile* on Fastidious Anaerobe Agar after 24 hour incubation (with permission from Dr J Brazier).

1.6 Infections

C. difficile is often implicated in a spectrum of diseases referred to as *C. difficile*-associated diarrhoea (CDAD) or *C. difficile* infection (CDI) which manifests as mild self-limiting antibiotic associated diarrhoea (AAD), antibiotic associated colitis (AAC) or a more serious life-threatening pseudomembranous colitis (PMC) with toxic megacolon and possible gut perforation (Figure 1.7; Figure 1.8 and Figure1.9). Common clinical findings in patients with CDI include diarrhoea, colitis with abdominal cramps, fever, leucocytosis (sometimes as high as 50,000-100,000 WBC/mm^3), and inflammation in the colonic biopsy. Although diarrhoea is the hallmark of CDI, it may be absent in severe disease as in toxic megacolon, ileus or secondary to colonic dysfunction. This presentation may occur in post-operative patients who are receiving narcotics for pain. Therefore, symptoms like unexplained fever, leucocytosis, and abdominal pain in patient with recent antibiotic exposure or surgery should raise the suspension of CDI, even in the absence of diarrhoea (Burke *et al.*, 1988; Bartlett and Gerding, 2008).

PMC is the most severe manifestation of the disease and is usually pancolitis affecting especially the distal colon and the rectum. However, right-sided colitis as well as small bowel involvement has been described (Jacobs *et al.*, 2001). PMC represents an advanced stage of the disease which is "nonspecific" but nearly diagnostic of *C. difficile* infection (Bartlett *et al.*, 1980). It is often associated with hypoalbuminaemia and sometimes with anasarca because it is protein-loosing enteropathy (Tedesco *et al.*, 1974).

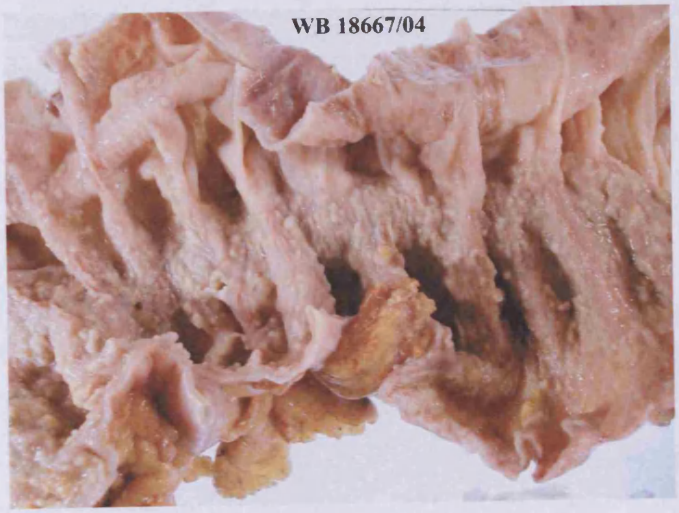


Figure 1.7. A picture of pseudomemranous colitis (with permission from Professor I Poxton)

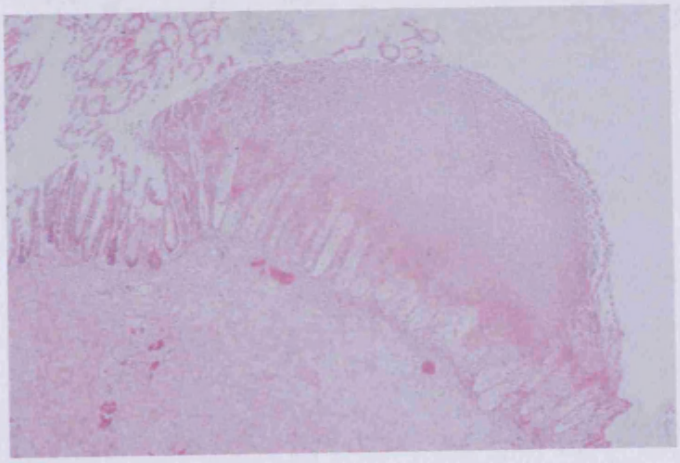


Figure 1.8. Histopathology section of the colon with PMC (with permission from Professor I Poxton)

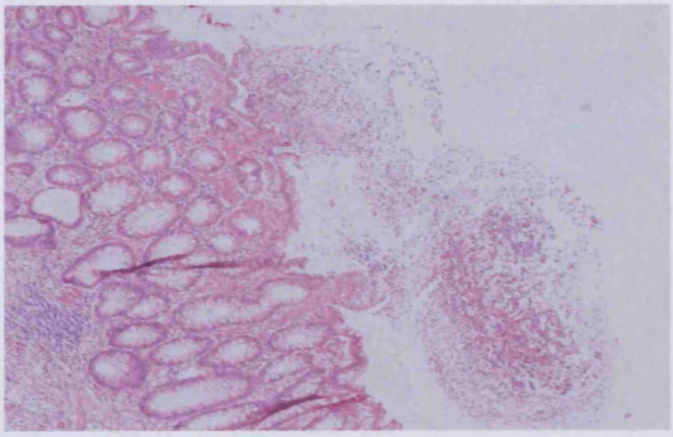


Figure 1.9. Histopathology section showing PMC (with permission from Professor I Poxton)

There is evidence of *C. difficile* involvement in extracolonic infections such as bacteremia, prosthetic device infections, pleuropulmonary infections with empyema, primary peritonitis, osteomyelitis, reactive arthritis, cellulitis, necrotizing fasciitis and visceral abscesses, e.g. in the spleen and pancreas (Jacobs *et al.*, 2001). It is also associated with outbreaks of diarrhoea and colitis in hospitalised adults receiving antibiotics (Pierce *et al.*, 1982; Tabaqchali *et al.*, 1984 b; Bartlett *et al.*, 1978).

C. difficile has been branded an emerging pathogen and an important causative agent of nosocomial diarrhoea. Evidence to this effect is demonstrated in reports of major outbreaks of diarrhoea in hospitals where the organism has been the sole culprit. The organism, or its toxin, has been identified in 8-10% of cases of nosocomial diarrhoea, while other common bacterial enteric pathogens, e.g. *Salmonella* spp., *Shigella* spp., *Klebsiella oxytoca* and *Campylobacter* spp. were rarely isolated (Fan *et al.*, 1993; Barbut *et al.*, 1995; Rohner *et al.*, 1997; Hogenauer *et al.*, 2006). It has been shown that recurrence of CDI occurs in 20-25% patients and half of these patients are due to relapses which arise as a result of germination of the sporulated *C. difficile* occurring post therapy (McFarland, 2008a).

1.7 Severity of the disease

The severity of CDI can be characterised by any of the following criteria: admission to intensive care unit for therapy of CDI or any of its complications; requirement for surgery, i.e., colectomy for toxic megacolon, perforation of the gut or refractory colitis; or death occurring within 30 days after the diagnosis of CDI if CDI is the primary or contributory cause (Kuijper *et al.*, 2006). Admission to the healthcare facility as an indicator of severe illness is subject to debate. Other criteria of the severity of the illness include raised leucocyte count, raised creatinine levels, or hypoalbuminaemia (Kuijper *et al.*, 2007). Raised leucocyte count (more than 2 x 10^{10}/L) and creatinine level (more than 200μmole) have been associated with adverse effect of recurrence of the disease (Pepin *et al.*, 2004). There are few reports on grading the severity of the disease according to the amount and consistency of the stool. In their report, Dubberke *et al.*, (2007b) were able to grade the severity of the diarrhoea according to the volume of the stool per a day. However, there were practical difficulties in grading the diarrhoeal stool according to the volume especially in very sick and confused patient.

1.8 Risk factors

Several risk factors for AAD have been described. They can be divided into 4 groups viz: (i) those that disrupt the normal colonic microflora e.g. antimicrobial agents and surgery; (ii) those related to host factors e.g. advanced age, impaired immune system, comorbidities, impaired immune system and low production of anti-toxin A and/or B; (iii) exposure to *C. difficile* spores from hospital environmental surfaces, infected room-mates and hand carriage by health-care personnel; and (iv) those related to microbial factors e.g. the strain type.

1.8.1 Antimicrobial agents

The most important risk factor that drives the development of CDI is prior use of antibiotic especially within the previous 8 weeks (Bouza *et al.*, 2005). Excessive and sometimes irrational use of antimicrobial agents are widely believed to be the main driving factor behind CDI, with differences in the risk depending on the class of antimicrobial agent (McDonald *et al.*, 2005). Differences in the risk of different antimicrobial agents have been postulated as being due to the differences in the properties of the antimicrobial agents e.g. the amount of the antibiotic in the gut and the duration of their effect on the gut flora (Spencer, 1998), their activity against *C. difficile*, and possibly their drug or its metabolite levels in the gut (Freeman *et al.*, 2003). Any antimicrobial agent may cause CDI, but administration of clindamycin, broad spectrum cephalosporins and broad spectrum penicillins are often implicated (Freeman and Wilcox, 1999; Thomas *et al.*, 2003).

Some antibiotics, particularly clindamycin (relative risk "RR", 9.0), cephalosporins (RRs range from 7.8 for cefaclor to 36.2 for cefotaxime) and β-lactams (RRs range from 2.0 to 22.2 to ampicillin and ampicillin–clavulanic acid) are associated with relatively high risk of *C. difficile* acquisition (Gerding *et al.*, 1995; Bartlett, 1994; Bignardi, 1998). However, Shek *et al.* 2000, did not find any evidence to support that increasing use of cephalosporins, more elderly patients or increasing length of hospital stay are major contributors to the rising incidence of CDI (Shek *et al.*, 2000).

Fluoroquinolones have been in use since 1988, but they have only recently been implicated as common causes of CDI (Loo *et al.*, 2005; Pepin *et al.*, 2005; Kazakova *et al.*, 2006; McCusker *et al.*, 2003). Gaynes *et al.*, attributed an outbreak of CDI at long-term care facility due to changing

their antibiotic formulary from levofloxacin to gatifloxacin which has an anti-anaerobic activity. In addition, the rate of CDI decreased after a switch back to levofloxacin coupled with the infection control measures (Gaynes *et al.*, 2004). The use of combination antibiotic therapy and long-term receipt of antibiotic therapy are also risk factors. CDI may also occur even in patients exposed to short term prophylactic antibiotic courses (Bartlett, 1994). Almost all antibiotics may predispose to the development of CDI (Mylonakis *et al.*, 2001), although it is less often associated with some - e.g., metronidazole, aminoglycosides, trimethoprim - and it very rarely occurs without antibiotics. It is hypothesised that antibiotics deplete the normal flora of the gut that normally provide colonization resistance (Farrell and LaMont, 2000). Ertapenem, a carbapenem with an antianaerobic activity, may be associated with increased risk of CDI (Itani *et al.*, 2006).

1.8.2 Age

Other predisposing factors that have been associated with *C. difficile* infections include old age. Elderly patients are at noticeably higher risk, with disease rate for patients ≥65 years of age as much as 20-fold higher than those for younger patients (Loo *et al.*, 2005; Pepin *et al.*, 2005). However, children older than 2 years are also known to develop CDI (McFarland *et al.*, 2000). Neonates and infants less than 2 years are often asymptomatic carriers of *C. difficile*, but rarely they may develop symptoms (McFarland, 2008b). During 2000-2003, there was a report from Canada which indicated that 200 young children (mean age 5.4 years) developed CDI, of which 23% of cases had serious disease that necessitate admission to the hospital and 31% of the patients had at least one recurrence (Morinville and McDonald, 2005).

1.8.3 Anti-acid medication

Other independent risk factors include proton pump inhibitors which may increase the risk by 3-fold (Dial *et al.*, 2004). It is presumed that the low acidic pH in the stomach may lead to destruction of the spores, but colonic receptors do exist in the colon for some proton pump inhibitor in animal models (Nakamura *et al.*, 1995) and clostridial spores are generally resistant to acidic conditions. However, other investigators have demonstrated that exposure to H_2 receptor antagonists and proton pump inhibitors are associated with elevated risk of CDI only in univariate analysis and that there is no increased risk after adjustment for comorbidities on multivariate analysis (Pepin *et al.*, 2005).

1.8.4. HIV infection

In the report by Sanchez *et al.*, (2005), *C. difficile* was found to be the most common bacterial cause of diarrhoea in HIV infected patients. It was found in 598 (53.6%) out of 1115 HIV-positive patients. The explanation for this high prevalence rate in HIV patients was, in part, related to the frequent use of prophylactic and therapeutic antimicrobial agents in this population and perhaps to the frequent visits to the health care facilities.

1.8.5 Host related factors

Several host related risk factors have been described with CDI such as prolonged hospital stay (McFarland 1995), recent gastrointestinal surgery, enema and stool softener (McFarland *et al.*, 1990), endoscopy, immunosuppressive therapy and anti-neoplastic drugs (Halim *et al.*, 1997).

Jiang *et al.* (2006) investigated the association between interleukin-8 (IL-8) promoter polymorphism and patients with CDI. They found that out of 42 patients with CDI, 39% were positive for the polymorphism in IL-8 compared to 16% and 17% of the control patients with *C. difficile* negative diarrhoea and patients without diarrhoea, respectively. The majority of these infections have been reported in the geriatric wards, haematology wards (Heard *et al.*, 1986; Delmee *et al.*, 1987; Tabaqchali and Wilks, 1992) and intensive care units (Foulke and Silva 1989; Samore *et al.*, 1994a).

1.9 Laboratory diagnosis

1.9.1 Specimen collection

Accurate diagnosis of CDI is important for the management of the patient, controlling the spread as well as generating surveillance data. *C. difficile* infection is suspected in adults or children ≥1 year of age with unexplained diarrhoea especially those who received antibiotics in the previous 2 months or whose diarrhoea begins 72 hours after admission to hospital (National *Clostridium difficile* Standards Group, 2004; Thielman, 2000). For optimal investigation of CDI, fresh stool specimen is the standard sample for diagnostic test. If this is not possible, the stool could be refrigerated or frozen. Storage at ambient temperature may lead to denaturation of the toxin (Brazier, 1993). However, multiple cycles of freezing/thawing have minimal effect on the viability of the *C. difficile* or its spores. Storage at 4°C has no discernible effect on the cytotoxin (HPA, 2009). But, storage at -20°C has detrimental effect on the cytotoxin and multiple cycles of freezing/thawing may adversely affect toxin titres (Freeman and Wilcox, 2003).

1.9.2 Cell cytotoxicity assay

Although there is no gold standard for the diagnosis of CDI, the cell cytotoxicity assay is the best available. It is the most sensitive and specific test available to detect toxin B (Wilkins and Lyerly, 2003) and can detect *C. difficile* toxin at less than 10 picogram levels (Bartlett and Gerding, 2008). It can be done on different cell lines such as Vero, HEP-2 or MRC-5 cell lines. It needs neutralization of the cytotoxin by *C. sordellii* antitoxin. The major disadvantages of the cell cytotoxicity assay are that it is technically demanding, requiring continuous maintenance of the cell line and support from the virology department and the need for neutralization of the positive cytopathic effect. In addition, it is expensive and has a relatively long turnaround time (24-48 h).

1.9.3 Stool culture

Although stool culture on selective media has high sensitivity (i.e. a test that detect positive results among patients with disease), the specificity (i.e. a test that detect ngative results among patients without disease) is low because of the rate of asymptomatic carriage of *C. difficile* especially among hospitalized patients. In order to increase the specificity of the culture, broth culture can be evaluated further by means of a cell cytotoxicity assay or enzyme immunoassay (EIA) (National *Clostridium difficile* Standards Group, 2004). Stool culture for *C. difficile* is rarely done in a routine diagnostic Microbiology Laboratory because of the relative long turnaround time (48-72h), and the fact that it is not specific for in vitro production of toxins. However, culture allows molecular typing of the organisms, monitoring of antibiotic susceptibility and molecular epidemiology.

1.9.4 Immunological assays

The main types of kit-based toxin detection are commercially available and these are: EIA, which gives coloured reaction in a microtitre well for a positive test, immunochromatography assays, which gives a coloured band or line in the substrate strip and common antigen tests that detect the enzyme glutamate dehydrogenase as an indicator of *C. difficile* infection in the stool. The current EIAs for detection of toxin A only, or both toxins A and B, in the stool are relatively insensitive as they can miss up to 40% of positive cases compared to culture (Delmee *et al.*, 2005) and cell cytotoxicity assays (Shanholtzer *et al.*, 1992). The initial EIA tests were for the detection of toxin A only. But, after the recognition that some isolates only produce toxin B, combined assays were introduced that detect both toxins. These assays are only 70-80% as sensitive as the cell cytotoxicity assay, which in turn is less sensitive than the stool culture (O'Connor *et al.*, 2001; Delmee *et al.*, 2005). Commercial EIAs give results within hours rather than days but have low sensitivity than culture and cell cytotoxicity assays.

The most common antigen test (known as the glutamate dehydrogenase test or GDH test) is an EIA that detects GDH enzyme. *C. difficile* produces GDH enzyme constitutively and it is easily detectable at all levels. The test has good sensitivity of 90-100% which is almost equivalent to a positive culture. It is rapid and not expensive. However, a positive GDH indicate the presence of the organism only and does not indicate the production of the toxins. Therefore, it can be used as screening test to detect the GDH-positive stool that need further testing by cell cytotoxicity, EIA for toxins or toxigenic culture. In addition, this enzyme is produced by other anaerobes thus reducing the specificity of the test (Brazier, 1995).

Commercially available tests for *C. difficile* toxins are the following: EIA for toxin A or both toxins A and B and immunochromatography for toxin A only. Toxin A EIA and immunochromatography can miss *C. difficile* strains that express only toxin B. However, they have rapid turnaround time (<1 h for immunochromatography and about 2 h for EIAs), have high specificity and easy to perform and commercially available by different suppliers (Sunenshine and McDonald, 2006). These methods can detect from 100-1000 pg of toxin (Yolken *et al.*, 1981; Viscidi *et al.*, 1984). EIA on 2-3 specimens will increase the cost as well as the diagnostic yield by 5-10% compared to one stool EIA (Manabe *et al.*, 1995). The sensitivities of the different EIA vary in different reports and they range from 85-95%. In the earlier studies, the sensitivity was 33.3-59.4% for toxin A and as low as 38% for toxins A and B in the recent studies (Bartlett and Gerding, 2008). Some laboratories use one method to screen for *C. difficile* in stool samples with subsequent testing for cytotoxin in samples with positive results. For example, some investigators have used two-step protocol in which common antigen assay (GDH) was used as screening test to exclude 75-90% of stool specimens that do not contain *C. difficile* (Ticehurst *et al.*, 2006). Then, the positive specimens were further tested by cell cytotoxicity assay to improve the specificity of the non-specific GDH result. This, theoretically, should reduce the cost by decreasing the number of cytotoxicity assays required while maintaining high specificity and sensitivity. But this approach will increase the turnaround time.

1.9.5 PCR amplification methods

PCR amplification tests using primers to detect toxin B and toxin A genes, 16S rRNA gene of *C. difficile* and toxin B sequencing have been developed (Thielman, 2000). But the clinical use in routine diagnostic laboratory is still not clear.

1.10 Clinical diagnosis

Patients with CDI often present with diarrhoea associated with history of antibiotic use. It is known that the diarrhoea occurs during or as late as 10 weeks after stopping the course of antibiotic (Tedesco, 1982). For mild to moderate disease, the patient may pass up to 10 loose stool per day (Bartlett *et al.*, 1980). The stool is usually watery, foul smelling and takes the shape of the container; rarely is there fresh blood in the stool. Recognisable accompanying features include fever, lower quadrant abdominal cramps and leukocytosis. *C. difficile* colitis may present with fever, leucocytosis, abdominal pain, colonic inflammation visualized by endoscopy for the presence of pseudomembranes or Computerized Tomography (CT) scan and the presence of thickening of the colonic wall, pericolonic stranding, the accordion sign or the double-halo sign (Kawamoto *et al.*, 1999; Sunenshine and McDonald, 2006). Infrequently, severe *C. difficile* infection may present without diarrhoea (Bruke *et al.*, 1988). This occurs when the infection causes paralytic ileus that does not allow the passage of the stool. This presentation may occur in post-operative patients who received antibiotics as well as narcotics for pain.

1.10.1 Role of endoscopy

The detection of PMC by colonoscopy is the preferred method of diagnosis because PMC, in up to one-third of the patients, can involve the right colon only and will not be detected by sigmoidoscopy. However, the sensitivity of endoscopy is only 51% in the presence of PMC (Bartlett and Gerding, 2008). As in any invasive procedure, complication is always a worry. An important complication of colonoscopy is perforation of the gut especially in patients with fulminant colitis (Longo *et al.*, 2004).

1.11 Epidemiology

During the first month of life, up to two-thirds of neonates may be colonized with *C. difficile* without producing any symptoms. This may be related to the acquisition of organism from hospital environment. Most reports show a wide variation in the isolation rate among neonates and infants ranging from 2 to 71% (Viscidi *et al.*, 1981; Larson *et al.*, 1982; Al-Jumaili *et al.*, 1984; Tabaqchali *et al.*, 1984a). The presence of toxin-producing organisms has also been demonstrated in this population, but the prevalence of colitis remains very low (Thielman, 2000). The body of evidence in the literature show that the carriage of *C. difficile* among healthy adults in western industrialized countries is uncommon and any association with community-acquired colitis is low and sporadic (Viscidi *et al.*, 1981). However, the carriage rate among healthy adults varies significantly in different countries, from 1.9-7% in western industrialized countries (Aronsson *et al.*, 1985; Bartlett 1979; Aronsson *et al.*, 1983) to 15.4% in Japan (Nakamura *et al.*, 1981) and 33.3% in Nigeria (Rotimi *et al.*, 1986). However, it is not clear if this carriage is a permanent or a temporary phenomenon. The rate of colonization increases with age. For

example, the rate of asymptomatic colonization has been reported as 7% of residents of long-term care facilities (Walker *et al.*, 1993), 14% of hospitalised elderly patients on acute medical wards and 20% of patients on chronic medical care wards (Rudenskey *et al.*, 1993). Also worthy of note is the finding that the rate of asymptomatic colonization is between 3-5 folds more common than in symptomatic patients (Samore 1993; McFarland *et al.*, 1989).

Some other studies have demonstrated the direct effect of *C. difficile* infection on the length of hospital stay and consequent enormous cost. Elegant studies by Riley *et al.*, in 1995 and Wilcox *et al.*, in 1996 showed that *C. difficile* infection in the hospital increased the length of hospital stay by 18 days in adults and 21.3 days in geriatric wards, respectively (Riley *et al.*, 1995; Wilcox *et al.*, 1996). The approximate additional cost of each *C. difficile* infection was reported as £4107 (Wilcox *et al.*, 1996). Fortunately, the mortality rate from CDI is relatively low, accounting for only 0.6% as reported by Olson *et al.* (Olson *et al.*, 1994). However, Eriksson and Aronsson (1989) have earlier reported a higher mortality rate in elderly CDI patients compared with controls (21% vs. 7%). In addition, when surgical intervention is involved particularly in the treatment of toxic megacolon or colon perforation, the mortality rate may increase to 35-50% (Morris *et al.,* 1990). In 2007, the Office of National Statistics reported 8324 death certificates with CDI listed as an underlying or contributory cause of death; in 49% it was the underlying cause. In the same year there were approx. 62,000 cases of CDI in England (National statistics, 2009).

It appears that the most important sources of *C. difficile* in a hospital and long-term care facility setting are symptomatic cases or asymptomatic carriers who are the main reservoirs of *C. difficile* in the hospital. The environment of these patients is also an important source (Dubberke *et al.*, 2007a). It has been found that environmental contamination in the rooms of patients with

diarrhoea is more than in rooms with asymptomatic carriers (49 vs. 29%) (McFarland *et al.*, 1989). In addition, it has been found that rooms occupied by *C. difficile*-negative patients were contaminated with *C. difficile*, in spite of routine cleaning, due to the spores of the organism that can persist for several months (Muligan *et al.*, 1979). However, a recent study by Dubberke *et al.* 2007a, showed that rooms housing a patient with CDI were more likely to be culture positive than non-CDI patients rooms (100% vs. 33% ; $P < 0.01$). Several neutral agents and chlorine-releasing agents have poor activity against *C. difficile* spores (Wilcox and Fawley, 2000). This is significant in outbreak situations as epidemic strains produce significantly more spores than non-epidemic strains and sporulation may be enhanced when the stools containing *C. difficile* are exposed to non-chlorine-based cleaning agents. Thus, enhancing the persistence of *C. difficile* spores in a hospital environment (Wilcox and Fawley, 2000).

Physical proximity to an asymptomatic carrier has been reported as an important risk factor for transmission with an attributable risk of 12% (Chang and Nelson, 2000). This may be related to the contamination of near-patient environment or movement of the patients between contaminated fomites, e.g. commodes. Transfer of patients between wards or institutions has also been implicated in the transmission of *C. difficile* (Safdar and Maki, 2002).

Direct or indirect contact represents the main route of *C. difficile* transmission, as the spores may persists in the environment for months or years and they show resistance to various environmental cleaners e.g. detergents and some disinfectants (Fawley and Wilcox, 2001; Wilcox *et al.*, 2003). Person-to-person transmission on hospital wards, particularly geriatric wards, as well as environmental contamination and carriage on the hands of hospital workers have been well documented (Mulligan *et al.*, 1979; Kim *et al.*, 1981; Malamou-Ladas *et al.*, 1983; Savage and Alford 1983; Heard *et al.*, 1986; McFarland *et al.*, 1989). Transmission occurs

principally via the faecal oral route as well as by direct contact with contaminated surfaces (Barbut and Petit, 2001). *C. difficile* has been isolated from the hands of hospital personnel taking care of patients with *C. difficile* infection. Brooks and colleagues have also suggested that transmission may occur via direct inoculation into the bowel by contaminated rectal thermometers (Brooks *et al.*, 1992). Direct transmission via the airborne route is unlikely to occur, but it has been suggested recently in a pilot study (Roberts *et al.*, 2008). However, the potential for the dispersal of *C. difficile* spores in air needs further exploration, especially as the organism has been found widely in the soil and the gut of many animals, and is a known cause of colitis in animals such as cats, dogs, horses, rodents and neonatal pigs (Avbersek *et al.*, 2009).

AAD is an important cause of morbidity and mortality in elderly hospitalised patients. Recently, there has been a dramatic rise in both the incidence and the mortality rate of CDI worldwide (Dallal *et al.*, 2002; Morris *et al.*, 2002; Pepin *et al.*, 2004; McDonald *et al.*, 2006). In a 1997 survey of 18 Canadian institutions, the mean incidence of CDI was 6 per 10,000 admissions, and 1.5% of the affected patients died as a direct or indirect effect of this complications (Hyland *et al.*, 2001; Miller *et al.*, 2002), while in 2004, the overall incidence had risen to 22.5 per 1000 admissions, and the 30-day attributable mortality rate to 6.9% (Loo *et al.*, 2005). In another recent survey of 8 European hospitals, the incidence of CDI was found to be 1.1 per 1,000 patient admissions (Barbut *et al.*, 2003a) compared to 0.1-2% reported earlier (Olson *et al.*, 1994; Bowen *et al.*, 1995). This rise in the incidence has been associated with the emergence of an epidemic strain of *C. difficile* (Ribotype 027 or BI/NAP1). Infection with this strain is associated with more severe disease and fluoroquinolone resistance (McDonald *et al.*, 2005; Pepin *et al.*, 2005; Bartlett 2006; Hubert *et al.*, 2007).

1.11.1 Typing methods

For epidemiological purpose, the typing method ideally should be characterized by its reproducibility, its discriminatory power, stability of the marker investigated and universal typability of the isolates (van Belkum *et al.*, 2007). Several phenotypic and molecular typing techniques have been used for *C. difficile*. The phenotypic methods used for *C. difficile* include the following: antibiogram, plasmid profiling, serogrouping, protein polyacrylamide gel electrophoresis of cell surface antigens and pyrolysis mass spectrometry (Brazier, 2001). On the other hand, the molecular typing methods include, methods utilizing enzyme restriction of genomic DNA (e.g. pulsed-field gel electrophoresis and restriction endonuclease analysis), methods dependent on PCR products (e.g. PCR ribotyping and multi-locus variable-number tandem-repeat analysis), methods using sequencing of selected genes (e.g. multilocus sequence typing and surface layer protein A gene sequence typing) and combination of enzyme restriction and PCR (e.g. amplified fragment length polymorphism) (Killgore *et al.*, 2008). Of all these, the PCR ribotyping method has become well established, reproducible, reliable and popular.

1.12 Epidemiology of *C. difficile* ribotype 027

A new era of *C. difficile* infection started early during this millennium when the frequency and severity of CDI suddenly increased across North America, Europe and Southeast Asia with devastating consequences. There were several outbreaks caused mainly, but not exclusively, by a newly recognised PCR ribotypes 027 strain, alternatively known as North American pulsed-field type 1 (NAP 1), restriction endonuclease type BI, toxinotype III or the hypervirulent strain

(depending on the typing method used). This hypervirulent strain carries genetic mutations in the *tcdC* toxin regulatory gene, binary toxin gene and fluoroquinolone resistant mutation.

1.12.1 Hypervirulent CDI in USA

In the USA, a recent cohort analysis of patients from the Nationwide Inpatient Sample, representing 1,000 hospitals across 35 states, found a doubling of the *C. difficile* colitis incidence rate from 261 cases per 100,000 discharged patients in 1993 to 546 cases per 100,000 discharged patients in 2003 (Ricciardi *et al.*, 2007) and over half of that increase occurred in the final 2 years of the study. Population-based estimates of *C. difficile* mortality rates rose more than fourfold, from 5.7 per million population in 1999 to 23.7 per million population in 2004 (Redelings *et al.*, 2007). NAP 1 (ribotype 027) *C. difficile* strains have been isolated and identified in at least 27 states as of April 2007 (CDC, 2007). In addition, it has been reported as the main cause of outbreaks in several states (McDonald *et al.*, 2005; Tan *et al.*, 2007).

1.12.2 Hypervirulent CDI in Canada

The Canadian Nosocomial Infection Surveillance Program (CNISP) surveyed 2,062 patients from 19 institutes in 8 provinces during 1997 (Miller *et al.*, 2002). The incidence of CDI was 5.9 cases per 1,000 patient admissions; 8% of these patients had complications with 1.5% mortality attributed directly or indirectly to CDI. The increase in the frequency and mortality of CDI was first identified in December 2002 (Weiss *et al.*, 2007) and later reported in 2004 (Eggertson and Sibbald, 2004). A study in Quebec, a French-speaking province of Canada, during the first 6

months of 2004 involving 12 hospitals determined an incidence of 22.5 per 1,000 patient admissions, with mortality rate of 6.9% (Loo *et al.*, 2005) i.e. fourfold compared to CNISP study. Although NAP1 was the dominant pathogen in that study, the mortality rate was related to the presence of the binary toxin and a partial deletion in the negative toxin regulator gene *tcdC*. A follow up CNISP survey of 34 hospitals across 9 provinces during November 2004 to April 2005 revealed an unchanged scenario in the overall mean of CDI incidence at 6.4 per 10,000 patient days (CNISP, 2007). However, the CDI incidence in Quebec of 11.9 per 10,000 patient days remained almost double the initial CNISP figure.

1.12.3 Hypervirulent CDI in Europe

A similar situation to that in North America has emerged in Europe. The NAPI/027 ribotype has been recognised as the cause of outbreaks across 9 European countries; namely, the UK (England, Wales, Scotland and Northern Ireland), Netherlands, Belgium, France, Austria, Germany, Finland, Luxembourg, and Ireland while sporadic cases have been detected in Austria, Norway, Poland, Hungry, Denmark and Spain (Kuijper *et al.*, 2008). The attributable mortality rate had increased 2.3-fold from 1999 to 2004.

1.12.4 Hypervirulent CDI in Asia

There is less epidemiologic information on *C. difficile* and CDI from the Asian countries. In a survey of 310 *C. difficile* isolates from different hospitals in Japan and Korea and from healthy infants from Indonesia, Rupnik *et al.*, (2003) discovered a wide variety of toxinotypes with no

027/NAP1 isolated; toxinotype 0 was predominant. An epidemic strain (PCR ribotype f 'smz') was identified in an analysis of 148 isolates from one Japanese teaching hospital. This strain was responsible for outbreaks in many hospitals (Sawabe *et al.*, 2007); only one 027/NAP1 isolate susceptible to fluoroquinolone and associated with community onset disease was found.

1.12.5 Hypervirulent CDI in the Middle East

So far, no 027/NAP1 strain has been found in any of the countries in the Middle East nor has the prevalence of *C. difficile* infection/colonisation been studied in any hospital setting in Kuwait, including the intensive therapy units (ICUs), haematology wards or burn units, which lack general guideline on the rational use of antibiotics. Nobody knows the antibiotic susceptibility pattern or the ribotypes of the strains of *C. difficile* circulating in our teaching hospitals with ICU facilities. Kuwait is a small country with a population of about 2.1 million, of which the Kuwaitis are one-third. The indigenes enjoy traveling abroad, both for holidays and for medical treatment in many European and American hospitals. It is not unknown for Kuwaitis who have travelled abroad to import multi-resistant bacteria. A recent example was a strain of mupirocin-resistant methicillin-resistant *Staphylococcus aureus*, imported from a UK hospital (Unpublished observation, Dr EE Udo). Although the acquisition rate of *C. difficile* in stool specimens of patients on admission to the intensive care units (ICUs) of Kuwait hospitals has been reported (Rotimi *et al.*, 2002), the prevalence, risk factors and molecular epidemiology of CDI has not been established.

1.13 Antibiotic susceptibility

Although metronidazole is the first line therapy for the treatment of CDI, current drug treatment of CDI focuses on both metronidazole and vancomycin. Early studies have demonstrated their equivalence of therapeutic outcomes (Mylonakis *et al.*, 2001; Bartlett 2006) and the recommendation of several health authorities like CDC, HPA, SHEA, IDSA favour the use of both antimicrobials. However, recent data have shown increased CDI rates, increase disease severity as well as higher risk of treatment failure and CDI recurrence after treatment with metronidazole (Musher *et al.*, 2005; McDonald *et al.*, 2006). In addition, the CDC has discouraged the use of vancomycin for the treatment of CDI in hospital settings in order to minimize the risk of development of vancomycin resistance in enterococci and staphylococci (HICPAC, 1995). Clinical Microbiology Laboratories do not usually culture for *C. difficile* and they do not perform antimicrobial susceptibility testing on *C. difficile* as they depend on the detection of toxin A and/or B for the laboratory diagnosis of CDI. Several studies have reported the full susceptibility of *C. difficile* to metronidazole and vancomycin. However, few reports have appeared indicating that some clinical strains demonstrating decreased susceptibility to these therapeutic agents have emerged Table 1.2; (Barbut *et al.*, 1999; Brazier *et al.,* 2001; Pelaez *et al.*, 2002). Recently, the number of isolates with vancomycin MIC of 4μg/ml has been found to increase from 2.7% to 21.6% in Scotland and the UK (Drummond *et al.*, 2003a; Mutlu *et al.*, 2007). This emphasizes the need for periodic monitoring of any emerging of resistance in *C. difficile* and to check the suitability of the current treatment as well as to investigate for new antimicrobial agents active against *C. difficile*, such as rifaximin, OPT-80, ramoplanin, rifalazil, tinidazole, linezolid and tigecycline.

Table 1.2. Resistance of *C. difficile* isolates to metronidazole

Country	Resistance to metronidazole	Reference
Hong Kong	1/100 (1%) clinical isolate with MIC = 64 μg/ml	Wong *et al.*, 1999
France	6/198 (3%) clinical isolates with MIC ≥8μg/ml	Barbut *et al.*, 1999
UK	One environmental isolate with MIC = 16μg/ml	Brazier *et al.*, 2001
Spain	26/415 (6.3%) clinical isolates with MIC ≥32μg/ml	Pelaez *et al.*, 2002
Israel	1/49 (2%) clinical isolate with MIC >256μg/ml	Bishra *et al.*, 2006
UK	21/86 (24.4%) among among clinical isolates ribotype 001	Baines *et al.*, 2008

In a recent report by Hecht and colleagues (Hecht *et al.*, 2007), they found that the most active agents *in vitro* were rifaximin, rifalazil, tizoxanide, nitazoxanide and OPT-80. In-vitro activity of linezolid was examined against 115 *C. difficile* isolates and found to have an MIC range of 0.03-4μg/ml, which was thought to be susceptible (Pelaez *et al.*, 2002). However, whether in-vitro activity correlates with in-vivo effect is a matter of debate especially as some of these agents, e.g., linezolid are absorbed and the levels in the colon are low.

Antimicrobial susceptibility patterns of 677 *C. difficile* isolates obtained from different hospitals in England 2007-2008 revealed significantly lower susceptibility to metronidazole in the more common PCR ribotypes (027, 106, 001) when compared to the less common ribotypes but all have MICs within the susceptible range (Brazier *et al.*, 2008). The same investigators found that all the isolates were susceptible to vancomycin. The more common ribotypes (027, 106, 001) were more resistant to moxifloxacin and erythromycin than the less common strains (Brazier *et al.*, 2008). There is no information on the antimicrobial susceptibility of clinical isolates in

Kuwait or indeed in the entire Middle Eastern countries. Information regarding the susceptibility of *C. difficile* is vital to the formulation of treatment guidelines for CDI in our country.

1.14 Treatment of CDI

This can discussed as current and new options.

Current treatment of CDI has traditionally been to discontinue the implicated antimicrobial agent or agents with supportive care that include rehydration, electrolytes replacement and avoiding the use of antiperistaltic agents, e.g. narcotics and loperamide, as they may predispose to the development of toxic megacolon by slowing down the clearance of *C. difficile* toxin from the gut (Bouza *et al.*, 2005). When the treatment with the precipitating antimicrobial agent has to be continued, then it is better to use antibiotics with low probability of causing CDI, such as sulphonamide, aminoglycosides, vancomycin, tetracycline, trimethoprim-sulfamethoxazole (Bricker *et al.*, 2005).

The traditional therapy for patients with moderate to severe CDI is either oral metronidazole (400 mg 8 hourly) or oral vancomycin (125mg 6 hourly) for 10-14 days (HPA, 2009). Metronidazole is the preferred agent for treatment according to the Health Protection Agency in UK (HPA) (HPA, 2009), the Infectious Disease Society of America (IDSA), the US Centres for Disease Control and Prevention (CDC) and the Society for Healthcare Epidemiology of America (SHEA) guidelines (Bartlett, 2006). Metronidazole is the preferred agent due to its low cost, comparability to vancomycin in clinical trials and it is less likely to promote the development and spread of vancomycin-resistant enterococci (VRE) (Gerding, 1997). However, a recent study has indicated that both metronidazole and vancomycin are equivalent in promoting vancomycin-

resistant enterococci during therapy of CDI (Al-Nassir *et al.*, 2008), which may provide reassurance about using vancomycin more often in the treatment of moderate and severe CDI. Treatment with metronidazole is complicated with high recurrence rate in 20-25% of the patients, usually occurring 1-10 days after discontinuation of the treatment (Kuijper *et al.*, 2006). Metronidazole has poor pharmacological profile in term of its absorption in the small intestine after oral therapy with only a detectable level found in the stool in the presence of diarrhoea (Bolton and Culshaw, 1986). As the diarrhoea improves, the level of metronidazole in the lumen of the colon drops, which may be a factor that leads to failure to respond to therapy or disease recurrence. In addition, some *C. difficile* isolates have been reported to be resistant to metronidazole in Spain and the UK (Brazier *et al.*, 2001; Pelaez *et al.*, 2002; Baines *et al.*, 2008). There are some recent evidence which indicated that metronidazole is associated with higher failure rate compared to vancomycin in the treatment of seriously ill patients (Musher *et al.*, 2005) and that there is a poor response to metronidazole if the antibiotic for the initial condition has to be continued (Modena *et al.*, 2006). These are in addition to an earlier report by Wilcox and Howe (1995) which showed that metronidazole has slower clinical response rate compared with oral vancomycin.

Although oral vancomycin is only approved by the US Food and Drug Administation (FDA) for CDI, many infectious disease authorities such as HPA (HPA, 2009) have suggested that vancomycin can be used for severe and serious forms of disease. It has an ideal pharmacological profile for treating colon infection as it is totally unabsorbed by the gut and it is found in the colon lumen at concentrations more than 100-fold higher than the MIC (Bartlett, 2006). All *C. difficile* isolates are susceptible to vancomycin *in vitro* except for one report from Spain which showed that about 3% of their isolates were intermediately susceptible to vancomycin with MICs

ranging between 8-16μg/ml (Pelaez *et al.*, 1998). However, the main problems with vancomycin are its high cost and possible promotion of acquisition of vancomycin-resistant enterococci, which of course, has been disputed by the recent report of Al-Nassir and his colleagues (2008).

There is enough evidence to show that both oral metronidazole and vancomycin are efficacious in the majority of patients with CDI. However, in cases of ileus or toxic megacolon, they may not respond because the antimicrobial agents may not reach the necessary site of the disease, i.e. the colon. Therefore, vancomycin may be delivered via retention enema or a long tube raised above the patient employing gravitational force (Bartlett, 2008). Another alternative therapy is the use of intravenous metronidazole and intravenous immunoglobulin. However, the success rate of this regimen is variable and total colectomy may be required, especially if the patient is seriously ill.

Another problem of therapy is relapse after stopping the therapy which may occur in 22-26% of patients, according to Bartlett (2008). They usually respond to retreatment because the majority of the recurrences are re-infection (Wilcox *et al.*, 1998). Some may have additional relapses with small proportion of these patients having repeated relapses that may necessitate several courses of antibiotics. Different methods have been used to treat several and repeated relapses. These include, tapering or pulse dosing of vancomycin (McFarland *et al.*, 2002), use of probiotics (*Saccharomyces boulardii*, *Lactobacillus rhamnosus*) (McFarland *et al.*, 2002), stool implants (Aas *et al.*, 2003), and immunotherapy with intravenous immunoglobulin (McPherson *et al.*, 2006). It is believed that all of these interventions work some of the time and none work all of the time (Bartlett 2008).

1.14.1 New therapies using established antibiotics

Antimicrobial agents other than metronidazole and vancomycin are being studied as potential treatment options for CDI such as bacitracin, fusidic acid, nitazoxanide, rifampicin and teicoplanin. The conclusion reached by Kuijper *et al.*, (2007) is that they are not superior to vancomycin or metronidazole. In a prospective randomized double-blind study, fusidic acid was used as a therapeutic alternative to metronidazole in the treatment of CDI by comparing the efficacy of both drugs. The cure rate and relapse rate were the same but resistance to fusidic acid was found in 1% pretreatment patients versus 55% of patients who remained culture positive after fusidic acid (Noren *et al.*, 2006).

1.14.2 Alternative therapies

Nitazoxanide is a new thiazolide used to treat some parasitic and protozoal infection and has high concentration in the colon. It has good *in vitro* activity against *C. difficile* (Senok and Rotimi 2008). It was studied in a randomized double-blind study in comparison to metronidazole (Musher *et al.*, 2006). The response to metronidazole was 57.6% compared to 65.8 and 74.3% for nitazoxanide for courses lasting 7 and 10 days, respectively. But there was no difference in term of recurrence rate in that study. Rifaximin is a rifampicin-like antibiotic that remains in the gut after oral administration. In an uncontrolled study reported by Johnson *et al.*, (2007), it was used to treat 8 patients with multiple recurrent CDI after completing 2 weeks therapy with vancomycin. It was found to be effective in 7 of the 8 patients. Ramoplanin (an oral non-absorbable lipoglycodepsipeptide that blocks peptidoglycan synthesis) and rifampicin are non-

inferior to the standard antimicrobial agents in phase II trials without serious effects (Pullman *et al.*, 2004; Lagrotteria *et al.*, 2006).

1.14.3 Adsorbent agents

Some workers have suggested the use of absorbent agents which bind to the *C. difficile* toxins thus preventing adherence and consequent damage to the intestinal epithelial cells. Several adsorbent agents have been used to treat CDI which include ion-exchange resin, e.g., cholestyramine and toxin binding polymers, e.g., tolevamer and Synsorb 90 (Senok and Rotimi, 2008). Oral cholestyramine has been used successfully to treat CDI (Moncino and Falletta, 1992). One of the drawbacks of the ion-exchange resin is their binding to the standard antibiotics used to treat CDI making the level of the antibiotics in the colon low. There is, therefore, the need to use these drugs with caution. Tolevamer is a polymer that binds and neutralizes *C. difficile* toxin A and B within the intestinal lumen. In a prospective multicentre double-blind study in patients with CDI, that compared low and high dosing tolevamer regimen with vancomycin, low dose tolevamer (3g/day) was found to resolve the diarrhoea in 67% of the patients compared to 83% for the higher dose tolevamer (6g/day) and 91% in vancomycin group (Louie *et al.*, 2006). However, there was no significant difference between these rates. The recurrence rate was similar among the treatment groups (23%, 10% and 19% for the low dose tolevamer, higher-dose tolevamer and vancomycin groups, respectively). In addition, it was well tolerated but associated with increased risk of hypokalemia (Senok and Rotimi, 2008). Non-toxigenic *C. difficile* strains have also been successfully used to treat two patients with multiple relapses (Seal *et al.*, 1987).

