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Detection of *Clostridium difficile* and Its Toxin A (TcdA) in Stool Specimens from Hospitalised Patients

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A b s t r a c t

The study has been carried out to determine the frequency of *C. difficile* recovery in stool cultures and the rate of *C. difficile* toxin A detection in faecal specimens of patients with nosocomial diarrhoea. Clinical specimens comprised 4414 stool samples collected from 1998 to 2002 from adult patients hospitalised in different wards of a university-affiliated hospital (1200 beds) and suspected of *C. difficile*-associated disease (CDAD). There have been 1308 (29.6%) specimens positive for *C. difficile* culture (15.1% in 1998, 29.5% in 1999, 33.8% in 2000, 31.2% in 2001 and 32.0% in 2002). The highest number of *C. difficile* strains was cultured from stool samples of patients hospitalised in the haematology/oncology ward (51.1% of all isolates), neurology (8.3%), nephrology (8.0%), gastrointestinal surgery (7.0%) and neurosurgery (6.2%) wards. The testing for *C. difficile* toxin A yielded 847 (19.2%) positive samples and 3567 (80.8%) toxin A-negative results. The percentage of *C. difficile* toxin A-positive samples was 29.4% in 1998, 17.5% in 1999, 23.2% in 2000, 17.1% in 2001 and 15.0% in 2002. In the analysed period we observed an increase in the number of stool specimens tested for *C. difficile* and an increase in the number of *C. difficile* culture-positive samples. A decrease in the number of *C. difficile* toxin A-positive samples was noted in the last 2 years of the study. This phenomenon may be due to an improved antibiotic policy of the hospital.

K e y w o r d s: *Clostridium difficile*, *C. difficile* toxin A, antibiotic-associated diarrhoea, pseudomembranous colitis

Introduction

In the recent years *Clostridium difficile* strains have been isolated with increasing frequency from the clinical specimens obtained from hospitalised patients (Wilcox and Smyth, 1998). It is therefore regarded as an emerging pathogen of the hospital-acquired infections. The bacterium spreads easily between patients due to transmission by the hospital environment or healthcare personnel. Furthermore, intensive therapy with broad-spectrum antibiotics and chemotherapeutic agents favour colonisation of the patients and subsequently development of disease. In healthy adults asymptomatic carriage rate is 2–3%, but upon hospitalisation increases to over 20%, especially if antibiotic therapy has been administered to the patient (Kyne *et al.*, 1998). In adults with nosocomial diarrhoea *C. difficile* is the most commonly detected agent (Decre *et al.*, 2000). Therapy with clindamycin or third generation cephalosporins has been predominantly reported as a predisposing factor to *C. difficile*-associated disease (Mylonakis *et al.*, 2001). Furthermore, the infection causes prolonged hospitalisation and significantly increases its costs, even by over 50% (Wilcox *et al.*, 1998; Wilcox and Dave, 2000; Kyne *et al*., 2002). *C. difficile* is an etiological agent of *C. difficile*-associated disease (CDAD). This entity comprises antibiotic-associated diarrhoea (AAD), antibiotic-associated colitis (AAC) and the most severe clinical presentation – pseudomembranous colitis (PMC), which

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Table I
Current methods for detection of *C. difficile* toxins and genes encoding *C. difficile* toxins

Toxin/toxin encoding genes	Detection methods
Toxin A (TcdA)	immunoenzymatic assay (EIA), latex test
Toxin B (TcdB)	tissue culture cytotoxicity assay (cell lines: Vero, McCoy's, *etc.*) cytotoxin neutralization assay (tissue culture + antitoxin)
Toxin A/toxin B (TcdA/TcdB)	immunoenzymatic assay (EIA)
Binary toxin (CDT)	not available
Toxin A gene (*tcdA*)	polymerase chain reaction (PCR)
Toxin B gene (*tcdB*)	polymerase chain reaction (PCR)
Binary toxin gene (*cdt*)	polymerase chain reaction (PCR)