1.14.4 Probiotics

Probiotics have been studied extensively in the treatment of various diseases including diarrhoea and CDI. The probiotics cover a variety of bacteria and yeast while the clinical efficacy against CDI have been linked to certain types, such as *Saccharomyces boulardii*, *Lactobacillus rhamnosus* GG and a non-toxigenic strain of *C. difficile*. The main principle of using these microorganisms is to restore the normal gut flora which is unfavorable for the proliferation of *C. difficile*. However, there are differences in the outcome of these clinical trials perhaps due to the different type of the probiotics and the regimen used. A recent meta-analysis of 6 randomized, placebo controlled trials published between 1994 and 2005 using probiotics combined with either metronidazole or vancomycin to treat CDI concluded that probiotics were effective in reducing the risk of CDI (McFarland 2006). However, these studies lack large and well designed trials to validate the routine clinical use of probiotics. Other meta-analysis failed to detect statistically significant efficacy in treating or preventing CDI (Pillai and Nelson, 2008). In addition, there are reports of fungaemia and bacteraemia in immunosuppressed patients who received probiotics (Schlegel *et al.*, 1998; Niault *et al.*, 1999).

1.14.5 Faecal enema

Faecal enema or transplant is another new option which appears to have caught the attention of clinical microbiologists and infectious disease experts. Fecal transplantation in humans has been used to replace the gut normal flora. This is used as the last option in patient with recurrent CDI refractory to the standard therapy. A fresh stool (30-50g) from a healthy donor is given in normal saline by rectal enema or orally via nasogastric tube or colonoscopy (HPA, 2009). It has been

successfully used in patients with recurrent CDI (Aas *et al.*, 2003). However, this type of treatment is aesthetically not appealing and carries the risk of transmission of various infectious agents present in the stool of "healthy donors".

1.15 Immunotherapy

1.15.1 Immunoglobulin

There are some reports documenting the use of intravenous immunoglobulin for the treatment of refractory CDI (Wilcox 2004; McPherson *et al.*, 2006). A dosage of 400mg/Kg of immunoglobulin given intravenously, as a stat dose, has been found to be beneficial in about two-third of intractable cases (HPA, 2009). Unfortunately, there are no randomized well controlled clinical trials on the use of immunoglobulin in CDI. Therefore, it may be difficult to evaluate its effectiveness in recurrent or severe CDI. It is not known, whether or not the efficacy of immunoglobulin is related to the concentration of antibodies to *C. difficile* toxins present in the pooled immunoglobulin or antibodies to different types of antigen. An immune concentrate made from cows immunized with inactivated *C. difficile* toxin and killed whole-cell *C. difficile* has been tested in a trial involving 77 patients with CDI (Young *et al.*, 2007). The patients were given anti-*C. difficile* concentrate for 2 weeks after 10 days treatment with vancomycin. Only 4 patients (6.3%) relapsed within 46 days post treatment. This therapeutic approach has been well tolerated without adverse effects. Administration of neutralizing human monoclonal antibodies against *C. difficile* toxin A in healthy volunteer adults was safe and well tolerated (Taylor *et al.*, 2008). The participants were followed for up to 56 days without any adverse reactions. A

randomized study of this monoclonal antibody to *C. difficile* toxin A in patients with CDI is worthy of further evaluation.

1.15.2 Vaccination

Vaccine strategies have been developed to protect newly admitted patients to various hospitals and other healthcare facilities from developing CDI. A parenteral *C. difficile* formalin-inactivated toxoid A and B vaccine was been shown to be highly immunogenic in Phase I clinical trials in 30 healthy adults (Kotloff *et al.*, 2001). The vaccine elicited fourfold rise in the neutralizing antibody to toxin A and B in all except one of the volunteers and serum antitoxin A IgA and IgG antibody responses were seen in all except one candidate. The conclusion was that the toxoid vaccine was safe and immunogenic. Other investigators (Ghose *et al.,* 2007) have found that transcutaneous immunization with formalin treated *C. difficile* toxin A was effective and induced systemic as well as mucosal immune response in mouse. Other bacterial components may be immunogenic and protective as O'Brien *et al.*, were able to demonstrate the ability of high titre rabbit anti-surface layer protein antibodies to attenuate the progress of the disease in hamsters infected with *C. difficile* (O'Brien *et al.*, 2005). However, Ni Eidhin and colleagues recently demonstrated that conventional adjuvant approaches were insufficient in terms of generating neutralizing antibody responses to *C. difficile* based on surface layer protein alone in mouse and hamster models (Ni Eidhin *et al.*, 2008).

1.16 Infection control and prevention

Control of *C. difficile* infections and outbreaks involved 4 strategies: (i) interruption of the spread of *C. difficile* spores as well as the vegetative bacteria; (ii) reduction in the burden of clinical disease by prompt diagnosis and treatment of symptomatic patients; (iii) reduction of the inciting agents by antimicrobial stewardship programs and (iv) reduction of *C. difficile* recurrences.

Interruption of the route of the transmission can be achieved by using disposable gloves and thermometers, contact precautions or isolation precautions for *C. difficile* positive patients and environmental disinfection.

1.16.1 Isolation precautions

Contact or isolation precaution is advised for any patient with CDI. It is recommended that the patient be nursed in a single room with a self-contained toilet and hand washing basin. Personal protective clothes e.g. gloves and gowns should be used when in contact with the patient or the patient's environment, and healthcare workers should wash their hands with soap and water before and after patient contact (HPA, 2009). If isolation in single rooms is not possible, then a cohort nursing in a bay with a solid partition or a door to separate the bay from the rest of the ward or a cohort ward may be considered (HPA, 2009). According to the HPA document, a dedicated cohort ward is preferable to a cohort bay in a ward because very strict supervision on the cleanliness of the toilet/commodes should be maintained to ensure staff contact precautions in these bays are observed (HPA, 2009). The cohorted patients have to be nursed and managed by designated staff in order to minimize the risk of cross-infection to other patients (Vonberg *et*

al., 2008a). It is prudent that the patients should be on isolation precautions until the bowel function return to normal for at least 48 h especially during an outbreak setting (Al-Barrak *et al.*, 1999).

1.16.2 Hand hygiene

Standard infection control policies emphasize on hand hygiene to prevent the transmission of any healthcare-associated infection. If the hands are visibly clean, alcohol-based product is useful. If the hands are visibly dirty or contaminated, soap-based hand washing is used. However, it must be emphasized that *C. difficle* spores are not killed by alcohol, therefore it is not recommended as a replacement for hand-washing in CDI. Chlorhexidine, iodophors, chloroxylenol or triclosan are not effective against *C. difficile* in the form of antiseptic hand-wash or hand-rub (WHO, 2007). In a report published by Barbut *et al.*, (2003b), 4% polyvidone soap was more effective in reducing *C. difficile* count than chlorhexidine or non-medicated soap. In addition, the above products are more effective than alcohol-based products.

Bacterial spores can be removed from the hands by the physical action of hand washing and rinsing, whether using non-antibacterial liquid soap or antiseptic substances (Samore *et al.*, 1996). There is a debate whether to use soap containing disinfectant or disinfectant-free soap as some studies have shown that there was no difference in the residual counts of *C. difficile* on the hands after using liquid soap or chlorhexidine gluconate (Bettin *et al.*, 1994). However, other studies have shown that there was a significant difference in the removal of the spores on the hands of healthcare workers (HCW) using chlorhexidine gluconate compared to non-disinfectant soap (McFarland *et al.*, 1989). Wearing gloves when caring for patients with CDI has been found

to reduce the degree of contamination of HCW's hands with the *C. difficile* spores, but with a rider that the hands are washed and dried after removal of the gloves (WHO, 2007). Because patient-to-patient transmission has been reported, hand washing with soap and water is highly recommended for the patients with CDI especially after using the toilet and before eating (Samore *et al.*, 1996).

1.16.3 Protective clothes

The use of disposable protective clothes, like disposable apron and gloves, for any physical contact with patients with CDI and patient's immediate environment or body fluids is highly recommended by a variety of health care authorities (HPA, 2009; Pittet *et al.*, 2006). The bare hands of HCW are vulnerable to contamination with *C. difficile* spores especially when hand hygiene practice is not followed properly (McFarland *et al.*, 1989). It is possible that contamination of the hands may occur during removal of the gloves; therefore, hand washing and drying is necessary after removal of the gloves (Doebbeling *et al.*, 1988). Gowns and aprons help to prevent contamination of the cloths of HCW by *C. difficile* and its spores (AL-Barrak *et al.*, 1999). Since contamination of HCW may occur during work, it is advised that appropriate aprons be worn during work (Perry *et al.*, 2001). Direct or indirect contamination from the environment have been documented by Perry and colleagues (Perry *et al.*, 2001) who found that the some nurses uniforms were contaminated with *C. difficile* spores before the work-shift and that some of these uniforms have been laundered at home.

1.16.4 Environmental cleaning

Environmental cleaning of the immediate surroundings of an infected patient is crucial in the control and prevention of CDI. The environment of a patient with CDI is known to be contaminated with *C. difficile* spores especially in patients with stool incontinence or symptomatic patients with large volume of liquid stool (Samore *et al.*, 1996). The spores often contaminate the floors, commodes, toilets, bedpan, bed railings and frames. However, it is not known whether the spores in the environment are the source of the infection or a sequelae of the CDI because of the clonal nature of infection (Fawley and Wilcox, 2001). *C. difficile* spores in the environment may persist for months or years because they can resist drying, heat and disinfection substances (Wilcox and Fawley, 2000; Wilcox *et al.*, 2003). Cleaning of an environment contaminated with *C. difficile* spores with detergent alone has been shown not to be sufficient means of decontamination (Verity *et al.*, 2001) but there is a need for use of sporicidal products (Fawley *et al.*, 2007). Hypochlorite-based disinfectant has been found to be very effective in decontaminating frequently touched surfaces especially as they are significantly less likely to enhance sporulation of *C. difficile* in vitro (Wilcox and Fawley, 2000). Some studies have demonstrated that hypochlorite solution, at a concentration of 1:1000ppm, used to clean the environment was associated with significant reduction of CDI compared with the use of detergent only (Wilcox *et al.*, 2003; Fawley *et al.*, 2007). However, it should be stressed that hypochlorite is corrosive to metal surfaces and does not remove organic matter. Quaternary ammonium compounds, although no longer used in hospital practice, have been shown to decontaminate the environment contaminated with *C. difficile* (Samore *et al.*, 1994).

In a recent report, hydrogen peroxide vapour was shown to reduce the environmental contamination with *C. difficile* effectively (Boyce, 2007). In that study, *C. difficile* isolation

reduced from 2.4% of swab cultures and 25.6% of sponge cultures to zero from both the swabs and sponge cultures after the use of hydrogen peroxide vapour. The incidence of new C. *difficile* infection dropped from 1.36 cases per 1,000 patient days to 0.84 cases per 1,000 patient days. However, major drawbacks for hydrogen peroxide vapour are that it is expensive, the rooms have to be sealed, and the rooms of the patients and staff location would have to be vacated for several hours. Although glutaraldehyde is effective in inactivation *C. difficile* spores, it not recommended for cleaning the environment due to safety issues (Vonberg *et al.*, 2008a). Peracetic acid 0.2%, which has replaced glutaraldehyde in most of the UK hospitals, is also effective in inactivation of *C. difficile* spores, although it has not been used for environmental decontamination (Vonberg *et al.*, 2008a).

According to the HPA (2009) report, environmental cleaning of the rooms of *C. difficile* patients has to be done at least once daily using chlorine containing cleaning agent (minimum of 1,000ppm available chlorine). It is also recommended in this report that all hospital wards should be cleaned at regular interval with maximal concentration of the disinfectant, particularly the frequently touched surfaces. Once faecal soiling occurs in the environment, cleaning staff have to be notified so that immediate cleaning can be done (Vonberg *et al.*, 2008a). The toilets and the items that can be soiled with stool e.g. commodes and bed pans, have to be cleaned thoroughly. Once a patient is discharged, terminal cleaning and disinfection of the room must be done thoroughly.

1.16.5 Cleaning of medical equipments

Medical equipments like rectal thermometers, oximeters, blood pressure cuffs may play role in the transmission of disease (Samore *et al.*, 1996; Brooks *et al.*, 1992). Therefore, it is ill-advised to use rectal thermometers on the wards or the ICUs (Bartlett, 2008). The recommendations of the European *C. difficile*-Infection Control Group and the European Centre for Disease Control and Prevention include, the use of dedicated blood pressure cuffs for each patient, possible use of disposable materials if feasible, and avoidance of using shared thermometers or, alternatively, using thermometers with disposable sheath (Vonberg *et al.*, 2008a). In addition, these bodies recommended that any equipment used on CDI patient should be cleaned carefully and disinfected with a sporicidal agent.

1.16.6 Antimicrobial stewardship

Antimicrobial stewardship programmes have been advocated for the control and prevention of CDI as the use of antimicrobial agents is the most important risk factor for CDI. Recently, Valiquette and colleages in 2007, reported that the incidence of CDI did not change after strengthening of infection control procedures, but it was reduced markedly after implementation of antibiotic stewardship programme (Valiquette *et al.*, 2007). Almost any antibiotic can predispose to CDI. For some time, the most common antibiotics are the third-generation cephalosporins, clindamycin and aminopenicillins (Freeman *et al.*, 1999; Thomas *et al.*, 2003). Therefore, aggressive restriction of high-risk antibiotics, reducing polypharmacy, prevention of long-term therapy and avoiding inappropriate prescribing are the first steps in reducing the high incidence of CDI. In addition, the use of automatic stop-dates, electronic prescribing, banning of

certain antibiotics and prescriber education and antibiotic policies will help in reducing CDI incidence (Davey, 2006). European *C. difficile*-Infection Control Group and the European Centre for Disease Control and Prevention recommend stopping any (non-*C. difficile*) antimicrobial treatment in a patient with CDI as soon as possible. There is evidence that shows that attempts to prevent the infection with prophylactic metronidazole or vancomycin will lead to increase in the rate of *C. difficile* carriage and infection (Bartlett, 2008).

1.16.7 Education programmes

Education of the staff and communication is an important way to limit the spread of *C. difficile.* Education should include information on basic pathogenesis, potential reservoirs, route of transmission, contamination of the environment, decontamination of the hands/surfaces and infection control measures. Training of the staff, including HCWs as well non-medical personnel involved in cleaning the environment is paramount to a successful prevention of spread of CDI. It is equally important to educate the visitors who enter the patient's room, especially on hand hygiene (Al-Barrak *et al.*, 1999).

1.16.8 Active surveillance

The use of active surveillance cannot be over-emphasized and it is highly recommended because it will detect any rise in the incidence of CDI, the severity of the disease and risk factors (Brazier and Duerden, 1998). It is useful especially in endemic areas where it may detect high baseline

rates of infection or significant variations in different locations that need more infection control intervention (Vonberg *et al.*, 2008a).

1.17 Objective

So far, no 027/NAP1 strain has been found in any of the countries in the Middle East nor has the prevalence of *C. difficile* infection/colonisation been studied in any hospital setting in Kuwait, including the intensive therapy units (ICUs), haematology wards or burn units, which lack general guideline on the rational use of antibiotics. Nobody knows the antibiotic susceptibility pattern or the ribotypes of the strains of *C. difficile* circulating in our teaching hospitals with ICU facilities. Although the acquisition rate of *C. difficile* in stool specimens of patients on admission to the intensive care units (ICUs) of Kuwait hospitals has been reported (Rotimi *et al.*, 2002), the prevalence, risk factors and molecular epidemiology of CDI has not been established

The main aims of my study were to investigate the nosocomial acquisition of *C. difficile* by new patients admitted into the ICUs of 4 major teaching hospitals in Kuwait namely: Mubarak Al Kabir hospital, Amiri hospital, Ibn Sina hospital and the Kuwait Cancer Control Centre (KCCC), investigate the epidemiology of the isolates and determine the prevalence of *C. difficile* infection (CDI) in the country.

Specific objectives were to

1. Isolate and identify strains of *C. difficile* isolated from the stools of all new patients admitted onto the ICUs and haematology wards of these centres, with or without symptoms.

2. Screen their stools for the presence of *C. difficile* toxin
3. Screen the immediate environment of the patients for the presence of *C. difficile*
4. Determine the antimicrobial susceptibility and resistance pattern of all isolates.
5. Type all the isolates by using the PCR-ribotyping technique.
6. Investigate the effects of various concentrations of antibiotics known to predispose to CDI and, also, of those used in its therapy, on the production of extracellular and intracellular toxin B by *C. difficile*.
7. To assess the prevalence of CDI in Kuwait over a 3-year period (2003-2005) as well as correlate this to molecular epidemiology.

CHAPTER 2

MATERIALS AND METHODS

2.1 Growth media used for *C. difficile*

2.1.1 Fastidious Anaerobe Agar

Fastidious Anaerobe Agar (FAA) was obtained from Lab M International Diagnostics Group (Bury, UK) and prepared according to the manufacturers instructions.

2.1.2 Cycloserine-Cefoxitine Egg Yolk Agar

Cycloserine-Cefoxitine Egg Yolk Agar (CCEYA) was obtained from Oxoid (Basingstoke, UK) and prepared according to the manufacturers instructions. The selective agents were cycloserine at a concentration of 25μg/ml and cefoxitin at 8μg/ml (Oxoid).

2.1.3 Cycloserine-Cefoxitine Fructose Agar

Cycloserine-Cefoxitine Fructose Agar (CCFA) was obtained from Oxoid (Basingstoke, UK) and prepared according to the manufacturers' instructions plus 6% horse blood. The selective agents were cycloserine at a concentration of 25μg/ml and cefoxitin at 8μg/ml (Oxoid).

2.1.4 Robertson Cooked Meat Media

Robertson Cooked Meat Media (RCM) was obtained from Oxoid (Basingstoke, UK) and prepared with 25ml of Fastidious Anaerobe Broth according to the manufacturers' instructions.

2.1.5 Fastidious Anaerobe Broth

Fastidious Anaerobe Broth was obtained from Lab M International Diagnostics Group (Bury, UK) and prepared according to the manufacturers instructions.

2.1.6 Brucella Agar

Brucella agar was obtained from Acumedia (Baltimore, USA) prepared according to the manufacturers instructions.

2.1.7 Nutrient Agar was obtained from Lab M International Diagnostics Group (Bury, UK) and prepared according to the manufacturers instructions.

2.2 Culture of stool and isolation of *C. difficile*

Stool/rectal swabs were inoculated onto various selective (CCEYA, CCFA) and enriched media (RCM) within 20 min of receipt in the laboratory. The process was carried out inside an anaerobic cabinet (Ruskinn Tech. Ltd, Guiseley, Leeds, UK) and incubation was for 48 h at 37°C in the presence of CO_2 5%, H_2 5% and N_2 90%. 500µl of the FAB broth culture from the RCM was heated for 10 min at 80°C (Brazier, 1995). Then, CCEYA and CCFA plates were inoculated with one loop of the heated broth and incubated for 48 h anaerobically in the anaerobic cabinet.

Figure 2.1. Stool culture during the first part of the study

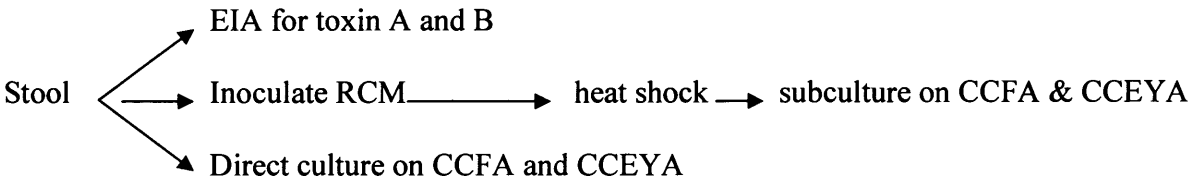
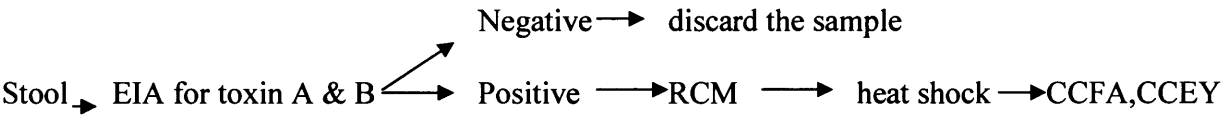


Figure 2.2. Stool culture during the second part of the study



2.3 Identification of *C. difficile*

Colonies of putative *C. difficile* usually fluoresce a yellow-green colour under long-wave ultraviolet light illuminator. Therefore, suspected colonies on CCEYA or CCFA were exposed to long-wave UV light (565nm). Representative colonies with characteristic smell, which fluoresced yellowish-green under UV light were identified by API 20A (bioMerieux, SA, France). All isolates were further confirmed as *C. difficile* at the Anaerobe Reference Laboratory, University Hospital of Wales, Cardiff, UK.

2.4 Toxin A and B detection

All strains were tested for toxin A/B production by the *C. difficile* TOX-A/B test, ELISA, for rapid detection of toxin A and B (TechLab, VPI Research Park, Blacksburg, Va., USA). The procedure was carried out according to manufacturers' instructions in package insert.

2.5 PCR ribotyping method

2.5.1 DNA extraction

All isolates were typed by the polymerase chain reaction (PCR) ribotyping technique previously described by O'Neill *et al.*, (1996). After obtaining a pure culture, a single colony was further subcultured on FAA supplemented with 6% horse blood and incubated for 24 h at 37°C in the anaerobic cabinet. DNA was extracted from a suspension of 10-12 colonies in 100 µl of 5% Chelex 100 (BioRad Laboratories, USA) by heating at 100°C for 10min. The cell suspensions were centrifuged for 10 min at 17000g to remove the cell debris and the supernatant was used as DNA template.

2.5.2. Polymerase Chain Reaction

PCR was performed with the following primers: P3 (CTGGGGTGAAGTCGTAACAAGG) and P4 (GCGCCCTTTGTAGCTTTGACC). Amplification was performed in a final volume of 100µl. The reaction mixture contained the following: 1.5mM $MgCl_2$, 10mM Tris HCl (pH 9), 50mM KCl, 0.1% Triton X-100, 2.5U taq polymerase, 200mM of each dNTP, 50pmol of each primer and 10µl of DNA template. The PCR programme was 35 cycles of denaturation at 95°C

for 1 min, annealing at 56°C for 1 min and extension at 72°C for 2 min. A positive control (*C. difficile* type 001 NCTC 11209) and negative control (sterile water) were included in each run.

2.5.3 Agarose gel electrophoresis:

The product was separated by electrophoresis in 3% metaphor agarose (FMC, Rockland, ME, USA) at 200 V for 3 h. DNA fragments were visualised by staining in 0.5μg/ml ethidium bromide. Gel data were analysed with GelCompar software (Applied Maths, Kortrijk, Belgium). The results were then compared with the library of PCR ribotypes already established at the Anaerobe Reference Unit in Cardiff, Wales, UK.

2.6 Determination of Minimum Inhibitory Concentration (MIC) by E test method

For MIC determination by E test, growth from a 48 h Fastidious Anaerobe Agar (FAA) culture suspended in Fastidiuos Anaerobe Broth (FAB) and adjusted to 1.0 McFarland turbidity standard was inoculated onto pre-reduced Brucella agar (Oxoid) plates supplemented with 5% horse blood, haemin 5μg/ml and menadione 1μg/ml. The E test strips of the antibiotic was applied onto the agar surface, after drying for 15 min, and then incubated in Anoxomat Anaerobic jars (MART) for 48 h at 37°C in an atmosphere of CO_2 10%, H_2 10%, N_2 80%. The MIC was read as the interception of the elliptical zone of inhibition. *C. difficile,* ATCC 9689 and ATCC 17857, and *C. perfringens* ATCC 13124, were included in each run as controls for antibiotic potency and media performance.

CHAPTER 3

NOSOCOMIAL ACQUISITION OF *CLOSTRIDIUM DIFFICILE* IN THE ICU SETTING

3.1 Introduction

The prevalence of *C. difficile* spores in the environment is relatively high in hospitals and long term facilities (McFarland *et al.*, 1989). For example the rate of colonization among hospitalized patients are 10-25% and 4-20% among residents in long term care facilities compared to 2-3% for healthy individuals in the general population (Bartlett, 1994; Simor *et al.*, 2002). Very few studies have looked at the rate of acquisition of *C. difficile* in an ICU setting and there is certainly no reported study on the prevalence of *C. difficile* in hospitalized patients neither in Kuwait nor in the entire Gulf state countries. This study was designed to determine the rate of nosocomial acquisition and the prevalence of *C. difficile* in the ICU patients admitted to the 4 main teaching hospitals in Kuwait. No reported study exists that has carried out this type of investigation in the Middle East.

3.2 Materials and Methods

3.2.1 Patients and samples

Consequative patients admitted to the four ICUs were examined for *C. difficile* infection. Freshly passed stool (rectal swabs, in Amies transport medium, if collection of stool was not possible) was taken from each patient for culture on the day of admission, 3 days post-admission and weekly thereafter until the patient was discharged or until diarrhoea secondary to *C. difficile*

developed. General demographic data including name, hospital number, room and bed location, age, sex, underlying disease, medications (antibiotics), nasogastric tube insertion, previous hospital admissions and length of hospital stay, were all obtained after informed consent mostly from the relatives.

3.2.2. Other samples

3.2.2.1 Environmental samples

A sterile swab pre-moistened with sterile normal saline was wiped over selected surfaces in the units e.g. bed sheets, mattress, bed edges, bed ledges, surface of the side table next to the patients, suction regulator, oxygen regulator, ventilator surfaces, IV stand and the floor under the bed. The samples were then inoculated into Robertson Cooked Meat (RCM; Oxoid, Basingstoke, UK) medium containing 25ml of Fastidious Anaerobe Broth (FAB; Lab M, Bury, UK) and onto Cycloserine-Cefoxitin Egg-Yolk Agar (CCEYA; Oxoid) and Cyclserine-Cefoxitin Fructose Agar (CCFA).

3.2.2.2. Hospital personnel

A sample group of doctors, nurses and physiotherapists caring for the patients with positive *C. difficile* cultures were investigated before and after contact with the patients. Their samples were taken by making contact with hand-surface imprint and fingernail impressions on selective CCEYA.

3.2.3 Transportation of the specimens

3.2.3.1 Stool samples

All stool/rectal swab samples were transported immediately, without any delay, to the Anaerobe Research Laboratory, Department of Microbiology in the Faculty of Medicine of Kuwait University, where the work was done. Samples from Amiri hospital, Ibn Sina hospital and Kuwait Cancer Control Centre (KCCC; about 16 km away) were collected personally or through a designated porter. When samples could not be sent immediately, they were kept in the refrigerator or frozen and then sent within the next 24-48 h. This method has been shown to preserve the spores in the stool samples (Brazier, 1995).

3.2.3.2 Biopsy specimen

Rectal/colonic biopsies were taken when necessary and sent in formalin immediately to the Department of Pathology where histopathological studies were carried out.

3.2.4 Culture media

Selective and non-selective media were used for the isolation of *C. difficile.* All samples were cultured on selective and non selective *C. difficile* media (Chapter 2.1).

Non-clostridial causes of diarrhoea were investigated using the following media: MacConkey agar (Oxoid), Salmonella Shigella agar (SS agar; Oxoid), Xylose Lysine Deoxycholate agar (XLD agar; Oxoid), Selenite F broth (Difco Laboratories, Detroit MI, USA), urease broth

(Becton Dickinson Microbiology System, Cockeysville, MD, USA) and Triple Sugar Iron slants (TSI slants; Difco).

3.2.5 Inoculation and isolation

All stool/rectal swabs were cultured for *C. difficile* on CCFA and CCEYA (Chapter 2 section 2.2; Figure 2.1) and on media for Salmonella and Shigella. Environmental and personnel samples were onto *C. difficile*-selective media only. In addition, the stool/rectal swabs were inoculated onto MacConkey agar and into Selenite F broth, and then incubated aerobically for 24 h at 37°C. After incubation, the Selenite F broth was subcultured onto Salmonella and Shigella agar (SSA; Oxoid) and XLD agar and incubated aerobically for 24 h. Non-lactose fermenting colonies with, or without, black centre were subcultured onto urea broth and TSI slants. Any suspected colonies on the MacConkey, XLD, and SSA were selected for full identification.

3.2.6 Identification

Any suspected colonies on the CCEYA or CCFA plates were selected for full identification (see chapter 2.3).

3.2.7 Toxin detection

All stool samples and the isolated strains were tested for toxin A/B production by the *C. difficile* TOX-A/B test, ELISA, for rapid detection of toxin A and B (section 2.4).

3.2.8 Statistical analysis

Statistical analysis was done by $\chi 2$ statistical test for univariate comparisons of proportions using the SPSS statistical package for windows and Z test for proportion.

3.3 Results

3.3.1 Patients and samples

A total of 430, 63, 88, and 341 consecutive patients admitted to the ICU of Mubarak Al Kabeer Hospital (ICU-1), Ibn Sina Burn Unit (ICU-2), Haematology wards of KCCC (ICU-3) and ICU of Amiri Hospital (ICU-4), respectively, who stayed in the ICUs for a minimum period of 3 days, were studied over a period one year (February 2001 – January 2002). The 4 hospitals represent the 3 points of a triangle with Ibn Sina and KCCC (about 100m apart) at the apex and Mubarak and Amiri hospitals at the bases, about 16 km apart in equidistant directions. The hospitals serve as university teaching hospitals and are 500, 300, 200 and 300-bedded, respectively.

3.3.2 Prevalence of nosocomial acquisition of *C. difficile*

A total of 922 patients were admitted into the four units over a period of one year. *C. difficile* was not isolated from any patients on admission. The overall prevalence rate of nosocomially acquired culture-positive *C. difficile* in the 4 hospitals was 95/922 (10.3%). The patient's characteristics are given in Table 3.1. The median number of days in the units was 11 days (range 4-147 days), with 73.5% for 4-10 days, 16% for 11-20 days, 3.5% for 21-30 days, 1.7%

for 31-40 days and 5.3% for more than 40 days. Analysis by each hospital ICUs showed that 44 (10.2%) out of 430 patients were positive in Mubarak Al-Kabeer Hospital ICU (ICU-1), 4 (6.3 %) out of 63 patients in Ibn Sina Hospital ICU (ICU-2), 16 (18.2%) out of 88 in KCCC ICU (ICU-3) and 31 (9.1%) out of 341 in Amiri Hospital ICU (ICU-4). There was no significant difference between acquisition rate in the four ICUs except between ICU-3 and ICU-4 where the acquisition was significantly higher in ICU-3 ($p < 0.013$ by z test for proportion). This may be related to the small number of positive patients in ICU-2. Out of the total of 95, 30 (32%) patients developed diarrhoea attributable only to *C. difficile* and the other 65 (68%) patients were symptom-free. The burn unit, ICU-2, deals with adults and children. However, the positive four patients were children whose age range between 0.7-2 years with a median of 1.5years. None of the adults had positive stool culture.

Table 3.1. General characteristics of the 95 hospital-acquired *C. difficile* culture-positive patients

Characteristics	Culture Positive patients			
	ICU-1 (No. = 44)	ICU-2 (No. = 4)	ICU-3 (No. = 16)	ICU-4 (No. = 31)
Age in years				
Range	0.3-73	0.7-2	16-64	0.3-84
Median	51	1.5	31	59
Days in the units(median)	14	6	47	6
Days before culture + ve (median)	6	5	22	4
No. of patients on antibiotics	41 (93.2)	4 (100)	16 (100)	21 (67.8)
No. of pts on multiple antibiotics	20 (45.5)	4 (100)	16 (100)	17 (54.8)
Underlying disease:				
Cardiovascular	17 (38.6)	0	5 (31.3)	19 (61.3)
Diabetes mellitus	6 (13.6)	0	3 (18.8)	12 (38.7)
Liver disease	9 (20.5)	0	2 (12.5)	2 (6.5)
Head trauma	12 (27.3)	0	0	0
Malignancy	3 (6.8)	0	16 (100)	5 (16.1)
COPD	11 (25)	0	0	9 (29)
Renal impairment	1 (2.3)	0	0	2 (6.5)
Burn	0	4 (100)	0	0
CNS	4 (9.1)	0	0	0
CVA	7 (15.9)	0	0	2 (6.5)
Sepsis	3 (6.8)	0	0	7 (22.6)
Patients with diarrhoea	18 (40.9)	0	5 (31.3)	7 (22.6)
Patients symptoms-free	26 (59.1)	4 (100)	11 (68.8)	24 (77.4)

Percentage is given in parentheses. CNS: central nervous system; CVA: cerebrovascular accident; COPD: chronic obstructive pulmonary disease. ICU-1: Mubarak hospital; ICU-2: Ibn Sina Hospital; ICU-3: Kuwait Cancer Control Centre (KCCC); ICU-4: Amiri hospital

3.3.3 Time of acquisition

The culture-positive patients stayed in the units for 4-147 days with a median length of stay of 11 days. Seventy (73.7%) of the 95 patients with nosocomial acquisition of *C. difficile* became culture-positive within 4-10 days, 12 (12.6%) within 11-20 days, 6 (6.3%) patients within 21-30days, 2 (2.1%) within 31-40 days and 5 (5.3%) more than 40 days. Over half (51/95; 53.7%) of the nosocomially infected patients acquired the organism within the first week of admission and by the end of the second week; another 20 (21.1%) patients had become positive. The remaining 24 patients acquired the organism after 15 days of admission. Thus, on the basis of time of acquisition and patient characteristics, two arbitrary groups of patients were identified: patients who acquired the organism within 2 weeks of admission in the ICU (designated as early acquisition) and those who acquired it after 2 weeks of admission (late acquisition).

Table 3.2 shows the clinical characteristics of patients with early and late nosocomial acquisition of *C. difficile*. The median ages of both groups were not significantly different ($P>0.08$). Fifty four (76%) and 16 (67%) of the patients in the early and late groups, respectively, were on cephalosporin therapy. Of the 54 and 16, 30 (55.6%) and 2 (12.5%), respectively, were on one class of cephalosporin therapy for one week. Thus, overall, the consumption of antibiotics such as the cephalosporins, metronidazole and meropenem had no observable significant effects on those who acquired the organism in the early or late groups. Comparing patients in the early and late groups for co-morbidity, use of nasogastric tube, endoscopy and surgery, patients in the late group had more severe underlying diseases and higher number of deaths (data not shown). There was no significant difference with insertion of nasogastric tube or surgery ($P>0.05$), although higher number of patients had endoscopy done in the early versus late onset group.

Table 3.2. Salient characteristics associated with the 95 patients with early and late hospital-acquired *C. difficile*

Characteristics	Early acquisition (n = 71)	Late acquisition (n = 24)
Median age, years	46	36
Cephalosporin	54 (76.1)	16 (66.7)
1 week*	30 (42.3)	2 (8.3)
>1 week*	24 (33.8)	14 (58.3)
Metronidazole	12 (16.9)	5 (20.8)
Meropenem	4 (5.6)	2 (8.3)
Erythromycin	7 (9.9)	0
Nasogastric tube	39 (54.9)	16 (66.7)
Endoscopy	9 (17.7)	2 (8.3)
Surgery	20 (28.2)	7 (29.2)

Percent of patients (in parenthesis); * duration of therapy

3.3.4 Prevalence of CDI

3.3.4.1 Case definition

A patient was defined as having CDI when there was diarrhoea with at least one positive *C. difficile* assay (culture and/or toxin A/B assay). Antibiotic-associated diarrhoea (AAD) was defined in this study as passage of six loose motions within 36 h following exposure to antibiotic and in whom other aetiological causes of diarrhoea had been excluded (McFarland *et al.*, 1989).

3.3.4.2 Prevalence of CDI

Of the 95 *C. difficile* culture-positive patients, 30 (31.6%) developed CDI. The characteristics of those patients with CDI and those who were symptom-free are shown in Table 3.3. There was a non-significant difference between the median ages of those with diarrhoea (47.95 years) and symptom-free (29.49 years) ($p>0.05$). All the 30 patients with CDI and 52 (80%) of those who did not have diarrhoea received antibiotics during admission to the ICU. A high proportion (28; 93.3%) of the 30 patients with diarrhoea were infected with toxigenic strains compared with 44 (67.7%) of the 65 symptom-free patients ($p>0.05$). However, *C. difficile* toxin A/B was present in the stool of the same 28 patients with diarrhoea but present in only 7 (10.8%) out of 65 of those who were symptom-free, which is statistically significant ($p<0.001$).

Table 3.3. Important characteristics of all the 95 patients with and without diarrhoea

Characteristics	With diarrhoea (n=30)	Without diarrhoea (n=65)
Age, (median years)	47.95	29.49
Antibiotics	30 (100)	52 (80)
Patients with tox + strains	28 (93.3)	44 (67.7)
Patients with NT strains	2 (6.7)	21 (32.3)
Patients with toxin in stool	28 (93.3)	7 (10.8)

Percentages are given in parenthesis. Tox + strains: toxigenic strains; NT: non toxigenic strains

3.3.5 The prevalence of CDI in each hospital ICU

The distribution of the patients with the three classical presentations of CDI, and toxigenic strains, in each hospital is shown in Table 3.4. Overall, the prevalence of CDI per ICU was 4.2% in ICU-1, 0% in ICU-2, 5.7% in ICU-3 and 2.1% in ICU-4. Analysis of the symptomatic (CDI) patients in each ICU revealed that the highest proportion of symptomatic cases was in ICU-1 (60%) followed at a distance by ICU-4 (23.3%) and ICU-3 (16.7%). No CDI case was seen among the patients in ICU-2. The 2 PMC cases recorded were in ICU-1 while the other forms of CDI were distributed almost evenly in ICUs 1, 3 and 4.

Table 3.4. Distribution of symptomatic *C. difficile*-positive patients by hospital

Characteristics	Number (%) patients in the ICUs				Total
	ICU-1 (n=18)	ICU-2 (n=0)	ICU-3 (n=5)	ICU-4 (n=7)	
Age, years (median)	52.4	0	23.0	52.8	
Pts. on antibiotic therapy	18 (100)	0	5 (100)	7(100)	30
Toxigenic strains^{+ve}	18 (100)	0	4 (80)	6 (85.7)	28
Faecal toxins^{+ve}	18 (100)	0	4 (80)	6 (85.7)	28
Patients with AAD	15 (83.3)	0	4 (80)	5 (71.4)	24
Patients with AAC	1 (5.6)	0	1 (20)	2 (28.6)	4
Patients with PMC	2 (11.1)	0	0	0	2

+ve: positive. AAD: antibiotic-associated diarrhoea; AAC: antibiotic-associated colitis; PMC: pseudomembranous colitis.

3.3.6 Hand carriage among healthcare providers in the unit

The hands of the healthcare workers (doctors, nurses, radiologists and respiratory therapists) in the ICUs were examined for the possibility of hand carriage and thus vehicle of transmission of *C. difficile*. Samples from the hands of 126 healthcare workers were cultured for *C. difficile* before and after exposure to patients whose cultures were positive. However, none were positive.

3.3.7 Environmental isolates

Environmental samples were examined for the possibility of cross-contamination and transmission. Of the 380 samples were taken, only 18 samples were culture-positive; 6 from ICU-1, none from ICU-2, 3 from ICU-3 and 9 from ICU-4.

3.4 Comments

In spite of the growing number of studies devoted to *C. difficile*-related diseases in the western countries, studies on *C. difficile*-associated diseases in the Middle East especially Kuwait are almost non-existent with a few exceptions (Rotimi *et al.*, 2002; Jamal *et al.*, 2002). This may be explained, in part, by the lack of expertise (until recently), technology and facilities for culturing anaerobic pathogens. Besides, the high proportion of asymptomatic carriers of *C. difficile* encountered in our hospitals (Prof VO Rotimi; personal communication) makes the interpretation of a positive culture equivocal. However, in a previous study reported by Akhter *et al.* (Akhter *et al.*, 1994) in nearby Riyadh, Saudi Arabia, *C. difficile* cytotoxin was detected in 9.5% of patients with acute gastroenteritis, suggesting that this organism might be an important enteric pathogen

in the Gulf and Middle East. To support this conjecture, is the fact that *C. difficile* has been isolated from the stool of symptomatic patients in Turkey (Soyletir *et al.*, 1996) and Israel (Rudensky *et al.,* 1993; Rivilin *et al.*, 1998; Boaz *et al.*, 2000).

The overall 10.3% prevalence of acquisition of *C. difficile* amongst patients admitted to the 4 ICUs, whose initial cultures were negative, reported in this study, is similar to the prevalence rate of 9.7% reported by Barbut *et al.*, (Barbut *et al.*, 1996) in various intensive care units in French hospitals but lower than the 30% reported elsewhere by Samore and colleagues in 1994 (Samore *et al.*, 1994b), although in a relatively smaller population of patients than the number investigated in this study. The acquisition rate of *C. difficile* patients in our burn unit ICU was 6.5% and none of them had *C. difficile*-associated diseases, unlike the study by Grube and colleagues, who found a prevalence of 9.8% CDI among 112 critically ill burned patients in their ICU (Grube *et al.,* 1987). Still and his colleagues (Still *et al.*, 2002) evaluated 1753 patients in their burn unit over 3-year period and reported 18 cases (1%) in whom *C. difficile* toxin was detected. A relatively high acquisition rate of 18.2% occurred in our haematology wards of KCCC (ICU-3), an experience much higher than the 2.6% rate reported by Tabaqchali and Wilks in 1992 (Tabaqchali and Wilks, 1992) in the absence of outbreak, and 15% reported by Wroblewska *et al.* (2005), similar to our setting, but lower than the 39.2% reported by Heard *et al.* (1986) in the presence of an outbreak. However, our finding was similar to the 16.6%, reported by Delmee *et al.* (1987).

A finding of toxin A/B positivity in the stool was significantly higher among infected patients with diarrhoea than among asymptomatic carriers. However, there were two cases of CDI from whom non-toxigenic *C. difficile* strains were isolated and who had no toxins in their stools. This may be explained by the relatively lack of sensitivity of the ELISA TOX A/B kits (O'Connor *et*

al., 2001). This is buttressed by the isolation of a toxin A-negative/toxin B-positive strain from a symptomatic patient without evidence of neither the organism nor the toxin in Amiri hospital. This was the only strain of such description that was isolated in this study. Anecdotal reports of previous studies have described such strains in asymptomatic children (Delmee *et al.*, 1988; Depitre *et al.*, 1993). However, in recent years, toxin-variable strains, such as those producing cytotoxin but not enterotoxin, have been described in symptomatic adults and appear to be more prevalent in Japan (Kato *et al.*, 1997), USA (Johnson *et al.*, 2001) and UK (Brazier *et al.*, 1999). In addition, an outbreak involving 16 patients, due to toxin variable *C. difficile,* has been described in Canada (Al-Barrak *et al.*, 1999). At the present time, from an epidemiological perspective, these toxin-variable strains do not constitute a problem in our hospitals.

Environmental contamination and carriage on the hands of the healthcare workers have been documented as playing a significant role in the transfer of *C. difficile* from patient to patient (Mulligan *et al.*, 1979; Kim *et al.*, 1981; Malamou-Ladas *et al.*, 1983; McFarland *et al.*, 1989). McFarland *et al.* (1989) found that carriage of *C. difficile* on the hands of healthcare providers nearly always involved the same type as the patients strain and sometimes occurred even after patient care practices thought ordinarily to pose little risk, e.g. daily patient assessment, physical examination or charting. It is noteworthy that in this present study, we did not recover any *C. difficile* from the hands of healthcare providers. This may reflect the compliance of our nursing and medical staff with hand washing hygiene which is enhanced by continuous eduction, demonstration of hand washing techniques and displaying posters at the clinical areas. Our finding is concordant with the reports of other workers in Israel (Rudensky *et al.*, 1993), in the UK (Malamou-Ladas *et al.*, 1983) and in the USA (Gerding *et al.*, 1986) who did not recover *C. difficile* from the hands of staff members caring for their *C. difficile* culture-positive patients.

The environment around some of the patients in 3 of the 4 ICUs was contaminated with *C. difficile*. The frequency of such contamination correlated with the patient's clinical status. It was highest around patients with diarrhoea, an observation supported by earlier reports (Mulligan *et al.*, 1979; Kim *et al.*, 1981; McFarland *et al.*, 1989). This contamination of the environment from asymptomatic carrier is of special concern because it involves patients whose carrier state usually remain undetected. Thus, these asymptomatic carriers, who are nursed without enteric precautions, constitute credible potential sources of cross-infection and perhaps outbreak of infection. The environmental prevalence of *C. difficile* was low in our ICUs. This may be related to the method for isolation and culturing of the organisms as we used enrichment broth. Other studies (Wilcox *et al.*, 2000b) showed that incorporation of lysozyme (5mg/l) into selective agar significantly increased the recovery of *C. difficile* from the environmental samples probably due to increased germination of dormant spores. In addition, Verity and colleages found that *C. difficile* was recovered significantly more frequently from swabs plated directly onto *C. difficile* selective media containing lysozyme than from enrichment broth (Verity *et al.*, 2001).

None of the conventional enteric pathogens was isolated in this study and our data showed that the main cause of nosocomial diarrhoea among ICU patients in Kuwait is *C. difficile*. This finding is in agreement with several earlier reports from elsewhere (Fan *et al.*, 1993; Rohner *et al.*, 1997). It therefore stands to reason to suggest that, in the western/developed countries, stool culture for common enteric pathogens need not be done for hospitalized patients who had stayed in the hospital for more than three days unless clinically or epidemiologically indicated.

CHAPTER 4

MOLECULAR TYPING OF *CLOSTRIDIUM DIFFICILE* ISOLATES

4.1 Introduction

C. difficile infection has increased dramatically over last decade especially in North America (Eggertson and Sibbald, 2004; Loo *et al.*, 2005), Europe (Kuijper *et al.*, 2008) and Asia (Rupnik *et al.*, 2003; Sawabe *et al.*, 2007). Several outbreaks due to the same genotype or closely related genotypes of *C. difficile* have been reported (Loo *et al.*, 2005; McDonald *et al.*, 2005). Therefore, it is important to trace the emergence and the spread of certain epidemic strains for infection control investigations and epidemiological studies.