can be fatal particularly in immunocompromised patients (Kato *et al*., 1991). Toxin A-producing strains of *C. difficile* have been mainly incriminated in these conditions. Majority of toxigenic *C. difficile* strains produce two types of toxins – A (TcdA) and B (TcdB). Some strains (7–8%) may produce a third toxin called binary toxin (CDT). The most sensitive and specific test available for diagnosis of *C. difficile* infection, which remains the "gold standard", is a tissue culture assay for cytotoxicity of toxin B (Decre *et al.*, 2000; Mylonakis *et al.*, 2001). However it is not used in most of routine laboratories, since it requires tissue culture facilities. Also detection of binary toxin is not done routinely so far. Recently an immunoassay has been developed to determine simultaneously the presence of both toxin A and toxin B in a clinical sample, however without their discrimination from one another (Decre *et al*., 2000; Aldeen *et al*., 2000). In hospitalised patients with severe diarrhoea immunoassays for detection of toxin A (enterotoxin) appear at present to be important laboratory tests helping the clinicians with the diagnosis of infections caused by *C. difficile* (Jacobs *et al*., 1996). In patients with hospital-acquired diarrhoea it is therefore necessary to test stool specimens for the presence of *C. difficile* toxin A (Gerding *et al*., 1995; Poutanen and Simor, 2004). Diagnostic methods used for detection of *C. difficile* toxins and toxin encoding genes are listed in Table I.

The aim of the study was to evaluate the frequency of recovery of *C. difficile* in culture and to determine the frequency of *C. difficile* toxin A detection in the stool specimens of patients hospitalised in a tertiary care hospital in view of an increasing number of cases of nosocomial postantibiotic gastrointestinal disorders.

Experimental

Materials and Methods

The study comprised retrospective analysis of faecal specimens from adult patients suspected on clinical grounds of CDAD. The patients were hospitalised in the Central Clinical Hospital in Warsaw (1200 beds) over a period of five years (1998–2002). Duplicate specimens were excluded. The samples were collected into sterile containers. Inoculation of culture media and testing for *C. difficile* toxin A was done within 5 hours of specimen collection. Whenever possible, both the culture of *C. difficile* and TcdA detection were done on clinical specimens comprised in the study.

Isolation of *C. difficile* strains. The stool samples were cultured for *C. difficile* by inoculation of Columbia blood agar containing cefoxitin, cycloserine and amphotericin B (CCCA medium). The plates were incubated at 37°C for 48 h in an anaerobic chamber "Heraeus" (85% N_2, 5% H_2 and 10% CO_2) and isolates identified by standard methods for these anaerobic bacteria (colony morphology, characteristic smell of the colonies, microscopic appearance of bacteria and their fluorescence in the UV lamp). The identification of *C. difficile* was confirmed with a latex agglutination assay for *C. difficile* antigen "Culturette Brand CD" test (Becton Dickinson).

***C. difficile* toxin A detection in stool samples.** The stool specimens were examined for the presence of *C. difficile* toxin A using a commercial immunoassay "*Clostridium difficile* toxin A test" (Oxoid, England).

Results

In total, 4414 samples have been cultured in the studied period (1998–2002). Out of them, 1308 specimens (29.6%) have yielded growth of *C. difficile*. This comprised 1053 (80.5%) samples from patients hospitalised in internal medicine wards and 255 (19.5%) specimens from surgical wards (Table II). Among

Table II
Number of *C. difficile* culture-positive stool specimens in patients hospitalised in 1998–2002 in various wards (N = 1308)

Ward		1998	1999	2000	2001	2002	Total	%
Internal medicine	haematology-oncology	36	110	123	180	219	668	51.1
	neurology	0	6	35	27	41	109	8.3
	nephrology	7	9	21	23	45	105	8.0
	vascular disorders	12	13	6	17	7	55	4.2
	gastroenterology	5	11	10	7	15	48	3.7
	other	9	20	14	9	16	68	5.2
Surgery	gastrointestinal surgery	3	14	18	34	22	91	7.0
	neurosurgery	7	11	20	26	17	81	6.2
	transplantation surgery	3	5	12	4	19	43	3.3
	surgical ICU	0	3	8	1	7	19	1.4
	other	1	5	4	5	6	21	1.6
Total		83	207	271	333	414	1308	100.0