This study was designed to determine the molecular relatedness of all the isolates obtained from the nosocomial acquisition of *C. difficile* study as well as the environmental strains, described in chapter 2, using the PCR ribotyping method.

4.2 Materials and Methods

4.2.1 Bacterial isolates

All the 95 clinical as well as the 18 environmental isolates obtained from the previous study described in chapter 3 were used for this study. The isolates, initially kept at -80°C, were lyophilised and shipped to the Anaerobe Reference Laboratory, University of Wales College of Medicine, Cardiff, UK where PCR ribotyping of all the isolates was carried out. The lyophilised

samples were reconstituted with addition of FA broth to the vials and the content inoculated onto a set of FAA plates to which 6% horse blood had been added. The inoculated plates were then incubated in the anaerobic cabinet in the presence of CO_2 10%, H_2 10%, N_2 80%, at 37°C for 48 h.

4.2.2 PCR-ribotyping method

All isolates were subjected to PCR ribotyping technique (section 2.5). The results were then compared with the library of PCR ribotypes already established at the Anaerobe Reference Unit in Cardiff, Wales, UK.

4.3 Results

The distribution of the PCR-ribotypes of the clinical and environmental isolates is outlined in Table 4.1 and Table 4.2, respectively as well as Figure 4.1.

4.3.1 PCR ribotype of the clinical isolates

As shown in Table 4.1, 32 distinct genotypically different DNA ribotypes were established among the 95 clinical isolates. Of these, ribotypes 097 (toxigenic), 078 (toxigenic) and 039 (non-toxigenic) were 3 distinct clones circulating in all the 4 hospitals. The commonest ribotype in Kuwait was 097 with a prevalence of 19%, followed by 039 representing 10.5%; 078, 9.5% and 076, 6.3%. The remaining 52 isolates belonged to diverse DNA ribotypes. In ICU-1, the

Table 4.1. Distribution of PCR-ribotypes and the toxigenic strains of *C. difficile* from patients in the four teaching hospital ICUs

PCR-Ribotypes	Toxin (A/B)	Number of isolates in each hospital ICU				Total (n=95)
		Mubarak (n=44)	Ibn Sina (n=4)	KCCC (n=16)	Amiri (n=31)	
002	+	0	1	1	0	2
010	-	3	0	0	0	3
012	+	2	0	0	0	2
013	+	1	0	0	0	1
014	+	1	0	1	1	3
017	-/+	0	0	0	1	1
020	+	1	0	0	0	1
026	-	1	0	0	0	1
029	+	1	0	1	0	2
035	-	1	0	0	0	1
039	-	6	1	1	2	10
045	+	0	0	0	2	2
046	+	1	0	0	0	1
051	+	0	0	3	0	3
054	+	1	0	0	0	1
056	+	2	0	0	3	5
064	+	1	0	0	0	1
070	+	0	0	1	0	1
076	+	3	0	0	3	6
077	+	0	0	3	0	3
078	+	4	1	2	2	9
081	+	1	0	0	0	1
094	+	2	0	1	0	3
097	+	3	1	1	13	18
098	+	0	0	0	2	2
105	+	2	0	0	0	2
113	-	0	0	1	0	1
128	-	5	0	0	0	5
129	+	1	0	0	0	1
131	+	1	0	0	0	1
140	-	0	0	0	1	1
141	-	0	0	0	1	1

ribotypes associated with diarrhoea in 18 symptomatic patients were ribotypes 078 (4, 22%), 076 (3, 17%), 012 (2, 11%), 056 (2, 11%), 094 (2, 11%), 097 (2, 11%), 105 (2, 11%) and 046 (1, 6%). Those associated with diarrhoea in ICU-3 were ribotypes 077 (2), 014 (1), 097 (1) and 113 (1), while those associated with diarrhoea in ICU-4 were ribotypes 017 (1), 039 (1) and 097 (5). Even though 078 and 097 were 2 of the 4 ribotypes found in the ICU-2, they were not associated with any symptoms.

All symptomatic patients with toxigenic ribotypes had detectable toxins in their stools, except 2 patients colonised with non-toxigenic ribotypes 039 and 113 who demonstrated no detectable level of toxin in their stools by the TOX A/B ELISA assay. The two PMC cases in ICU-1 were infected with ribotype 078. One AAC case in each of ICU-1 and ICU-3 were infected with ribotypes 046 and 097, respectively. Two AAC cases in ICU-4 were infected with ribotype 097.

4.3.2 PCR ribotype of the environmental isolates

As demonstrated in Table 4.2, the 18 environmental isolates were assigned to 9 genotypically distinct ribotypes (6 toxigenic and 3 non-toxigenic); the predominant types were 010 (3), 078(3) and 097 (4). Ribotypes 078 and 097 were isolated primarily from the environment of symptomatic patients infected with the same ribotype in ICU-1 and ICU-4, respectively. Ribotype 010 was isolated from the environment of asymptomatic patients in ICU-1. Ribotype 105 associated with diarrhoea in ICU-1 was absent in its environment but present in the environment of ICU-3 and ICU-4 where it did not contribute to disease process. Ribotypes 001 and 144 were present in the environment of ICU-4 but were not isolated from any patient in the

unit. Similarly, ribotypes 120 and 125 were isolated from the floor of ICU-3 but were not isolated from the patients in this unit.

Table 4.2. Distribution of the PCR-ribotypes and the toxigenic strains of *C. difficile* in the environment of the 4 teaching hospital ICUs

PCR-Ribotypes	Toxin (A/B)	Number of isolations in each hospital				Total (n = 18)
		ICU-1 (n=6)	ICU-2 (n=0)	ICU-3 (n=3)	ICU-4 (n=9)	
001	+	0	0	0	1	1
010	-	3	0	0	0	3
056	+	0	0	0	2	2
078	+	3	0	0	0	3
097	+	0	0	0	4	4
105	+	0	0	1	1	2
120	+	0	0	1	0	1
125	-	0	0	1	0	1
144	-	0	0	0	1	1

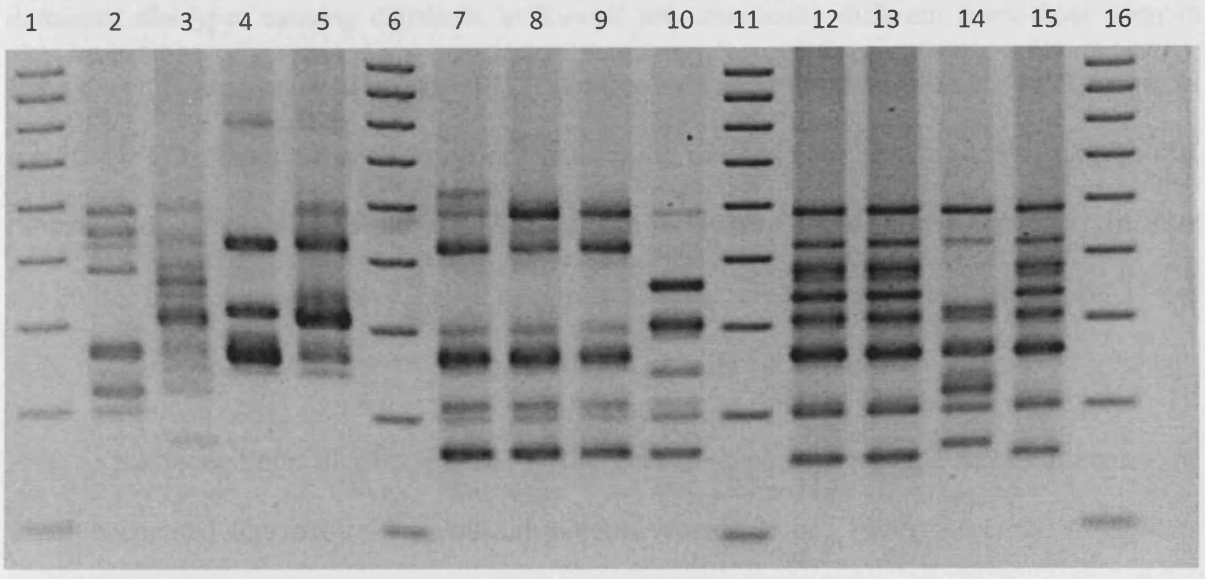


Figure 4.1. PCR ribotype profiles of *Clostridium difficile* isolated in Kuwait from 2001 to 2002. Lanes 1,6,11,16 = 100bp; Lane 2 = 139; Lane 3 = 140; Lane 4 = 14; Lane 5 = 070; Lane 7 = 055; Lane 8 = 001; Lane 9 = reference strain NCTC 11209 (ribotype 001); Lane 10 = 017; Lane 12 = 097; Lane 13 =097; Lane 14 = 056; Lane 15 = 097.

4.4 Comments

The ribotyping of isolates from all patients in the 4 ICUs revealed some interesting epidemiological findings. Our data showed that the 95 culture-positive patients harboured 32 different highly diverse PCR-ribotypes of *C. difficile*. Three major different DNA clones (PCR-ribotypes 097, 078 and 039) were detected among the patients in all hospitals. All other isolates were assigned to 29 distinct and diverse types. Ribotype 097 was the single most prevalent type and was responsible for about 19% of the CDI while ribotype 078 was responsible for about

9.5% of the CDI. Thus, ribotypes 097 and 078 were responsible for over one third of the cases of CDI seen. For full epidemiological point of view, this is an interesting finding in that the dominant ribotypes causing diarrhoea in Kuwait are completely different from those seen in Europe as it is demonstrated in Table 4.3 (Martirosian *et al.*, 1995; Stubbs *et al.*, 1999; Urban *et al.*, 2001). Fifty five percent of infections seen in the UK hospitals are caused by ribotype 001 (Stubbs *et al.*, 1999), while ribotype 087 accounted for 39% of all isolates in Hungary (Urban *et al.*, 2001). In another study in a Polish maternity hospital, all environmental isolates and 11 out of 31 neonatal isolates belonged to ribotype 001 (Martirosian *et al.*, 1995).

Prior to the recognition of ribotype 027 in the UK, ribotype 001 was the most common type which accounted for 55% of hospitalized patients (Stubbs *et al.*, 1999). Recently, *C. difficile* ribotype 106 has become the predominant strain in England, accounting for 26% while ribotypes 027 and 001 account for 25% each (HPA, 2006). However, it appears that the epidemiology of prevalent ribotypes continues to change rapidly in the UK, with ribotype 027 assuming a higher proportion (Figure 4.2). A more recent report in England has demonstrated that of 2,084 *C. difficile* isolates, 42% were ribotype 027, 19% were ribotype 106 and only 10% as ribotype 001 (Kuijper *et al.*, 2008). In Scotland, ribotype 106 accounts for 55% of all isolates while type 001 for 21%; ribotype 078 has emerged in 4 isolates. Presently, in Ireland, the commonest ribotypes are 001 (35%), 106 (11.6%) followed by type 078 (8.3%) (Kuijper *et al.*, 2008). In a recent outbreak in the Netherlands, 25.3% of patients with CDI were due to type 027 (Goorhuis *et al.*, 2007). In all these countries the predominant ribotypes are different from the ribotypes present in our hospitals. Even in these hospitals the predominant ribotypes differ. In Mubarak hospital, ribotype 078 was predominant while ribotype 097 was the commonest in Amiri hospital. Ribotype 078 is an interesting finding in that this ribotype is mostly associated with animal

origin in the UK and Netherlands (Goohuis *et al.*, 2007). All the patients from whom this strain was isolated had no history of animal contact. Neither ribotype 001 nor the hypervirulent 027 was detected in this series in our hospitals. The single ribotype 001 found in our study was from the environment in Amiri hospital. The absence of these strains in Kuwait may explain the absence of outbreak situations in our country.

Table 4.3. Distribution of various *C. difficile* ribotypes in different countries

Country	Percentages of ribotypes	Reference
Hungry	39%, ribotype 087; 20% for 012; 12.3% for 001	Urban *et al.*, 2001
Poland	35.5%, ribotype 001	Martirosian *et al.*, 1995
Scotland	55%, ribotype106; 21% ribotype 001	Kuijper *et al.*, 2008
Ireland	35%, ribotype 001; 11.6% for 106; 7.8% for 078	Kuijper *et al* 2008
Netherland	25.3%, ribotype 027	Goorhuis *et al.*, 2007
England	55%, ribotype 001	Stubbs *et al.*, 1999
England	26% for 106; 25% for 001, 25% 027	HPA, 2006
England	42% for 027; 19% for 106; 10% for 001	Kuijper *et al.*, 2008

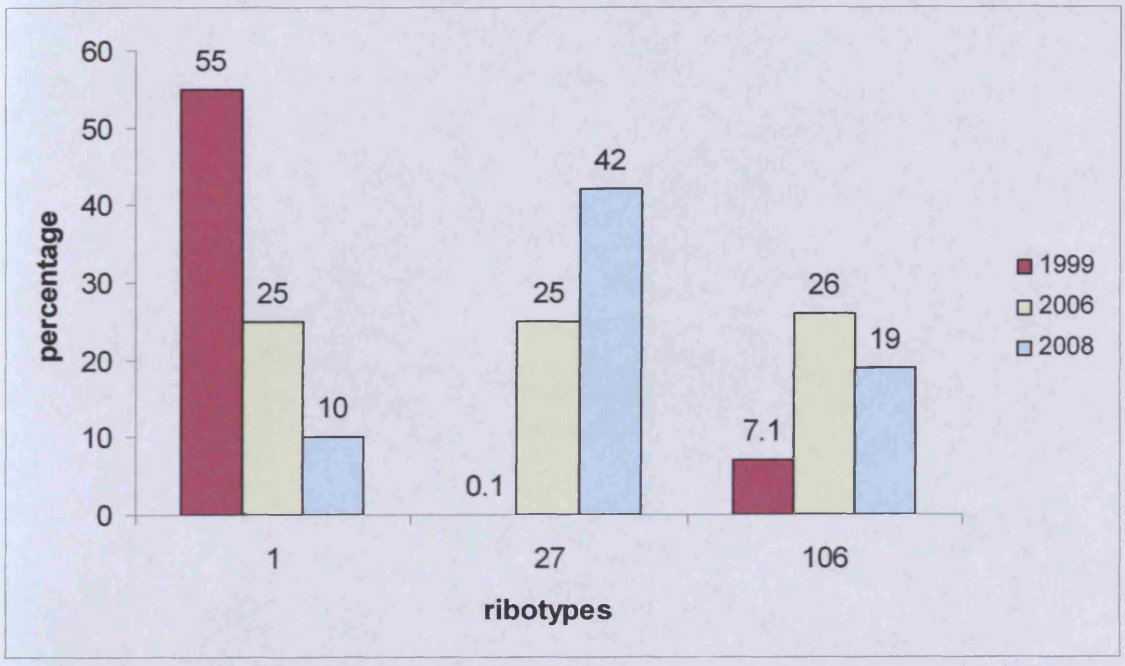


Figure 4.2. Distribution of the three different *C. difficile* ribotypes over time in England (Stubbs *et al.*, 1999; HPA, 2006; Kuijper *et al.*, 2008)

The environmental strains were heterogeneous in each hospital. The 18 isolates were assigned to 9 different ribotypes. The environment of 2 patients each in ICU-1 and ICU-4 was contaminated by the same ribotype as in the patient clinical samples. Thus, confirming that the patient's environment is a potential source of *C. difficile* and cross-contamination may contribute to the acquisition of nosocomial CDI (Cohen *et al.*, 2000b). In some cases there was no correlation between environmental and patient's isolates. For instance, the environmental isolates in ICU-3 and some in ICU-4 differed from those in the patients. Cohen *et al.* (1997) also found none of the environmental *C. difficile* types among the isolates from the patients nor did Simore *et al.*, (1993) in a survey of *C. difficile* infection in a long-term care facility found any cross-transmission in

their institution. In conclusion, *C. difficile* isolates in Kuwait are of different ribotypes from those circulating in the UK or Europe.

CHAPTER 5

ANTIBIOTIC SUSCEPTIBILITY PATTERN OF CLINICAL AND ENVIRONMENTAL *CLOSTRIDIUM DIFFICILE* ISOLATES

5.1 Introduction

Metronidazole is currently the first choice to treat CDI due to its lower potential for selection of vancomycin-resistant enterococci (VRE) and its approval by several health institutions, like HPA and IDSA, and for economical reasons. The role of antimicrobial susceptibility testing of anaerobes including *Clostridium difficile* has been questioned in the past decade. Several diagnostic microbiology laboratories do not perform antimicrobial susceptibility testing for anaerobes in general, and specifically for *C. difficile*, as the main method of the diagnosis in routine diagnostic laboratory is dependent on toxin detection rather than culture. In addition, anaerobes have predictable susceptibility pattern to most antibiotics with anti-anaerobic activity as well as lack of simple method for testing and are bedevilled with delay in getting pure culture. On several occasions, there is little correlation between clinical outcome and the antimicrobial susceptibility testing. In Kuwait routine antibiotic susceptibility testing of anaerobes in the clinical laboratories is not usually done. However, resistance of anaerobes to anti-anaerobic drugs is on the increase, even to drugs with excellent anti-anaerobic activity like metronidazole. Recently, *C. difficile* resistant to metronidazole (Pelaez *et al.*, 1997; Brazier *et al.*, 1999; Pelaez *et al.*, 2002a; Bishara *et al.*, 2006) and intermediate susceptibility to vancomycin (Pelaez *et al.*, 2002a) have been reported. In addition, earlier in 2005, treatment failure or poor outcome with metronidazole treatment for CDI was reported by Musher *et al.*, (2005).

Empirical therapy of anaerobic infections is mainly based on data derived from periodic surveillance studies, which are not always up-to-date. These data also suffer from great

variations reported over time and from different geographical area. Reports on the susceptibility of *C. difficile* to a variety of antibiotics are scarce and far between. This study was designed to determine the antibiotic susceptibility pattern of the clinical as well as the environmental isolates of *C. difficile* obtained in the two periods of this project.

5.2 Materials and Methods

5.2.1 Bacterial isolates

A total of 151 *C. difficile* isolates, comprising the 95 clinical and 18 environmental isolates obtained during the first phase of this study, February 2001- January 2002, described in chapter 2 and the 38 clinical isolates obtained in the second phase from January 2003 – December 2005, described in chapter 5, were included in this study. They were stored at -80°C and subcultured twice on Fastidious Anaerobe agar (FAA; Lab M) before use to ensure purity.

5.2.2 Antibiotic susceptibility testing (AST)

The AST was performed to determine the minimum inhibitory concentrations (MICs) of 16 antibiotics against the clinical and environmental isolates using the E test (AB Biodisk, Solna, Sweden) and agar dilution methods. The following antibiotics were tested by the E test according to the manufacturer's instruction: amoxicillin-clavulanic acid, ampicillin, cefotaxime, cefoxitin, cefuroxime, clindamycin, imipenem, linezolid, meropenem, metronidazole, penicillin, piperacillin, piperacillin-tazobactam, teicoplanin and vancomycin. For MIC determination by E test, see chapter 2.6.

Trovafloxacin (Pfizer, Inc., Groton, Conn., USA) susceptibility was determined by the agar dilution method recommended by the National Committee for Clinical Laboratory Standards (NCCLS, 2001). Briefly, serial two-fold dilutions of trovafloxacin were incorporated into FAA, supplemented with 5% horse blood, to give final antibiotic concentrations of 0.03-256 μg/ml. An inoculum was prepared in Fastidious Anaerobe Broth (FAB) from a 48-h FAA culture. The suspension was adjusted to 1.0 McFarland turbidity standard. Then the inoculum was applied onto the surface of pre-reduced antibiotic-containing FAA plate supplemented with 5% horse blood, with a 35-prong Steers applicator that delivered 10 μl (10^5cfu/ml) per spot. The plates were then incubated in Anoxomat Anaerobic jar system (Model WS800: MART Microbiology BV, Lichtenvoorde, Netherlands) in an atmosphere of CO_2 10%, H_2 10%, N_2 80%, for 48 h at 37°C. Anaerobiosis was checked by inclusion of a nutrient agar plate culture of *Pseudomonas aeruginosa* and a chemical indicator (Oxoid). After incubation, the MIC was recorded as the lowest concentration of each antibiotic that inhibited visible growth of the organism.

C. difficile, ATCC 9689 and ATCC 17857, and *C. perfringens* ATCC 13124, were included in each run as controls.

5.2.3 Statistical method

Fisher's exact test was used to test proportion for the respective variables. The probability level of <0.05 was considered as significant.

5.3 Results

5.3.1 The antimicrobial susceptibility of the isolates

The MICs of the 16 antibiotics tested, as shown in Figures 5.1, 5.3 and 5.5 (Appendix Tables 5.3.1, 5.3.2 and 5.3.3), are presented as the concentrations that killed 50% (MIC_{50}), 90% (MIC_{90}) of the isolates and the percentage of resistant isolates in Figure 5.2, Figure 5.4 and Figure 5.6.

5.3.2 Clinical isolates (2001-2002)

This data is summarized in Figure 5.1 and Figure 5.2 where the percentage of susceptibility is demonstrated as opposed to percentage of resistance (Appendix; Table 5.3.1). As shown in Figure 5.1 (Appendix; Table 5.3.1), among the 95 isolates tested in the first part of the study, amoxicillin-clavulanic acid, ampicillin, linezolid, meropenem, metronidazole, piperacillin, teicoplanin, trovafloxacin and vancomycin demonstrated excellent in vitro activities against all isolates of *C. difficile* with MIC_{90}s of 0.38, 1.5, 2.0, 1.5, 0.19, 4, 0.25, 4 and 0.75 μg/ml, respectively. There was no statistically significant difference between the susceptibility of toxigenic and non-toxigenic strains. One non-toxigenic strain had decreased susceptibility to vancomycin and teicoplanin with MICs of 3 and 2μg/ml, respectively. Another 2 isolates had decreased sensitivity to vancomycin with MIC of 2 μg/ml but fully susceptible to teicoplanin.

All the isolates were resistant to cefuroxime, 97.5% were resistant to cefoxitin and 92.7% were resistant to cefotaxime (Appendix; Table 5.3.1). Nearly half of the isolates (46/95; 48.4%) were resistant to clindamycin. Of these 46 clindamycin-resistant isolates, 23 (50%) exhibited high-level resistance, MIC >256 μg/ml. The MIC of clindamycin for the remaining 23 isolates ranged

between 6 and 32 μg/ml. Although the MIC_{90} to penicillin was 1.5 μg/ml, two isolates with MICs of 6 and >32 μg/ml, were seen. Of interest, 91.4% and 4.8% of the isolates were resistant to imipenem and meropenem, respectively, with MIC_{90}s of >32 and 1.5 μg/ml, respectively. Eight strains had MIC of imipenem ≤4 μg/ml and one had MIC of 12 μg/ml.

Figure 5.1. MIC_{50} amd MIC_{90} of 95 clinical isolates of *C. difficile* against 16 antibiotics

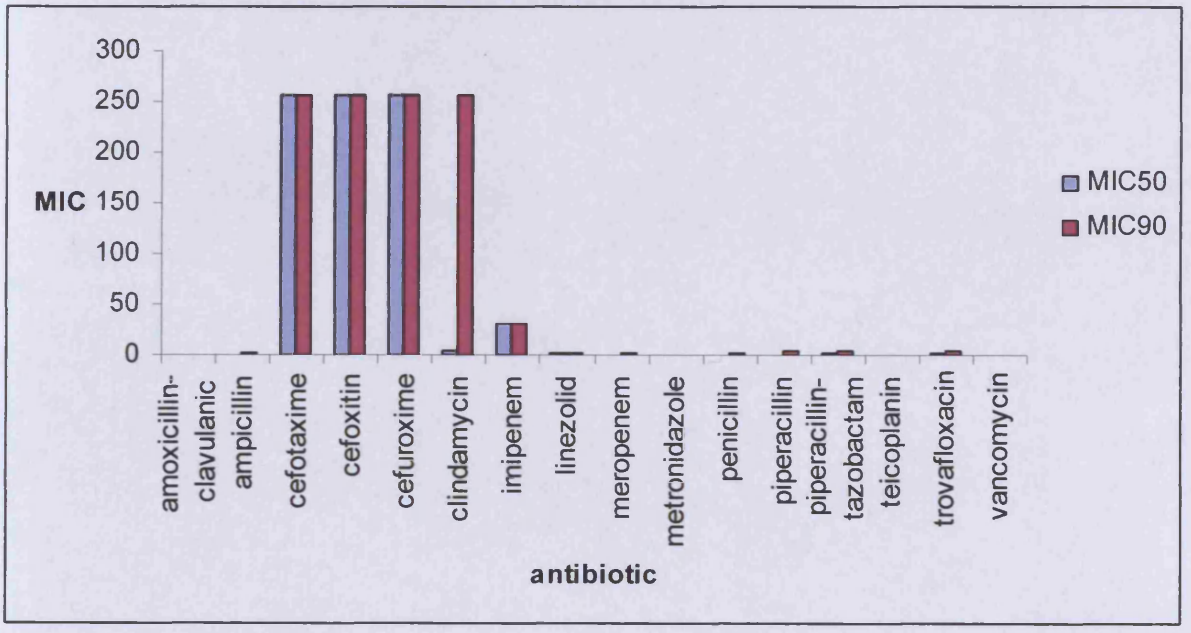
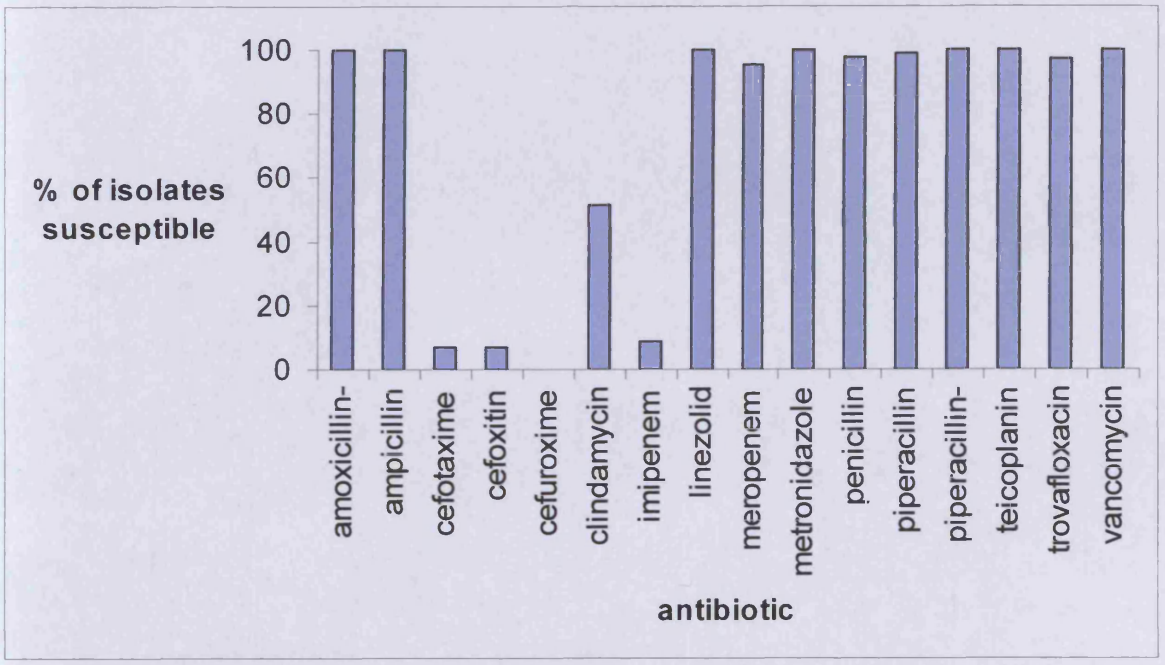


Figure 5.2. Percentage of susceptibility of 95 clinical isolates of *C. difficile* against 16 antibiotics



5.3.3 Environmental isolates (2001-2002)

This data is summarized in Figure 5.3 and Figure 5.4 where the percentage of susceptibility is demonstrated as opposed to percentage of resistance (Appendix; Table 5.3.2). Figure 5.3 and Figure 5.4 (Table 5.3.2; Appendix) show the susceptibility pattern of the environmental isolates. Compared with those of the clinical isolates the environmental strains were relatively less resistant, especially against cefotaxime with MIC_{90} of 83.3% and clindamycin with MIC_{90} of 38.8%. The environmental isolates were more resistant to meropenem with a higher MIC_{90} value of >32 μg/ml and percentage resistant at 50%. All isolates were susceptible to the penicillins, glycopeptides, linezolid, trovafloxacin and piperacillin-tazobactam with MIC_{90}s ranging from 0.19 – 4.0 μg/ml.

Figure 5.3. MIC_{50} and MIC_{90} of 18 environmental isolates of *C. difficile* to 16 antibiotics

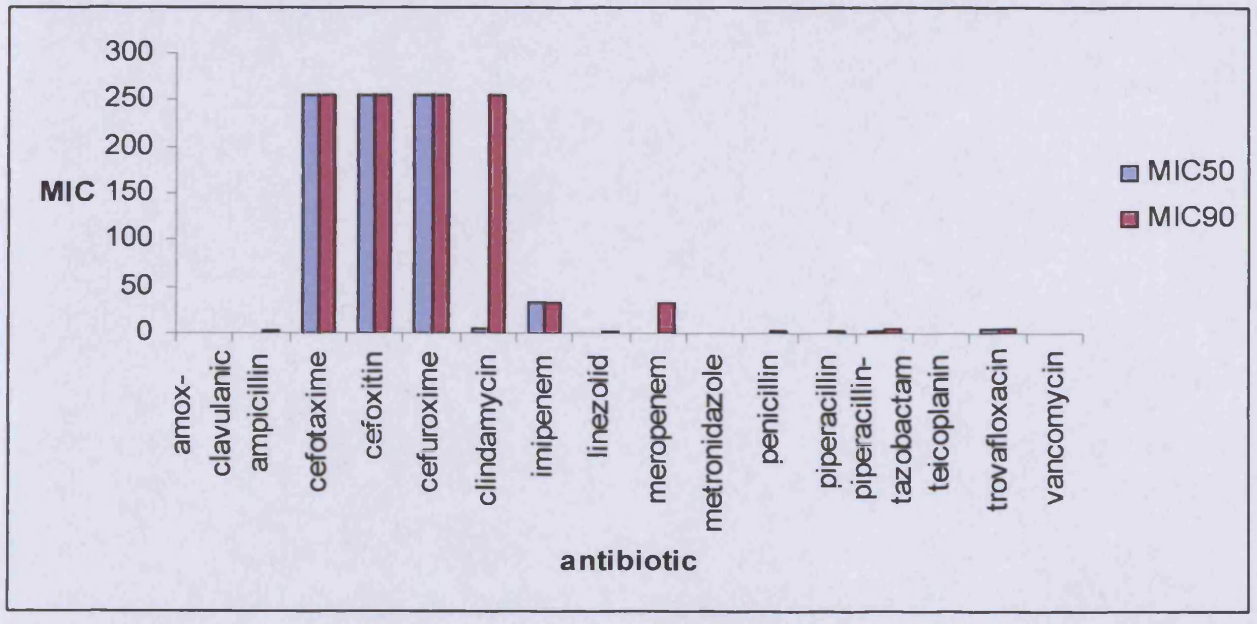
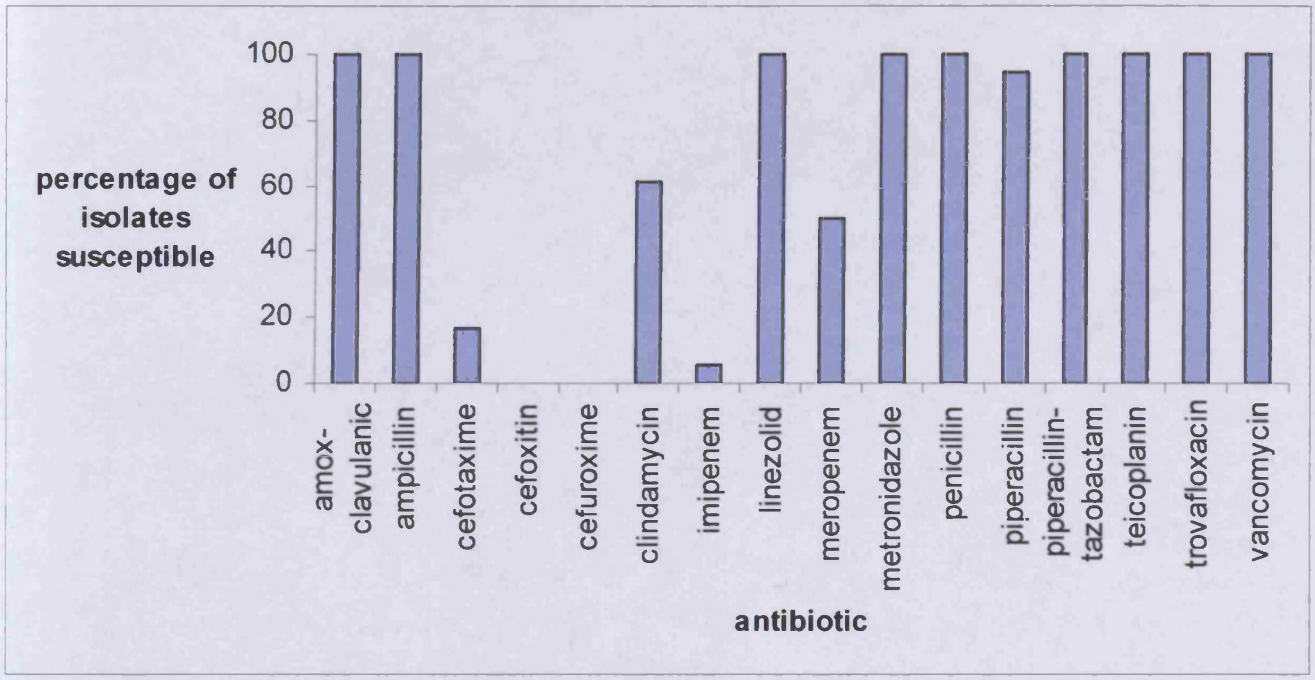


Figure 5.4. Percentage of susceptibility of 18 environmental isolates of *C. difficile* to 16 antibiotics



5.3.4 Clinical isolates (2003-2005)

This data is summarized in Figure 5.5 and Figure 5.6 where the percentage of susceptibility is demonstrated as opposed to percentage of resistance (Appendix; Table 5.3.3). Figure 5.5 and Figure 5.6 (Table 5.3.3; Appendix) show the MIC_{50} and MIC_{90} and percentage resistance to the 16 antibiotics tested. The resistance rates of these latter isolates were similar to those of the earlier years, although resistance rates to meropenem, penicillin and piperacillin were higher at 21.4, 16.6 and 11.9%, respectively. Three (7.9%) of the 38 isolates were resistant trovafloxacin and 33 (86.8%) to imipenem. One toxigenic strain had decreased susceptibility to vancomycin and teicoplanin with MICs of 3 and 2µg/ml, respectively.

Figure 5.5. MIC_{50} and MIC_{90} of 38 clinical isolates of *C. difficile* collected in 2003-2005

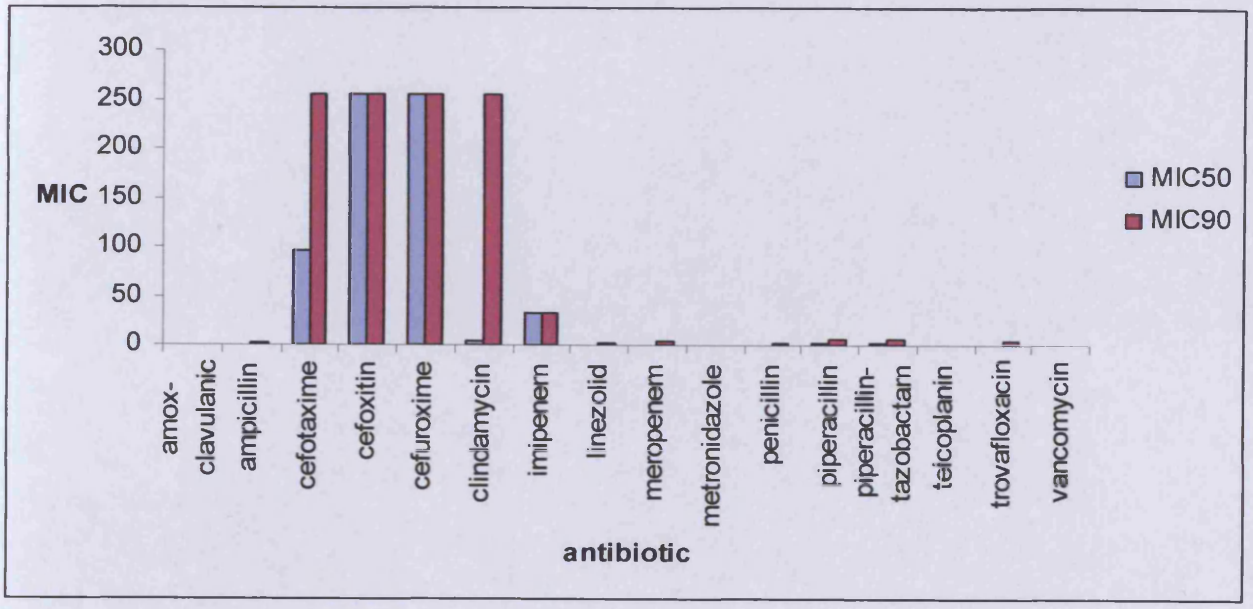
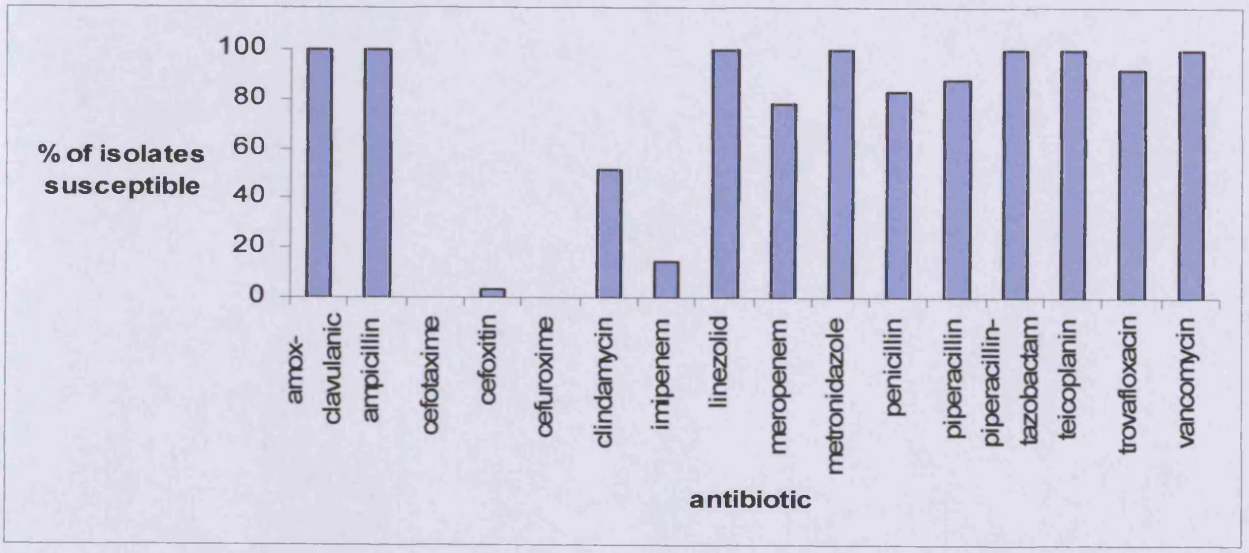


Figure 5.6. Percentage of susceptibility of 38 clinical isolates of *C. difficile* collected in 2003-2005



5.3.5 Prevalence of multi-drug resistant isolates

The prevalence of multi-drug resistant (MDR) clinical isolates of *C. difficile* in 2001-2002 and 2003-2005 was virtually the same and for that reason only the analysis of the isolates in 2001-2002 is demonstrated in Table 5.1, Table 5.2 and Table 5.3.

A total of 74 (77.9%) of the clinical isolates were multiply resistant, i.e. resistance to two or more classes of antibiotics. The analysis of individual isolates in relation to multiple resistance, and toxin or non-toxin production, is shown in Table 5.1. Out of the 74 multi-resistant strains, only one was resistant to 4 antibiotics (cefoxitin, clindamycin, imipenem and trovafloxacin) and one was resistant to cefoxitin, imipenem and trovafloxacin; these 2 strains were toxin-producers. Another 39 (52.7%) were resistant to a different combination of 3 antibiotics (cefoxitin, clindamycin and imipenem); 25 (64.1%) of these were toxigenic and 14 non-toxigenic. Resistance to cefoxitin and imipenem was noted in another 31 (41.9%) strains, of which 25 (80.6%) were toxigenic. The distribution of the multiply resistant clinical isolates by hospital ICUs is shown in Table 5.2. A total of 39 (52.7%) of the multiply resistant strains were from ICU-1 compared with 3 (4.1%) in ICU-2, 14 (18.9%) in ICU-3 and 18 (24.3%) in ICU-4. The ratio of the isolation rates of the multiply resistant toxigenic strains to non-toxigenic strains was, in ICU-1, 1.6:1, ICU-2, 1:1, ICU-3, 13:1 and ICU-4, 3.5:1.

5.3.6 Correlation between MDR strains and CDI

The correlation between the MDR strains and symptomatic or asymptomatic patients is shown in Table 5.3. Twenty two (73.3%) of the 30 symptomatic patients were infected with multiply resistant strains and 54 (83%) of 65 asymptomatic patients were colonised by resistant strains, a difference that did not attain statistical significance, $p>0.05$.

Table 5.1. Antibiotic resistance profile of 74 multiply resistant clinical isolates of *C. difficile*

Resistance groups	Number of isolates		Total number
	Toxigenic (53)	Non-toxigenic (21)	(n = 74)
Fox, clind, imip, trov	1	0	1
Fox, imip, trov	1	0	1
Fox, clind, imip	25	14	39
Fox, trov	0	1	1
Fox, imip	25	6	31
Fox, clind	1	0	1

Fox = cefoxitin, clind = clindamycin, imip = imipenem, trov = trovafloxacin

Table 5.2. Distribution of the 74 multiply resistant clinical isolates of *C. difficile* in the four different intensive therapy units (ICUs)

Resistance group	Number of isolates in:								Total (No.= 74)
	ICU-1		ICU-2		ICU-3		ICU-4		
	T	NT	T	NT	T	NT	T	NT	
Fox, clind, imip, trov	0	0	0	0	0	0	1	0	1
Fox, imip, trov	0	0	0	0	1	0	0	0	1
Fox, clind, imip	17	12	0	0	4	0	4	2	39
Fox, trov	0	0	0	0	0	1	0	0	1
Fox, clind	0	0	1	0	0	0	0	0	1
Fox, imip	7	3	1	1	8	0	9	0	31

T = toxigenic; NT = non-toxigenic; Fox = cefoxitin; clind = clindamycin; imip = imipenem; trov = trovafloxacin

Table 5.3. Resistant profiles of toxigenic *C. difficile* isolated from symptomatic and asymptomatic patients

Resistant groups	No. (%) patients that were	
	Symptomatic (n = 30)	Asymptomatic (n = 65)
Fox, clind, imip, trov	1 (3.3)	0
Fox, imip, trov	0	1 (1.5)
Fox, clind, imip	9 (30)	28 (43)
Fox, trov	0	1 (1.5)
Fox, imip	12 (40)	23 (35.3)
Fox, clind	0	1 (1.5)

Fox = cefoxitin, clind = clindamycin, imip = imipenem, trov = trovafloxacin

5.4 Comments

C. difficile is an important cause of hospital-acquired diarrhoea and PMC which has a mortality rate that ranges from 15-30% (Bartlett 2008). Therefore, it is important to be abreast of the susceptibility pattern of local isolates to the antibiotics that are available for the treatment of disease caused by this organism. Although susceptibility testing of *C. difficile* is not often done and generally not relied upon for clinical-decision making, resistance to anti-anaerobic drugs is gradually emerging.

Highlights form this study includes the result of susceptibility testing of our strains showing that 90% of the strains were inhibited by 0.75μg/ml of vancomycin and 0.25μg/ml of teicoplanin. Although 4 and 2 clinical isolates had reduced susceptibility to vancomycin (2-3μg/ml) and

teicoplanin (2μg/ml), all of them were inhibited by concentrations that did not exceed 3 and 2 μg/ml, respectively. However, these upper limits were more than the concentrations reported in other studies (Bartolini *et al.,* 1990; Biavasco *et al.*, 1991; Barbut *et al.*, 1999; Newsom *et al.*, 1995). Resistance to vancomycin has been reported in Spain by Pelaez *et al.* (1997; 2002a) although all of intermediate resistance. The rise in vancomycin MIC is also noticed in other studies e.g. in Scotland, where all of the *C. difficile* were susceptible to vancomycin but there was increase in the number of *C. difficile* with MIC of 4μg/ml from 2.7% (5/186) of the isolates (Drummond *et al.*, 2003a) to 21.6% (25/116) in another study (Mutlu *et al.*, 2007). Therefore, there is need for constant monitoring of vancomycin susceptibility of *C. difficile* in our hospitals.

Metronidazole was the most active of all the antibiotics. All the isolates were inhibited by a concentration that did not exceed 2μg/ml. This is in contrast to studies that have reported some degree of decreased susceptibility to metronidazole (Pelaez *et al.*, 1998, Brazier *et al.*, 2001) and to the single isolate that was completely resistant to metronidazole with MIC of 64μg/ml and >256μg/ml (Wong *et al.*, 1999; Bishara *et al.*, 2006). These resistant strains appear to be more common in patients with recurrent *C. difficile* diarrhoea (Pelaez *et al.*, 1998). Barbut *et al.* in 1999, described six *C. difficile* clinical isolates with MICs of metronidazole ranging from 8-32μg/ml among 198 isolates recovered in France in 1991 and in 1997. But 5 out of 6 strains were non-toxigenic isolates. Another Spanish study reported metronidazole resistance (MIC, >16μg/ml) in 6.3% of 415 clinical *C. difficile* isolates (Pelaez *et al.*, 2002a). In spite of these reports, the incidence of metronidazole-resistant strains remains very low as was demonstrated in our present study. Recently however, Baines *et al.* (2008) demonstrated the emergence of reduced susceptibility to metronidazole in 24.4% of *C. difficile* ribotype 001 isolates from their institution in Leeds, UK. They used the spiral gradient end-point analysis and the resistance was

further confirmed by agar incorporation method. Although metronidazole and vancomycin are equally effective for the treatment of CDI (Olson *et al.*, 1994; Teasley *et al.*, 1983; Kelly *et al.*, 1994), metronidazole has been the drug of choice in Kuwait for the past one decade. This is primarily because of its good in-vitro susceptibility, cost and risk of emergence of vancomycin-resistant enterococci (VRE). Despite the efficacy of teicoplanin when compared to vancomycin, its high cost prevents its use as the drug of choice.

In this study, susceptibility to the ß-lactam antibiotics was largely predictable and essentially similar to other reports (Chow *et al.*, 1985; Goldstein *et al.*, 1999; Nord 1996; Bourgault *et al.*, 2006). The resistance to penicillin in our study was 2.4%, in the first phase of the study but rose to about 17% in the second phase. A much earlier report in Italy by Panichi *et al.* (1990), similar to ours, found a penicillin resistance rate (14%) much higher than ours in first phase study but about the same in the second phase. A very striking finding was the consistent high resistance rate to imipenem while meropenem demonstrated relatively good activities against the clinical strains in the first phase and seemingly high resistance rate (21%) in the second phase. In general, carbapenems are regarded as alternative drugs of choice after metronidazole for therapy of infections involving anaerobes because of their excellent in-vitro and in-vivo activities. This rather unexpected finding contrasts with the finding of Panichi and colleagues (Panichi *et al.*, 1990) who showed that imipenem had excellent activity against their isolates. This finding deserves detailed investigation at the molecular level which, of course, is currently outside the scope of this project. However, imipenem-resistant *C. difficile* is not entirely unknown as attested to by Jones as well as John and Brazier in Cardiff, UK (Jones 1985; John and Brazier, 2005). The data presented in the study also demonstrated the ineffectiveness of the cephalosporins

against *C. difficile*. All these go to show a particular spectrum of antibiotic resistance that distinguishes this organism from other clostridia.

The resistance rate of over 48% to clindamycin in this study was higher than the one reported by Wongwanich *et al.* (1996) and Bourgault *et al.* (2006), but lower than those reported by Chow *et al.* (1985), Levet (1988), Panichi *et al.* (1990), Nord *et al.* (1993), Barbut *et al.* (1999) and Mutlu *et al.* (2007).

Susceptibility of *C. difficile* to the quinolones has always been poor, especially to the first and second generation agents, such as norfloxacin and ciprofloxacin (Chow *et al.,* 1985; Nord *et al.*, 1993; Bourgault *et al.*, 2006). However, trovafloxacin, a new fluoroquinolones, has good in-vitro activity against most Gram-positive and Gram-negative anaerobes. In this study, the resistance to trovafloxacin by the clinical isolates in both phases of the study was at acceptable levels; none of the environmental strains was resistant. This is in agreement with Wilcox and his colleagues who found that trovafloxacin was highly active against *C. difficile* by MIC determination (Wilcox *et al.,* 2000a). In general, there was no significant difference in the susceptibility of the environmental isolates compared with the clinical isolates except with meropenem to which almost 50% were resistant.

CHAPTER 6

INFLUENCE OF ANTIBIOTIC EXPOSURE ON CELL-BOUND AND CELL-FREE CYTOTOXIN PRODUCTION BY *CLOSTRIDIUM DIFFICILE*

6.1 Introduction

CDI is often associated with antibiotic exposure leading to depletion of normal gut flora and impairment of colonization resistance. Previous studies have shown that subinhibitory concentration of clindamycin and cephradine stimulated enterotoxin production (Honda *et al.*, 1983) while tetracycline did not induce both toxins. In addition, clindamycin and cephradine increase toxin A production (Onderdonk *et al.*, 1979). Recently, it has been demonstrated that exposure of *C. difficile* to sub-lethal concentration of various antibiotics such as vancomycin, metronidazole, amoxicillin, clindamycin, cefoxitin and ceftriaxone has no consistent relationship between growth and toxin A production (Drummond *et al.*, 2003b). Emerson *et al.*, used microarray technology to analyse the transcriptional responses of *C. difficile* 630 strain to environmental shock and to the growth in the presence of subinhibitory concentrations of antibiotics (amoxicillin, clindamycin and metronidazole) (Emerson *et al.*, 2008). Amoxicillin and clindamycin increased the transcription of ribosomeal protein genes and changed the transcription of genes encoding surface-associated proteins, while exposure of *C. difficile* to subinhibitory concentration of metronidazole resulted in minor changes in transcription patterns.

The aim of this study was to investigate the effects of various concentrations of antibiotics known to predispose to CDI and, also, of those used in its therapy, on the production of extracellular toxin B by *C. difficile*.

The specific objectives were to:

1. Investigate the production of extracellular toxin B by quantitative measurement of the cytotoxic effect on Vero cell line;
2. Investigate and quantify the cytotoxic effect of cell-bound toxin on the Vero cell lines
3. Compare the quantitative cytotoxic activities of cell-free and cell-bound toxin B produced by Kuwait toxigenic strains with toxigenic strain from the UK.

6.2 Materials and methods

6.2.1 *Clostridium difficile* strains

Six strains of *C. difficile* were used for this study. These were: strains 233D ribotype 078 & K34A ribotype 097 (two local toxigenic strains isolated from the stool samples of patients with pseudomembranous colitis (PMC) from Mubarak Al Kabeer Hospital; strain 175 ribotype 039 (a local non-toxigenic strain isolated from a stool sample of a patient with suspected antibiotic-associated diarrhoea, ADD); strain A11A ribotype 017 (a toxin A negative/toxin B positive strain from a patient with ADD from Amiri hospital, Kuwait); strain 362C ribotype 046 (a local toxigenic strain isolated from a patient with antibiotic-associated colitis (AAC) in Mubarak Hospital) and a strain of ribotype 001, associated with an outbreak of AAD in the UK, obtained from Prof. BI Duerden and Dr Jon Brazier of the Anaerobe Reference Unit, Cardiff, UK.

6.2. 1 Media and reagents

The following media and reagents were used throughout the experiments: Brucella agar (Unipath, Basingstoke, UK), Anaerobe broth (Unipath), Brain Heart Infusion broth (BHI) (Difco,

Becton, Dickinson and Company, Sparks, MD, USA), Phosphate Buffered Saline (PBS; ICN Biomedicals, Inc., OH, USA), Liver broth (Oxoid Ltd, Basingstoke, Hampshire, England), Minimum Essential Medium (D-MEM; Gibco, Invitrogen, Paisley, Scotland, UK), foetal bovine serum (FBS; Gibco), amphotericin B (Gibco), penicillin-streptomycin sulphate (Gibco).

6.2.3 Susceptibility testing

Five antibiotics were chosen for the study: vancomycin and metronidazole, two agents that are used for the treatment of CDI, and three agents that are associated with precipitation of the disease: ampicillin, cefotaxime and clindamycin. The susceptibility of the isolates to these antibiotics was determined by the E test (section 2.6).

6.2.4 Cytotoxin detection

6.2.4.1 Inoculum standardization

The number of viable bacteria in the inoculum was determined by a modified Miles and Misra method (Brown *et al.*, 1989). *C. difficile* strains were grown overnight in liver broth. Then the neat broth culture was serially diluted in ten-fold steps from neat to 10^{-8} with BHI. Three drops (20µl) from each dilution were dropped on dry blood agar from a height of 1.5-2cm. The drops were allowed to dry and then incubated in anaerobic jars at 37°C overnight. The colonies in the area of each drop were counted, added together and divided by 3 to find the average. Then the count was multiplied by 50, to convert it to 1ml, and by the dilution factor to get the final bacterial count/ml in each diluted broth culture.

Each of the diluted broths was centrifuged at 3,500 g for 5min and the supernatant filtered through a sterile membrane filter with 0.45μm pore size. The filtrates were then assayed for cytotoxicity.

The deposit was washed twice with 3ml of 50 mM PBS and centrifuged for 3,500 g for 5 min and then the supernatant was discarded. The washed sediment was sonicated with an ultrasonic homogenizer (Labsonic U, B. Braun, Model 1254, Melsungen AG, W Germany) for 5 min. The cellular debris was removed by centrifugation at 3,500 g for 10 min. The supernatant was filtered through a sterile membrane filter, 0.45μm size, and assayed for cytotoxicity. An inoculum containing 10^{11} CFU/ml which produced the highest cytotoxic activity was then used throughout the cytotoxin detection experiments.

6.2.4.2 Cytotoxin detection assay

In a study for cytotoxin detection assays, *C. difficile* toxigenic strain 233D (ribotype 078) and a non-toxigenic strain 175 (ribotype 039) (as a control) were seeded onto blood agar and incubated anaerobically for 48 h at 37°C. Then, a loopful of each strain was inoculated into 10ml of sterile liver broth, and incubated anaerobically overnight. This was diluted 1:10 corresponding to 10^{11} CFU/ml after which it was exposed to differing concentrations of the test antibiotics. Concentration of antibiotics used in this study corresponded to the MIC, 1/2, 1/4, 1/8, 1/16, 1/32 and 1/64 MICs. Antibiotics were prepared in sterile distilled water with reference to the highest concentration required (MIC). Doubling dilutions were then made in 10ml of freshly prepared sterile BHI broth in 6 different bottles per concentration containing the specific concentrations of the test antibiotics and the bottles were labelled to correspond to the MIC values as above per

each day of incubation. An inoculum of 100 µl containing approximately 10^{11} CFU/ml of the inoculated liver broth was added to each bottle. An antibiotic-free broth culture was used as control for each strain for baseline comparison. The inoculated broth cultures were then incubated for 1, 2, 3, 4, 5 and 7 days in an anaerobic gas chamber (Bactron Anaerobic Chamber, model IV, Sheldon Manufacturing Inc., Cornelius, Oregon 97113) at 37°C. At the end of each incubation period two types of cytotoxin were assayed, viz: cell-free cytotoxin and cell-bound cytotoxin.

6.2.4.3 Cell-free supernatant cytotoxin detection

After incubation of each bottle of the broth cultures for each period (1-5 and 7 days), 5-ml samples were centrifuged at 3,500 x g for 5 min. The supernatant was filtered through a sterile membrane filter (Millex-AH, Millipore, Carrigtwchill, Co. Cork, Ireland) with 0.45µm pore size, and then assayed for cytotoxicity.

6.2.4.4. Cell-bound cytotoxin detection

The remaining 5ml of the broth cultures was centrifuged as above and the sediment washed twice with 3ml of 50mM PBS (pH 7.0) by centrifugation for 5 min at 3500 g and discarding the supernatant. The washed sediment was sonicated with an ultrasonic homogenizer (Labsonic U, B. Braun, Model 1254, Melsungen AG, W. Germany) for 5 min. The cellular debris was removed by centrifugation at 3,500 g for 10 min. The supernatant was filtered through a sterile membrane filter, 0.45 µm pore size (Millex-AH), and assayed for cytotoxicity.

6.2.4.5. Assay for cytotoxicity

For this experiment, monolayer of Vero cells were used. 50µl of a Vero cell suspension containing 200,000 cells/ml of growth media was dispended into each well of a micro test plate containing 96 wells of 7-mm diameter. The growth medium was made up of 500ml of Minimal Essential Media (D-MEM), 10% foetal bovine serum (FBS), 5ml of amphotericin B (Gibco) 1µg/ml, 5ml of penicillin 100 Unit/ml and streptomycin sulphate (Gibco) 100µg/ml. After incubation for 24 h at 37°C in CO_2 5% atmosphere, each well had a confluent monolayer of cells. Serial twofold dilutions of each filter-sterilised broth culture supernatant fluid and the sonicated effluent were made with maintenance media (500ml of D-MEM, FBS 2%, amphotericin B 1µg/ml and penicillin 100 Unit/ml-streptomycin sulphate 100µg/ml). A 50 µl volume of undiluted sample and subsequent dilutions were introduced into each well of confluent monolayer of cells. The dilution series was 1:40, 1:80, 1:160, 1:320, 1:640, 1:1280, 1:2560 and 1:5120 for the control, the supernatant and the sonicated effluent. The inoculated Vero cells were incubated at 37°C in CO_2 5% and examined at 24 and 48 h for cytotoxic changes, defined as rounding of the cells, changes in morphology or partial loss of adherence. The highest dilution resulting in complete rounding of the cells was taken as the number of cytotoxic units (CU)/50-µl sample (Nakamura *et al.*, 1982; Nakamura *et al.*, 1981) and the results were expressed as CU/ml.

All the remaining strains were subjected to the same procedures. At the end of 7^{th} day incubation, the mean CUs/ml were calculated and tabulated.

6.3 Results

6.3.1 Minimum inhibitory concentrations

The MICs of the 5 antibiotics tested against the 6 strains investigated are shown in Table 6.1.

Table 6.1. MICs (μg/ml) of selected antibiotics against clinical strains of *C. difficile*

Antibiotics	MIC (μg/ml) of antibiotics against strains:					
(breakpoints; μg/ml)	078	039	097	017	046	001
Clindamycin (4)	8.0	>256	>256	>256	4.0	8.0
Metronidazole (8)	0.25	0.06	0.06	0.25	0.25	0.25
Vancomycin (4)	0.5	1.0	1.0	1.0	0.5	2.0
Ampicillin (8)	0.5	0.5	1.0	1.0	1.0	2.0
Cefotaxime (32)	>256	>256	>256	>256	>256	>256

6.3.2 Inoculum standardisation

Tables 6.2a and 6.2b demonstrate the inoculum at which cell-free and cell-bound cytotoxin production was maximal below the neat broth. Thus, inoculum of 10^{11} cfu/ml was used throughout the experiments.

Table 6.2a. Cytotoxic activity (CU/ml) versus dose (CFU/ml) of the cell-free broth culture of *C. difficile*

Viable count (CFU/ml)	CU/ml of strain											
	078		039		097		017		046		001	
	24h	48h	24h	48h	24h	48h	24h	48h	24h	48h	24h	48h
10^{12} (Neat)	128	256	0	0	32	32	128	256	32	32	256	512
10^{11}	64	128	0	0	16	32	16	32	16	16	32	64
10^{10}	0	0	0	0	0	0	16	16	0	0	0	0
10^{9}	0	0	0	0	0	0	0	0	0	0	0	0
10^{8}	0	0	0	0	0	0	0	0	0	0	0	0
10^{7}	0	0	0	0	0	0	0	0	0	0	0	0
10^{6}	0	0	0	0	0	0	0	0	0	0	0	0

Table 6.2b. Cytotoxic activity (CU/ml) of cell-bound cytotoxin versus dose (CFU/ml) of *C. difficile*

Viable count (CFU/ml)	CU/ml of strain											
	078		039		097		017		046		001	
	24h	48h	24h	48h	24h	48h	24h	48h	24h	48h	24h	48h
10^{12} (Neat)	256	512	0	0	256	512	2048	2048	256	256	1024	1024
10^{11}	16	16	0	0	16	16	256	512	16	16	128	128
10^{10}	0	0	0	0	0	0	0	0	0	0	0	0
10^{9}	0	0	0	0	0	0	0	0	0	0	0	0
10^{8}	0	0	0	0	0	0	0	0	0	0	0	0
10^{7}	0	0	0	0	0	0	0	0	0	0	0	0
10^{6}	0	0	0	0	0	0	0	0	0	0	0	0

6.3.3 Quantitative cytotoxic activity of *C. difficile*, strain 233D (ribotype 078)

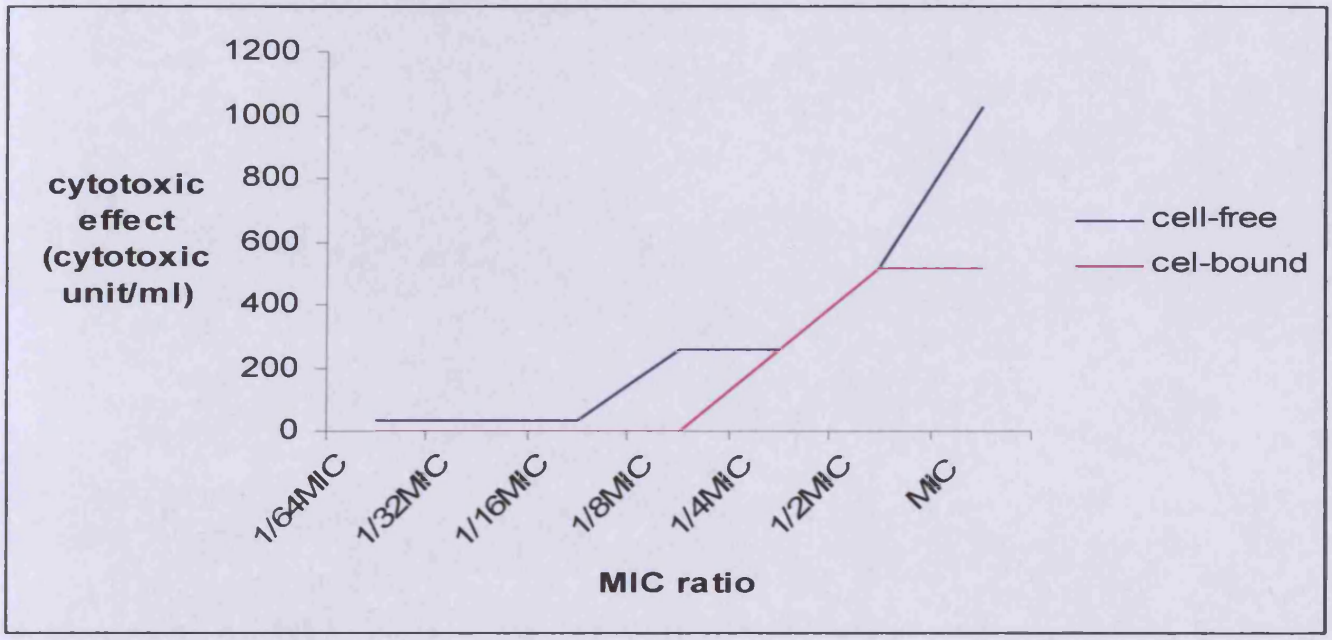
A study for cell-bound and cell-free cytotoxin production was carried out with the toxigenic strain *C. difficile* 233D (078) on the Vero cell line. The test was done for induction of cytotoxin production in the presence of inhibitory and sub-inhibitory concentrations of cefotaxime, ampicillin, metronidazole, vancomycin or clindamycin. Induced intra- and extra-cellular cytotoxin was determined after incubation with the antibiotics for 1, 2, 3, 4, 5 and 7 days. The results are shown in Figure 6.1 and Figure 6.2 (Appendix; Tables 6.3.1a and 6.3.1 b to 6.3.10a and 6.3.10b). The non-toxigenic strain 175 039 did not produce any effect in the Vero cell lines.

The graphs in this chapter show representative results for cytotoxic activity after exposure to an antibiotic for 3 days and Vero cells assays read after 24 hours. Full details of the results over 7 days and of Vero cells assays read after 48 hours are in the Appendix.

6.3.4 The effect of exposure to cefotaxime (CTX)

As shown in Figure 6.1 (Appendix; Table 6.3.1a and Table 6.3.1b), post-exposure extra-cellular cytotoxin production could be demonstrated on Vero cell lines after incubation for 24 and 48 h. Cefotaxime induced twice (32 CU/ml) the level of activity obtained with the control antibiotic-free broth culture (16 CU/ml) on day 1 at the MIC and ½ MIC of cefotaxime, through a 4-fold increase (64 CU/ml), to an 8-fold rise (128 CU/ml) at 1/32 MIC. The CU reached a 16-fold increase, 2048 CU/ml, on the 5^{th} day of exposure at concentration from 1/8 MIC down to 1/64 MIC. Peak induction, 64-fold increase (2048 CU/ml), was reached on day 7 from the MIC to the lowest cefotaxime concentration of 1/64 MIC.

Figure 6.1. Cytotoxic activity (CU/ml) of cell-free and cell-bound cytotoxin of *C. difficile* strain 233D (ribotype 078) post-exposure to **cefotaxime** after 3days incubation on Vero cell line



After incubation on Vero cell lines for 48h (Appendix; Table 6.3.1b) the quantity of induced cell-free cytotoxin increased two-fold from MIC exposure to 1/8 MIC on day 1. This trend was observed through all the days of incubation with the antibiotic up till day 7.

The effect of exposure to cefotaxime on cell-bound cytotoxic activity was not as pronounced as it was with the cell-free cytotoxic activity. As shown in Figure 6.1 (Appendix; Tables 6.3.2a and 6.3.2b), the cell-bound cytotoxic activities became noticeable only at relatively low concentrations (1/16 MIC) of the antibiotic on days 1 and 2 post-exposure after incubation on Vero cells for 24 and 48 h. Even on days 3, 4, 5 and 7, there were only 2-8-fold increases over the cytotoxic activity of the control filtered broth culture at 1/32 – 1/64 MIC exposures after 24 h incubation and twice these levels, at the same concentrations, after 48 h incubation.

6.3.5 The effect of exposure to ampicillin (AMP)

The effect of exposure to ampicillin on cell-free cytotoxic activity, shown in Figure 5.2 (Appendix; Tables 6.3.3a and 6.3.3b), was very striking from day 1 cultures after incubation with Vero cells for 24 and 48 h. Maximum induction, 2048 CU/ml, was seen at 5 and 7 days post-AMP exposure. This represented a 32-fold increase over the antibiotic-free broth culture filtrate control. This high degree of induction, particularly on days 5 and 7, was observed at all levels of inhibitory and sub-inhibitory MICs.

Figure 6.2. Cytotoxic activity (CU/ml) of cell-free and cell-bound cytotoxin of *C. difficile* strain 233D (ribotype 078) post- exposure to **ampicillin** after 3 days incubation on Vero cell line

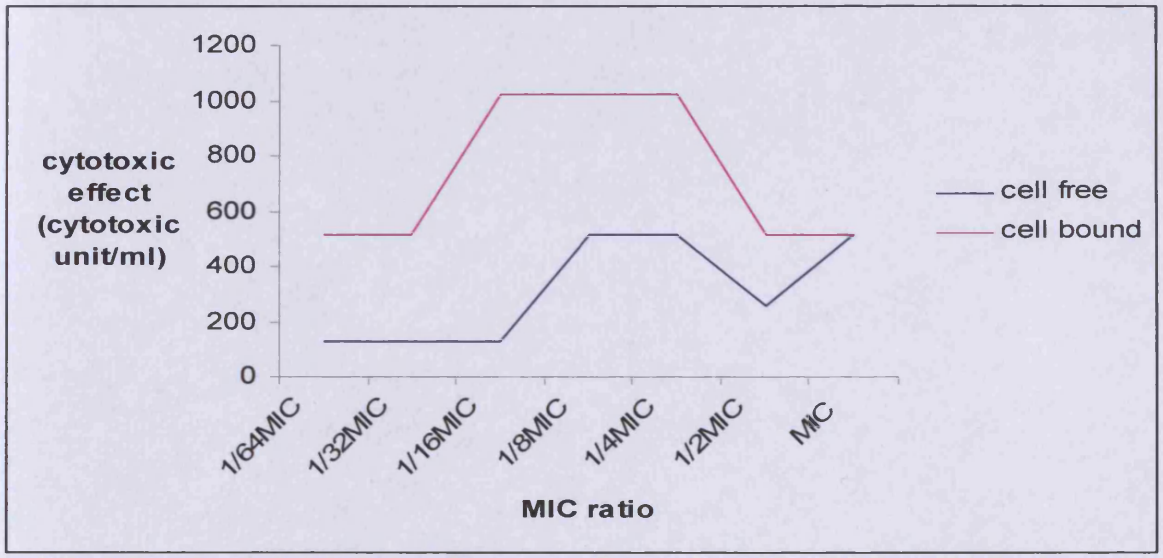


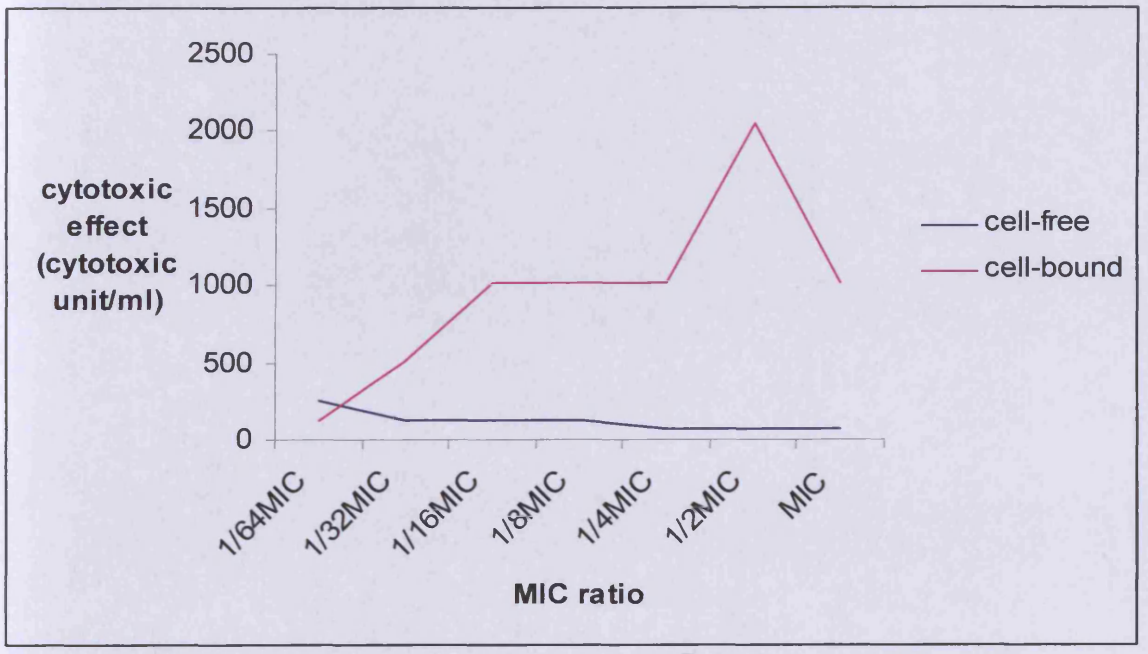
Figure 6.2 (Appendix; Tables 6.3.4a and 6.3.4b) show the effect of ampicillin on cell-bound cytotoxin induction. The drug had no effect on days 1 and 3. However, the maximum effects, 1028 and 2048 CU/ml, were observed on days 3 and 4 when compared with the 32 and 64 CU/ml, respectively, of control antibiotic-free sonicated effluent culture after incubation with Vero cells for at 24 and 48 h.

6.3.6 The effect of exposure to metronidazole (MTZ)

The effects of exposure to metronidazole are shown in Figure 6.3 (Appendix; Tables 6.3.5a, 6.3.5b, 6.3.6a and 6.3.6b). As can be seen in Appendix ;Tables 6.3.5a and 6.3.5b, the cytotoxic effects were 2-4-fold higher than the control filtered broth culture supernatant on days 1 and 2 for ½ MIC down to the 1/64 MIC for the cell-free cytotoxin and increased to 4-8-fold on days 3 and 4, reaching the maximum of 32-fold increase on day 5, after 24 h incubation of supernatants on Vero cell lines. The same observation was noticed after 48 h incubation although the maximum cytotoxic effects, 2048 CU/ml, were also determined on day 7.

The effect of MTZ on the cell-bound cytotoxin was more pronounced than the effects on cell-free cytotoxin (Appendix; Table 6.3.6a and 6.3.6b) on days 1-3, reaching maximum activities of 2048 CU/ml, i.e. 128-fold increase, after incubation for 24 and 48 h. The level of activity decreased to 256 and 512 CU/ml (4-8 folds) on days 5 and 7 at the MIC and higher sub-inhibitory MICs.

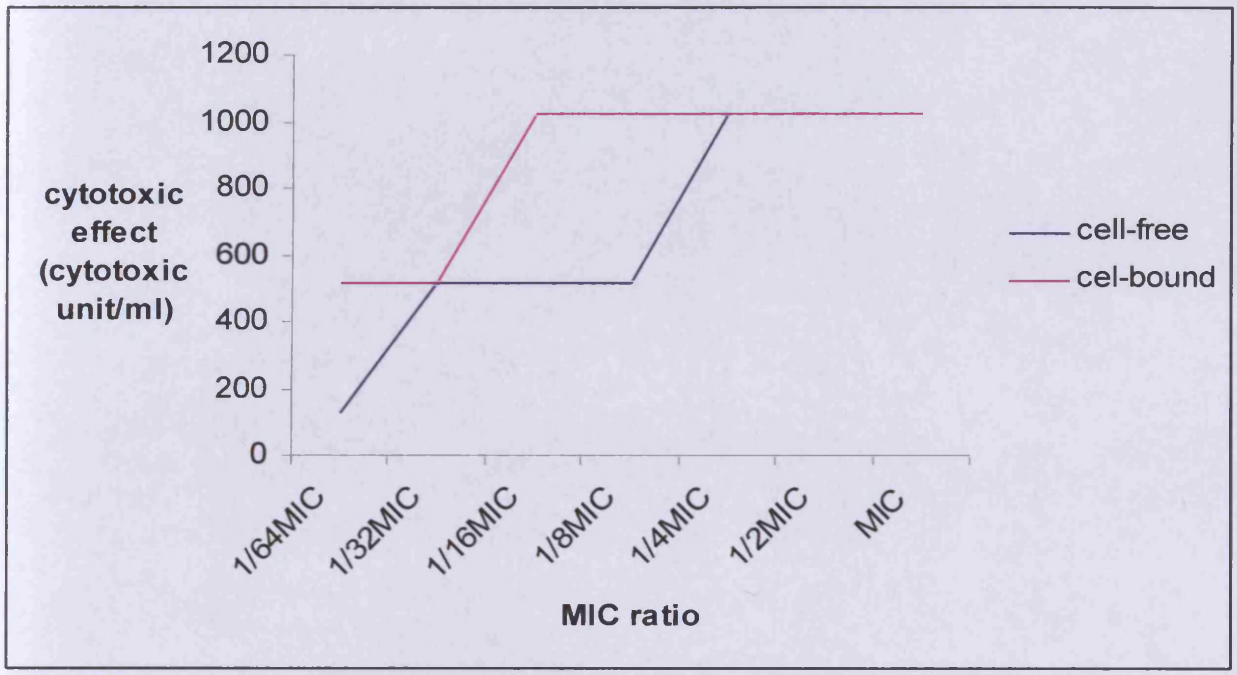
Figure 6.3. Cytotoxic activity (CU/ml) of cell-free and cell-bound cytotoxin of *C. difficile* strain 233D (ribotype 078) post- exposure to **metronidazole** after 3 days incubation on Vero cell line



6.3.7 Effect of exposure to vancomycin

Figure 6.4 (Appendix; Tables 6.3.7a and 6.3.7b, and 6.3.8a and 6.3.8b show the amount of cell-free and cell-bound CUs produced by strain 233D ribotype 078 pre- and post- exposure to vancomycin on the Vero cell line. Vancomycin induced cell-free cytotoxin maximally on days 4 and 5 where it caused a 16-32 fold rise in the extracellular cytotoxin production compared to the control when incubated for 24 h on Vero cells but much higher levels of 1028 and 2048 CU/ml (32-64 - fold rise), on days 3, 4 and 5 after incubation for 48 h. On day 7, at both incubation periods, the activities dropped to below that of the control broth culture after incubation for 24 h or the same level after 48 h except at 1/32 and 1/64 MICs when 1024 CU/ml was recorded.

Figure 6.4. Cytotoxic activity (CU/ml) of cell-free and cell-bound cytotoxin of *C. difficile* strain 233D (PCR ribotype 078) post- exposure to **vancomycin** after 3 days incubation on Vero cell line

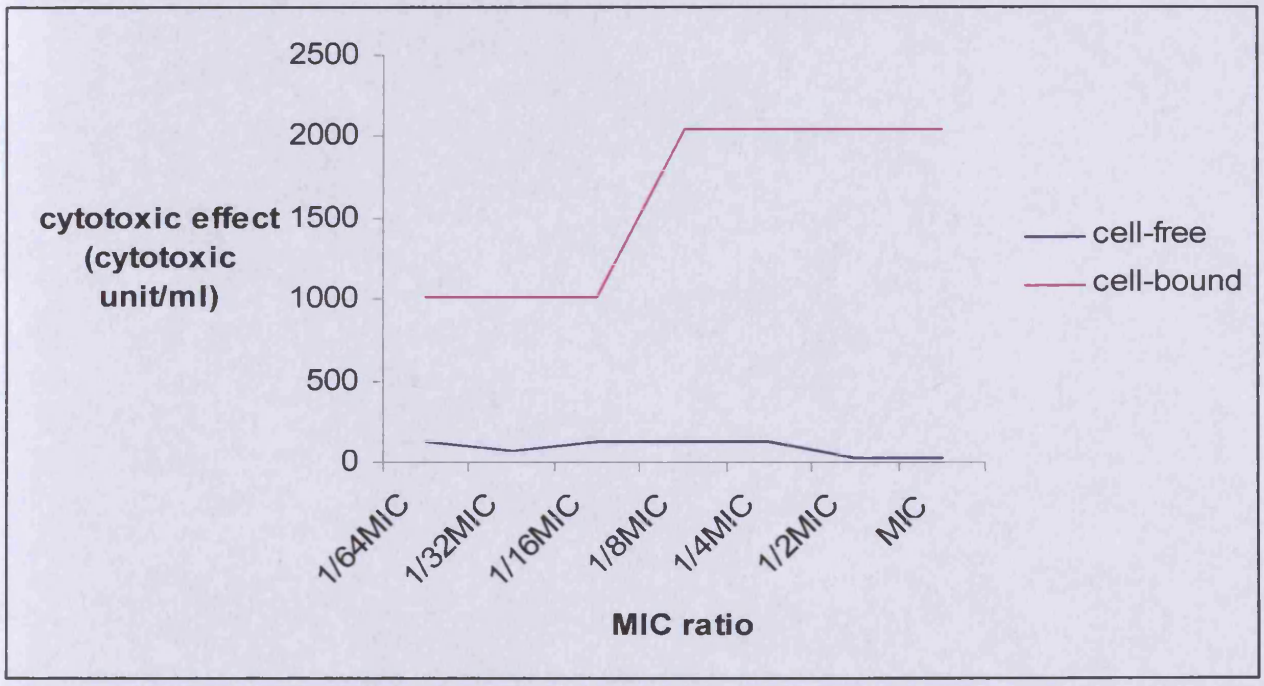


At days 1, 2, and 3, vancomycin induce an increase in cell-bound cytotoxin by 32 to 64-fold at MIC and ½ MIC, then maximally increasing with further sub-inhibitory concentration to 128 - fold at 1/64 MIC while decreasing to 2-fold increases on days 4, 5 and 7 after incubation for 24 h with Vero cells (Appendix; Table 6.3.8a). A similar trend (Appendix; Table 6.3.8b) was observed after incubation for 48 h but at a higher level of activity, i.e. 2048 CU/ml, was achieved with higher MICs on days 1-3, decreasing to 4-32 fold on days 4 and 7.

6.3.8 Effect of exposure to clindamycin

Figure 6.5 (Appendix; Tables 6.3.9a and 6.3.9b, and 6.3.10a and 6.3.10b) show the amount of cell-free and cell-bound CU produced by strain 233D (ribotype 078) pre- and post- exposure to clindamycin on the Vero cell line. The maximum cytotoxic effect of 2048 CU/ml was produced on days 5 and 7 after 24 and 48 h incubation on Vero cells, although representing only a 16-32-fold increase in the extracellular cytotoxin activity. The effects were minimal on day 1 but increased by 2 to 4-fold on days 2 – 4 after both incubation periods on the Vero cell line, i.e. clindamycin hardly caused any induction of cell–free cytotoxin on day 1 except at ½ and ¼ MIC. The effect of clindamycin on cell-bound cytotoxin was more pronounced on days 1 – 4 after incubation for both 24 and 48 h.

Figure 6.5. Cytotoxic activity (CU/ml) of cell-free and cell-bound cytotoxin of *C. difficile* strain 233D (ribotype 078) post- exposure to **clindamycin** after 3 days incubation on Vero cell line



As shown in Figure 6.5 and (Appendix; Tables 6.3.10a and 6.3.10b), the effects of clindamycin were 64-fold higher than the control on days 1, 2, 3 and 4 after incubation for 24 and 48 h. These activities decreased to a 16-fold rise on days 5 and 7 after incubation for both periods.

6.3.9 The mean cytotoxic activity of all strains after exposure to the various antibiotics

The analysis of the data for the remaining 4 strains was done by calculating the mean of the cytotoxic activities produced on all post-exposure days to the antibiotics along with those of *C. difficile* strain 233D (078).

6.3.10 Mean effect of cefotaxime on cytotoxin induction

Figure 6.6 (Appendix; Table 6.3.11a) shows that the increase in the cytotoxic activities of cell-free cytotoxin produced by *C. difficile* strain 233D 078 was linear from MIC to 1/64MIC. There was an overall increase from the pre-exposure level of cell-free cytotoxin of 66.6 CU/ml to post-CTX exposure levels of 368.0 CU/ml (5.5x) at the MIC to 1050.6 CU/ml (15.7x) at 1/64MIC after incubation with Vero cells for 24h. After incubation for 48h, the levels increased 2-fold over the 24 h incubation values from 48.0 CU/ml to 448.0 CU/ml (9.3x) at the MIC and 1322 CU/ml (27.5x) at 1/64MIC.

The effects on the cell-bound cytotoxic activities were not as marked as shown in Figure 6.7 (Appendix; Table 6.3.11b). On day 1, there was no increase in cytotoxin production at the MIC after incubation for 24 or 48 h. The increase observed at ½ MIC was marginal, 1.3x the control but this rose gradually to 7.0x the control at 1/64 MIC after incubation for 24 h. After incubation

for 48h, there was no increase from the pre-exposure level at the MIC and ½ MIC. However, there were gradual increase from 106.6 CU/ml (1.4x) at ¼ MIC to 789.3 CU/ml (10.2x) at 1/64 MIC.

Following exposure of strain A11A 017 to CTX, there was a massive increase in the cell-free cytotoxin (Figure 6.6; Appendix; Table 6.3.11a). Cell-free cytotoxin production increased by 16.5 times at the MIC and 128 times at 1/64 MIC after incubation for 24h with Vero cells. The effects were 64-fold more than the control from the MIC to 1/64 MIC after incubation for 48h. For cell-bound cytotoxin, the effect of exposure to CTX was considerably less. It was 2.8 and 32 times the control at the MIC after incubation for 24 and 48 h and 20 and 18 times the control at 1/64 MIC following incubation for 24 and 48 h.

Cefotaxime failed to induce cell-free or cell-bound cytotoxin in strain K34 097 at the MIC and ½ MIC following incubation for both periods, but CTX induced cell-free cytotoxin at 1/4 MIC by 8-fold and gradually through 1/16 MIC (10x) to 1/64 MIC (16x) after incubation for 24 and 48h. The effect on cell-bound cytotoxin was about the same following incubation at both 24 and 48 h.

With strain 362C 046, CTX induced cell-free cytotoxin at ½ MIC by 8-fold compared to the control and the level increased between 1/8-1/16 MIC, dropping gradually at 1/32 MIC; these values were higher than the control after incubation with Vero cells for 48h.

Cefotaxime did not induce cell-free cytotoxin from the UK *C. difficile* strain, ribotype 001 after incubation with Vero cells for 24h. However, after incubation for 48 h, the activity levels of cell-bound cytotoxin increased 5 times over the control at ½ MIC and by 24-fold at ¼ MIC. These values decreased to 12-fold at lower sub-inhibitory MICs.

CTX did not induce any cell-free or cell-bound cytotoxin in the non-toxigenic strain 175 039 in the presence or absence of CTX.

Figure 6.6. Mean cytotoxic activity (CU/ml) of cell-free cytotoxin of *C. difficile* post exposure to **cefotaxime** by all test strains

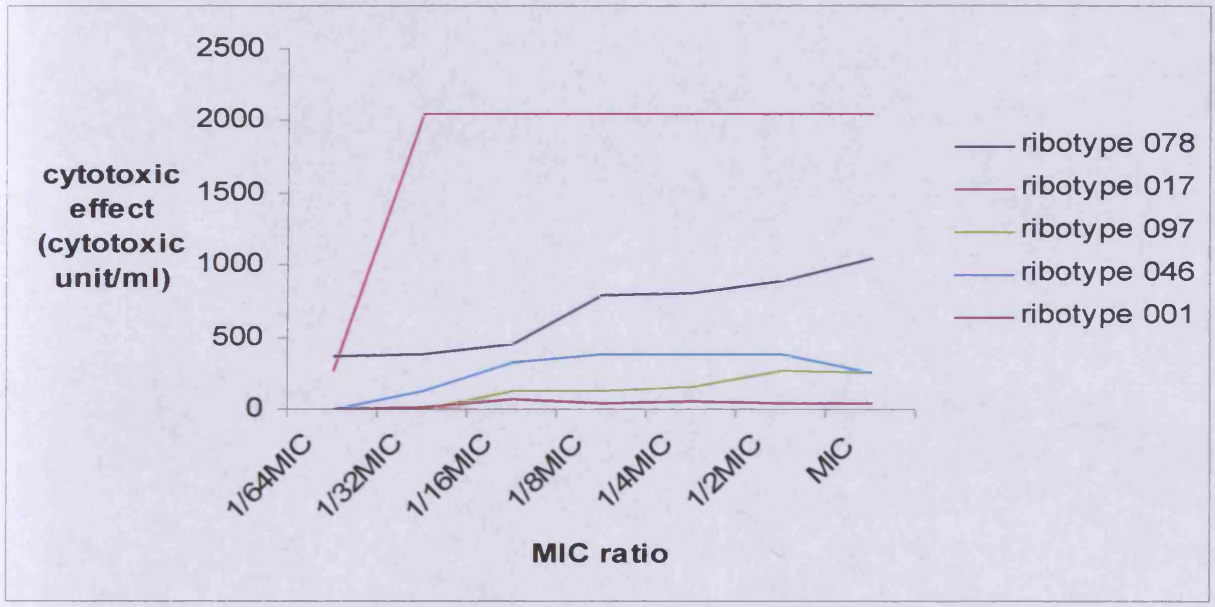
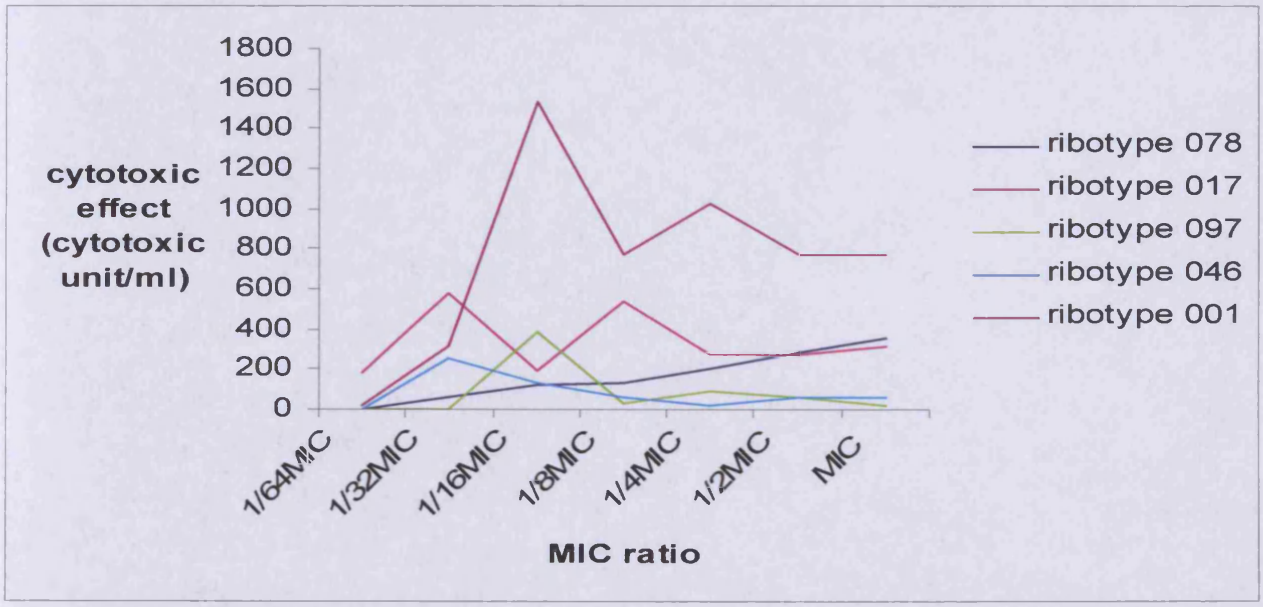


Figure 6.7. Mean cytotoxic activity (CU/ml) of cell-bound cytotoxin of *C. difficile* post exposure to **cefotaxime** by all test strains



6.3.11 The Mean cytotoxic activity of all strains after exposure to ampicillin

Cell-free cytotoxin activity: The cytotoxic activity of the supernatant of all tested *C. difficile* strains is shown in Figure 6.8 (Appendix; Table 6.3.12a). For strain 233D 078, the induction of extracellular cytotoxin after exposure to ampicillin started at the MIC (0.5 μg/ml) where it was about 18.5-fold higher than the control. Then the cytotoxic activity increased reaching its highest level of 1088 CU/ml and 960 CU/ml between ½ MIC and ¼ MIC; and then declined with subsequent sub-inhibitory concentrations.