Table III
Detection of *C. difficile* toxin A (TcdA) in faecal samples of patients hospitalised in 1998–2002 (N = 847)

Year	Total number of samples	Number of toxin A -positive samples	Percentage
1998	551	162	29.4
1999	702	123	17.5
2000	802	186	23.2
2001	1067	182	17.1
2002	1292	194	15.0
Total	4414	847	19.2

samples positive for *C. difficile* in culture predominated specimens from haematology-oncology (51.1%), neurology (8.3%), nephrology with dialysis unit (8.0%), gastrointestinal surgery (7.0%) and neurosurgery (6.2%) wards (Table II). There was an increase in the number of positive culture results over these years – 83, 207, 271, 333 and 414, respectively (Table II). This corresponded to the following percentages of culture-positive samples: 15.1%, 29.5%, 33.8%, 31.2% and 32.0% in the consecutive years of the analysed period.

The results of testing for *C. difficile* toxin A done on 4414 samples are shown in Table III. In total there were 847 (19.2%) toxin A-positive samples and 3567 (80.8%) toxin A-negative results. Over five analysed years the percentage of toxin A-positive samples was 29.4% in 1998, 17.5% in 1999, 23.2% in 2000, 17.1% in 2001 and 15.0% in 2002.

Discussion

C. difficile is considered as the most frequent etiological agent of nosocomial diarrhoea occurring in hospitalised patients, spreading easily to the environment, the hands of the health care workers and subsequently to other patients, particularly in large hospitals. In the recent years there was a steady increase in the frequency of *C. difficile*-associated diseases, which accounted for up to 15% of outbreaks of hospital-acquired diarrhoea (Djuretic *et al*., 1999; Zadik and Moore, 1998). Between 1992–1997 in the UK there has been 2.6-fold rise in culture-positive reports, while the corresponding increase in toxin-positive reports was approximately 9-fold (Wilcox and Smyth, 1998). An increase in the number of culture-positive specimens has also been recorded in our institution (Table II). We observed nearly 5-fold increase in *C. difficile* culture-positive samples during the study period (83 in 1998 and 414 in 2002).

Diseases caused by *C. difficile* are related to the increased morbidity and mortality of elderly patients, as well as patients hospitalised in the renal medicine and chest medicine wards (Wilcox and Smyth, 1998;

Kyne *et al*., 1998; Wilcox *et al*., 1998; Zadik and Moore, 1998; Boswell *et al*., 1998). In our study strains of *C. difficile* were isolated mainly from patients in haematology-oncology ward, followed by nephrology/ renal unit patients, neurology, gastrointestinal surgery and neurosurgery wards (Table I). This points to high risk areas for nosocomial spread of *C. difficile* strains (Blot *et al*., 2003). In the wards included in our study most commonly used antimicrobial agents comprised cephalosporins of the 3rd generation (ceftriaxone, ceftazidime) and 4th generation (cefepime), carbapenems, amoxicillin/clavulanate, metronidazole and fluconazole.

Standard laboratory methods for diagnosing these infections include stool culture and identification of bacterial isolate, faecal toxin detection and *C. difficile* antigen detection. PCR technique can also be used for the rapid identification of toxigenic *C. difficile* (Kato *et al*., 1991). It has been reported that culture for *C. difficile* was positive in 30% of stool samples from patients with nosocomial diarrhoea (Pituch *et al*., 2000). In our study the frequency was similar – 29.6% of positive specimens overall for the analysed period. We also observed a steady increase in the number of *C. difficile* culture-positive results from 1998 to 2002. This could be ascribed to emergence of *C. difficile* in hospital-acquired infections as well as increased awareness of the clinicians of this etiology of diarrhoea.