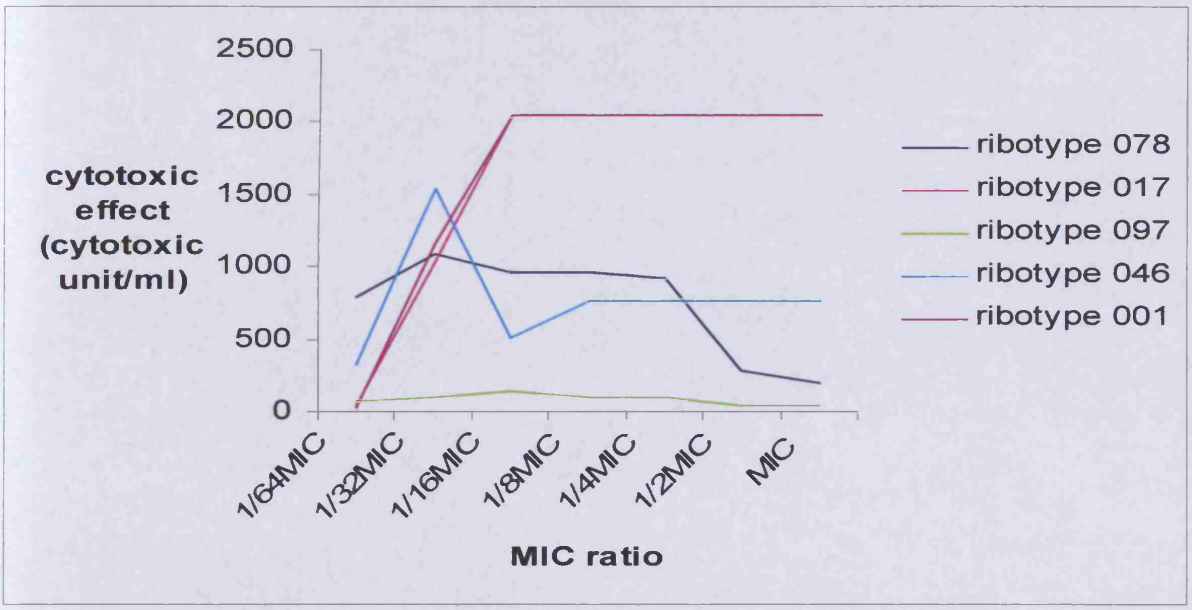
The induction of cell-free cytotoxin in strain A11A 017 by ampicillin when incubated with Vero cells for 24 h was about 2.5-fold increasing to 128-fold higher than the control at ¼ MIC. This was the same after incubation for 48 h on Vero cells.

With the cell-free filtered supernatant of strain K34A 097 exposed to various concentrations of ampicillin, there was a 2-fold increase at the MIC which increases slightly to 3 X at ½ MIC and 4.5-fold at ¼ MIC. Thereafter, the levels dropped back to 3-fold at 1/8 and 1/16 MIC and then to 1.5-fold at 1/32 MIC.

Ampicillin started to induce a rise in cell-free cytotoxin production by strain 362C 046 at the MIC then remained at the same high level (24-32fold higher than the control).

For the UK ribotype 001 strain, ampicillin did not induce cell-free cytotoxin at the MIC value but the cytotoxic activity increased by 18-fold at ½ MIC and 32-fold for ¼ MIC down to 1/64 MIC after incubation for 24h. After incubation with Vero cells for 48h the values were the same except at ½ MIC when it was 32-fold instead of 18-fold after 24 h.

Figure 6.8. Mean cytotoxic activity (CU/ml) of cell-free cytotoxin of *C. difficile* post exposure to **ampicillin** by all test strains



Cell-bound cytotoxin: The cytotoxic activity of the sonicated cell effluents of all the tested strains before and after exposure to ampicillin is shown in Figure 6.9 (Appendix; Table 6.3.12b). For strain 233D 078, the cytotoxic activity of the intracellular cytotoxin started to rise at the MIC and reached a maximum at 1/8 MIC (15 X the control) then dropped gradually with further dilution of the ampicillin.

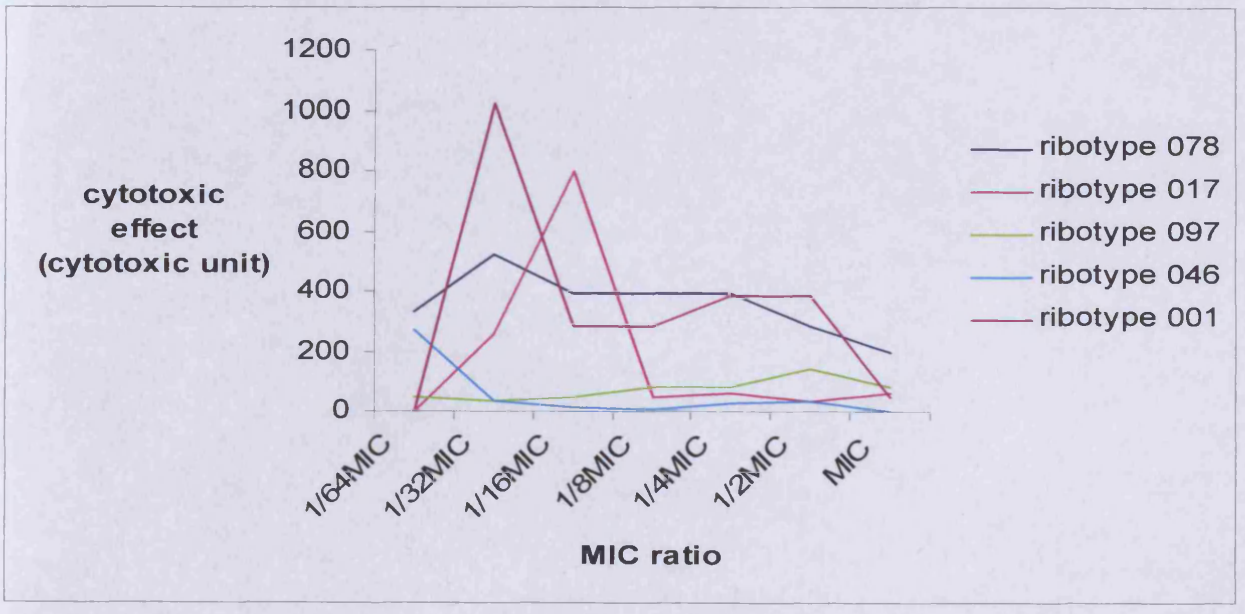
There was no apparent change in the cytotoxic activity of the cell-bound cytotoxin after exposure to ampicillin for strain A11A 017, except at 1/4 MIC where the rise was 3-fold higher (800.0 CU/ml) than the antibiotic free control (256CU/ml).

Ampicillin induced cell-bound cytotoxin production by strain K34A 097 in almost a linear fashion and reached a maximum at 1/16 MIC (37-fold higher than the control) after incubation with Vero cells for 48 h.

With strain 362C 046, ampicillin induced cell-bound cytotoxin production maximally at the MIC level - 272 (17-fold) and 544 CU/ml (32-fold), after incubation for 24 and 48 h. Thereafter the activities decreased sharply to the antibiotic-free levels.

Ampicillin did not induce cell-bound cytotoxin production in the strain of ribotype 001 at the MIC but induced high levels of cytotoxin, 1024.0 (16-fold) and 1536.0 CU/ml (24-fold), at ½ MIC, after incubation for 24 and 48 h. The levels then dropped to 4.5-fold at ¼ and 1/8 MICs and 6-fold at 1/16 and 1/32 MICs after incubations for 24 and 48h.

Figure 6.9. Mean cytotoxic activity (CU/ml) of cell-bound cytotoxin of *C. difficile* post exposure to **ampicillin** by all test strains



6.3.12 The Mean cytotoxic activity of all strains after exposure to metronidazole

Cell-free cytotoxin (Figure 6.10 and Appendix; Table 6.3.13a): For strain 233D 078, in the presence of metronidazole, the cell-free cytotoxin was greatly increased at the MIC by about 8.8 –fold, increasing to about 11-fold at ¼ MIC after incubation on Vero cells for 24 h compared to the control. But this effect was more apparent after incubation for 48 h.

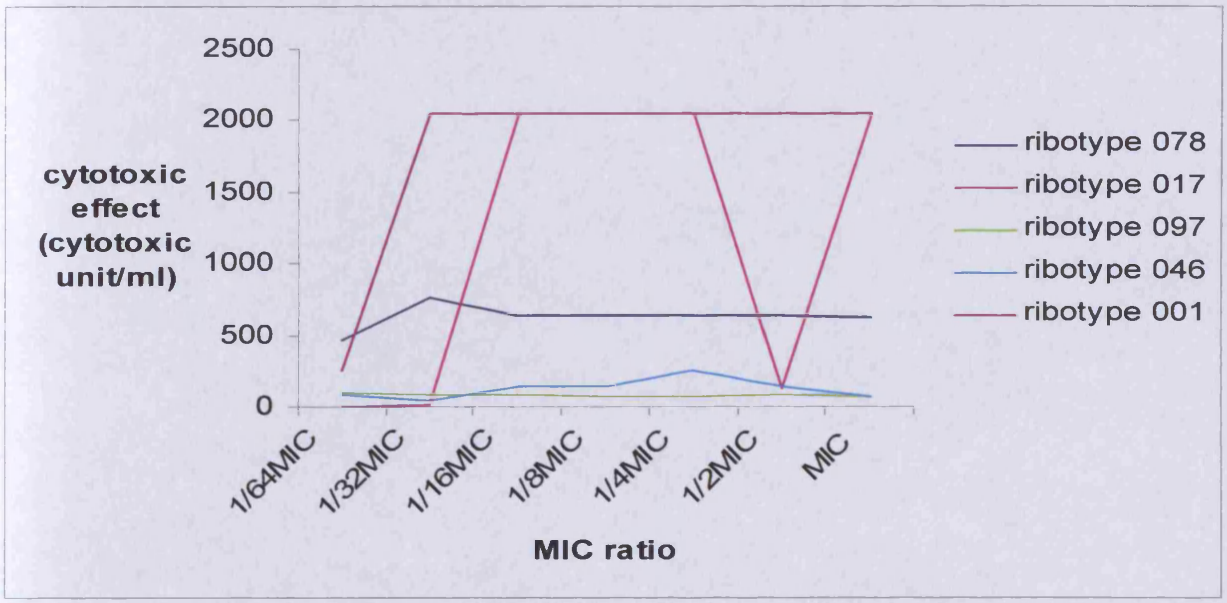
For the cell-free supernatant of A11A 017, there was a 128-fold rise in the cytotoxic activity at ¼ MIC and it remained at the same level throughout subsequent sub-inhibitory concentrations of MZ incubation for 24 and 48 h.

With the cell-free supernatant of K34A 097, there was a 6-fold rise in cytotoxin production starting at the MIC with further drop, but above the antibiotic free control.

With the strain 362C 046, there was a rise in the cytotoxic activity of the broth control from 16 to 80.0 CU/ml at the MIC, reaching a maximum of 256 CU/ml (16-fold) at 1/16 MIC and then dropping gradually to 136 and 64 CU/ml at 1/32 and 1/64 MIC, respectively.

Metronidazole induced the production of cell-free extracellular toxin at all concentrations. There was hardly any apparent change in the toxin secretion in the supernatant of broth cultures of the type 001 isolate after incubation on Vero cells for 24 and 48 h.

Figure 6.10. Mean cytotoxic activity (CU/ml) of cell-free cytotoxin of *C. difficile* and post exposure to **metronidazole** by all test strains



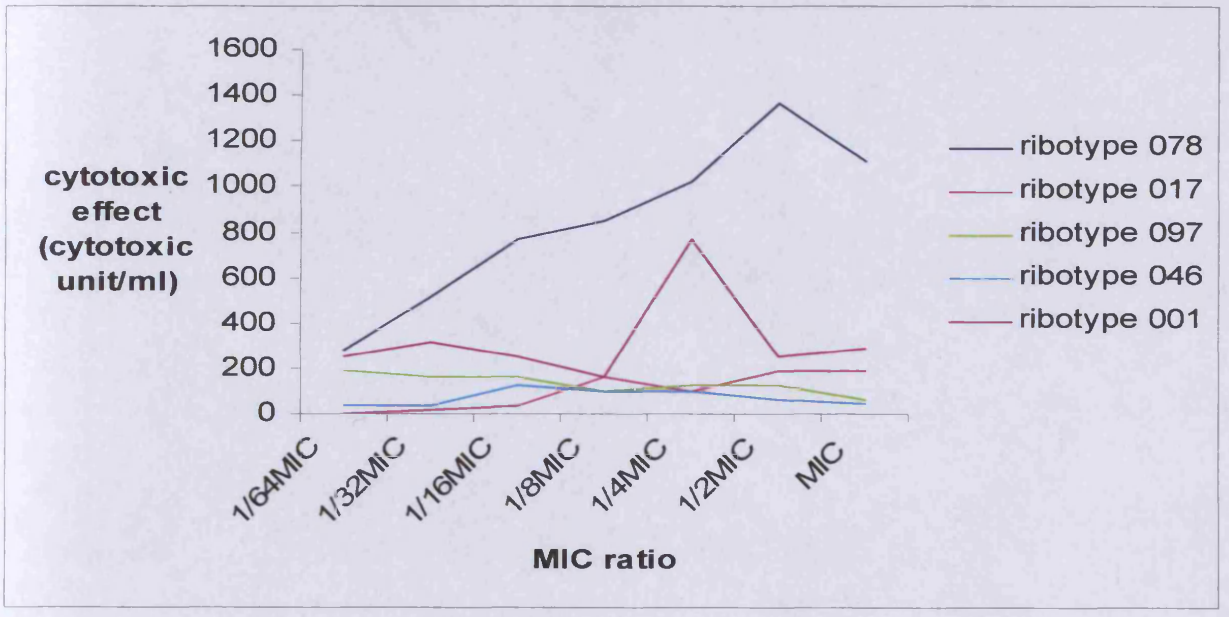
Cell-bound cytotoxin: Figure 6.11 (Appendix; Table 6.3.12b) show the amount of cell-bound cytotoxic unit (CU/ml) produced by various *C. difficile* strains pre and post-exposure to MZ. For strain 233D 078, in the presence of MZ, the cell-bound cytotoxin was increased at the MIC by about 4-fold and this increased greatly to about 23-fold at 1/32 MIC after incubation for 24 h compared to the control. But this effect was more apparent after incubation for 48 h from a 19-fold increase at the MIC to a 44-fold increase at 1/64 MIC.

For strain A11A 017, metronidazole increased the production of cell-bound cytotoxin production at 1/2 MIC by 1.5-fold compared to the control and reached maximum levels at 1/32 and 1/64 MICs (3-fold) after incubation with Vero cells for 24 h but at 1/8 MIC after incubation for 48 h, the level increased to 8.5-fold higher than the control.

With strain 362C 046 and ribotype 001, metronidazole induced cell-bound cytotoxin production in a similar manner at all MICs; twice the cytotoxic activities were observed at the MICs of both

strains. These increased to 6-fold at 1/8 and 1/16 MICs after incubation for 24 h for the 362C 046 strain. For the 001 strain, the maximum activity was 6-fold at 1/16 MIC after incubation for 24 h and 8-fold at the same MIC after incubation for 48h.

Figure 6.11. Mean cytotoxic activity (CU/ml) of cell-bound cytotoxin of *C. difficile* post exposure to **metronidazole** by all test strains.

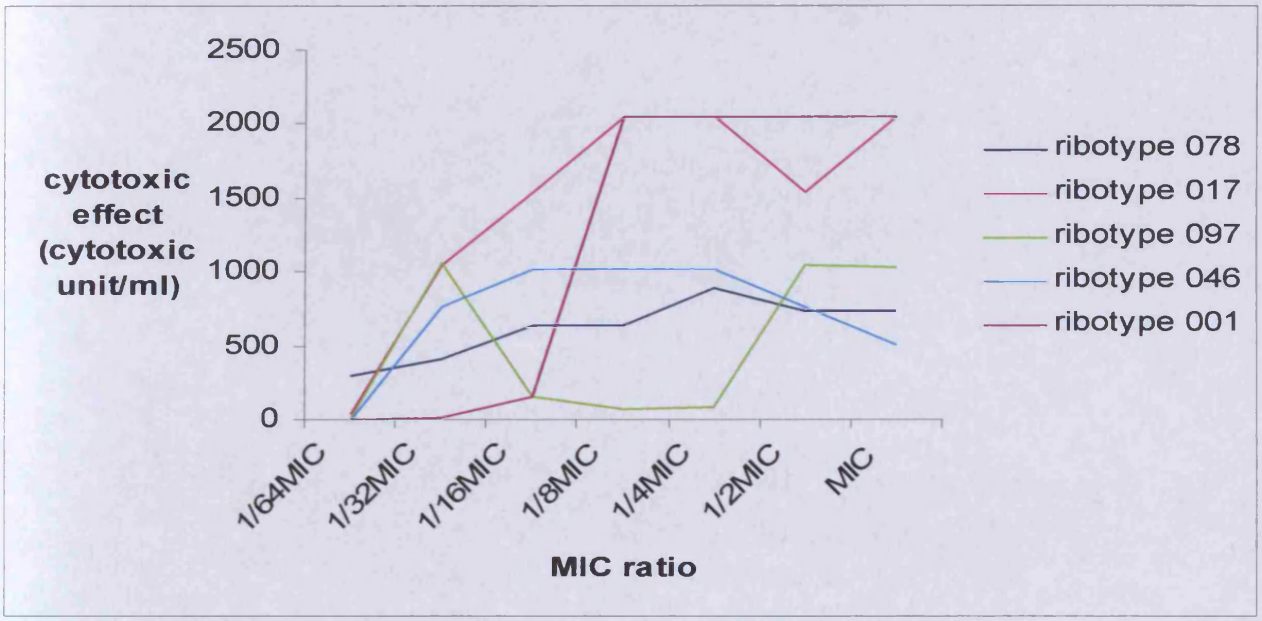


6.3.13 The Mean cytotoxic activity of all strains after exposure to vancomycin

Cell-free cytotoxin: Figure 6.12 (Appendix; Table 6.3.14a) shows the amount of cell-free CU/ml produced by all toxigenic of *C. difficile* pre- and post– exposure to vancomycin. Strain 233D 078, produced cell-free cytotoxin at the MIC 6-fold higher than the control and this increased in a linear way to 17.7-fold at 1/16 MIC after incubation for 24h. After incubation for 48h, there was greater increase of 24.3-fold at the MIC and this reached the maximum level of 30.6-fold at 1/32 MIC. The cytotoxic activity of the cell-free filtrate of strain A11A 017 was

1040 CU/ml (8-fold) at the MIC increasing to 2048 CU/ml (16-fold) at 1/8, 1/16 and 1/64 MIC after incubation for 24h. There was no difference in the level of activity after incubation for 48h. With the K34 097 strain, there was an increase of cell-free cytotoxin of 33-fold over the control at ½ MIC after incubation for 24h and this dropped to 5-fold at ¼ MIC. After 48 h, the cytotoxic activity was the same. Strain 362C 046, produced no cytotoxin at the MIC but produced 768.0 CU/ml (24-fold) at ½ MIC, increasing to 1024 CU/ml (32-fold) at ¼ to 1/16 MIC after incubation for 48h. A similar trend was observed after incubation for 48h. With ribotype 001, there was no production of cytotoxin at the MIC but it produced a sub-control level at ½ MIC. The level at ¼ MIC was 1536 CU/ml (24-fold rise), increasing to 2048 CU/ml (32-fold rise) from 1/8 to 1/64 MIC after incubation 24h. The same levels of activity were observed after incubation for 48h.

Figure 6.12. Mean cytotoxic activity (CU/ml) of cell-free cytotoxin of *C. difficile* post exposure to **vancomycin** by all test strains



Cell-bound cytotoxin Figure 6.13 (Appendix; Table 6.3.14b): The mean cell-bound cytotoxin levels of strain 233D 078 were 282.6 (11.7-fold rise) and 618.6 CU/ml (15.4-fold) after exposure to the MIC (0.5µg/ml) of vancomycin after incubation for 24 and 48h. Thereafter, there was a linear increase to 901.3 CU/ml (21.4-fold) at 1/64 MIC after incubation for 24h and 1536.0 CU/ml (38.3-fold) after incubation for 48h. Strain A11A 017 produced a relatively high level of constitutive cytotoxin in the absence of antibiotic measuring 128 and 256 CU/ml after incubation for 24 and 48 h, respectively. There was no induction of cell-bound cytotoxin after exposure to vancomycin above the control levels after incubation for 24 and 48h.

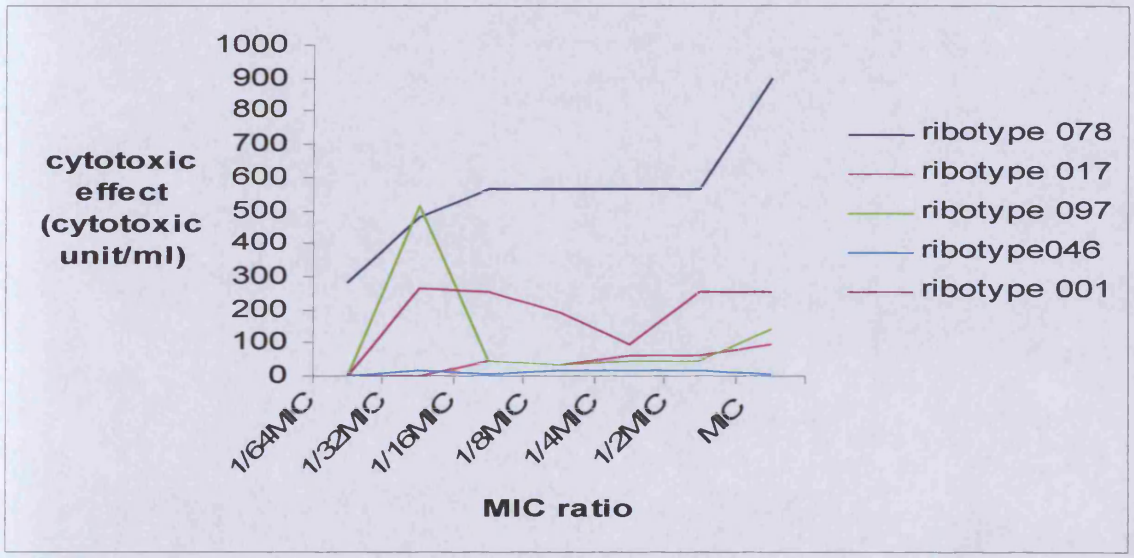
Vancomycin did not induce the production of cell-bound cytotoxin in K34A 097 at the MIC (1µg/ml) but did at sub-inhibitory concentrations. The level increased to 512 CU/ml (32-fold)

after exposure to vancomycin at ½ MIC (0.5μg/ml) following incubation for 24 h. After subsequent exposure to sub-inhibitory concentrations, there was a decrease in the cell-bound toxin to 32 CU/ml (2-fold) at 1/8MIC after incubation for 24h and this then increased gradually to 48 (3-fold) and 144 CU/ml (9-fold) at 1/16, 1/32 and 1/64 MIC, respectively. The cytotoxic activity was enhanced by prolonged incubation in Vero cells for 48 h.

Exposure of strain 362C 046 to vancomycin did not result in induction of appreciable cytotoxin production at the level of MIC, ¼ MIC and 1/64 MIC, as shown in Appendix; Table 6.3.8b after 24 h incubation. However, it induced the cell-bound cytotoxin at low level of 24 CU/ml compared to the control (16U/ml) after 48 h incubation.

Exposure of ribotype 001 strain to vancomycin did not result in the induction of cytotoxin production at the MIC (0.5μg/ml). Then there was a relatively high rise in the cytotoxic activity to 264 CU/ml (4-fold) at ½ MIC after incubation for 24h. Almost the same level of activity was observed after incubation for 48h.

Figure 6.13. Mean cytotoxic activity (CU/ml) of cell-bound cytotoxin of *C. difficile* post exposure **to vancomycin** by all test strains



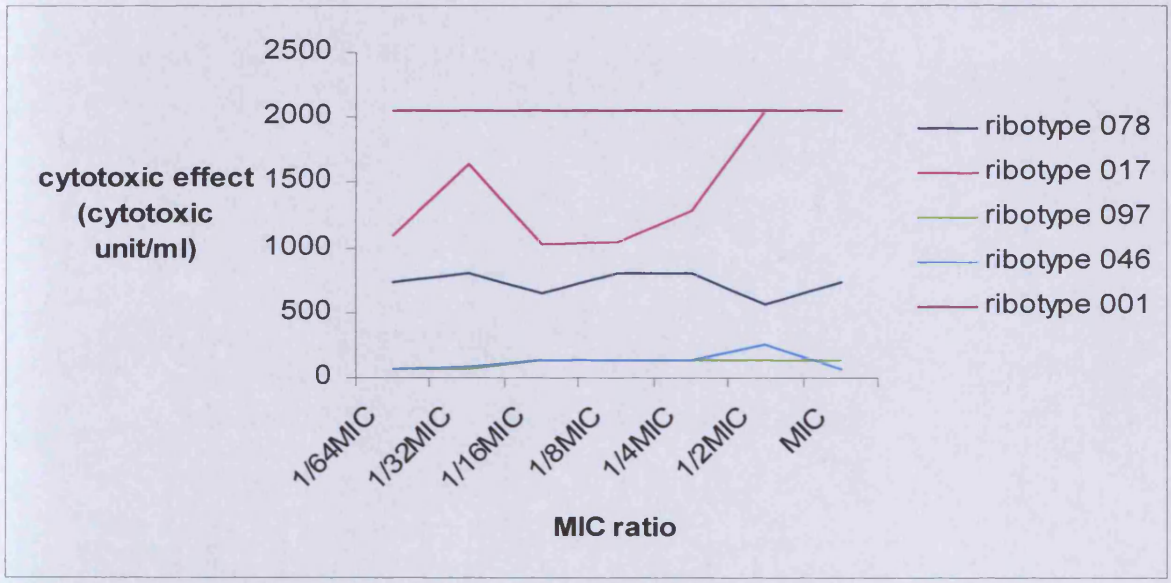
6.3.14 The Mean cytotoxic activity of all strains after exposure to clindamycin

Cell-free cytotoxin: Figure 6.14 (Appendix; Table 6.3.15a) show the mean cell-free CU/ml produced by the *C. difficile* strains pre and post-exposure to clindamycin. Clindamycin induced cell-free cytotoxin production by strain 233D 078 of 736 CU/ml (12-fold) at the MIC after incubation for 24h. The level increased further to 810.6 CU/ml (13-fold), compared to the control, at 1/8 and 1/16 MIC then dropped to the original level at 1/64MIC. There was little difference with the values obtained after incubation 48h.

The induction of cell-free cytotoxin by strain A11A 017 was highest (16-fold rise) at 1/32 and 1/64 MIC after incubation for 24h and after 48 h. With the K34A strain 017, there was only a slight rise (2-4-fold rise) at the MIC and other sub-inhibitory MICs after incubation for 24h. The values after incubation for 48h were 4 – 8 fold increase. Strain 362C 046 produced the highest

induced cytotoxin, 256 CU/ml (8-fold), at 1/32 MIC after incubation for 24h. After 48 h, the highest cytotoxic activities, 264 CU/ml (8-fold rise), were at 1/16 and 1/32 MICs. For ribotype 001, the cytotoxic activity of the cell-free filtrates was high, 2048 CU/ml (16-fold), from the MIC through all the sub-inhibitory concentrations, after incubation for 24 and 48h.

Figure 6.14. Mean cytotoxic activity (CU/ml) of cell-free cytotoxin of *C. difficile* post exposure to **clindamycin** by all test strains



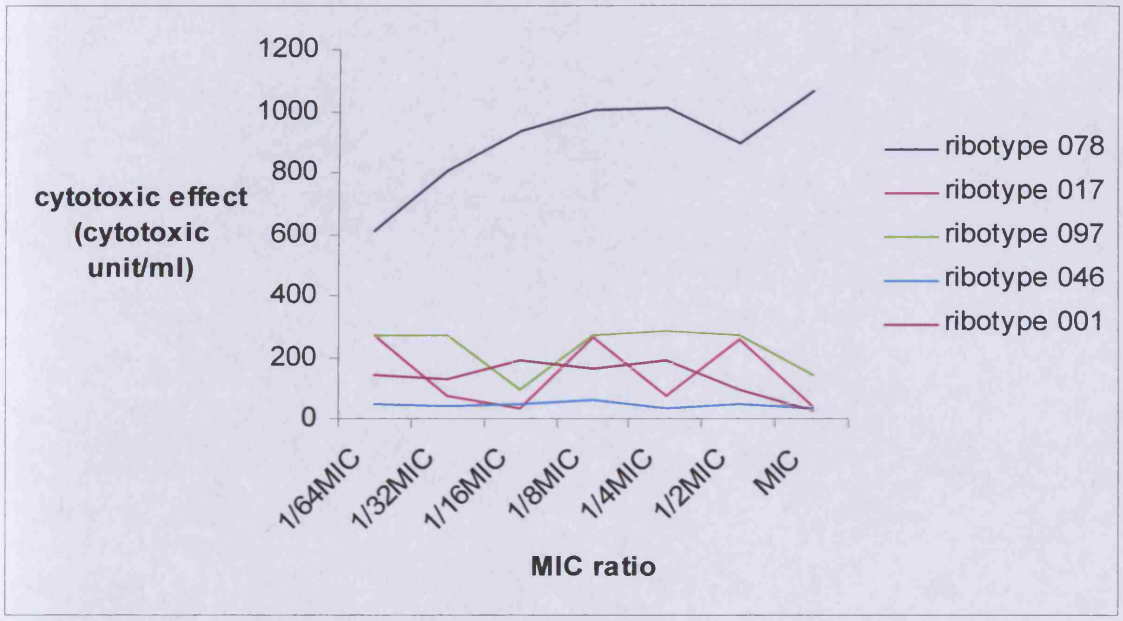
Cell-bound cytotoxin Figure 6.15 (Appedix; Table 6.3.15b): For strain 233D 078, induction of cell-bound cytotoxic activity after exposure to clindamycin at the MIC, was about 17-fold and 12-fold higher than the control after incubation with Vero cells for 24 and 48 h, respectively. Then it increased gradually reaching a maximum of 30-fold and 25-fold rises at 1/64 MIC, after incubation for 24 and 48h.

For strain A11A 017, clindamycin induced cell-bound cytotoxin maximally at the MIC by about 17-fold and 66-fold higher than the control after incubation for 24 and 48h, respectively. With K34A 097, the cytotoxic activities were 272 (17-fold) and 160 (10-fold) CU/ml at the MIC after incubation for 24 and 48h, respectively. The highest activities of 288 (18-fold) CU/ml and 320 (20-fold) CU/ml were observed at 1/16 MIC after incubation for 24 and 48h, respectively.

For strain 362C 046, the maximum activity of 64 CU/ml (4-fold) induction was at 1/8 MIC after incubation for 24h. After 48 h, the maximum activity of 96 CU/ml (6-fold) was detected at ¼, 1/8 and 1/32 MIC.

Exposure of ribotype 001 to clindamycin induced cell-bound cytotoxin production of 144 CU/ml (2-fold) at the MIC, after incubation for 24 and 48h. The maximum activities were 192 CU/ml (3-fold) at ¼, 1/16 MIC and 256 CU/ml (4-fold) at 1/16 MIC, after incubation for 24 and 48h.

Figure 6.15. Mean cytotoxic activity (CU/ml) of cell-bound cytotoxin of *C. difficile* post exposure to **clindamycin** by all test strains



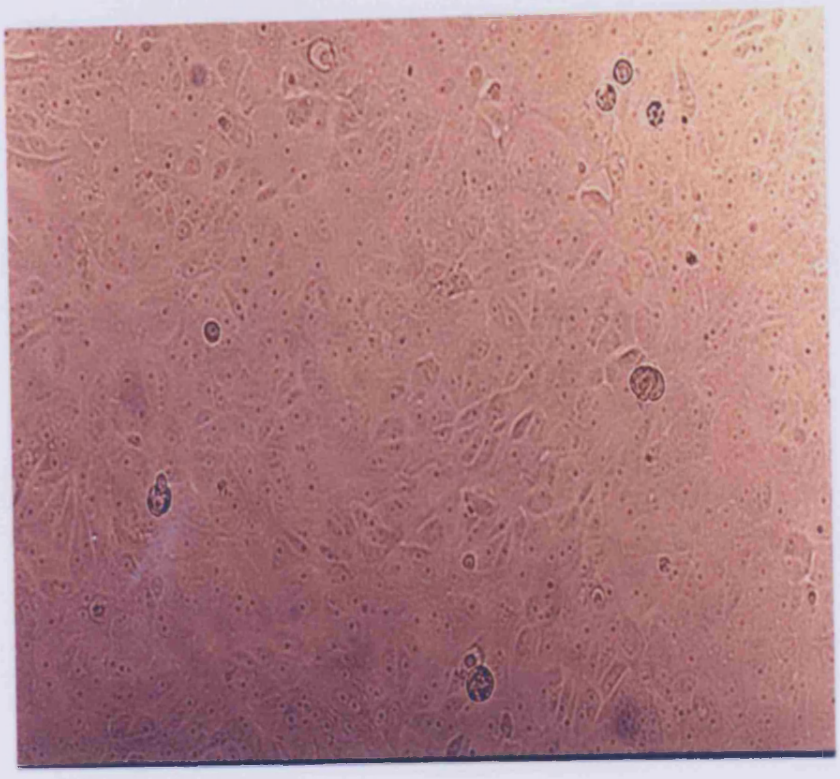


Figure 6.16. Uninoculated Vero Cells after 24 hour incubation (magnification X 40)

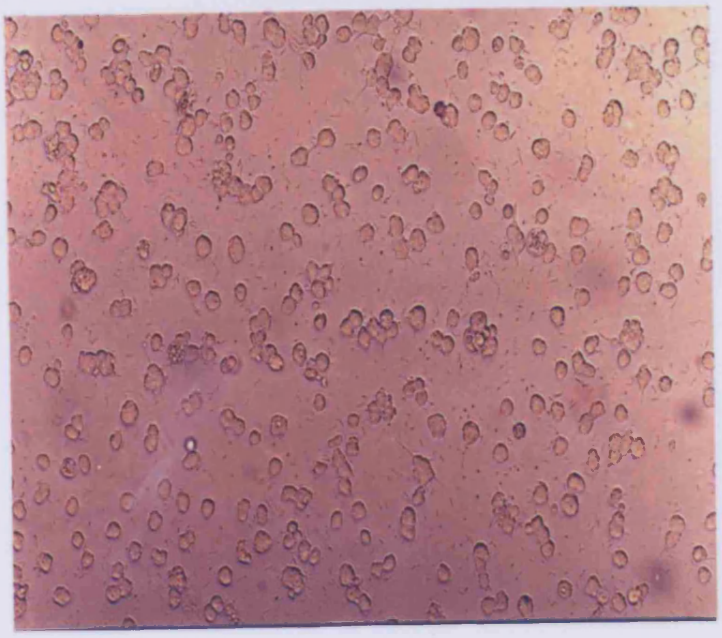


Figure 6.17. Cytopathic effect of *C. difficile* cytotoxin B on Vero Cells demonstrated by rounding of the cells after 24 hour incubation (magnification X 40)

6.4 Comments

Production of CDI is not only enhanced by antibiotics use but also by physiological changes that affect the pathogenicity of *C. difficile* (Lorian and Gemmell, 1994). Previously, Onderdonk *et al.*, (1979); Honda *et al.*, (1983) and Drummond *et al.*, (2003b) reported that certain antibiotics enhance synthesis of toxin A and/or cytotoxin B, which are the main virulence factors. Onderdonk and colleaques in 1981, compared the effect of clindamycin and its metabolites in a hamster model of *C. difficile*-associated colitis and they did not find any correlation between the clindamycin potency and the $AACD_{50}$ (toxin lethal to 50% of the animals). Other investigators described a human gut model of *C. difficile* infections and they demonstrated that *C. difficile* germinate and produce cytotoxin in response to clindamycin (Freeman *et al.*, 2005), cefotaxime with and without its metabolites, desacetylcefotaxime (Freeman *et al.*, 2003), metronidazole (Freeman *et al.*, 2007) and fluoroquinolones such as ciprofloxacin and levofloxacin (Saxton *et al.*, 2009). Recently, Baines and his colleagues demonstrate that vancomycin reduced the vegetative forms and cytotoxin titres of epidemic strains of *C. difficile* but did not have any anti-spore activity (Baines *et al.*, 2009). In addition, the same workers showed that vancomycin was more effective than metronidazole in reducing *C. difficile* PCR ribotype 027 numbers and cytotoxin titre (Baines *et al.*, 2009). In contrast, germination and cytotoxin production was not observed in the human gut model after exposure to piperacillin-tazobactam or tigecycline (Baines *et al.*, 2005; Baines *et al.*, 2006).

This study focused on the effects of MIC and sub-inhibitory concentrations of 5 different antibiotics, including those used for treatment and those known to predispose to CDI occurrence, on the production of cytotoxin B by 6 different strains of *C. difficile*. The study showed clearly that there is a heterogeneous relationship between antibiotic exposure and toxin B production

intracellularly and extracellularly by the toxigenic strains of *C. difficile*. The non-toxigenic strain from a symptomatic patient with diarrhoea, strain 175, did not produce toxin B inside or outside the cell in the absence or presence of any antibiotic. This may be related to the absence of the genes that produce the toxin or the absence of other virulence factors.

The results showed that certain strains, when exposed to the MIC and sub-inhibitory concentrations of certain antibiotics, are capable of producing high level of cytotoxin compared to the antibiotic-free control. This may be due to the stress that an organism experiences in the presence of antibiotics. Early reports suggested that the stress may induce extracellular toxin production. For example, Onderdonk and colleagues reported that raised temperature leads to higher cytotoxin production and toxin production increased in the presence of sub-inhibitory concentration of vancomycin and penicillin (Onderdonk *et al.*, 1979). A study by Karlsson and colleagues (Karlsson *et al.*, 2003) showed that temperature may act as a controlling factor for the expression of toxin A and TcdD. Therefore, toxin production may be enhanced by environmental stress. Emmerson *et al.*, showed exposure of *C. difficile* to environmental stress e.g. heat shock and acid shock lead to upregulation of certain genes that allow the vegetative form of *C. difficile* to tolerate this type of environmental stress. In addition, *C. difficile* is respond to oxidative stress by upregulation of electron transporters (Emmerson *et al.*, 2008). The same invistigators found that exposure to amoxicillin, clindamycin increased the transcription of ribosomal protein genes and altered transcription of genes encoding surface-associated proteins, while minor changes in the transcription occurred after exposure to metronidazole (Emmerson *et al.*, 2008).

In this study, strains A11A 017 and 001 were the most responsive to antibiotic induction of cytotoxin production. Strain A11A 017 is an isolate from an AAD patient and was not a unique isolate; indistinguishable isolates were cultured from other patients. Strain 001 is a strain

associated with CDI outbreaks in the UK. Clindamycin and ampicillin were the most potent inducers of cytotoxin in our hands. They provoked an increase in the cell-free as well as the cell-bound toxin in all toxigenic strains. Metronidazole and vancomycin were almost at the same level as inducers as the former two antibiotics. Surprisingly, cefotaxime induced the least amount of cytotoxic activity on all the strains. However, the cytotoxic effect of the cell-free toxin was more than the cell-bound toxin, particularly after induction with clindamycin, except with strain 233D 078 which produced more cell-bound cytotoxin than extracellular toxin on exposure to clindamycin, vancomycin and metronidazole.

Vancomycin induced more extracellular than intracellular cytotoxin production in all toxigenic isolates except for isolate 233D 078 in which vancomycin induced more intracellular than extracellular cytotoxin production. This may indicate that vancomycin increases cytotoxin release from within the cell rather than enhances synthesis for *C. difficile* 233D strain 078.

Metronidazole induced more cell-free than cell-bound cytotoxin for all toxigenic isolates except isolates 233D 078 and K34A 097 where cell-bound was greater than the cell-free cytotoxin. These 2 isolates were isolated from patients with PMC. However, ampicillin induced more cytotoxin extracellularly rather than intracellularly for all toxigenic *C. difficile* strains. Cefotaxime induced more cell-free than cell-bound cytotoxin B from all toxigenic strains except *C. difficile* ribotype 001 and K34A 097where the cell-bound toxin was more than the cell-free toxin.

There does not appear to be any correlation between the severity of the symptoms and the level of cytotoxin produced in Vero cell line. For example, cefotaxime induced more cytotoxic effect in Vero cells from A11A 017 which caused only AAD in contrast to strain K34A 097 that was

associated with PMC. Moreover, ampicillin induced more cytotoxic effect on Vero cells by strain A11A 017 isolated from a patient with diarrhoea (AAD) than strain 362C 046 isolated from a patient with colitis (AAC) and strain 233D 078 isolated from a PMC case. This suggests that the quantity of the cytotoxin produced is not enough to explain the gut pathology caused by the different strains of *C. difficile.*

The results showed that there is a general relationship between antibiotics and toxin production by *C. difficile.* All isolates showed increase in cytotoxin production after exposure to antibiotics but there is no consistent pattern and the response to different doses varied considerably. Antibiotics that are used for treatment and those that precipitate the disease have different effects on different *C. difficile* isolates, therefore the relationship may be complex. The effects of sub-inhibitory concentration of antibiotic that predispose to CDI development may suppress the normal gut flora partially and allow colonization and growth of *C. difficile* and may affect the level of toxin produced.

CHAPTER 7

ANALYSIS OF PREVALENCE, RISK FACTORS, AND MOLECULAR EPIDEMIOLOGY OF CDI IN KUWAIT

7.1 Introduction

The rate of community-acquired CDI may be underestimated if patients with atypical risk factors and no history of recent healthcare admission are not tested for *C. difficile* toxin. The previous study reported in chapter 2 suggests that CDI is the most common cause of hospital-acquired diarrhoea amongst patients admitted to our hospital ICUs although no outbreak has so far been reported in Kuwait. The findings in that study led us to believe that CDI may be under-reported in Kuwait hospitals in general. With that in mind the present second study was designed to investigate the prevalence, epidemiology and risk factors of CDI in our hospitals.

7.2 Materials and Methods

7.2.1 Patients

Stool specimens from all hospitalised patients and patients seen as outpatients with diarrhoea were requested to be sent to the Anaerobe Reference laboratory, Faculty of Medicine, Kuwait University from all the government hospitals in Kuwait. This was approved by the local ethics committee. The Reference Laboratory normally receives all stool samples from inpatients and outpatients in all Kuwait hospitals requesting for investigation of *C. difficile* and its toxins. Data were collected over a 3-year period, from January 2003 to December 2005. Inpatients with *C.*

difficile toxin A/B positive were evaluated for development of CDI, healthcare-related acquisition and molecular analysis. Data for each in-patient were collected from the patients' medical records regarding date of admission, duration of hospital stay, the date of onset of diarrhoea, antibiotic used 2 months prior to onset of disease, use of nasogastric tube, immunosuppressive therapy, recent surgical intervention, antibiotic used for treatment of the CDI, white blood cell count, temperature, renal function test (i.e. urea and creatinine), diagnosis with the underlying disease, type of CDI and the outcome.

7.2.2 Control patients

Control group data were collected for assessing the risk factors. The control group consisted of patients from whom diarrhoeal faecal samples were examined for *C. difficile* toxin A/B but who were negative for *C. difficile* toxin. They were matched to cases by age (with stratified groups of 2-20, 21-40, 41-60, 61-70, 71-80 and >81years), gender and date (within 2 months of the case becoming symptomatic).

7.2.3 Case definition

C. difficile-associated diarrhoea was defined as: (i) a minimum of 6 loose bowel motions in 36 h, (ii) a positive stool culture and/or cytotoxin assay for *C. difficile* and/or endoscopic evidence of PMC, and (iii) no other explanation for the presence of the diarrhoea (Samore *et al.*, 1994a).

7.2.4 Detection of *C. difficile* infection

Stool samples were investigated for *C. difficile* infection following Figure 2.2, section 2.4 and section 2.2

7.2.5 PCR ribotyping

The PCR ribotyping of all the isolates was done (section 2.5). All the isolates from these cases were included in this typing exercise to determine the ribotypes responsible for CDI and to estimate the prevalence of ribotype 027.

7.2.6 Statistical analysis

Fisher's exact test was used to test proportion between cases and controls for the respective variables. The probability level of <0.05 was considered as significant.

7.3 Results

7.3.1 Prevalence of nosocomial CDI

A total of 697 patients with diarrhoea were investigated over a period of 3 years as follows: 175 in 2003, 218 in 2004 and 304 in 2005. Of these 73 (10.47%) met the diagnosis of CDI. Out of these 73, 56 (76.7%) were hospital-acquired and 17 (23.3%) were from outpatient clinics (either from outpatient clinics in the hospitals or clinics run by the general practitioners). Thus, the

prevalence of hospital-acquired CDI was 8.03% over the study period. The prevalence of hospital-acquired CDI in 2003, 2004 and 2005 were, 17/175 (9.7%), 17/218 (7.8%) and 22/304 (7.2%), respectively. Patient's demographic data are shown in Table 7.1. Over half (55.4%) of hospital acquired CDI were male and less than half (44.6%) were females. Around 59% of the patients were Kuwaiti and over 41% were non-Kuwaitis. About one-quarter (23.2%) of the patients were the aged 41-60 years, another 23.2% were 71-80 years and one-fifth (19.6%) were aged 21-40 years and only 9% were where those aged 61-70 years. Only 14.2% of the patients were between the ages of 2-20 years. Those above the age of 81years accounted for only 10.7% of the total patients. Analysis of the patients from the clinics with CDI revealed that 41.1% were between the age of 21-40 years and 35.3% between 41-60years. A total of 78.5%, 17.8% and 3.5% patients developed AAD, AAC and PMC, respectively.

7.3.2 Length of hospital stay

Only the 56 in-patients and 56 controls were included in the subsequent analysis. As demonstrated in Table 7.2, for the health-care associated CDI, the median duration in the hospital wards was 26 days (range between 5-135 days). Almost one-quarter (23.2%) of the cases stayed in the hospital for 20-29 days, about one-fifth (21.4%) for 10-19 days and 17.8% for 30-39 days. Only 3.5% of the patients stayed for more than 90 days. However, for the controls, the median number of days spent in the hospital was 18 days (range 5-99 days). Nearly one-third (28.5%) of the controls stayed in the hospital for 10-19 days, about one-quarter (26.7%) stayed between 4-10 days and 16% between 20-29days. Only 3.5% of the controls stayed for more than 90 days. There was no statistically significant difference between the total duration of the stay in the hospital and the development of CDI between the patients and the controls.

Table 7.1. Demographic data for patients with CDI over 3 year period

	Hospital acquired (56 patients)	Community acquired (17 patients)
Sex		
Female	25 (44.6%)	8 (47.1%)
Male	31 (55.35%)	9 (52.9%)
Nationality		
K	33 (58.9%)	12 (70.6%)
NK	23 (41.1%)	5 (29.4%)
Age		
2-20years	8 (14.2%)	1 (5.8%)
21-40years	11 (19.6%)	7 (41.1%)
41-60years	13 (23.2%)	6 (35.3%)
61-70years	5 (9%)	1 (5.8%)
71-80years	13 (23.2%)	1 (5.8%)
≥80years	6 (10.7%)	1 (5.8%)

K: Kuwaiti; NK: non-Kuwaiti

7.3.3 Time to acquisition

As demonstrated in Table 7.2, patients with CDI stayed in the hospital between 4 and 129 days with median of 10 days before the onset of the disease. Half of the patients with CDI stayed in the hospital for less than 10 days, one third (32.1%) stayed between 10-19 days and only 5.3% of the patients stayed between 20-29 days. Only 2 patients (3.5%) stayed for 119 and 129 days before the onset of the disease. One patient (1.7%) in each of the following categories stayed between 40-49 days, 50-59 days and 60-89 days. However for the controls, they stayed in the hospital for 4-90 days with a median of 12 days before the onset of the diarrhoea. About one-third (33.9%) of the controls developed diarrhoea between 4-10 days after being admitted to the hospital and another one-third (32.1%) after 10-19 days. Fourteen percent of the controls stayed for 20-29 days before the onset of the diarrhoea and another 14% stayed for 30-49 days. Only two of the control patients stayed for more than 90 days. There was no statistical difference between the duration of the stay in the hospital before the onset of the disease and the development of CDI.

Table 7.2. Risk factors for patients with CDI regarding hospital stay and time of presentation

	No (%) of patients (n = 56)	No (%) of controls (n = 56)	*P*-value
Duration of hospital stay (days)			
<10	8 (14.2)	15 (26.7)	0.160
10-19	12 (21.2)	16 (28.5)	0.513
20-29	13 (23.2)	9 (16)	0.476
30-39	10 (17.8)	7 (12.5)	0.599
40-49	6 (10.7)	3 (5.3)	0.489
50-59	2 (3.5)	3 (5.3)	1.000
60-89	3 (5.3)	1 (1.7)	0.618
≥90	2 (3.5)	2 (3.5)	1.382
Days after initial presentation			
<10 days	28 (50)	19 (33.9)	0.125
10-19 days	18 (32.1)	18 (32.1)	1.160
20-29 days	3 (5.3)	8 (14.2)	0.203
30-39 days	2 (3.5)	4 (7.1)	0.679
40-49 days	1 (1.7)	4 (7.1)	0.364
50-59 days	1 (1.7)	0 (0)	1.000
60-89 days	1 (1.7)	1 (1.7)	1.505
≥90 days	2 (3.5)	2 (3.5)	1.382

7.3.4 Predisposing Factors

As demonstrated in Table 7.3, patients with CDI were significantly more likely than the control to have been exposed to immunosuppressive drugs (28.5% patients vs. 10.7% controls, P = 0.031) and parenteral feeding via naso-gastric tube (26.7% for the patients vs. 8.9% for the

controls, $P<0.05$). Although over one-third (30.3%) of the patients had major surgery within 2 months of the onset of the disease compared with 16% of the controls, the difference was statistically non-significant.

A statistically significant difference was observed between patients who had fever of >38°C compared with the controls; 51.7% vs 30.3%, respectively ($P = 0.034$). Only one patient and non of the controls had leucopenia with WBC < $1x1^9$/L. The majority of the patients (60.7%) had raised WBC of between 10-19.9 $x10^9$/L compared to 35.7% of the controls ($P=0.014$). Comparatively, 11 (19.6%) of the patients vs 34 (60.7%) of the controls had normal WBC ($P<0.05$). Two (3.5%) patients had marked leucocytosis of >$30x10^9$/L.

Analysis of the renal function tests did not reveal any statistically significant difference between the patients and controls.

Table7.3. Risk factors for the clinical presentation of patients with CDI

Risk factor	No. (%) of patients	No. (%) of control	*P*-value
Use of NGT	15 (26.7)	5 (8.9)	**0.025**
Surgery in previous 2 months	17 (30.3)	9 (16)	0.116
Immunosuppression	16 (28.5)	6 (10.7)	**0.031**
Fever >38°C	29 (51.7)	17 (30.3)	**0.034**
WBC count			
<1 x10^9/l*	1 (1.7)	0 (0)	1.000
<9.9 x10^9/l	11 (19.6)	34 (60.7)	**0.001**
10-19.9 x10^9/l	34 (60.7)	20 (35.7)	**0.014**
20-29.9 x10^9/l	8 (12.5)	2 (3.6)	0.094
>30x10^9/l	2 (3.6)	0 (0)	0.494
RFT			
Normal	38 (67.9)	46 (82.1)	0.126
Abnormal (↑)	18 (32.1)	10 (17.9)	0.126

NGT, nasogastric tube; WBC, white blood cells; RFT, renal function test; aplastic anaemia*

7.3.5 Outcome of the disease

Table 7.4 shows the impacts of CDI on the patients. Seven (12.5%) out of 56 patients died compared to 5 (8.9%) of controls without CDI. There was no significant difference between patients and controls regarding survival or death. The number of survivals were higher in the in-patients group than the controls, but the difference was statistically not significant (100% vs. 91.7%, respectively; P = 0.546). Exposure to specific antimicrobial therapy was significantly more prevalent in the in-patients with CDI than the control patients (73.2% vs. 35.9; P = 0.001). Patients and controls were treated with one of the following antimicrobial regimens:

metronidazole alone, vancomycin alone, both metronidazole and vancomycin, both metronidazole and ciprofloxacin or no antimicrobial treatment. Over half of CDI patients were treated with metronidazole alone compared to only one-fifth of the controls (53.5% vs 21.4%, $P<0.001$). Only four (7.1%) patients were treated with vancomycin alone and seven (12.5%) were treated with both metronidazole and vancomycin. None of the patients were treated with both metronidazole and ciprofloxacin compared to 8 (14.2%) controls. None of the control group received vancomycin with or without metronidazole. One-fifth (20%) of the patients who received metronidazole alone died compared to two (16.7%) controls. Only one patient (14.2%) died after being treated with both metronidazole and vancomycin. However, none of the patients who received vancomycin alone or no treatment died.

Table 7.4. The outcome and treatment of CDI and controls

	No (%) of patients	No (%) controls	*P*-value
Outcome			
Survive	49 (87.5)	51 (91)	0.761
Death	7 (12.5)	5 (8.9)	
Treatment			
Metronidazole	30 (53.5)	12 (21.4)	**0.001**
Survival	24 (80)	10 (83.3)	1.000
Death	6 (20)	2 (16.7)	
Vancomycin	4 (7.1)	0 (0)	0.118
Survival	4 (100)	NA	
Death	0 (0)	NA	
Metronidazole followed by vancomycin	7 (12.5)	0	**0.013**
Survival	6 (85.7)	NA	
Death	1 (14.2)	NA	
No therapy	15 (26.8)	36 (64.1)	0.001
Survival	15 (100)	33 (91.7)	0.546
Death	0 (0)	3 (8.3)	
Ciprofloxacin and metronidazole	0 (0)	8 (14.2)	0.006
Survival	0 (0)	8 (100)	-
Death	0 (0)	0 (0)	

7.3.6 Prior therapy with antibiotic

A total of 53 (95%) of the 56 patients with CDI received antimicrobial therapy 2 months prior to the onset of the disease compared to 49 (87.5%) of the 56 controls. As shown in Table 7.5, the third-generation (ceftriaxone, cefotaxime or ceftazidime) and fourth-generation (cefepime) cephalosporins were the commonest antimicrobial agent received by both the patients and controls. They were used alone (73% vs 42%) or in combination with amikacin (5.4% vs 8.9%) for the patients and the controls. Although it appears that cephalosporins usage was the most common previous therapy, the difference did not reach a statistically significant level when compared with the controls ($P = 0.112$). The second most common antibiotic administered was clindamycin which was used for 10 (17.8%) of the patients followed by ciprofloxacin (14.3%) and meropenem (14.3%). Five (8.9%) patients had used metronidazole prior to the onset of the disease but none of them used vancomycin. In contrast to the patients, the second common antibiotics used by the controls after third-generation cephalosporins were cefuroxime (21.4%) and piperacillin/tazobactam (21.4%).

Table 7.5. Antibiotics used 2 months prior to onset of disease

	No. (%) of patients (n=56)	No. (%) of controls (n=56)
Cloxacillin	6 (10.7)	4 (7.1)
Amoxicillin-clavulanic acid	2 (3.6)	0 (0)
Amikacin + cefotaxime	3 (5.4)	5 (8.9)
Cefuroxime	5 (8.9)	12 (21.4)
3^{rd}, 4^{th} generation cephalosporin	41 (73.2)	24 (42.9)
Metronidazole	5 (8.9)	7 (12.5)
Erythromycin	5 (8.9)	9 (16.1)
Ciprofloxacin	8 (14.3)	5 (8.9)
Meropenem	8 (14.3)	4 (7.1)
Piperacillin/tazobactam	4 (7.1)	12 (21.4)
Clindamycin	10 (17.9)	7 (12.5)
Vancomycin	0 (0)	3 (5.4)
No antibiotics	3 (5.4)	7 (12.5)

7.3.7 Underlying morbidity

The underlying diseases in the patients and controls are presented in Table 7.6. The main underlying diseases in the patients were cerebro-vascular accidents and ischemic heart diseases, diabetes mellitus and pneumonia recorded in 24 (42.9%), 15 (26.8%) and 12 (21.4%), respectively compared to 19 (33.9%), 5 (8.9%) and 9 (16.1%) of the controls, respectively.

Twice as many of the patients compared with the controls had septicaemia (4, 7.1% vs 2, 3.6%) and malignancy was found exclusively in 3 (5.4%) of the patients. Because of the small number these figures were not subjected to statistical analysis.

Table 7.6. Underlying morbidity in patients and control

	Patients (n=56)	Controls (n=56)
Pneumonia	12	9
Cerebro-vascular accidents and ischemic heart disease	24	19
Road traffic accidents	3	7
Septicaemia	4	2
Pancreatitis	2	2
SLE	3	2
Renal failure	5	4
Diabetes mellitus	15	5
Urinary tract infection	2	1
Peripheral vascular disease	0	4
Chronic obstructive airway disease	3	4
Inflammatory bowel disease	3	2
Solid organ tumour	1	3
Kidney transplant	0	1
Diabetic ketoacidosis	1	2
Liver cirrhosis	3	3
Short bowel syndrome	1	0
Septic arthritis	1	0
Myasthenia gravis	1	0
Aplastic anaemia	1	0
Underlying malignancy	3	0

SLE, systemic lupus erythematosus

7.3.8 Severity of the disease

Table 7.7 shows the mortality rate of all patients with CDI. Of the 56 patients with the diagnosis of CDI, 7 (12.5%) had severe form of the disease according to the study case definition of severe disease. Three (5.4%) of our patients developed CDI while in ICU for the initial management of their medical conditions. The ages of the seven patients were 44, 56, 64, 71, 75, 76 and 79 years with a median of 71 years. The severe disease was more frequent among those who were above 70 years where the mortality rate reached 30.7%.

Table 7.7. Deaths of inpatients with laboratory confirmed CDI, 2003-2005

Age group,	Total no of patients	No. of death	% of deaths within age group
<50 years	25	1	4
51-60 years	7	1	14.2
61-70 years	5	1	20
71-80 years	13	4	30.7
81-90 years	6	0	0
Total	56	7	12.5

Table 7.8, shows the variables associated with mild and severe CDI. The median age of all patients with CDI was 53 years. Patients with severe CDI were older than those with mild disease (71 vs 51years). More than half (57%) of the patients with severe CDI were above the age of 70 years while only about one third (30.6%) of patients with mild disease were above the age of 70 years. The ratio of male to female patients with and without severe disease is almost the same (1.3 vs. 1.2). Previous surgery within 2 months before the onset of the disease did not differ between patients with or without severe disease. However, more patients with severe disease used nasogastric tube feeding compared to the patients with mild or non-severe disease (57% vs. 22.4%). More patients with severe disease had WBC > 20,000/μl compared to those with mild disease (42.9% vs. 10.2%). There is not much difference between patients with or without disease regarding the underlying medical illness or malignancy.

Table 7.8. Analysis of the results of 56 patients with and without severe disease

Variable	Severe CDI (No = 7)	Non severe CDI (No = 49)
Age (median)	71	50:52
Age >70years	4 (57%)	15 (30.6%)
Male: Female ratio	4:3	27:22
Immunosuppresion	0	16 (32.7%)
NGT feeding	4 (57%)	11 (22.4%)
Previous surgery	2 (28.6%)	15(30.6%)
WBC>20,000/µl	3 (42.9%)	5 (10.0%)
CVA	4 (57%)	20 (40.8%)
DM	3 (42.9%)	15 (30.6%)
Pulmonary disease	1 (14.2%)	11 (22.4%)
Renal disease	1 (14.2%)	4 (8.2%)
Underlying cancer	0	4 (8.2%)

NGT, naso-gastric tube; WBC, white blood cells; CVA, cerebro-vascular accident; DM, diabetes mellitus

7.3.9 PCR ribotypes

The distribution of the PCR-ribotypes of the clinical isolates of *C. difficile* in this second phase prevalence and epidemiological study is outlined in Table 7.9 and Figure 7.1. Although there are 56 patients with CDI from whom *C. difficile* toxin A/B were demonstrated in the stool samples, only 38 (67.9%) *C. difficile* strains were isolated. These 38 isolates were subjected to PCR ribotyping. As shown in Table 32, 16 distinct genotypically different DNA ribotypes were established among the 38 clinical isolates. Two new ribotypes were added to the library,

ribotypes 195 (toxigenic) and 196 (non-toxigenic). Of these 38 isolates, ribotypes 002 (toxigenic; 18.4%), 001 (toxigenic; 15.7%), 126 (toxigenic; 10.5%) and 140 (non-toxigenic; 10.5%) were the four most common distinct clones. The remaining 17 isolates belonged to diverse ribotypes which are 003 (3, 7.8%), 014 (2, 5.3%), 057 (2, 5.3%), 195 (2, 5.3%), 005 (1, 2.6%), 029 (1, 2.6%), 056 (1, 2.6%), 083 (1, 2.6%), 107 (1, 2.6%), 159 (1, 2.6%), 177 (1, 2.6%) and 196 (1, 2.6%). The PCR ribotypes for those patients with severe disease were the following: 196 (non-toxigenic, new type), 083, 056, 126, 001 and 002 while the strain from the last patient with severe disease did not grow. The two patients with PMC were infected with ribotypes 056 and 001. No PCR-ribotype 027, 017 or 078 were encountered in this series.

Table 7.9. Distribution of *C. difficile* PCR ribotypes between 2003-2005.

PCR ribotype	Toxin A/B	Total no. (%) of strains (n = 38)
001	+/+	6 (15.7)
002	+/+	7 (18.4)
003	+/+	3 (7.9)
005	+/+	1 (2.6)
014	+/+	2 (5.3)
029	+/+	1 (2.6)
056	+/+	1 (2.6)
057	+/+	2 (5.3)
083	+/+	1 (2.6)
107	+/+	1 (2.6)
126	+/+	4 (10.5)
140	-/-	4 (10.5)
159	+/+	1 (2.6)
177	+/+	1 (2.6)
195*	+/+	2 (5.3)
196*	-/-	1 (2.6)

*New PCR-ribotypes added to the existing library in Cardiff, Wales, UK

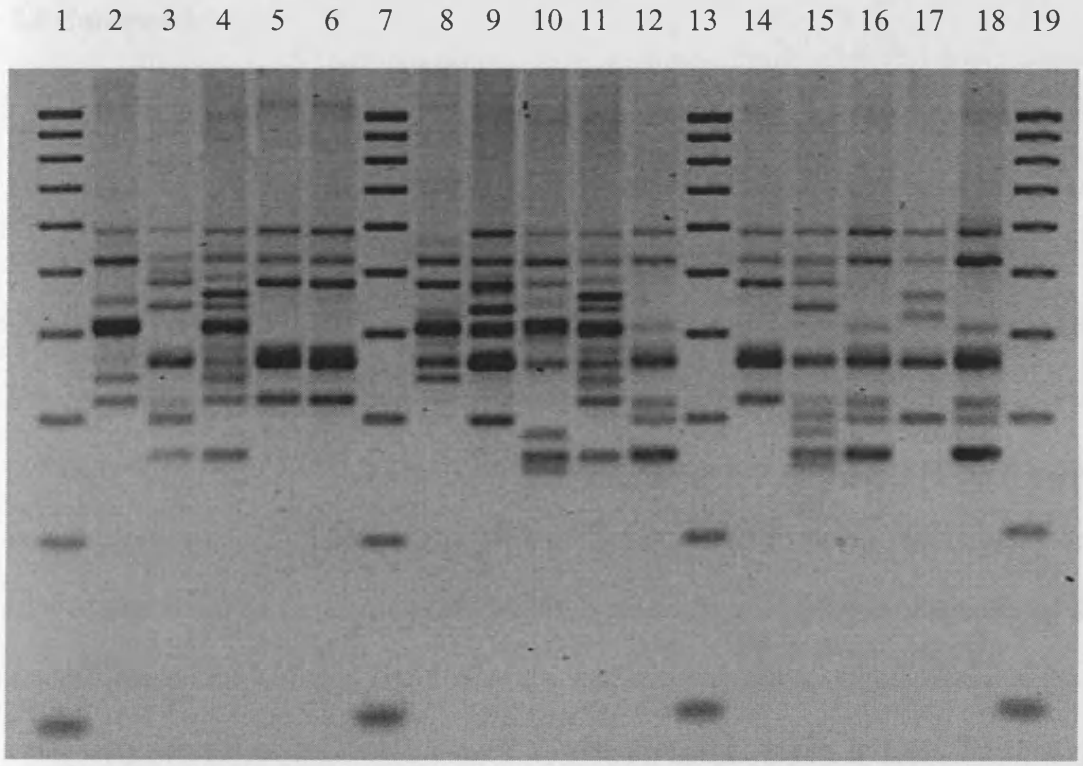


Figure 7.1. PCR ribotype profiles of *Clostridium difficile* isolated in Kuwait from 2003 to 2005. Lanes 1,7,13,19 = 100bp; Lane 2 = 057; Lane 3 = 083; Lane 4 = 140; Lane 5 = 002; Lane 6 = 002; Lane 8 = 126; Lane 9 = 195; Lane 10 = 003; Lane 11 = 140; Lane 12 = 001; Lane 14 = 002; Lane 15 = 107; Lane 16 = 001; Lane 17 = 029; Lane 18 = NCTC 11209 (type 001)

7.4 Comments

CDI is not a reportable disease in Kuwait and infection rates as well as disease epidemiology are not clear except perhaps in the intensive care units where documented case have been reported previously (Rotimi *et al.*, 2002). The present study extended the previous ICU experience and assessed the prevalence and epidemiology of all hospital-acquired CDI over a 3-year period. In this study, the data suggest that the prevalence of CDI decreased from year to year with an overall prevalence of 8.03% which is lower than was reported earlier in a specific hospital setting in Kuwait (Rotimi *et al.*, 2002). The low prevalence of the disease in Kuwait may be related to lack of awareness on the part of the clinicians concerning the clinical diagnosis of the disease. Another reason may be due to the use of a less sensitive toxin immunoassay in the Reference Laboratory instead of the tissue culture cytotoxin assays which is more laborious and time-consuming. In addition to lack of awareness on the part of our clinicians, the low prevalence may also be related to good antibiotic control policy that is in place in Kuwait hospitals as well as the limited use of the new fluoroquinolones, e.g. gatifloxacin which has been associated with outbreaks of CDI (Gaynes *et al.*, 2004). It is conceivable that the strict infection control guideline requiring the use of soap and water as the primary means of hand hygiene in Kuwait hospitals may have helped to limit the spread of infection as soap is more effective in removing the spores than alcohol-based hand rub (WHO, 2007).

That the overall low prevalence of CDI in Kuwait is now a documented evidence that the disease is not yet a big problem here, particularly when compared with the prevalence rates in the Western countries such as the UK (Durai, 2007; HPA 2009), USA (McDonald *et al.,* 2006), Canada (Loo *et al.*, 2005) and Israel (Samra *et al.*, 2002). In Canada, the incidence of CDI was 22.5 per 1000 admissions during 2004 (Loo *et al.*, 2005). Indeed recent data from these countries

have demonstrated high incidence of CDI of 100-450/10,000 admissions among hospitalised patients. This has been associated with the emergence of infections due to the hypervirulent strain of ribotype 027/NAP1 (McDonald *et al.*, 2005; Pepin *et al.*, 2005; Loo *et al.*, 2005; Kuijper *et al.*, 2008). So far no such strain has been isolated in Kuwait. Even compared with some developing countries, like Peru, our prevalence rate is much lower than the reported prevalence of 35.5% (Garcia *et al.*, 2007). However, the only one report to emerge from the Middle East also demonstrates equally low prevalence rate such as the rate 9.7% in Jordan (Shehabi *et al.*, 2001).

It is well known that *C. difficile* infection can occur in the community as well as hospital (Wilcox *et al.*, 2008) as it is widely disseminated in the environment and has been isolated from the soil, water and faeces of many wild and domestic animals (Brazier, 1998). This current study revealed that 17 (23.3%) positive stool samples for *C. difficile* toxin A/B were from patients attending the clinics. This is a very high rate and an interesting finding as there has been no data about the incidence or prevalence of community-acquired CDI from Kuwait. It is therefore an important observation worthy of a follow-up study. This is buttressed by a recent report from the UK that estimated the prevalence of *C. difficile* cytotoxin positivity in urban and semi-urban areas in the community as 2.1% (Wilcox *et al.*, 2008). Another report from the Centers for Disease Control and Prevention estimated that the minimum annual incidence of community-acquired CDI in Philadelphia area between July 2004 and June 2005 was 7.6 cases /100,000 populations (CDC, 2005). Price and colleagues recently reported the incidence of CDI within 48 hour of admission to the hospital as 4.0 cases /10,000 patient-days as compared to 7.0 cases /10,000 patient days for nosocomial CDI (Price *et al.*, 2007). All these reports suggest a low level of CDI in the community and so whether Kuwait's situation is a paradox remains to be investigated.

As previously pointed out by Asha *et al.*, 2006, age of 70 years and above is an important risk factor for CDI. Our data showed that 42.9% of the CDI patients were above 60 years out of which over 79% were aged 71 years and above. Overall, 33.9% of patients with CDI were above the age of 71 but finding 23.2% in a relative younger age group of 41-60 years group was surprisingly high. Although concordant with the findings of McDonald *et al.* 2006, who reported a rate several fold higher in persons above the age of 65 (228/100,000) with the next highest rate in the 45-65 year olds 40/100,000), the rate in the latter group was much lower than the present study. The possible reasons that may explain the association between age and CDI are the increased exposure to health care facilities, the use of antimicrobial agents and reduced stomach acidity due to possible use of H_2 blockers or proton pump inhibitors which are associated risk factors for CDI (Asha *et al.*, 2006). Eight (14.2%) of our patients were children between the age of 2-20 years. Finding CDI in this very young age is supported by a previous report published by Benson *et al.* (2007) which reported a much higher proportion of children with CDI above the age of 2 years.

The association between the length of hospital stay (≥7 days) and CDI development has been described repeatedly (Asha *et al.*, 2006; Garcia *et al.*, 2007; Manian *et al.*, 2007). However, prolonged hospital stay, as found in this study, was not necessarily a significant risk factor as the median number of days spent in the hospital before the onset of the disease was 10 days versus 12 days for the control. This is also buttressed by the fact that 50% of the patients developed CDI within 4-10 days of admission to the hospital, thereby nullifying the notion of prolonged hospital stay as a significant risk factor, at least not in Kuwait.

Several other risk factors have been described previously, such as naso-gastric tube feeding, immuno-suppression, surgery within 2 months of the onset of the disease, antacid, proton pump

inhibitors and antibiotic therapy. In this study, nasogastric tube feeding and immunosuppressive drugs were statistically significant risk factors. This is in accord with previous reports (McFarland, 1995; Halim *et al.*, 1997). It is postulated that tube feedings may help nosocomial transmission of *C. difficile* spores in the hands of healthcare providers. Unlike other studies (McFarland, 1995; Halim *et al.*, 1997), recent operations and length of hospital stay were not statistically significant risk factor in this study.

Exposure to antimicrobial agents is also considered as an important precipitant factor for CDI. Broad spectrum cephalosporins and clindamycin are considered to be high risk antibiotics and the main drivers of CDI (Freeman and Wilcox, 1999). Recent data (Freeman *et al.*, 2003) suggested that the broad-spectrum cephalosporins may stimulate the production of toxin by *C. difficile*. Experiment described in chapter 4 of this study seems to support this assertion. Seventy three percent of our patients had history of exposure to third or fourth-generation cephalosporin compared to only 42.9% of the controls, a finding that was statistically not significant (P=0.112). This is in contrast to the findings of Loo *et al.*, (2005) and Asha *et al.* (2006). However, our data is similar to the report by Weiss *et al.* (2007), who did not find any correlation between the quantity of cephalosporin consumption and the magnitude of CDI outbreak development. Clindamycin was the second most common antibiotic used among our patients. However, there was also no statistically significant difference between patients and controls which is similar to the findings by Loo *et al.* (2005). But contrasting to a report by Weiss *et al.* (2007), there was no association between CDI cases and clindamycin consumption. The newer fluoroquinolones, e.g. gatifloxacin which has been associated with increased risk of CDI (Loo *et al.*, 2005; Gaynes *et al.*, 2004), is not available in Kuwait although consumption of older ones such as ciprofloxacin and norfloxacin is relatively high (Eggleston *et al.*, in press). However, only 8 patients used

ciprofloxacin which was not of statistical significance compared with the control. Aminoglycoside, e.g. amikacin, was used by only three CDI patients. It is therefore not considered as a trigger antibiotic for CDI because anaerobes are intrinsically resistant to it and it does not disrupt the normal anaerobic flora. Piperacillin/tazobactam is the second most commonly used antibiotic (21.4%) by the control patients but used by only 7.1% of our patients.

It is interesting to note that all of the fifteen patients with CDI who did not receive proper antimicrobial therapy for their illness survived and 6 out of 30 patients who received metronidazole died. This may reflect the severity of the illness i.e. those who died had severe illness that necessitate initiation of metronidazole while those who had mild illness did not require antimicrobial therapy. In addition, those who died had other co-morbidities that increased the risk of death. Recent report had questioned the efficacy of metronidazole to treat CDI which may be related to its poor pharmacological profile or resistance to metronidazole (Musher *et al.*, 2005, Baines *et al.*, 2008). The latter speculation is out of the question as all our *C. difficile* were susceptible to metronidazole as will be discussed in chapter 6.

The PCR ribotypes of the *C. difficile* isolates of the second part of the study differ from those that were isolated previously (Rotimi *et al.*, 2003). Between 2001-2002, ribotypes 097,039 and 078 were the most common ribotypes isolated from CDI patients. However, the most common ribotype isolated between 2003-2005 were type 002 followed by type 001. Although type 001 was the second commonest PCR-ribotype during the 2003-2005 study, it was isolated only from one environmental sample in the 2001-2002 study of the CDI acquired in the ICUs. PCR-ribotype 027 was not isolated during 2003-2005 study and this may partially explain the mild illness from CDI seen in Kuwaiti hospitals and absence of major outbreaks among the hospitals. Different PCR-ribotypes (196, 083, 056, 126, 001 and 002) were associated with severe disease

among our patients with severe CDI. These finding suggests that the clinician in Kuwait may be able to link the risk of severe outcome with clinical data e.g. age, nasogatric tube feeding and WBC without individual genotyping.

Although nearly half (44.6%) of our patients were less than 50 years of age, they had the least number of death rate (4%). This may be related to better immune system than elderly patients. As expected advanced age was associated with higher death rate (30.7%) than the younger patients. Death due to severe CDI in our patients was more among those above 71 years, a finding which is similar to those of other studies (Loo *et al.*, 2005; Henrich *et al.*, 2009). We did not find any association between immunosuppression or underlying malignancy with severity of the disease similar to the findings of Henrich *et al.*, (2009). Patients with severe disease were more likely to have been exposed to nasogastric tube feeding than the controls, a finding similar to that of Henrich *et al.*, (2009) and Loo *et al.*, (2005). Laboratory markers e.g. raised leucocytes counts above 20,000/μl and increased creatinine level have been correlated with poor outcome for patients with CDI. Our study showed that raised leucocytes count but not creatinine level was associated with the severity of the disease.

In conclusion, CDI appears to occur at a relatively low level in hospitals in Kuwait and the prevalence of CDI in patients attending outpatient clinics is even much lower although a conclusion like this is not well supported by the methodology used in the study. The prevalence in hospitals is decreasing as demonstrated by trend over 3 year period. PCR-ribotypes 002 and 001 are the predominant ribotypes circulating in Kuwait right now. Type 027 has not reach Kuwait yet. Advanced age and use of enteral feeding as well as raised leucocyte count is associated with severe outcome of CDI in Kuwait. The physicians need to maintain high index of suspicion for CDI in Kuwait and there is a need for continuous surveillance to better understand

the changing epidemiology of CDI. Therefore, more educational programmes should be directed to all physicians concerning the role of *C. difficile* as an important enteric pathogen especially in hospitalised patients.

CHAPTER 8

GENERAL DISCUSSION

C. difficile is the most important cause of health-care associated infectious diarrhoea and prior antibiotic exposure is regarded as the most important risk factor for the development of diarrhoea (Barbut *et al.*, 1996). Some patients may remain without symptoms after exposure to the organism, while others may have diarrhoea ranging from mild diarrhoea to colitis to fulminant PMC. The illness may become severe requiring colectomy, intensive care unit admission or lead to death. *C. difficile* may be transmitted directly or indirectly as its spores may survive for long periods of time and can resist environmental challenges, detergents and some disinfectants.

The data generated from this study have shown that *C. difficile* is the most common cause of hospital-acquired diarrhoea in Kuwait in the study carried out in 2001-2002 and the evidence from this study showed that it causes sporadic infections rather than major outbreaks in our hospital ICU setting. The overall prevalence of health-care acquisition of *C. difficile* amongst patients admitted to the four ICUs during this period was 10.3%. This overall acquisition rate may appear to be low but a breakdown of the rate into different hospital prevalence reveals that individual rates may be higher that those from similar settings elsewhere. For example, the acquisition rate was lowest in the burn unit (ICU-2) with only 6.5% of patients being colonised but this rate is much higher than the 1% rate reported by Still *et al.* (2002) in a similar setting. Similarly, the highest acquisition rate of 18.2% obtained in the haematology wards (ICU-3) of KCCC was higher than the 15% reported earlier by Wroblewska *et al.*, (2005).

In general hospital-acquired *C. difficile* infection in the ICUs occurred in only 30 (31.6%) out of 95 patients and the remaining 65 patients were asymptomatic carriers. This goes to show that in special units, like the ICUs, of a hospital these asymptomatic patients may act as the reservoir for the dissemination of the organism. During the 2001-2002 study, the majority of the 30 symptomatic patients (93%) were caused by toxin A/toxin B-positive strains with the exception of one patient in Amiri hospital with toxin A-negative/toxin B-positive strain (ribotype 017); The remaining 2 symptomatic patients were infected by toxin-negative *C. difficile* isolates. Finding a toxin A-/toxin B+ isolate at the time was interesting as several outbreaks of infection due to toxin A-/toxin B+ strains have been reported in Canada and Poland (Al-Barrak *et al.*, 1999; Kuijper *et al.*, 2008). This strain seems to have disappeared as no such strain was isolated in the most recent study that looked at the general prevalence of CDI in a hospital-wide study across the country (see chapter 7).

The majority of literature evidence indicates that the major risk factors for CDI are antibiotic exposure, advanced age and hospitalisation. In the four ICUs (1-4) studied, the median ages were 51, 1.5, 31 and 59 years, respectively. This finding reveals a much lower age of acquisition of infection than the ones that have been reported by other investigators (McFarland *et al.*, 1990; Loo *et al.*, 2005) which shows that the average age of acquisition of CDI in Kuwait is below the average elsewhere. This may be explained by the lower average age of patients usually admitted to the ICUs of our hospitals. In the same study, the majority of the patients with early and late acquisition of *C. difficile* were exposed to the cephalosporins and few patients were exposed to metronidazole and meropenem. This finding is supported by reports from other countries which have shown that cephalosporins, clindamycin and fluoroquinolones are important risk factors for the development of CDI (Bartlett, 1994; Gerding *et al.*, 1995; Bignardi, 1998; Loo *et al.*, 2005).

The environment is said to play an important part in the maintenance and transmission of the organism in the hospital setting. Although the environment of the ICUs was contaminated with *C. difficile*, there was little epidemiological link to the development of CDI as is revealed by PCR-ribotyping study described in chapter 4. Another important source of transmission investigated in this study was the hands of health care workers in the ICUs. Unlike other investigators who isolated *C. difficile* from the hands of their health care workers (McFarland *et al.*, 1989), *C. difficile* was not isolated from the hands of any of our staff members in the 4 ICUs.

It would appear that the PCR ribotypes of the *C. difficile* circulating in Kuwait and associated with CDI are not stable. During 2001-2002, 95 culture-positive patients had 32 different PCR ribotypes. The main ones were ribotype 097, which account for 27% of the isolates, and ribotype 078 which account for 13% of all the isolates. But during the 2003-2005 study, 38 culture-positive isolates had 16 different PCR ribotypes. The commonest ribotypes described during this period were ribotypes 002 (18.4%) and 001 (15.7%) followed by 126 and 140. There were no 097 or 078. The explanation for this change is not clear. During the first part of the study, ribotype 001 was detected from only one sample from the environment of ICU-4 in Amiri hospital whereas during the second part, it was the second most common riboytpe among the patients. Similarly, type 002 was isolated from only 2 patients (2.1%) in the previous study but became the predominant type during the second part of the study. This finding tends to compare with the situation in Europe although at a lower level. For instance, in UK, 55% of the infections in hospitals were due to ribotype 001 (Stubbs *et al.*, 1999) in the earlier years but recently the situation has changed. For example, between April 2007- February 2008, the commonest ribotypes in England were 027 (42%), 106 (19%) and 001 accounting for only 10% (Kuijper *et al.*, 2008). Also, in Scotland, in 2006, the commonest PCR ribotype was type 106 (55%)

followed by 001 at a distant 21%. However, ribotype 001 seems to still maintain its edge in Northern Ireland, where, during a survey between September and December 2006, it remains the commonest ribotypes (35%) followed by 106 (11.6%) and 078 (8.3%). Ribotype 027 has also surfaced in Northern Ireland (Kuijper *et al.*, 2008).

During the initial part of the study, ribotype 078 which was the second most common toxigenic strain isolated from the CDI patients, was not isolated from any of the 38 culture-positive patients during the 2003-2005 study. PCR ribotype 078 has emerged recently in Scotland and it accounts for 6.3% of Belgium isolates (Kuijper *et al.*, 2008). The most frequently isolated PCR ribotypes in The Netherlands are ribotypes 027, 014, 001 and 078 (Goorhuis *et al.*, 2007; Goorhuis *et al.*, 2008) while the most frequent PCR ribotype in Poland is type 017 which accounts for 45% of their tested isolates (Pituch *et al.*, 2006).

In the experiment on the induction of *C. difficile* cytotoxin production by MIC and sub-inhibitory concentrations of antibiotics, it was clear that there is a heterogeneous relationship between antibiotic exposure and intra- and extra-cellular toxin production by the toxigenic strains. Certain strains of *C. difficile* when exposed to MIC and sub-inhibitory concentrations of certain antibiotics are able to produce high level of cytotoxin. In this experiment, ampicillin and clindamycin were the most potent inducers of cytotoxin production followed by metronidazole and vancomycin. Surprisingly, cefotaxime induced the least amount of the cytotoxin activity. Thus, suggesting that the quantity of the cytotoxin produced is not enough to explain the gut pathology caused by the different strains of *C. difficile*. This may be due to the stress that an organism experiences in the presence of antibiotics. Early reports suggested that the stress may induce extracellular toxin production. As an example, Onderdonk *et al.* (1979) showed in their study that raised temperature lead to increased cytotoxin production and toxin production

increased in the presence of sub-inhibitory concentration of vancomycin and penicillin which essentially agrees with the findings of the present study. Therefore, toxin production may be enhanced by environmental stress as well as by exposure to antibiotics. The results of the experiment also showed that there is no uniform relationship between antibiotics and toxin production by *C. difficile* as antibiotics that are used for treatment and those that precipitate the disease may have different effects on different *C. difficile* isolates, therefore suggesting that the relationship may be complex. It can be speculated that the effects of sub-inhibitory concentration of antibiotic that predispose to CDI development may suppress the normal gut flora partially and allow colonisation and growth of *C. difficile.*

In the study of the susceptibility of the *C. difficile* strains it was shown that all the isolates were uniformly susceptible to ampicillin, amoxicillin-clavulanic acid, linezolid, metronidazole piperacillin-tazobactam, vancomycin and teicoplanin. In this study, metronidazole and vancomycin remained highly active in vitro. Although 4 and 2 clinical isolates had raised MIC to vancomycin (2-3μg/ml) and teicoplanin (2μg/ml), all of them were inhibited by concentrations that did not exceed 3 and 2 μg/ml, respectively, i.e. they were within the susceptible range. Other findings similar to this have been reported by Drummond *et al.* (2003a) and Mutlu *et al.* (2007). However, in vitro activity does not necessarily correlate with the in vivo activity or the therapeutic outcome for the patients. For example, during treatment of severely sick patients with CDI, metronidazole was associated with higher failure rate compared to vancomycin (Musher *et al.*, 2005) and that there was a poor response to metronidazole if the antibiotic for the initial condition has to be continued (Modena *et al.*, 2006).

In this study, there was no isolation of *C. difficile* resistant to linezolid unlike in Israel where 2% of the isolates are resistant to linezolid (Bishara *et al.*, 2006). It must be emphasised that despite

the excellent in vitro activity, experience with the use of linezolid therapy in CDI is limited and more trials are needed to make any of such recommendation especially as there is report of treatment failure with intravenous linezolid in the therapy of patients with CDI (Lazzarini and De Lalla, 2003). This drug has also been known to contribute to the development of *C. difficile* colitis with fatal outcome (Zabel and Worm, 2005).

A very important highlight of this study is the discrepancy observed between imipenem and meropenem susceptibility in the two phases. A large proportion of the isolates were resistant to imipenem and sparing meropenem, particularly in the first phase study. The rise in meropenem resistance from 4.8% to 21.4% in the first and the second part of the study respectively is also worthy of note. As both drugs are carbapenems with identical mode of action and spectrum of activity it is not clear at this time what the mechanism of resistance might be. While meropenem resistance has not been detected among *C. difficile* isolates, imipenem resistance has been reported (John and Brazier, 2005; Bougault *et al.*, 2006) which is in support of the findings reported in the thesis.

The rate of resistance to clindamycin by the local Kuwait isolates is exceedingly high (48%), much higher than the previously reported 14.7% resistance rate in Canada by Bourgault *et al.*, (2006). The unrestricted use of clindamycin in the country may eventually lead to selection of *C. difficile* and overgrowth in the gut and it is predicted that more cases of CDI will follow. Another note-worthy finding was marked increase in the isolation of penicillin-resistant strains in the 2003-2005 study which was 16.6% compared to 2.4% in 2001-2002. This may be due, in part, to that fact that a relatively fewer number of isolates were tested in the latter study.

During the period 2003-2005, the overall prevalence of hospital-acquired CDI was low (8%) which is much lower than that reported in other countries like UK (Durai, 2007), Canada (Loo *et al.*, 2005) and USA (McDonald *et al.*, 2006). Our hospitals are noted for good infection control practices and strict adherence to published policies. This may probably be a plausible explanation for this low prevalence of CDI. On the other hand, it may be that cases are been under diagnosed because of the use of a less sensitive technique for the diagnosis of CDI such as ELISA for toxin A and toxin B (TechLab Tox A/B) that is the only available tool in the Anaerobe Reference Laboratory. Although this assay has a specificity of 98%, its sensitivity of 78% is rather too low compared to the cell cytotoxicity assay (O'Connor *et al.*, 2001).

As expected in a general hospital, CDI is often associated with advanced age. About one-third of our patients in the second part of the study were above the age of 71 years and one-quarter between 41-60 years. This is essentially similar to the findings of Loo *et al.*, (2005) and Henrich *et al.*, (2009). Exposure to entral feeding via nasogastric tube and immunosuppressive medication, like chemotherapy or steroids, was significantly associated with CDI in our study which contrasts the report of Loo *et al.*, (2005) who did not demonstrate a significant association between these risk factors and CDI. However, these findings in the present study are concordant with previous reports (McFarland, 1995; Halim *et al.*, 1997). Patients with severe disease are more likely to have been exposed to nasogastric tube feeding than the controls, a finding similar to that of Loo *et al.*, (2005) and Henrich *et al.*, (2009). It is conceivable that entral feeding via the tube may help nosocomial transmission of *C. difficile* spores that are present in the hands of healthcare providers during care of the patient. Unlike other studies (McFarland, 1995; Halim *et al.*, 1997), recent operations and length of hospital stay were not statistically significant risk factor in this study. About two-thirds of our patients with CDI had leucocyte counts between

10,000-19,000 x 10^9/L compared to the controls. Laboratory markers, such as raised leucocyte counts (leucocytosis) above 20,000/μl and increased creatinine level have been correlated with poor outcome for patients with CDI. Our study showed that raised leucocyte count but not creatinine level was significantly associated with the severity of the disease.

In conclusion, *C. difficile* is acquired in our hospital ICUs by over one out of 10 patients and the prevalence of CDI in Kuwait is at a relatively low level. Cases of CDI was also seen among patients attending the outpatient clinics although with a much lower prevalence rate than it is in the hospital setting. It is suggested that the use of less sensitive methodology for detecting *C. difficile* toxin, lack of awareness on the part of the physicians, restrictive use of fluoroquinolones (only ciprofloxacin is readily available in Kuwait) and adherence to infection control guidelines and policies may be responsible for the relatively low prevalence rate. The prevalence in hospitals is decreasing as demonstrated by trend over 3 year period. The previous predominant PCR-ribotypes, 097 and 078 in the earlier study, have been replaced by 002 and 001 as the predominant ribotypes now present in Kuwait. It is reassuring that ribotype 027 has not yet reached Kuwait. Advanced age and use of entral feeding as well as raised leucocyte count, are associated with severe outcome of CDI in Kuwait. *C. difficile* strains from Kuwait appear to be uniquely resistant to imipenem with a high resistance rate to clindamycin. Strains implicated in aetiology of CDI are capable of inducing extra- and intra-cellular cytotoxin production after exposure to sub-inhibitory concentrations of certain antibiotics. To keep the prevalence of CDI at acceptable low level, the physicians must maintain high index of suspicion for CDI in Kuwait and there is a need for continuous surveillance to better understand the changing epidemiology of CDI. Educational programmes should be directed to all physicians concerning the role of *C. difficile* as an important enteric pathogen especially in hospitalised patients.