The culture lacks however specificity due to the possible faecal carriage of non-toxigenic isolates, therefore many laboratories rely on toxin detection rather than culture for the diagnosis of *C difficile* infection (Wilcox *et al*., 1998). There have been reports that examined stool samples were positive for toxin A in 5.5% in community-acquired diarrhoea and up to 22% in nosocomial diarrhoea (Wilcox and Smyth, 1998; Pituch *et al*., 2000; Fedorko *et al*., 1999; Miller *et al*., 2002). In our study this value was 19.2% in samples from patients with possible CDAD.

An immunoassay for the detection of toxin A of *C. difficile* is an easy and rapid method in comparison to other techniques (direct examination of the sample, culture and testing for *C. difficile* antigen) used for diagnosis of these infections (Fedorko *et al*., 1999). Detection of *C. difficile* toxin A has proved to be of diagnostic importance also in our study (Table III). Toxin A-producing *C. difficile* appears to be an emerging pathogen in patients hospitalised in our hospital, particularly in the haematology-oncology ward (Table II). The discrepancy between the increase in the number of culture-positive samples (Table II) and a relative (expressed in %) fall in the number of toxin A-positive results (Table III) may have resulted from the fact, that clinicians are more aware of this etiology of diarrhoea. Therefore, more patients were detected who were colonised with *C. difficile* in the gastrointestinal tract, while diarrhoea could be due to other reasons (other bacteria, viruses, fungi, protozoa). Mixed diarrhoeal infections are also observed (Rokosz *et al*., 2002).

However, recent reports have shown that *C. difficile* strains negative for toxin A and positive for toxin B (A-B+), as well as strains producing binary toxin alone, may also be virulent and cause clinical symptoms (Alfa *et al*., 2000; Stubbs *et al*., 2000; Wilcox and Fawley, 2001). Brazier and coworkers reported the frequency of 3% of A-B+ strains in over 1300 isolates from 35 hospitals (Brazier *et al*., 1999). However, up to 28–31% of *C. difficile* strains may have a mutant toxin A gene (Al-Barrak *et al*., 1999; Pituch *et al*., 1999). Toxin A-negative isolates of *C. difficile* cultured from human stools usually contain a small deletion of 1.8 kb within the repetitive regions of the *tcdA* gene (van den Berg *et al*., 2004). Webb argues, that prevalence of A-B+ strains is highly variable, ranging in many reports from 0.2% up to 48% in a paediatric population (Webb, 2000). In our study some cases might be due to toxin A-negative toxin B-positive and/or binary toxin-positive strains, because we did not test for toxin B and binary toxin at the time the study was conducted. Therefore development of laboratory tests for routine use, which could rapidly detect three known *C. difficile* toxins becomes a necessity. At present results obtained by different methods should be used in conjunction with patient history when making a diagnosis of *C. difficile* infection.

Control of *C. difficile* infections requires avoidance of unnecessary antibiotic use, especially clindamycin, third generation cephalosporins and other agents, which show the greatest association with *C. difficile* disease (Mylonakis *et al*., 2001, Zadik and Moore, 1998). A tight restriction of their use is therefore needed. We recorded a decrease in the hospital expenses on antibacterial agents during the study period, from 25% in 1998 to approximately 20% in 2002, calculated as a percentage of the total medical costs of the hospital. This might have also contributed to less cases of CDAD recorded recently, in comparison to the previous years. A change in antibiotic policy and implementation of standard infection control measures reduced the incidence of *C. difficile* symptomatic infections (Wilcox and Smyth, 1998; Wilcox *et al*., 1998; Boswell *et al*., 1998; Khan *et al*., 2003; Riley, 2004). Combined approach, involving effective infection control measures, the use of rapid and sensitive techniques for laboratory diagnosis, as well as prudent use of antibiotics, is necessary to reduce morbidity and mortality due to *C. difficile*-associated infections in hospitalised patients.

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