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Appendix

Table 5.3.1. Susceptibility of 95 clinical isolates of *C. difficile* against 16 antibiotics

Antibiotic	Minimum inhibitory concentration (MIC) in μg/ml			
(breakpoint, μg/ml)	MIC_{50}	MIC_{90}	Range	% resistant
Amox/clav (8)	0.125	0.38	0.016-0.75	0
Ampicillin (8)	0.25	1.5	0.016-4	0
Cefotaxime (32)	>256	>256	24->256	92.7
Cefoxitin (32)	>256	>256	0.25-256	97.5
Cefuroxime (32)	>256	>256	256-256	100
Clindamycin (4)	4	>256	0.016->256	48.4
Imipenem (8)	>32	>32	0.064->32	91.4
Linezolid (4)	2.0	2.0	0.5-4.0	0
Meropenem (8)	0.5	1.5	0.012-32	4.8
Metronidazole (8)	0.047	0.19	0.016-0.94	0
Penicillin (4)	0.75	1.5	0.006-32	2.4
Piperacillin (32)	1	4	0.016-256	1.2
Pip/taz (64)	2	4	0.016-16	0
Teicoplanin (2)	0.125	0.25	0.047-2	0
Trovafloxacin (4)	2	4	0.5-64	2.4
Vancomycin (4)	0.5	0.75	0.125-3	0

Amox/clav= amoxicillin-clavulanic acid; Pip/taz = piperacillin-tazobactam.

Table 5.3.2. Susceptibility of 18 environmental isolates of *C. difficile* to 16 antibiotics

Antibiotic	Minimum inhibitory concentration (MIC) in μg/ml			
(breakpoint, μg/ml)	MIC_{50}	MIC_{90}	Range	% resistant
Amox/clav (8)	0.25	0.38	0.064-0.5	0
Ampicillin (8)	0.75	1.5	0.19-2	0
Cefotaxime (32)	>256	>256	24->256	83.3
Cefoxitin (32)	>256	>256	256->256	100
Cefuroxime (32)	>256	>256	256->256	100
Clindamycin (4)	4	>256	1.5-256	38.8
Imipenem (8)	>32	>32	3->32	94.4
Linezolid (4)	0.5	2.0	0.01-4.0	0
Meropenem (8)	1	>32	0.094-32	50
Metronidazole (8)	0.094	0.19	0.023-.38	0
Penicillin (4)	0.75	1.5	0.25-3	0
Piperacillin (32)	1	2	0.125-256	5.5
Pip/taz (64)	3	4	0.032-8	0
Teicoplanin (2)	0.125	0.19	0.094-0.38	0
Trovafloxacin (4)	4	4	1-4	0
Vancomycin (4)	0.5	0.75	0.125-0.75	0

Amox/clav = amoxicillin-clavulanic acid; Pip/taz = piperacillin-tazobactam.

Table 5.3.3. Susceptibility of 38 clinical isolates of *C. difficile* collected in 2003-2005

Antibiotic	Minimum inhibitory concentration (MIC) in μg/ml			
(breakpoint, μg/ml)	MIC_{50}	MIC_{90}	Range	% resistant
Amox/clav (8)	0.38	0.75	0.094-1.5	0
Ampicillin (8)	0.75	1.5	0.19-2.0	0
Cefotaxime (32)	96	>256	24->256	100
Cefoxitin (32)	>256	>256	0.25->256	97
Cefuroxime (32)	>256	>256	256->256	100
Clindamycin (4)	4	>256	0.16->256	48
Imipenem (8)	32	>32	0.064->3	86.8
Linezolid (4)	0. 5	2.0	0.125-4.0	0
Meropenem (8)	0.5	4.0	0.003-16	21.4
Metronidazole (8)	0.19	1.0	0.016-2.0	0
Penicillin (4)	0.5	1.5	0.016-12	16.6
Piperacillin (32)	3	6	0.25-24	11.9
Pip/taz (64)	3	6	0.016-24	0
Teicoplanin (2)	0.125	0.25	0.032-2.0	0
Trovafloxacin (4)	0.5	4	0.25-32	7.9
Vancomycin (4)	0.5	0.75	0.15-3.0	0

Amox/clav = amoxicillin-clavulanic acid; Pip/taz = piperacillin-tazobactam.

Table 6.3.1a. Cytotoxic activity (CU/ml) of cell-free cytotoxin of *C. difficile* strain 233D (ribotype 078) pre- and post- exposure to **cefotaxime** after 24 h incubation on Vero cell line

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 7
Control	16	32	64	128	128	32
MIC*	32	32	32	32	32	2048
1/2MIC	32	32	32	32	128	2048
1/4MIC	64	32	32	32	512	2048
1/8MIC	64	32	256	256	2048	2048
1/16MIC	128	128	256	256	2048	2048
1/32MIC	128	128	512	512	2048	2048
1/64MIC	32	128	1024	1024	2048	2048

*MIC = >256μg/ml

Table 6.3.1b. Cytotoxic activity (CU/ml) of cell-free cytotoxin of *C. difficile* strain 233D (ribotype 078) pre- and post- exposure to **cefotaxime** after 48 h incubation on Vero cell line

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 7
Control	16	16	32	64	128	32
MIC*	128	128	128	128	128	2048
1/2MIC	128	128	128	128	256	2048
1/4MIC	128	256	512	256	2048	2048
1/8MIC	128	512	512	512	2048	2048
1/16MIC	256	512	512	512	2048	2048
1/32MIC	256	512	1024	1024	2048	2048
1/64MIC	256	512	1024	1024	2048	2048

*MIC = >256μg/ml

Table 6.3.2a. Cytotoxic activity (CU/ml) of cell-bound cytotoxin of *C. difficile* strain 233D (ribotype 078) pre- and post- exposure to **cefotaxime** after 24 h incubation on Vero cell line

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 7
Control	16	32	64	64	64	64
MIC*	0	0	0	0	0	0
1/2MIC	0	0	0	64	256	64
1/4MIC	0	0	0	64	256	32
1/8MIC	0	16	256	256	256	32
1/16MIC	128	32	256	256	512	64
1/32MIC	128	32	512	512	512	32
1/64MIC	256	64	512	512	512	256

*MIC = >256μg/ml

Table 6.3.2b. Cytotoxic activity (CU/ml) of cell-bound cytotoxin of *C. difficile* strain 233D (ribotype 078) pre- and post- exposure to **cefotaxime** after 48 h incubation on Vero cell line

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 7
Control	16	64	64	128	128	64
MIC*	0	0	0	0	0	0
1/2MIC	0	0	0	64	256	128
1/4MIC	0	0	0	64	512	64
1/8MIC	0	32	512	512	256	128
1/16MIC	512	512	512	512	512	256
1/32MIC	512	512	1024	512	512	128
1/64MIC	1024	1024	1024	512	512	128

*MIC = >256μg/ml

Table 6.3.3a. Cytotoxic activity (CU/ml) of cell-free cytotoxin of *C. difficile* strain 233D (ribotype 078) pre- and post- exposure to **ampicillin** after 24 h incubation on Vero cell line

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 7
Control	16	16	32	64	64	64
MIC*	256	128	128	128	2048	2048
1/2MIC	128	128	128	512	2048	2048
1/4MIC	128	128	128	2048	2048	2048
1/8MIC	128	512	512	512	2048	2048
1/16MIC	128	256	512	512	2048	2048
1/32MIC	128	256	256	512	2048	2048
1/64MIC	64	256	512	512	2048	2048

*MIC = 0.5µg/ml

Table 6.3.3b. Cytotoxic activity (CU/ml) of cell-free cytotoxin of *C. difficile* strain 233D (ribotype 078) pre- and post- exposure to **ampicillin** after 48 h incubation on Vero cell line

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 7
Control	16	16	64	64	64	64
MIC*	512	128	256	256	2048	2048
1/2MIC	256	256	1024	1024	2048	2048
1/4MIC	256	512	512	2048	2048	2048
1/8MIC	256	512	1024	512	2048	2048
1/16MIC	256	512	1024	512	2048	2048
1/32MIC	256	512	512	512	2048	2048
1/64MIC	128	256	512	512	2048	2048

*MIC = 0.5µg/ml

Table 6.3.4a. Cytotoxic activity (CU/ml) of cell-bound cytotoxin of *C. difficile* strain 233D (ribotype 078) pre- and post- exposure to **ampicillin** after 24 h incubation on Vero cell line

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 7
Control	16	32	32	64	64	128
MIC*	32	32	512	1024	256	128
1/2MIC	16	32	512	1024	1024	512
1/4MIC	16	32	1024	1024	128	128
1/8MIC	16	32	1024	1024	128	128
1/16MIC	16	32	1024	1024	128	128
1/32MIC	32	32	512	1024	64	64
1/64MIC	0	32	512	512	64	64

*MIC = 0.5µg/ml

Table 6.3.4b. Cytotoxic activity (CU/ml) of cell-bound cytotoxin of *C. difficile* strain 233D (ribotype 078) pre- and post- exposure to **ampicillin** after 48 h incubation in Vero cell line

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 7
Control	16	32	32	64	64	128
MIC*	128	128	2048	2048	512	512
1/2MIC	64	64	1024	2048	512	512
1/4MIC	32	32	1024	2048	512	512
1/8MIC	16	32	2048	2048	512	512
1/16MIC	16	32	2048	2048	256	256
1/32MIC	16	32	2048	2048	128	256
1/64MIC	16	64	2048	2048	128	128

*MIC = 0.5µg/ml

Table 6.3.5a. Cytotoxic activity (CU/ml) of cell-free cytotoxin of *C. difficile* strain 233D (ribotype 078) pre- and post- exposure to **metronidazole** after 24 h incubation on Vero cell line

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 7
Control	16	16	64	64	64	128
MIC*	0	0	256	256	256	2048
1/2MIC	32	32	128	256	2048	2048
1/4MIC	32	32	128	512	2048	1024
1/8MIC	32	64	128	512	2048	1024
1/16MIC	64	64	64	512	2048	1024
1/32MIC	64	64	64	512	2048	1024
1/64MIC	32	32	64	512	2048	1024

*MIC = 0.25µg/ml

Table 6.3.5b. Cytotoxic activity (CU/ml) of cell-free cytotoxin of *C. difficile* strain 233D (ribotype 078) pre- and post- exposure to **metronidazole** after 48 h incubation on Vero cell line

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 7
Control	16	32	32	64	64	128
MIC*	16	32	256	256	256	2048
1/2MIC	32	64	128	256	2048	2048
1/4MIC	32	64	256	512	2048	2048
1/8MIC	64	256	256	1024	2048	2048
1/16MIC	64	128	128	1024	2048	1024
1/32MIC	64	64	128	512	2048	2048
1/64MIC	32	32	128	512	2048	2048

*MIC = 0.25µg/ml

Table 6.3.6a. Cytotoxic activity (CU/ml) of cell-bound cytotoxin of *C. difficile* strain 233D (ribotype 078) pre- and post- exposure to **metronidazole** after 24 h incubation on Vero cell line

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 7
Control	16	16	64	128	64	64
MIC*	2	512	128	512	256	256
1/2MIC	1024	512	512	512	256	256
1/4MIC	1024	1024	1024	512	512	512
1/8MIC	1024	1024	1024	1024	512	512
1/16MIC	2048	1024	1024	1024	512	512
1/32MIC	2048	1024	2048	2048	512	512
1/64MIC	2048	1024	1024	1024	1024	512

*MIC = 0.25μg/ml

Table 6.3.6b. Cytotoxic activity (CU/ml) of cell-bound cytotoxin of *C. difficile* strain 233D (ribotype 078) pre- and post- exposure to **metronidazole** after 48 h incubation on Vero cell line

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 7
Control	16	16	32	64	64	64
MIC*	2048	1024	1024	256	256	256
1/2MIC	2048	1024	1024	512	256	256
1/4MIC	2048	2048	1024	512	256	512
1/8MIC	2048	2048	512	512	1024	1024
1/16MIC	2048	2048	2048	2048	1024	1024
1/32MIC	2048	2048	2048	2048	1024	1024
1/64MIC	2048	2048	2048	2048	1024	1024

*MIC = 0.25μg/ml

Table 6.3.7a. Cytotoxic activity (CU/ml) of cell-free cytotoxin of *C. difficile* strain 233D (ribotype 078) pre- and post- exposure to **vancomycin** after 24 h incubation on Vero cell line

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 7
Control	16	64	64	64	64	32
MIC*	0	128	128	1024	512	0
1/2MIC	128	256	512	1024	512	16
1/4MIC	128	128	512	2048	1024	16
1/8MIC	128	128	512	2048	1024	16
1/16MIC	64	128	1024	2048	2048	16
1/32MIC	32	256	1024	1024	1024	1024
1/64MIC	32	256	1024	1024	1024	1024

*MIC = 0.5µg/ml

Table 6.3.7b. Cytotoxic activity (CU/ml) of cell-free cytotoxin of *C. difficile* strain 233D (ribotype 078) pre- and post- exposure to **vancomycin** after 48 h incubation on Vero cell line

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 7
Control	32	32	64	64	32	32
MIC*	0	1024	1024	2048	2048	0
1/2MIC	256	512	1024	2048	2048	64
1/4MIC	256	256	2048	2048	2048	32
1/8MIC	256	512	2048	2048	2048	32
1/16MIC	128	256	2048	2048	2048	32
1/32MIC	64	512	2048	2048	2048	1024
1/64MIC	64	256	2048	2048	2048	1024

*MIC = 0.5µg/ml

Table 6.3.8a. Cytotoxic activity (CU/ml) of cell-bound cytotoxin of *C. difficile* strain 233D (ribotype 078) pre- and post- exposure to **vancomycin** after 24 h incubation on Vero cell line

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 7
Control	16	16	64	64	64	32
MIC*	512	512	512	128	32	0
1/2MIC	1024	1024	512	128	128	64
1/4MIC	1024	1024	1024	128	128	64
1/8MIC	1024	1024	1024	128	128	64
1/16MIC	1024	1024	1024	128	128	64
1/32MIC	1024	1024	1024	128	128	64
1/64MIC	2048	2048	1024	128	128	32

*MIC = 0.5µg/ml

Table 6.3.8b. Cytotoxic activity (CU/ml) of cell-bound cytotoxin of *C. difficile* strain 233D (ribotype 078) pre- and post- exposure to **vancomycin** after 48 h incubation on Vero cell line

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 7
Control	16	32	64	64	32	32
MIC*	1024	1024	1024	512	128	0
1/2MIC	1024	1024	1024	256	256	128
1/4MIC	2048	2048	2048	512	512	128
1/8MIC	2048	2048	2048	1024	1024	1024
1/16MIC	2048	2048	2048	256	256	128
1/32MIC	2048	2048	2048	256	256	128
1/64MIC	2048	2048	2048	1024	1024	64

*MIC = 0.5µg/ml

Table 6.3.9a. Cytotoxic activity (CU/ml) of cell-free cytotoxin of *C. difficile* strain 233D (ribotype 078) pre- and post- exposure to **clindamycin** after 24 h incubation on Vero cell line

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 7
Control	32	32	32	64	64	128
MIC*	0	64	128	128	2048	2048
1/2MIC	64	64	64	512	2048	2048
1/4MIC	64	128	128	512	1024	2048
1/8MIC	0	128	128	512	2048	2048
1/16MIC	0	128	128	512	2048	2048
1/32MIC	0	32	32	256	1024	2048
1/64MIC	0	32	32	256	2048	2048

*MIC = 8.0μg/ml

Table 6.3.9b. Cytotoxic activity (CU/ml) of cell-free cytotoxin of *C. difficile* strain 233D (ribotype 078) pre- and post- exposure to **clindamycin** after 48 h incubation on Vero cell line

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 7
Control	32	32	32	64	128	128
MIC*	0	128	128	128	2048	2048
1/2MIC	64	128	128	512	2048	2048
1/4MIC	64	128	128	512	2048	2048
1/8MIC	0	128	128	512	2048	2048
1/16MIC	0	128	128	512	2048	2048
1/32MIC	0	32	64	512	2048	2048
1/64MIC	0	32	128	256	2048	2048

*MIC = 6.0μg/ml

Table 6.3.10a. Cytotoxic activity (CU/ml) of cell-bound cytotoxin of *C. difficile* strain 233D (ribotype 078) pre- and post- exposure to **clindamycin** after 24 h incubation on Vero cell line

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 7
Control	16	32	32	64	32	32
MIC*	0	1024	1024	1024	512	64
1/2MIC	1024	1024	1024	1024	512	256
1/4MIC	1024	1024	1024	2048	256	256
1/8MIC	128	1024	2048	2048	512	256
1/16MIC	128	1024	2048	2048	1024	256
1/32MIC	0	0	2048	2048	1024	256
1/64MIC	0	1024	2048	2048	1024	256

*MIC = 8.0μg/ml

Table 6.3.10b. Cytotoxic activity (CU/ml) of cell-bound cytotoxin of *C. difficile* strain 233D (ribotype 078) pre- and post- exposure to **clindamycin** after 48 h incubation on Vero cell line

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 7
Control	32	64	64	64	64	32
MIC*	0	2048	1024	1024	1024	256
1/2MIC	2048	2048	2048	2048	1024	256
1/4MIC	2048	2048	2048	2048	512	512
1/8MIC	2048	2048	2048	2048	1024	512
1/16MIC	256	2048	2048	2048	1024	512
1/32MIC	256	2048	2048	2048	1028	512
1/64MIC	2048	2048	2048	2048	2048	512

*MIC = 6.0μg/ml

Table 6.3.11a. Mean cytotoxic activity (CU/ml) of cell-free cytotoxin of *C. difficile* pre- and post exposure to **cefotaxime** by all test strains

MIC & sub-Inhibitory MICs	Cytotoxic activity (CU/ml) of strains									
	078		017		097		046		001	
	24h	48h	24h	48h	24h	48h	24h	48h	24h	48h
Control	66.6	48.0	16.0	32.0	16.0	32.0	16.0	16.0	64.0	64.0
>256	368.0	448.0	264.0	2048.0	0	0	0	0	1.2	1.2
128	384.0	469.3	2048.0	2048.0	0	0	128.0	128.0	16.0	16.0
64	453.3	832.0	2048.0	2048.0	128.0	128.0	320.1	560.4	76.8	76.8
32	784.0	960.0	2048.0	2048.0	128.0	266.3	384.3	760.3	38.4	38.4
16	810.6	981.3	2048.0	2048.0	160.3	290.4	384.3	760.3	51.2	51.2
8	896.0	1152.0	2048.0	2048.0	272.4	290.4	384.3	640.3	38.4	51.2
4	1050.6	1322.0	2048.0	2048.0	256.1	256.0	256.0	380.4	38.4	38.4

Table 6.3.11b. Mean cytotoxic activity (CU/ml) of cell-bound cytotoxin of *C. difficile* pre- and post exposure to **cefotaxime** by all test strains

MIC & sub-Inhibitory MICs	Cytotoxic activity (CU/ml) of strains									
	078		017		097		046		001	
	24h	48h	24h	48h	24h	48h	24h	48h	24h	48h
Control	50.6	77.3	16.0	32.0	16.0	32.0	16.0	16.0	64.0	64.0
>256	0	0	182.3	1024.0	0	0	0	0	24.0	32.0
128	64.0	74.6	576.1	1536.3	0	0	256.0	512.0	320.0	320.0
64	117.3	106.6	192.3	768.2	384.2	512.0	128.0	128.3	1536.1	1536.1
32	136.0	240.0	544.0	1152.1	28.0	320.0	64.0	64.0	768.1	768.1
16	208.6	469.3	272.3	576.3	96.0	232.3	24.0	48.4	1024.0	1024.0
8	288.0	704.0	272.3	528.2	64.4	128.1	64.0	96.0	768.3	1024.0
4	352.0	789.3	320.0	576.0	24.0	48.0	64.0	64.0	768.3	768.3

Table 6.3.12a. Mean cytotoxic activity (CU/ml) of cell-free cytotoxin of *C. difficile* pre- and post exposure to **ampicillin** by all test strains

MIC & sub-Inhibitory MICs	Cytotoxic activity (CU/ml) of strains									
	078		017		097		046		001	
	24h	48h	24h	48h	24h	48h	24h	48h	24h	48h
Control	42.6	48.0	16.0	32.0	32.0	32.0	32.0	32.0	64.0	64.0
4	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	32.0	32.0
1	-	-	40.0	48.0	64.0	128.0	320.0	560.0	1152.0	2048.0
0.5	789.3	874.0	1036.0	1040.0	96.0	256.0	1536.0	1024.0	2048.0	2048.0
0.25	1088.0	1109.3	2048.0	2048.0	144.0	384.0	512.0	760.0	2048.0	2048.0
0.125	960.0	1237.3	2048.0	2048.0	96.0	256.0	768.0	1024.0	2048.0	2048.0
0.06	960.0	1066.6	2048.0	2048.0	96.0	192.0	768.0	1024.0	2048.0	2048.0
0.03	917.0	1066.6	2048.0	2048.0	48.0	160.0	768.0	1024.0	2048.0	2048.0
0.015	289.5	981.3	2048.0	2048.0	48.0	160.0	768.0	1024.0	-	-
0.007	197.3	917.3	-	-	-	-	-	-	-	-

- , not tested at the corresponding MIC

Table 6.3.12b. Mean cytotoxic activity (CU/ml) of cell-bound cytotoxin of *C. difficile* pre- and post exposure to **ampicillin** by all test strains

MIC & sub-Inhibitory MICs	Cytotoxic activity (CU/ml) of strains									
	078		017		097		046		001	
	24h	48h	24h	48h	24h	48h	24h	48h	24h	48h
Control	56.0	56.0	256.0	512.0	16.0	16.0	16.0	16.0	64.0	64.0
4	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	0	0
1	-	-	0	0	48.0	32.0	272.0	544.0	1024.0	1536.0
0.5	330.6	896.0	256.0	256.0	32.0	96.0	32.0	48.0	288.0	244.0
0.25	520.0	704.0	800.2	276.0	48.0	80.0	16.0	16.0	288.0	320.0
0.125	392.0	693.0	48.0	80.0	80.0	160.0	8.0	16.0	384.0	768.0
0.06	392.0	861.3	64.0	160.0	80.0	600.0	24.0	24.0	384.0	256.0
0.03	392.0	776.0	32.0	40.0	144.0	288.0	32.0	32.0	48.0	48.0
0.015	288.0	754.6	64.0	160.0	80.0	160.0	0	8.0	-	-
0.007	200.0	738.6	-	-	-	-	-	-	-	-

- , not tested at the corresponding MIC

Table 6.3.13a. Mean cytotoxic activity (CU/ml) of cell-free cytotoxin of *C. difficile* pre- and post exposure to **metronidazole** by all test strains

MIC & sub-Inhibitory MICs	Cytotoxic activity (CU/ml) of strains									
	078		017		097		046		001	
	24h	48h	24h	48h	24h	48h	24h	48h	24h	48h
Control	53.0	45.3	16.0	16.0	16.0	32.0	16.0	16.0	32.0	64.0
0.5	-	-	-	-	-	-	-	-	256.0	2048.0
0.25	469.3	477.3	0	8.0	-	-	80.0	80.0	2048.0	2048.0
0.125	757.3	784.0	8.0	24.0	96.0	160.0	48.0	80.0	2048.0	2048.0
0.06	629.3	826.6	2048.0	2048.0	80.0	96.0	144.0	144.0	2048.0	2048.0
0.03	634.6	949.3	2048.0	2048.0	80.0	288.0	136.0	136.0	2048.0	2048.0
0.015	629.3	903.6	2048.0	2048.0	64.0	128.0	256.0	516.0	2048.0	2048.0
0.007	629.3	810.0	128.0	2048.0	72.0	144.0	136.0	264.0	2048.0	2048.0
0.0035	618.6	800.0	2048.0	2048.0	80.0	144.0	64.0	256.0	-	-
0.0017	-	-	-	-	64.0	128.0	-	-	-	-

- , not tested at the corresponding MIC

Table 6.3.13b. Mean cytotoxic activity (CU/ml) of cell-bound cytotoxin of *C. difficile* pre- and post exposure to **metronidazole** by all test strains

MIC & sub-Inhibitory MICs	Cytotoxic activity (CU/ml) of strains									
	078		017		097		046		001	
	24h	48h	24h	48h	24h	48h	24h	48h	24h	48h
Control	58.0	42.6	64.0	128.0	16.0	16.0	16.0	16.0	128.0	128.0
0.5	-	-	-	-	-	-	-	-	256.0	512.0
0.25	277.3	810.0	0	0	-	-	32.0	64.0	320.0	640.0
0.125	512.0	753.3	16.0	16.0	192.0	384.0	32.0	32.0	256.0	384.0
0.06	768.0	1066.6	40.0	96.0	160.0	160.0	128.0	192.0	160.0	320.0
0.03	853.0	1194.6	160.0	1088.0	160.0	192.0	96.0	160.0	768.0	1024.0
0.015	1024.0	1706.6	96.0	320.0	96.0	320.0	96.0	128.0	256.0	256.0
0.007	1365.3	1706.6	192.0	512.0	128.0	384.0	64.0	128.0	288.0	284.0
0.0035	1109.3	1877.3	192.0	640.0	128.0	192.0	48.0	48.0	-	-
0.0017	-	-	-	-	64.0	96.0	-	-	-	-

- , not tested at the corresponding MIC

Table 6.3.14a. Mean cytotoxic activity (CU/ml) of cell-free cytotoxin of *C. difficile* pre- and post exposure to **vancomycin** by all test strains

MIC & sub-Inhibitory MICs	Cytotoxic activity (CU/ml) of strains									
	078		017		097		046		001	
	24h	48h	24h	48h	24h	48h	24h	48h	24h	48h
Control	50.0	42.1	128.0	256.0	32.0	32.0	32.0	32.0	64.0	128.0
2	-	-	-	-	-	-	-	-	0	0
1	-	-	48.0	48.0	16.0	64.0	-	-	16.0	16.0
0.5	298.6	1024.0	1040.0	1056.0	1056.0	1088.0	0	0	1536.0	1536.0
0.25	408.0	992.0	1536.0	2048.0	160.0	320.0	768.0	760.0	2048.0	2048.0
0.125	642.0	1114.6	2048.0	2048.0	64.0	128.0	1024.0	1024.0	2048.0	2048.0
0.06	642.0	1157.3	2048.0	2048.0	80.0	160.0	1024.0	1024.0	2048.0	2048.0
0.03	888.0	1093.3	1536.0	2048.0	1040.0	1056.0	1024.0	1024.0	2048.0	2048.0
0.015	730.0	1290.6	2048.0	2048.0	1032.0	1000.0	768.0	1024.0	-	-
0.007	730.5	1248.0	-	-	-	-	512.0	1024.0	-	-

- , not tested at the corresponding MIC

Table 6.3.14b. Mean cytotoxic activity (CU/ml) of cell-bound cytotoxin of *C. difficile* pre- and post exposure to **vancomycin** by all test strains

MIC & sub-Inhibitory MICs	Cytotoxic activity (CU/ml) of strains									
	078		017		097		046		001	
	24h	48h	24h	48h	24h	48h	24h	48h	24h	48h
Control	42.0	42.1	128.0	258.0	16.0	16.0	16.0	16.0	64.0	128.0
2	-	-	-	-	-	-	-	-	0	0
1	-	-	0	0	0	0	-	-	264.0	272.0
0.5	282.6	618.6	0	8.0	512.0	1024.0	0	0	256.0	384.0
0.25	480.0	618.6	48.0	160.0	48.0	144.0	16.0	16.0	192.0	384.0
0.125	565.3	1216.0	32.0	160.0	32.0	96.0	8.0	8.0	96.0	96.0
0.06	565.3	1536.3	62.0	192.0	48.0	48.0	16.0	24.0	256.0	256.0
0.03	565.3	1130.6	62.0	192.0	48.0	86.0	16.0	24.0	256.0	256.0
0.015	565.3	1130.6	96.0	192.0	144.0	288.0	16.0	24.0	-	-
0.007	901.3	1536.0	-	-	-	-	8.0	8.0	-	-

- Not tested at the corresponding MIC

Table 6.3.14a Mean cytotoxic activity (CU/ml) of cell-free cytotoxin of *C. difficile* pre- and post exposure to **clindamycin** by all test strains

MIC & sub-Inhibitory MICs	Cytotoxic activity (CU/ml) of strains									
	078		017		097		046		001	
	24h	48h	24h	48h	24h	48h	24h	48h	24h	48h
Control	58.6	69.3	128.0	258.0	32.0	32.0	32.0	32.0	128.0	128.0
256	-	-	1088.8	1152.0	64.0	128.0	64.0	136.0	-	-
128	-	-	1536.0	2048.0	64.0	128.0	82.0	160.0	-	-
64	-	-	1024.0	1024.0	136.0	136.0	144.0	144.0	-	-
32	-	-	1040.0	1088.0	136.0	136.0	136.0	264.0	-	-
16	-	-	1280.0	2048.0	136.0	144.0	136.0	136.0	-	-
8	736.0	746.6	2048.0	2048.0	136.0	264.0	256.0	264.0	2048.0	2048.0
4	800.0	821.3	2048.0	2048.0	136.0	264.0	72.0	136.0	2048.0	2048.0
2	650.6	821.3	-	-	-	-	-	-	2048.0	2048.0
1	810.6	810.6	-	-	-	-	-	-	2048.0	2048.0
0.5	810.6	810.6	-	-	-	-	-	-	2048.0	2048.0
0.25	565.3	778.6	-	-	-	-	-	-	2048.0	2048.0
0.0125	736.0	757.3	-	-	-	-	-	-	2048.0	2048.0

- , not tested at the corresponding MIC

Table 6.3.16b. Mean cytotoxic activity (CU/ml) of cell-bound cytotoxin of *C. difficile* pre- and post exposure to **clindamycin** by all test strains

MIC & sub-Inhibitory MICs	Cytotoxic activity (CU/ml) of strains									
	078		017		097		046		001	
	24h	48h	24h	48h	24h	48h	24h	48h	24h	48h
Control	34.6	69.3	16.0	16.0	16.0	16.0	16.0	16.0	64.0	64.0
256	-	-	272.0	1056.0	272.0	160.0	48.0	64.0	-	-
128	-	-	72.0	136.0	272.0	288.0	40.0	40.0	-	-
64	-	-	32.0	64.0	96.0	96.0	48.0	96.0	-	-
32	-	-	264.0	1040.0	272.0	288.0	64.0	96.0	-	-
16	-	-	72.0	272.0	288.0	320.0	32.0	48.0	-	-
8	608.0	896.0	256.0	384.0	272.0	288.0	48.0	96.0	144.0	144.0
4	810.0	1578.6	40.0	160.0	144.0	160.0	32.0	32.0	128.0	192.0
2	938.6	1536.0	-	-	-	-	-	-	192.0	192.0
1	1002.6	1621.3	-	-	-	-	-	-	160.0	192.0
0.5	1088.0	1322.6	-	-	-	-	-	-	192.0	256.0
0.25	896.0	1332.6	-	-	-	-	-	-	96.0	128.0
0.0125	1066.6	1792.0	-	-	-	-	-	-	24.0	24.0

- , not tested at the corresponding MIC

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In vitro activity of 15 antimicrobial agents against clinical isolates of *Clostridium difficile* in Kuwait

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Abstract

A total of 73 clinical isolates of *Clostridium difficile* isolated from stool/rectal swabs of patients admitted to the intensive care units at Mubarak Hospital, Ibn Sina Hospital Burn unit and Haematology wards at the Kuwait Cancer Control Centre, were investigated for their susceptibility to 15 antibiotics using the Etest. Amoxycillin–clavulanic acid, ampicillin, meropenem, metronidazole, penicillin, piperacillin, piperacillin/tazobactam, teicoplanin and vancomycin had excellent activities with MIC_{90}s of 0.38, 0.5, 1, 0.19, 1.5, 2, 3, 0.25 and 0.75 mg/l, respectively. Of the 73 *C. difficile* isolates, 86% were resistant to imipenem (MIC_{90} > 32 mg/l) and almost 97% were resistant to trovafloxacin (MIC_{90} > 256 mg/l). Forty eight percent of the isolates were resistant to clindamycin. A total of 18 isolates were highly clindamycin-resistant with an MIC of > 256 mg/l; 10 of these were toxin producers. Multiple antibiotic resistance (two or more antibiotics) was noted in 63 isolates. These were more common among the toxigenic strains than the non-toxigenic strains by a ratio of 2.5:1.

Keywords: *Clostridium difficile*; Clinical isolates; Antibiotic susceptibility

1. Introduction

Bartlett and his colleagues positively identified *Clostridium difficile* as an aetiological agent of pseudomembranous colitis (PMC) in 1978 [1]. It soon became obvious after this pioneering work that the organism was responsible for a spectrum of disease referred to as *C. difficile*-associated diarrhoea (CDAD) ranging from mild, self-limiting diarrhoea to potentially life-threatening PMC. This organism is now recognized as a major nosocomial pathogen all over the world [2]. The diseases it causes, when they occur in the hospital setting, have contributed significantly to extended hospital stay and increased the cost [3,4].

Metronidazole and vancomycin, given orally for 10 days, are the drugs of first choice for the treatment of CDAD. Several clinical trials have established that these two antibiotics have equivalent efficacy, particularly in the therapy of mild to moderate diseases [5–7] although sometimes accompanied by high relapse rates. Teicoplanin, another potent glycopeptide, is equally efficacious as vancomycin [8].

The role of antibiotic susceptibility testing of anaerobes in general and of *C. difficile* specifically, has been questioned over the past decades. Mainly because of the predictable sensitivity pattern of anaerobes to most antibiotics with anti-anaerobic activity as well as lack of simple method for testing, delay in getting pure culture and little correlation between the clinical outcome and the antibiotic susceptibility testing results [9,10]. In our country, routine antibiotic susceptibility testing of anaerobes is not usually done. However, resistance of anaerobes to anti-anaerobic antibiotics is on the increase, even to drugs with excellent anti-anaerobic activity like metronidazole [11–13], imipenem [14,15] and β-lactam-β-lactamase inhibitors [16]. In a recent report from Spain, Pelaez et al., [17] described an increase in the number of clinical isolates of *C. difficile* with decreased susceptibility to metronidazole and the emergence of strains with decreased susceptibility to vancomycin thus emphasizing the importance of antibiotic susceptibility testing of anaerobic bacteria, in

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particular *C. difficile*, in different geographical areas. This prompted us to investigate the susceptibility of clinical isolates of *C. difficile* to establish a base-line susceptibility pattern to 15 antibiotics and assess the level of resistance of our isolates to established anti-anaerobic agents.

2. Material and methods

2.1. Bacterial strains

Clinical isolates of *C. difficile* obtained from symptomatic and asymptomatic ICU patients in three large teaching hospitals in Kuwait (Mubarak, Ibn Sina and Kuwait Cancer Control Center (KCCC), Hospitals) over a period of 2 years were studied. The isolates were identified as *C. difficile* by their characteristic yellow/green fluorescence under an ultraviolet (UV) illuminator, Gram-stain morphology, horse-stable smell and reactions in the API 20A (BioMerieux, SA, France). Two reference quality control strains, *C. difficile* ATCC 9689 and ATCC 17857, and *C. perfringens* ATCC 13124, were included in each run.

2.2. Susceptibility testing

The susceptibility of the isolates was determined by estimating the minimum inhibitory concentrations (MICs) of the 15 antibiotics using the Etest (AB Biodisk, Slona, Sweden) and agar dilution method. The 15 antibiotics tested are listed in Table 1. The activity of trovafloxacin (Pfizer, Inc., Groton, CT) was determined by the agar dilution method recommended by the National Committee for Clinical Laboratory Standards (NCCLS, 1997) [18]. Briefly, serial two-fold dilutions of trovafloxacin were incorporated into Fastidious Anaerobe agar (FAA; Lab M, Bury, UK) with final antibiotic concentrations of 0.03–256 mg/l. An inoculum was prepared in Anaerobe broth (Difco, USA) from a 48 h FAA culture. The suspension was adjusted to 0.5 McFarland turbidity standard. Then the inoculum was applied onto the surface of pre-reduced antibiotic-containing agar using a 35-prong Steers applicator that delivered 10 μl (10^5 CFU) per spot. The plates were incubated in anaerobic jars for 48 h at 37 °C. Anaerobiosis was checked by inclusion of a nutrient plate inoculated with *Pseudomonas aeruginosa* and a chemical indicator. After incubation, the MIC was recorded as the lowest concentration of each antibiotic that inhibited visible growth of the organisms.

For the MIC determination by Etest, a 48 h bacterial growth suspended in Anaerobe broth and adjusted to no. 1.0 McFarland turbidity standard was inoculated onto reduced brucella agar plates (Unipath, Basingstoke, UK) supplemented with 5% horse blood, haemin 5 μg/ml and menadione 1 μg/ml. The Etest strips of the antibiotics, other than trovafloxacin, were applied onto the agar surface, after drying for 15 min, and then incubated in anaerobic jars containing 10% CO_2, 90% H_2, for 48 h at 37 °C. The MIC was read as the interception of the elliptical zone of inhibition with the strip. *C. difficile* and *C. perfringens* control strains were included in each run as control of the media and the susceptibility test.

All the isolates were tested for toxin A and B production using commercial *C. difficile* TOX-A/B TEST Kit (Tech Lab, VPI Research Park, Blacksburg, VA) according to the manufacturer's package insert.

Table 1
Susceptibility of the 73 clinical isolates of *C. difficile* to 15 antibiotics

Antibiotics (breakpoint, mg/l)	MIC (mg/l)			
	Range	MIC_{50}	MIC_{90}	% Resistant
Amox/clav (8)	0.047–0.5	0.125	0.38	0
Ampicillin (8)	0.094–0.75	0.25	0.5	0
Cefotaxime (32)	24– > 256	96	> 256	100
Cefoxitin (32)	0.25– > 256	> 256	> 256	97
Cefuroxime (32)	> 256	> 256	> 256	100
Clindamycin (4)	0.16– > 256	4	> 256	48
Imipenem (8)	0.064– > 32	32	> 32	86
Meropenem (8)	0.032–1.0	0.75	1.0	0
Metronidazole (8)	0.023–0.19	0.094	0.19	0
Penicillin (4)	0.125–1.5	0.5	1.5	0
Piperacillin (32)	0.094–3	1.5	2	0
Piperacillin/tazo (64)	0.047–6	2.0	3	0
Teicoplanin (2)	0.032–2	0.125	0.25	0
Trovafloxacin (4)	0.5– > 256	32	64	97
Vancomycin (4)	0.125–3	0.5	0.75	0

Amox/clav, amoxycillin–clavulanic acid; tazo, tazobactam.

3. Results

A total of 73 clinical isolates (52 from Mubarak hospital, 4 from Ibn Sina and 17 from the KCCC), obtained over a period of 2 years, were studied. Forty-five (62%) of them were toxigenic. Fifteen of the 73 isolates were from symptomatic patients and the rest were from patients who became colonized after admission to the hospital. The MICs of the antibiotics tested, shown in Table 1, are presented as the concentrations that killed 50% (MIC_{50}) and 90% (MIC_{90}) of the isolates. Amoxycillin–clavulanic acid, ampicillin, meropenem, metronidazole, penicillin, piperacillin, piperacillin-tazobactam, teicoplanin and vancomycin had excellent activity against all isolates of *C. difficile* with MIC_{90}s of 0.38, 0.5, 1.0, 0.19, 1.5, 2.0, 3.0, 0.25 and 0.75 mg/l, respectively. One isolate had decreased susceptibility to vancomycin and teicoplanin with MICs of 3 and 2 mg/l, respectively.

As was expected, 71 (97%) of our 73 strains were resistant to cefoxitin and all them were resistant to cefotaxime and cefuroxime. Almost half of the *C. difficile* isolates were resistant to clindamycin (35/73; 48%). Of these 35 resistant isolates, 18 (51%) exhibited high-level clindamycin resistance with MIC >256 mg/l. The MIC of clindamycin for the remaining 17 isolates was 6–24 mg/l. High-level resistance to clindamycin was present in 10 of the 18 high-level resistant strains and they were toxigenic. Of interest, 63 (86%) and 71 (97%) were resistant to imipenem and trovafloxacin with MICs of >32 and 64 mg/l, respectively. Nine had imipenem MIC of $\leqslant 8$ mg/l and the remaining one strain had an MIC of 12 mg/l.

Analysis of individual isolates in relation to multiple resistance, i.e. resistance to two or more antibiotics, and toxin or non-toxin production, is shown in Table 2. A total of 27 isolates were resistant to 4 antibiotics (cefoxitin, clindamycin, imipenem and trovafloxacin) of which 15 were toxigenic and 12 non-toxigenic strains. Of the 25 strains resistant to a combination of cefoxitin, imipenem and trovafloxacin, 24 (96%) were toxigenic strains. Table 3 shows the distribution of the toxigenic strains that were multiply resistant among the symptomatic and asymptomatic patients. More isolates exhibiting resistance to 4 antibiotics came from the symptomatic patients than from the asymptomatic patients by a ratio of 2.75:1, whereas those resistant to 3 or fewer antibiotics were isolated predominantly from the asymptomatic patients.

Table 2
Antibiotic resistance profile of 63 multiply resistant *C. difficile* isolates

Resistance groups	Number of isolates		
	Toxigenic	Non-toxigenic	Total no.
Cefox, Clind, Imip, Trovafl	15	12	27
Cefox, Imip, Trovafl	24	1	25
Cefox, Clind, Trovafl	1	–	1
Cefox, Clind, Imip	2	–	2
Cefox, Trovafl	3	–	3
Imip, Trovafl	–	5	5

Cefox, cefoxitin; Clind, clindamycin; Imip, imipenem; Trovafl, trovafloxacin.

Table 3
Resistance profiles of the toxigenic isolates from symptomatic and asymptomatic patients

Resistance group	No. patients that were	
	Symptomatic	Asymptomatic
Cefox, Clind, Imip, Trovafl	11	4
Cefox, Imip, Trovafl	3	21
Cefox, Clind, Trovafl	1	0
Cefox, Clind, Imip	0	2
Cefox, Trovafl	0	3
Imip,Trovafl	0	5

A comparison was also made between antibiotic usage and multiple resistance. The average number of antibiotics prescribed per patient was 4.0 (range 0–11) for an average duration of 8.4 days (range 1–22) per prescription. The commonest antibiotics prescribed were cephalosporins (21%), metronidazole (17%), meropenem (14%), amikacin, vancomycin and teicoplanin (7% each) and imipenem (3%). Trovafloxacin has never been used in our hospitals before. Over 50% of the 63 multiply resistant isolates were from patients treated with the cephalosporins, meropenem and metronidazole.

4. Discussion

C. difficile is an important cause of nosocomial diarrhoea [19,20] and PMC, has a mortality rate that ranges from 15 to 30% [21]. It is therefore prudent to be abreast of the susceptibility pattern of local isolates to the antibiotics that are available for the treatment of the disease caused by the organism. The data from our present study show that 90% of the strains were inhibited by 0.75 mg/l of vancomycin and 0.25 mg/l of teicoplanin; all strains were inhibited by concentrations that did not exceed 3 mg/l and 2 mg/l, respectively, with the MICs distributed over a narrow range. These upper limits are higher than the concentrations reported in other studies [12,22–25]. However, only one strain with decreased susceptibility to vancomycin (MIC, 3 mg/l) and teicoplanin (2 mg/l) was isolated. This strain was from a symptomatic patient with CDAD and thus calls for vigilance and close monitoring of the susceptibility of future isolates in our hospitals.

Metronidazole was the most active of all the antibiotics. All isolates were inhibited by a concentration that did not exceed 0.19 mg/l, and with a narrow range of MIC distribution. This is in contrast to studies that have reported from 3–20% decreased susceptibility to metronidazole [5,17]. Wong et al [26] has also recently described a single isolate from a collection of 100 strains studied that had MIC of 64 mg/l. Despite these reports, the incidence of metronidazole resistant strains remains very low. Our finding suggests that metronidazole should remain the drug of choice for the therapy of CDAD in our hospitals. This is without prejudice to the fact that metronidazole and vancomycin are equally effective for treatment of *C. difficile*-associated disease [5,27,28]. In addition to the excellent in vitro activity, cost and concern regarding the emergence of vancomycin-resistant enterococci [27–29] support its choice as a preferred drug. Even though teicoplanin is as effective as vancomycin [8,30] its high cost precludes it as the drug of first choice.

All our isolates were susceptible to ampicillin, penicillin and amoxycillin-clavulanic acid but resistant to the cephalosporins (cefotaxime, cefuroxime and cefoxitin) with MIC_{90}s of more than 256 mg/l, a finding consistent some earlier reports [31,32]. Although, imipenem and meropenem have very good activity against anaerobes in general, there was a big difference in their in vitro activities against our *C. difficile* isolates. While meropenem showed excellent activity against all isolates of *C. difficile,* over three-quarters of the strains had high-level resistance to imipenem. In a previous review of 46 isolates by Jones [14], quite a few had MIC_{90} of more than 10 mg/l. The explanation for the discrepancy in susceptibility of our isolates to meropenem and imipenem is a subject of further investigation.

The resistance rate of our isolates to clindamycin is much lower than the rate reported elsewhere [33]. Nearly 25% of the isolates showed high-level clindamycin resistance and over half of these were toxigenic. These strains were isolated from patients in two of our three hospitals. Unlike a previous report [34] where a specific, highly clindamycin-resistant strain of *C. difficile* caused large outbreaks of diarrhoea in four hospitals in the United States, our isolates did not appear to have been associated with any particular outbreak. Only half of them were isolated from sporadic cases of CDAD. The majority of the non-toxigenic strains were susceptible to clindamycin. Susceptibility of *C. difficile* to the quinolones has always been poor, particularly to the first and second-generation agents, such as norfloxacin and ciprofloxacin [31,32]. Although in general, trovafloxacin has good anti-anaerobic activity against most of the Gram-positive and Gram-negative anaerobes, in our study trovafloxacin had very poor activity against *C. difficile* with about two-thirds of the strains showing high-level resistance. Our data indicates that this drug can not be considered for use in the treatment of disease caused by this organism.

Further analysis of the strains according to toxin production and multiple resistance revealed that all the toxigenic, more than the non-toxigenic strains, by a ratio of 2.5:1, were resistant to two or more antibiotics. Resistance to combinations of cefoxitin, imipenem and trovafloxacin, cefoxitin, clindamycin and imipenem, cefoxitin, clindamycin and trovafloxacin were exclusively observed in the toxigenic group. These results cast new light into the relationship between toxigenic strains and resistance grouping. While *C. difficile* isolates are routinely resistant to the cephalosporins and imipenem, resistance to clindamycin is less common. The finding of these multiple resistant strains in our hospitals is therefore an important one, particularly as those strains resistant to four antibiotics were associated more with symptomatic patients than asymptomatic patients by a ratio of 2.75:1. A study of larger number of isolates will be needed to confirm this observation. The use of these antibiotics and the development of CDAD are currently being investigated.

CDAD is almost unknown in the absence of antibiotic use and it has been demonstrated that the risk of this disease among hospitalized patients increases with the use of clindamycin and the presence of clindamycin-resistant strains [34] as well as the use of cephalosporins [35].

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Prevalent PCR ribotypes of clinical and environmental strains of *Clostridium difficile* isolated from intensive-therapy unit patients in Kuwait

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Ninety-five isolates of *Clostridium difficile* from symptomatic and asymptomatic patients and 18 from their environment in the intensive-therapy units (ITUs) of four teaching hospitals in Kuwait were typed by PCR amplification of rRNA intergenic spacer regions (PCR ribotyping). A total of 32 different ribotypes was detected among the clinical isolates. The predominant ribotypes from the clinical isolates were types 097 and 078, which accounted for ~ 40 % of all isolates in the ITUs in Kuwait. Ribotypes 097 (toxigenic), 078 (toxigenic) and 039 (non-toxigenic) were three distinct clones that were circulating in all four hospitals. Ribotypes 097, 078 and 076 (i.e. 50 % of isolates from symptomatic patients) were the predominant isolates associated with *C. difficile*-associated disease (CDAD). The environmental isolates belonged to a diverse range of ribotypes, with no particular types common to all the hospitals. Ribotype 078 was found only in the patient environment in Mubarak hospital, while ribotype 097 was restricted to Amiri hospital. The hospital environment occupied by symptomatic as well as symptom-free patients was contaminated with *C. difficile*. Eight new strains that did not match any in the PCR ribotype library established at the PHLS Anaerobe Reference Unit, Cardiff, UK, were assigned ribotypes 105, 125, 128, 129, 131, 134, 140 and 141. These findings show that the isolates associated with CDAD in Kuwait are different from those found in the UK and some other European countries.

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INTRODUCTION

Clostridium difficile is an anaerobic, Gram-positive, spore-forming bacillus. It is often associated with a spectrum of diseases, referred as *C. difficile*-associated disease (CDAD), which manifest as self-limiting antibiotic-associated diarrhoea (AAD), antibiotic-associated colitis (AAC) and pseudomembranous colitis (PMC) with toxic megacolon and possible gut perforation. Rarely, it can present as extra-intestinal infections, such as arthritis, osteomyelitis, soft-tissue infection and bacteraemia (Levett, 1986). CDAD is an important clinical problem because it is often acquired by hospitalized patients, and *C. difficile* is associated with outbreaks of diarrhoea and colitis in hospitalized adults receiving antibiotics (Bartlett *et al.*, 1978; Djuretic *et al.*, 1999).

Abbreviations: AAC, antibiotic-associated colitis; AAD, antibiotic-associated diarrhoea; CDAD, *Clostridium difficile*-associated disease; ITU, intensive-therapy unit; PMC, pseudomembranous colitis.

In spite of major efforts to control the spread of CDAD in hospitals and nursing homes, this organism has remained a major problem worldwide, and it continues to be responsible for endemic and epidemic nosocomial diarrhoea (McFarland *et al.*, 1989; Johnson *et al.*, 1990; Barbut *et al.*, 1996). *C. difficile*, or its toxins, has been identified in 8–10 % of cases of nosocomial diarrhoea, while other common bacterial enteric pathogens, *Salmonella*, *Shigella* and *Campylobacter* spp., are rarely isolated (Fan *et al.*, 1993; Rohner *et al.*, 1997).

It appears that the most important sources of *C. difficile* in a hospital setting are symptomatic patients and asymptomatic carriers who are the main reservoirs of *C. difficile* in the hospital. The environment of these patients is also an important source. There is evidence that environmental contamination in rooms of patients with diarrhoea is substantially greater than in rooms with asymptomatic patients (49 vs 29 %) (McFarland *et al.*, 1989). In addition, rooms currently occupied by *C. difficile*-negative patients

can, in spite of routine cleaning, be contaminated with *C. difficile* spores that can survive for several months in the hospital environment. Person-to-person transmission on hospital wards, especially geriatric wards, as well as environmental contamination and carriage on the hands of hospital workers have been documented (Kim *et al.*, 1981; Malamou-Ladas *et al.*, 1983; Savage & Alford, 1983; McFarland *et al.*, 1989). Transmission occurs mainly via the faecal oral route and direct contact with contaminated surfaces (Barbut & Petit, 2001).

Epidemiological studies of *C. difficile* strains isolated from different environmental and clinical sources involve detailed comparison of the different isolates from all sites. Several typing schemes have been developed to determine the relatedness of strains of *C. difficile* associated with infections. However, of these, PCR ribotyping offers several advantages over the other methods because it is highly discriminative, reproducible and relatively rapid and easy to perform (O'Neill *et al.*, 1996; Stubbs *et al.*, 1999).

Our main aim was to study the epidemiology of *C. difficile* isolates from patients admitted into the intensive-therapy units (ITUs) of the four main teaching hospitals in Kuwait by PCR ribotyping.

METHODS

Bacterial isolates from patients. Stool samples were collected from all patients admitted to the ITUs of Mubarak Al-Kabeer hospital (ITU-1), Ibn Sina Burn Unit (ITU-2), haematology wards of Kuwait Cancer Control Centre (ITU-3) and Amiri hospital (ITU-4) who had stayed in the units for a minimum period of 3 days. The study was carried out over a period of 1 year (February 2001–January 2002). Freshly passed stool (rectal swabs, in Amies transport medium, if collection of stool was not feasible) was taken on the day of admission and weekly thereafter until the patient was discharged or developed diarrhoea secondary to *C. difficile* infection/colonization.

Environmental and other samples. A sterile swab, pre-moistened with sterile normal saline, was wiped over selected surfaces in the units, e.g. bed sheets, mattress, bed edges, bed ledges, surfaces of the side table next to the patients, suction regulator, oxygen regulator, ventilator surfaces, IV stand and the floor under the bed. Hands of doctors, nurses and physiotherapists who had had contact with the positive cases were cultured, by contact agar plates, for evidence of *C. difficile* contamination.

Inoculation, isolation and identification. The stool and environmental samples were inoculated into Robertson cooked meat (RCM) medium containing 25 ml fastidious anaerobe broth (FAB; Lab M) and incubated anaerobically for 48 h at 37 °C. Five-hundred microlitres of the FAB from the RCM was heated for 10 min at 80 °C. Next, cycloserine-cefoxitin egg yolk agar (CCEYA; Oxoid) and cycloserine-cefoxitin fructose agar (CCFA; Oxoid) plates were inoculated with one loop of the heated broth and incubated anaerobically for 48 h in an anaerobic chamber (H_2 10 %, CO_2 10 %, N_2 80 %). Isolates that were Gram-positive bacilli with characteristic horse-dung smell and fluoresced yellowish-green under long-wave UV light (365 nm) were selected and their identity was confirmed as *C. difficile* by API 20A (bioMérieux).

Toxin detection. Single colonies were subcultured on pre-reduced Columbia agar base (Oxoid) supplemented with 5 % horse blood, vitamin K and haemin and incubated at 37 °C under anaerobic conditions for toxin A detection. Toxin A was detected by ELISA (TOX-A) kits (Tech Lab); the procedure was carried out according to the manufacturer's instructions. Toxin B was detected by cytotoxicity assay on Vero cells. Production of cytopathic effects by filtered supernate of *C. difficile* RCM broth culture on Vero cells indicated toxin B production. Toxins A and B were detected in the stool by the TOX-A/B kits (Tech Lab).

PCR ribotyping. All isolates were typed by the PCR ribotyping method described by O'Neill *et al.* (1996). Briefly, after obtaining a pure culture, a single colony was subcultured on fastidious anaerobe agar (FAA; Lab M) supplemented with 6 % horse blood and incubated for 24 h at 37 °C. DNA was extracted from a suspension of 10 colonies in 100 µl 5 % Chelex 100 (Bio-Rad) by heating at 100 °C for 10 min. Cell debris was removed from the suspension by centrifugation for 10 min at 17 000 *g*. The supernate obtained was then used as the DNA template. Primers P3 (5′-CTGGGGTGAAGTCGTAACAAGG) and P4 (5′-GCGCCCTTTGT AGCTTTGACC) were used for the PCR amplification. The reaction mixture (final volume, 100 µl) contained 1·5 mM $MgCl_2$, 10 mM Tris/HCl (pH 9), 50 mM KCl, 0·1 % Triton X-100, 2·5 U *Taq* polymerase, 200 mM of each dNTP, 50 pmol of each primer and 10 µl DNA template. The PCR programme was 35 cycles of denaturation at 95 °C for 1 min, annealing at 56 °C for 1 min and extension at 72 °C for 2 min. A negative control was included in each run. Amplification products were concentrated to a final volume of 25 µl by heating at 75 °C for 90 min before electrophoresis at 200 V, 100 A in Metaphore agarose gel (FMC) for 3 h at room temperature. DNA fragments were then visualized by staining the gel for 20 min in 0·5 % ethidium bromide. Gel images were analysed with GelCompar image analysis software (version 4.0; Applied Maths). Our results were then compared with the library of PCR ribotypes already established at the PHLS Anaerobe Reference Unit, Cardiff, UK.

RESULTS

C. difficile was isolated from 95 of 922 patients (430 in ITU-1, 63 in ITU-2, 88 in ITU-3 and 341 in ITU-4) screened during this period. Thirty (31·6 %) of these 95 patients were symptomatic with CDAD; two patients had PMC, four had AAC and 24 had AAD. The overall incidence of *C. difficile*-positive patients was 10·3 %, with incidence in each hospital being 10·5 % for ITU-1, 6·3 % in ITU-2, 18 % in ITU-3 and 9·6 % in ITU-4. The organism was isolated from 18 of 380 environmental samples; six from ITU-1, three from ITU-3 and nine from ITU-4.

The distribution of PCR ribotypes among the clinical and environmental isolates is shown in Tables 1 and 2, and the banding of representative ribotypes is demonstrated in Fig. 1. Of the 95 isolates, 72 (75·8 %) were toxin-producers, while 23 (24·2 %) were non-toxigenic. A total of 28 (38·9 %) of the 72 toxigenic and two (8·7 %) of the 23 non-toxigenic isolates were associated with diarrhoea. As shown in Table 1, 32 distinct, genotypically different ribotypes were identified among the 95 clinical isolates. Of these, three distinct clones were detected in all four hospitals, ribotypes 097 (toxigenic), 078 (toxigenic) and 039 (non-toxigenic). The remaining 58 isolates belonged to a set of diverse ribotypes. In ITU-1, the ribotypes associated with diarrhoea in the 18 symptomatic patients were ribotypes 078 (4, 22 %), 076 (3, 17 %), 012 (2, 11 %), 056 (2, 11 %), 094 (2, 11 %), 097 (2, 11 %), 105 (2, 11 %) and 046 (1, 6 %). The five isolates associated with

Table 1. Distribution of PCR ribotypes and toxigenic strains of *C. difficile* in four teaching hospital ITUs

Ribotype	Toxin (A/B)	ITU-1	ITU-2	ITU-3	ITU-4	Total
002	+	0	1	1	0	2
010	–	3	0	0	0	3
012	+	2	0	0	0	2
013	+	1	0	0	0	1
014	+	1	0	1	1	3
017	–/+	0	0	0	1	1
020	+	1	0	0	0	1
026	–	1	0	0	0	1
029	+	1	0	1	0	2
035	–	1	0	0	0	1
039	–	6	1	1	2	10
045	+	0	0	0	2	2
046	+	1	0	0	0	1
051	+	0	0	3	0	3
054	+	1	0	0	0	1
056	+	2	0	0	3	5
064	+	1	0	0	0	1
070	+	0	0	1	0	1
076	+	3	0	0	3	6
077	+	0	0	3	0	3
078	+	4	1	2	2	9
081	+	1	0	0	0	1
094	+	2	0	1	0	3
097	+	3	1	1	13	18
098	+	0	0	0	2	2
105	+	2	0	0	0	2
113	–	0	0	1	0	1
128	–	5	0	0	0	5
129	+	1	0	0	0	1
131	+	1	0	0	0	1
140	–	0	0	0	1	1
141	–	0	0	0	1	1
Total		44	4	16	31	95

Table 2. Distribution of PCR ribotypes and toxigenic strains of *C. difficile* in the environment of four teaching hospital ITUs

Ribotype	Toxin (A/B)	ITU-1	ITU-2	ITU-3	ITU-4	Total
001	+	0	0	0	1	1
010	–	3	0	0	0	3
056	+	0	0	0	2	2
078	+	3	0	0	0	3
097	+	0	0	0	4	4
105	+	0	0	1	1	2
120	+	0	0	1	0	1
125	–	0	0	1	0	1
144	–	0	0	0	1	1
Total		6	0	3	9	18

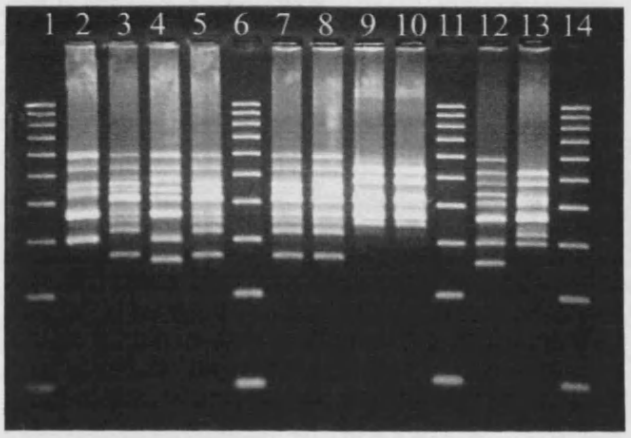

Fig. 1. Representative PCR ribotype profiles of *C. difficile* isolated from patients in Kuwait. Lanes: 1, 6, 11 and 14, 100 bp ladder; 2, ribotype 029; 3, 039; 4, 097; 5, 039; 7, 039; 8, 039; 9, 078; 10, 078; 12, 097; 13, 051.

diarrhoea in ITU-3 represented ribotypes 077 (2, 40 %), 014 (1, 20 %), 097 (1, 20 %) and 113 (1, 20 %), while the seven associated with diarrhoea in ITU-4 represented ribotypes 017 (1, 14·3 %), 039 (1, 14·3 %) and 097 (5, 71·4 %).

All symptomatic patients with toxigenic ribotypes had detectable toxins in their stools; two patients with non-toxigenic ribotypes 039 and 113 had no detectable level of toxin in their stools. Two PMC cases in ITU-1 were infected with ribotype 076. Single AAC cases in ITU-1 and ITU-3 were infected with ribotypes 046 and 097. Two AAC cases in ITU-4 were infected with ribotype 097.

As demonstrated in Table 2, the 18 environmental isolates were assigned to nine genotypically distinct ribotypes (six toxigenic and three non-toxigenic); the predominant types were 010 (3), 078 (3) and 097 (4). Ribotypes 078 and 097 were isolated primarily from the environment of symptomatic patients infected with the same ribotypes in ITU-1 and ITU-4, respectively. Ribotype 010 was isolated from the environment of asymptomatic patients in ITU-1. Ribotype 105, associated with diarrhoea in ITU-1, was absent in its environment but present in the environment of ITU-3 and ITU-4, where it did not contribute to CDAD. Ribotypes 001 and 144 were present in the environment of ITU-4, but were not isolated from any patient in the unit. Similarly, ribotypes 120 and 125 were isolated from the floor of ITU-3, but were not isolated from the patients in the unit.

No *C. difficile* was isolated from the hands of healthcare providers.

DISCUSSION

In spite of the growing number of studies devoted to CDAD in Western countries, studies on CDAD in the Middle East

are lacking, especially in Kuwait, where information on the incidence of *C. difficile* carriage and CDAD is almost non-existent. This is partly as a result of inertia in anaerobic bacteriology prompted, until recently, by lack of expertise, technology and facilities for culturing anaerobic pathogens. We have, in the recent past, encountered a relatively high proportion of asymptomatic carriers of *C. difficile* in our hospitals, and the interpretation of positive cultures was equivocal. *C. difficile* or its toxin has been reported in one study from nearby Saudi Arabia (Akhter *et al.*, 1994), in Turkey (Soyletir *et al.*, 1996) and in a few reports from Israel (Rudensky *et al.*, 1993; Rivlin *et al.*, 1998; Boaz *et al.*, 2000). However, this is the first report on PCR ribotyping of *C. difficile* strains isolated from symptomatic and asymptomatic patients in the Middle East.

The data generated from the present study showed that all 95 and 18 *C. difficile* isolates respectively originating from patients and their environment were typable by the PCR ribotyping method, and they revealed some interesting epidemiological findings. Secondly, none of the patients admitted to the ITUs carried *C. difficile* into the units on admission. Thus, an incidence rate of hospital-acquired *C. difficile* infection/colonization of 10·3 % was established for the four hospital ITUs in Kuwait, ranging from 6·3 to 18 % in different hospitals. Interestingly, the 95 *C. difficile* culture-positive patients harboured 32 different, highly diverse PCR ribotypes. Three different DNA clones (PCR ribotypes 097, 078 and 039) were detected among patient isolates in all hospitals. The other isolates were assigned to 29 distinct and diverse types. Ribotype 097 was the single most prevalent type and was responsible for about 27 % of CDAD, while ribotype 078 was responsible for about 13 % of CDAD. Thus, ribotypes 097 and 078 were responsible for over one-third of the cases of CDAD seen. From an epidemiological point of view, this is an interesting finding, in that the dominant ribotypes causing diarrhoea in Kuwait are completely different from those seen in Europe, particularly the UK (Stubbs *et al.*, 1999), Hungary (Urban *et al.*, 2001) and Poland (Martirosian *et al.*, 1995). Fifty-five per cent of *C. difficile* infections seen in UK hospitals are caused by ribotype 001 (Stubbs *et al.*, 1999), while ribotype 087 accounted for 39 % of all isolates in Hungary (Urban *et al.*, 2001). In the Polish study, all the environmental isolates and 11 of 31 neonatal isolates belonged to ribotype 001.

It is noteworthy that one of our patients in ITU-4 who had AAD was infected with ribotype 017, which is toxin-variable (toxin A-negative, B-positive). He gave a history of travel to Thailand and he was hospitalized there for unrelated illness. An outbreak of toxin A-negative, B-positive *C. difficile* associated with diarrhoea has been reported in a Canadian tertiary-care hospital (Al-Barrak *et al.*, 1999). This is not our experience in Kuwait so far, as the single isolate was restricted to only one hospital and one patient. Why some patients have more marked symptoms than others and some strains are more associated with outbreaks of CDAD in different countries is unclear. Systemic symptoms are caused mainly by toxin-mediated inflammatory mediators released in the colon (Dallal *et al.*, 2002). Thus, it is conceivable that the ability of the host to mount an effective antibody-mediated response to the *C. difficile* toxin plays a major role in this regard. However, the bacterial factors involved in the geographical differences seen in the two locations are worthy of further investigation.

The environmental strains were heterogeneous in each hospital. The 18 isolates were assigned to nine different ribotypes. The environment of two patients each in ITU-1 and ITU-4 was contaminated by the same ribotypes as found in the patients' clinical samples, confirming that the patient's environment is a potential source of *C. difficile* and that cross-contamination may play an important role in the acquisition of nosocomial CDAD (Cohen *et al.*, 2000). In some cases, there was no correlation between the environmental and patient isolates. For instance, the environmental isolates in ITU-3 and some in ITU-4 differed from those found in the patients. Our finding is not an isolated one. Earlier, Cohen *et al.* (1997) found none of the environmental *C. difficile* genotypes among isolates from patients, and Simor *et al.* (1993) found no cross-transmission in their institution in a survey of *C. difficile* infection in a long-term care facility.

In conclusion, the prevalent PCR ribotypes of *C. difficile* strains circulating in Kuwait hospitals are different from those found in many hospitals in Europe and are also different from those associated with CDAD in many patients. Further work is needed to elucidate the factors responsible for the geographical differences and the ability of one strain to cause more systemic symptoms than another.

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Correlation of Multidrug Resistance, Toxinotypes and PCR Ribotypes in *Clostridium difficile* Isolates from Kuwait

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Summary

***Clostridium difficile* is a common cause of nosocomial diarrhea. Its role in community-acquired diarrhea is also becoming an important public health concern. Hardly any studies have correlated strain ribotypes, toxinotypes and multidrug resistant (MDR) profiles. To investigate these characteristics, 65 *C. difficile* isolates obtained from stool samples of patients whose cultures were negative on admission but became positive after 48 h of admission to the ICUs of our hospitals were studied to determine the prevalent ribotypes, toxinotypes and their relationship with the MDR profiles using ELISA/cytotoxicity assays, PCR and Etest methods. The toxin-producing strains were toxinotyped by the PCR-RFLP technique. Of the 65 isolates, 42 (64.6%) were toxigenic (T). The isolates were of diverse ribotypes but types 097, 078, 056 and 039 (NT) were predominant. Thirty (71.4%) of 42 T and 13 (56.5%) of 23 NT strains were multiresistant to 3 or more antibiotics. Only 3 toxinotypes (0, "V-like" and XII) were encountered. Of the 42 T strains, 30 (71.4%) were of toxinotype 0, and 12 belonged to variant toxinotypes: 4 (9.4%) to toxinotype XII and 8 (19%) to "V-like" toxinotype in which amplified B1 PCR fragments was amplified as expected for toxinotype V but the A3 PCR fragment could not be amplified. The 43 MDR strains were assigned to 3 arbitrary resistance groups; groups 1, 11 and III. The most prevalent isolates (37; 86.1%) were in group II. Of the predominant T ribotypes (097, 078 and 056), c. 62% clustered in group II. Although the number of strains toxinotyped was small, ribotyping and toxinotyping correlated well with the published literature, except for 078 with a novel "V-like" toxinotype. Antibiogram was not as clear-cut.**

Key words: **Toxinotypes, ribotypes, resistance pattern, *Clostridium difficile*.**

INTRODUCTION

Clostridium difficile is regarded worldwide as one of, if not, the most common cause of nosocomial diarrhea. Diarrhea produced by this organism is usually mild and self-limiting often referred to as antibiotic-associated diarrhea (AAD) but more severe forms of the disease can arise in the form of *C. difficile*-associated colitis (CDAC) and life-threatening pseudomembranous colitis (PMC), particularly in patients in intensive care units (ICU) and other intensive therapy units in hospitals. These spectra of the diseases are mediated by bacterial toxin production and usually driven by antibiotic use. *C. difficile* produces two major virulence factors: toxin A (TcdA, an enterotoxin) and toxin B (TcdB, a cytotoxin). They are the largest bacterial toxins known, with molecular weights of 308 kDa and 279 kDa, respectively. Using a toxinotyping method based on PCR-RFLP analysis of a 19.6kb region encompassing the *C. difficile* pathogenicity locus PaLoc, 20 different toxinotypes (I to XX) have been established.[1,2] These include variants of *C. difficile* that harbor deletions,

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insertions, polymorphic restriction sites in one or more of the genes within the PaLoc, but still generate functional TcdA and TcdB toxins.

Many different methods have been used to analyze the genetic relatedness among *C. difficile* isolates during nosocomial outbreaks. Experience with PCR ribotyping, that uses specific primers complimentary to the 3' end of 16S rRNA gene and the 5'end of the 23S rRNA gene to amplify the variable-length intergenic spacer region, appears to have gained worldwide acceptance with relatively easy application in epidemiological studies.[3] Currently, over 190 ribotypes have been established and the database is available at the Anaerobe Reference Laboratory at the University of Wales Hospital, Cardiff, UK.

The results of various typing methods have demonstrated the heterogeneity of *C. difficile* showing that the organism consists of several groups. Not all members of these groups are associated with disease. However, some have been associated with particular outbreaks. Recent experience indicates that the organism is not uniformly susceptible to the same groups of antibiotics, in particular showing unusually consistent resistance to imipenem but not meropenem.[4] So far, the relationship between the different groups (toxinotypes and ribotypes) and antibiotic resistance pattern has not been well studied.

The aim of this study was to investigate and correlate PCR-ribotypes, toxinotypes and antibiotic resistance patterns in clinical strains of *C. difficile* isolated from the stools of patients in 4 teaching hospitals in Kuwait.

MATERIALS AND METHODS

Bacterial strains: A total of 65 clinical isolates of *C. difficile* obtained earlier[5] during a study on the acquisition of *C. difficile* by ICU patients in our hospital were included in this study. These were isolates obtained from stool samples of both symptomatic and asymptomatic patients 48 h post-admission to the Intensive Care Units (ICU) of 4 major teaching hospitals in Kuwait.

Growth conditions and toxin production: Isolates were grown on Fastidious Anaerobe Agar (FAA; Lab M) supplemented with 5% horse blood in anaerobic atmosphere at 37°C. Detection of toxin A production was achieved by using the ELISA (Tox-A) kits (TechLab, BioConnections, Leeds, UK) and the procedure was carried out according to manufacturer's instruction. Toxin B was detected by cytotoxicity assay on Vero cells by assessing the cytopathic effects produced by filtered supernatant of *C. difficile* broth culture (Robertson Cooked Meat broth; Unipath, Basingstoke, UK) on the Vero cell lines.

PCR ribotyping and toxinotyping: The isolates were ribotyped as described earlier.[3,6] Briefly, a single colony was subcultured on FFA supplemented with 5% horse blood and incubated overnight at 37°C. Crude nucleic acid template was prepared by resuspension of 10 colonies in 100µl of Chelex-100 (Bio-Rad) and boiling for 10 min. The suspension was centrifuged at 15,000xg to remove cell debris. The supernatant was then used for PCR ribotyping. DNA fragments were visualized by staining the Metaphore agarose gel for 20 min in 0.5% ethidium bromide. Gel images were analyzed with GelCompar image analysis software (version 4.0; Applied Maths). PCR-RFLP technique was used for the toxinotyping of isolates according to previously described methods.[7,8] Briefly, DNA was extracted with Chelex 100 (Bio-Rad Laboratories, USA). Subsequently, the strains were screened for variation in the first 3kb of *tcdB* (PCR fragment B1) and the 3kb fragment spanning the repetitive region of *tcd A* (PCR fragment A3) by PCR amplification as described previously.[7] Then, digestion with restriction enzymes *HincII* and *AccI* (for fragment B1) and *EcoRI* (for fragment A3) was done as described previously.[1] The toxinotype was then determined according to the combination of restriction patterns in the PCR fragments B1 and A3.[1,2]

Antimicrobial susceptibility testing (AST): AST was performed by determining the minimum inhibitory concentrations (MICs) of cefoxitin, clindamycin, imipenem, meropenem, and metronidazole using the Etest (AB, Biodisk, Slona, Sweden) method. Briefly, a 48-h bacterial growth culture suspended in anaerobe broth and adjusted to 1.0 McFarland turbidity standard was inoculated onto a reduced Brucella agar plate (Unipath, Basingstoke, UK) supplemented with 5% horse blood, hemin 5 µg/ml and menadione 1 µg/ml. The Etest strips of the tested antibiotics were applied onto the agar surface, and then incubated in anaerobic jars containing 10% CO_2, 90% H_2 for 48 h at 37°C. The MIC was read as the interception of the elliptical zone of inhibition with the strip. Trovafloxacin MIC was determined by agar dilution method recommended by the Clinical Laboratory Standards Institute (CLSI; formerly NCCLS).[9] The multiresistance (MDR) profile was based on resistance to 3 or more classes of antibiotics.

RESULTS

Ribotyping, toxinotyping and antibiotic susceptibility of C. difficile

Of 65 clinical isolates analyzed, 42 (64.6%) were toxin-producers (T) while 23 (35.4%) were non-toxigenic (NT). Thirty (46.2%) isolates out of 65 were from patients with diarrhea. A total of 28 (66.7%) of 42 toxigenic and 2 (8.7%) of the 23 non-toxigenic isolates were associated with diarrhea. The predominant ribotypes detected were ribotypes 097(T), 078 (T), 056 (T) and 039 (NT), which represented over 44% of the isolates (representative ribotypes are shown in *Figure 1*).

The 42 T strains, including those from symptomatic patients, were toxinotyped; 30 (71.4%) were toxinotype 0 and 12 (28.6%) belonged to variant toxinotypes: 4 (9.4%) to toxinotype XII and 8 (19%) to

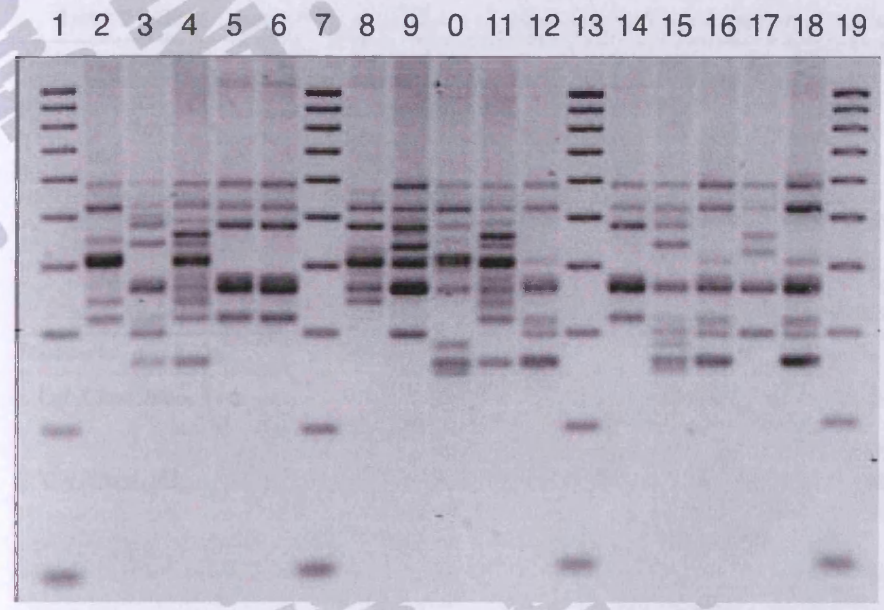

FIGURE 1 - PCR ribotype profiles of representative clinical isolates of *Clostridium difficile*.
Lanes 1,7,13,19 = 100bp; Lane 2 = 056; Lane 3 = 097; Lanes 4 and 11= 077; Lanes 5, 6 and 14 = 078; Lane 8 = 120; Lane 9 = 076; Lane 10 = 002; Lane 12 = 001 (non-toxinotyped environmental isolate); Lane 15 = 105; Lane 16 = 001; Lane 17 = 129; Lane 18 = NCTC 11209 (type 001)

"V-like" toxinotype in which amplified B1 PCR fragments suggested toxinotype V but the A3 PCR fragment could not be amplified (See *Table 1*). Of the 30 strains with toxinotype 0, 22 (82%) were associated with diarrhea. These strains were PCR ribotypes 097, 076, 012, 077, 094, 105, 014, 078, and 046. However, some strains of PCR ribotype 097 also belonged to more than one toxinotype (0, V).

All of the isolates were susceptible to metronidazole and meropenem. As shown in *Table 2*, 43 out of 65 (66.2%) of the clinical isolates were multiply resistant, i.e. resistant to 3 or more antibiotics. Their resistance pattern was divided arbitrarily into 3 groups according to various combinations of resistance profiles: group 1, resistance to cefoxitin (C), clindamycin (C), imipenem (I) and trovafloxacin (T) i.e. (CCIT); group II, CCI; group III, CIT. The analysis of individual isolates in relation to the multiresistance group, and toxin or non-toxin production, showed that out of the 43 multiresistant isolates, only 3 (7%) were resistant to 4 antibiotics (CCIT; all T strains) while 37 (86%) and 3 (7%) were resistant to CCI and CIT, respectively. The strains resistant to CCIT and CIT were all toxigenic strains but only 24 (64.8%) of the 37 resistant to CCI were toxin-producers. All the MDR-strains had common resistance to imipenem and cefoxitin.

Correlation between ribotypes, toxinotypes and antibiotic susceptibility:

Table 3 shows the 3 antibiotic resistance groups and the associated corresponding ribotypes. Of the prominent toxigenic ribotypes, 6 (75%) of the 8 ribotype 078 were clustered in group II CCI resistance pattern and the remaining 2 in group I. Three (75%) each of the 4 ribotypes 056 and 076, and 4 (57.1%) of 7

TABLE 1 - *PCR ribotypes and toxinotypes of the toxigenic C. difficile strains from symptomatic and non-symptomatic patients.*

PCR ribotypes (n.)	Toxinotypes	Comments
078 (8)	"V-like"*	078 normally belongs to toxinotype V
097 (7)	0	Some strains (not these ones) were toxinotype V
076 (4)	0	
056 (4)	XII	They do not have genes for binary toxin
077 (3)	0	
002 (2)	0	
014 (2)	0	
094 (2)	0	
105 (2)	0	
106 (2)	0	
012 (1)	0	
013 (1)	0	
054 (1)	0	
120 (1)	0	
129 (1)	0	
131 (1)	0	

*In these strains, B1 fragment was amplified which showed RFLP as expected for toxinotype V but A3 PCR fragment was not amplified which is unusual for toxinotype V.
039 (NT) was Cdd3+ but there was no amplification of PaLoc.

TABLE 2 - *Distribution of the multiresistant groups among the toxigenic and non-toxigenic C. difficile strains.*

Arbitrary grouping	Resistance grouping	N. of T strains	N. of NT strains	Total (%)
I	Cef, Clind, Imip, Trov	3	0	3 (7)
II	Cef, Clind, Imip	24	13	37 (86)
III	Cef, Imip, Trov	3	0	3 (7)

Cef: cefoxitin; Clind: clindamycin; Imip: imipenem; Trov: trovofloxacin; T: toxigenic strains; NT; non-toxigenic

TABLE 3 - *Antibiotic resistance groupings, PCR ribotypes and toxinotype distribution of toxigenic C. difficile isolates.*

Resistance grouping	PCR ribotyping (no.)	Toxinotype	Total n. (%) (n=30)
I. Cef, Clind, Imip, Trov	078 (2)	"V-like"	3 (10)
	105 (1)	0	
II. Cef, Clind, Imip	014 (2)	0	24 (80)
	054 (1)	0	
	056 (3)	XII	
	076 (3)	0	
	077 (1)	0	
	078 (6)	"V-like"	
	094 (2)	0	
	097 (4)	0	
	129 (1)	0	
	131 (1)	0	
III. Cef, Imip, Trov	013 (1)	0	3 (10)
	076 (1)	0	
	077 (1)	0	

Cef: cefoxitin; Clind: clindamycin; Imip: imipenem; Trov: trovofloxacin.

ribotype 097 were also in group II. These strains were associated with symptomatic CDI patients. The majority of those that were toxinotyped in the 3 resistance groups were of toxinotype 0.

DISCUSSION

The identification of *C. difficile* as a major cause of hospital-acquired diarrhea has led to the application of various methods for typing this organism and many laboratories have thus focused on genotyping methods. In this study, we correlated the relationship between antibiotic resistance, PCR-ribotypes and toxinotypes in *C. difficile* isolates in Kuwait. Some interesting findings are worth highlighting. For example, the main toxinotypes in our country were 0, XII and "V-like" toxinotypes. So far no previous study exists on the prevalence and distribution of toxinotypes in Kuwait or in all Gulf State countries. This is in contrast with the Brussels and Anaerobe Reference Unit collections in Cardiff which already share toxinotypes from I to XV.[1] Six (toxinotypes I, III, IV, VIII, IX and XII) of these 15 toxinotypes have been described among the Japanese strains. In addition, 11 various toxinotypes have been described in Asia (isolates from Japan, Korea and Indonesia).[2] Approximately 71% of the toxinotyped isolates in our study belonged to the "0" toxinotype. This relatively high prevalence of 0 toxinotype is similar to the findings of Geric *et. al.*,[7] who reported a prevalence of 80.4% among their clinical *C. difficile* isolates. In that study, 11.1% belonged to various other toxinotypes. A much higher prevalence of toxinotype 0 has been reported by other workers. Mutlu *et al.*[10] in Scotland reported a prevalence rate of 96.6% toxinotype 0 among their *C. difficile* isolates, with 2 toxinotype V isolates and single isolates for each of toxinotypes I, IV and XIII. The prevalence rate of 28.6% of variant (not 0) toxinotypes in our study is comparable to the findings of others in Europe (21.5-25%) [1,2,11] and Asia (23.5%).[2] Worthy of note in our report is the finding of a novel "V-like" toxinotype in ribotype 078, which differs from the V type in that the B1 PCR fragment was amplified but the A3 fragment was not.

Analysis of the strains according to the resistance pattern revealed that toxigenic strains were more resistant than non-toxigenic strains. Almost two-thirds (66.2%) of the resistant strains were MDR strains. Although this appears to be an alarming finding, its clinical relevance is in doubt as none of the strains were resistant to metronidazole or vancomycin, the drugs of choice for therapy of CDI. However, the observation is worth noting as the therapeutic options for infections caused by particular toxinotypes, such as toxinotypes 0 and "V-like" strains, become severely limited. Resistance to a combination of cefoxitin, clindamycin, imipenem and trovafloxacin, and cefoxitin, imipenem and trovafloxacin were exclusively seen in the toxigenic strains. In our study, only three of the isolates were resistant to 4 antibiotics and 2 of them were of ribotype 078 with "V-like" toxinotype and the other one was ribotype 105 belonging to toxinotype 0. None of our isolates were resistant to metronidazole and meropenem. However, recently Baines and his colleagues in Leeds, UK [12] have demonstrated the emergence of reduced susceptibility to metronidazole in 24.4% of *C. difficile* that belonged to ribotype 001. Ribotype 001 is often associated with outbreak of CDI in the UK but this ribotype never featured in our patients; only one strain of ribotype 001 has so far been found in the ICU environment of Amiri hospital in our country.[13]

The most predominant ribotypes (097, 078 and 039) in our series were essentially similar to the ribotypes in our previous study.[13] These ribotypes were different from those seen in the UK,[6] Hungary[14] and Poland.[15] In Hungary, the main ribotype is 087 while in Poland ribotype 001 is the predominant one. Prior to the recognition of ribotype 027 in the UK, ribotype 001 was the most common type which accounted for 55% of hospitalized patients.[6] Recently, *C. difficile* ribotype 106 has become the predominant strain in England, accounting for 26% while ribotypes 027 and 001 account for 25% each.[16] However, it appears that the epidemiology of prevalent ribotypes continues to change rapidly in the UK, with ribotype 027 assuming a higher proportion. A more recent report in England has demonstrated that of 2,084 *C. difficile* isolates, 42% were type 027, 19% were type 106 and only 10% as type 001.[17] However, in Scotland, ribotype 106 accounts for 55% of all isolates while type 001 for 21%. Ribotype 078, the most prevalent in our series, has emerged in only 4 isolates. The most recent report from Ireland showed that the commonest ribotypes are 001 (35%), 106 (11.6%) followed by type 078 (8.3%).[17] In a recent outbreak in the Netherlands, 25.3% of patients with CDI were due to type 027 which belonged to toxinotype III.[18] Interestingly, these reports contrast sharply with the findings in Kuwait where the prevalent ribotypes are 078 and 097. More and more of 078 are associated with animal origin although we did not investigate its association with any animal in this study.

In conclusion, the results obtained from our study demonstrate that the strains of *C. difficile* in Kuwait are complex and heterogeneous. Although characterization by toxinotyping or antibiotic resistance patterns appears to suggest that the majority of our strains may be closely related, the use of other typing method—especially PCR ribotyping—demonstrates a wide variation among the strains.

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Influence of antibiotic exposure on cell-bound and cell-free *Clostridium difficile* cytotoxin production

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Introduction:

Clostridium difficile is a Gram-positive anaerobic bacillus often incriminated in hospital-acquired diarrhoea (1,2) which represents a spectrum of diseases known as *C. difficile*-associated diseases (CDAD). This disease is mediated by elaboration of toxins A and B by the organism in the gut (2,3). CDAD is driven by a variety of environmental factors, including exposure to antibiotics. The objective of this study is to quantitatively determine the effects of different concentrations of antibiotics on cell-bound and cell-free cytotoxin B production.

Methods:

Ampicillin, cefotaxime, clindamycin, metronidazole and vancomycin were tested. These antibiotics are either incriminated as trigger drugs in CDAD or used for its therapy. Three toxigenic strains, **233D, A11A & 362C**, isolated from clinical cases, pseudomembranous colitis (PMC), antibiotic-associated diarrhoea (AAD; Tox A-/B+) and antibiotic-associated colitis (AAC), respectively, and a negative control non-toxigenic strain (175) were used for the study. The strains were grown in liver broth and brain heart infusion containing MICs and sub-MICs of the 5 antibiotics. Filtered cell-free supernatant and supernatant of sonicated cells (Ultrasonic Homogenizer Labsonic, Meisungen AG, Germany) were tested for cytotoxic activity (CA) on Vero cell lines. Positive result was represented by complete rounding of the cells after exposure to the extracts for 24 h. See Fig 1 (negative control) & Fig 2 (positive test).

Figure 1: Uninoculated Vero Cells

Figure 2: Cytopathic effect of *C. difficile* cytotoxin B on Vero Cells

Results:

***Effect on PMC-causing strain 233D (Figure 3)*:**
•Clindamycin, on the average, induced the highest of cell-bound cytotoxin at all MIC and sub-MIC concentrations. The average cytotoxic activity (CA) was 915.7 µg/ml which is 27-folds higher than the control, followed by metronidazole and vancomycin, 14.5- and 13.3- folds higher than the control, respectively.
•Ampicillin and cefotaxime induced more cell-free cytotoxin than the cell-bound cytotoxin, 17.4 and 10.1-fold versus 6.4 and 2.3-fold, respectively.

***Effect on AAD-causing strain A11A (Figure 4)*:**
•Cefotaxime, ampicillin, metronidazole, vancomycin and clindamycin induced more cell-free cytotoxin than the cell-bound toxin. The cell-free cytotoxin were 112-, 101-, 84-, 11.5- and 11.2- folds higher than the control; while the cell-bound cytotoxin were only 21-, 0.7-, 1.5-, 0.33- and 9-fold higher than the control, respectively.

***Effect on AAC-causing strain 362C (Figure 5)*:**
•Exposure to ampicillin, vancomycin, cefotaxime, metronidazole and clindamycin also induced more cell-free cytotoxin than the cell-bound cytotoxin. The cell-free cytotoxin were 24.2-, 22.8-, 16.5-, 7.7- and 3.9-folds higher than the control; while the cell-bound cytotoxin were 3.4-, 0.7-, 5.3-, 4.0- and 2.7- folds higher than the control, respectively.

Effect on non-toxigenic strain 175
•The non-toxigenic strain 175 did not induce any cytotoxic activity in the presence or absence of antibiotic.

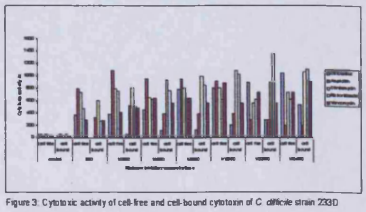

Figure 3: Cytotoxic activity of cell-free and cell-bound cytotoxin of *C. difficile* strain 233D

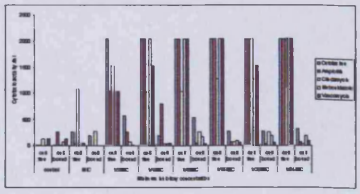

Figure 4: Cytotoxic activity of cell-free and cell-bound cytotoxin of *C. difficile* strain A11A

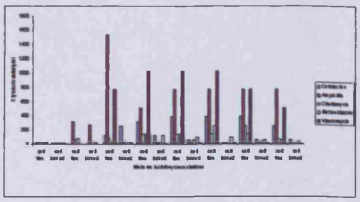

Figure 5: Cytotoxic activity of cell-free and cell-bound cytotoxin of *C. difficile* strain 362C

Conclusions

•Both the antibiotics that drive CDAD as well as those used in its therapy induced appreciable production of cell-free and cell-bound cytotoxic activities on contact with disease-producing *C. difficile*.
•Induction of both cell-free and cell-bound cytotoxin was equally high after exposing PMC strain 233D to the 5 antibiotics. It is noteworthy that induction of cell-bound cytotoxin was more prominent with this strain than the other non-PMC strains
•The highest cytotoxic activities occur mostly at sub-inhibitory concentrations.
•There appears to be no uniform relationship between antibiotic class and toxin production by *C. difficile*.
•There is no correlation between the severity of the illness and the level of cytotoxin production on Vero cells, except PMC and high induction of cell-bound cytotoxin.

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