

Molecular diagnosis and genotyping of Clostridium difficile

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Citation

Berg, R. J. van den. (2007, November 21). *Molecular diagnosis and genotyping of Clostridium difficile*. Retrieved from https://hdl.handle.net/1887/12445

Version:	Corrected Publisher's Version
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Downloaded from:	https://hdl.handle.net/1887/12445

Note: To cite this publication please use the final published version (if applicable).

Molecular diagnosis and genotyping of *Clostridium difficile*

Proefschrift

ter verkrijging van

de graad van Doctor aan de Universiteit Leiden,

op gezag van Rector Magnificus prof.mr. P.F. van der Heijden,

volgens besluit van het College voor Promoties

te verdedigen op woensdag 21 november 2007

klokke 13.45 uur

door

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geboren te Velsen in 1978

Promotiecommissie

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This work was supported by a grant from the Foundation Microbiology Leiden.

Cover design by Jenno van Belle

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In dulci iubilo

Voor degenen die ik liefheb

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Chapter 1

General introduction

Clostridium difficile

Clostridium difficile was first discovered by Hall and O'Toole (Hall, 1935) in faecal samples of healthy newborn infants. The infants were without symptoms of gastrointestinal disease. The new anaerobic bacterium was named *Bacillus difficilis*, due to the difficulty of growth on conventional media. They found *Clostridium difficile* to be a Gram-positive, spore-forming rod that grows anaerobically. The produced spores are non-bulging and are located subterminal. The injection of animals with a heat-labile toxin produced by the bacterium resulted in rapid death, from which was concluded that the bacterium was toxinogenic. The pathogenicity in mammals was first described in germ-free rats that developed transient diarrhoea after an inoculation with *C. difficile* (Hammarstrom, 1969).

The first description of pseudomembranous colitis (PMC), although not yet recognized as such, was in 1893 by Finney as a post-operative complication of a severe case of "diphteric colitis" shortly after gastrojejunostomy for an obstructive peptic ulcer in a young woman (Finney, 1893). In 1974 an association between this disease and patients receiving clindamycin was reported. A prospective study on 200 patients treated with clindamycin was performed and resulted in 41 patients with diarrhoea and 20 patients with PMC (Tedesco, 1974). The first recognition of *C. difficile* as the cause of PMC was in 1977, were experiments showed that clindamycin-associated colitis was due to a clindamycin-resistant *C. difficile* strain (Bartlett, 1977). *C. difficile* as the cause of PMC in humans was reported in 1978 (Bartlett, 1978).

The bacterium belongs to the division *Firmicutes*, family of the *Clostridiaceae* and the genus *Clostridium*. *C. difficile* is a bacillus of approximately 3-5 μ m in length. In Gram-staining, the bacteria present as long Gram-positive rods, with subterminal spores. However, sporulation is absent when grown on most *C. difficile* specific media. Following culture, *C. difficile* can be identified by its characteristic smell (horse manure), yellow-green fluorescence under long wave ultraviolet light, and/or by a latex slide agglutination test that reacts with cell wall antigens (Brazier, 1998). A rapid biochemical identification of *C. difficile* specific agar (George, 1979; Aspinall, 1992), specifically after ethanol-shock pre-treatment

(Borriello, 1981), positive for the production of L-proline-aminopeptidase (Fedorko, 1997; Garcia, 1997) and positive for hydrolysis of aesculin (Hafiz, 1976). In susceptibility assays, *C. difficile* is sensitive to metronidazole and vancomycin and can have a decreased susceptibility to antibiotics, such as: erythromycin, clindamycin, tetracycline, rifampin, ciprofloxacin and chloramphenicol (Barbut, 1999; Cheng, 1999). More recently, high numbers of resistance against fluoroquinolones has been found in specific epidemic strains (Muto, 2005; Drudy, 2006, Chapter 8).

Clinical disease and epidemiology

The illness associated with C. difficile ranges from mild diarrhoea to lifethreatening colitis (Bartlett, 2002). Typical clinical features include diarrhoea, lower abdominal pain and systemic symptoms such as fever, anorexia, nausea and malaise. Fulminant colitis occurs among 1%-3% of patients and is characterized by signs and symptoms of severe toxicity with fever and diffuse abdominal pain and distension. Cases of fulminant colitis could result in fatal disease, due to bowel perforation and peritonitis (Koss, 2006). PMC represents an advanced stage of disease, and although considered "nonspecific," it is highly suggestive of C. difficile infection. In endoscopy, pseudomembranes can be detected, presented by loss of the mucosal folds and plaques with hemorrhagic spots and deep ulceration. Severely ill patients may have little or no diarrhoea as a result of toxic dilatation of the colon (toxic megacolon) and paralytic ileus that may result from loss of colonic muscular tone. Toxic megacolon may be associated with severe sepsis and multiple organ dysfunctions (Dobson, 2003). Mortality associated with toxic megacolon is high, ranging from 25% to 40%. Recurrent diarrhoea is seen in 5%-40% of patients. CDAD is mainly known as a nosocomial disease in health-care facilities.

Although recent antibiotic treatment is one of the most important risk factors, the host and environmental factors may also play a role. The major risk factors are older age (>60 yrs; Karlstrom, 1998) and duration of hospitalization, but also the underlying disease severity (Kyne, 1999; Kyne, 2002; Asha, 2006). In patients older than 60 years, the CDAD incidence among 5133 cases was ten-fold higher than younger patients (Karlstrom, 1998). In a study on 73 patients in an outbreak situation, the mean age was 74 years. A significant association with

increasing age was shown (p<0.001; Kyne, 1999). Compared to 3-7% of outpatients, hospitalization resulted in colonization rates of 13-30% (Clabots, 1992; Bartlett, 1992; Viscidi, 1981; McFarland, 1989), after a mean duration of hospitalization of 19 days (McFarland, 1989). After two weeks of hospitalization, 13% of 634 cultured patients acquired C. difficile, whereas after four weeks of hospitalization this was shown to be 50% (Clabots, 1992). Surgery was shown to be associated with 100% mortality in four of 73 patients. In the same study, low Barthel and AMT scores (associated with severity of underlying diseases) at the onset of symptoms were significantly associated with the severity of CDAD. Patients with more severe disease were shown to have four underlying conditions, compared to patients with mild CDAD (Kyne, 1999). Most antibiotic-associated C. difficile cases are caused by treatment with clindamycin and cephalosporins (Thomas, 2003; McFarland, 1990; Chang, 2000; Kelly, 1994; Gerding, 1995; Fekety, 1993; Bartlett, 1981). An increased risk for CDAD after cephalosporin (RR 2.07) and penicillin (RR 3.62) treatment was shown by McFarland et al. (McFarland, 1990) after correction for age and severity of disease. Chang et al. showed a RR of 4.22 for development of CDAD after clindamycin treatment, after correction for age and length of hospitalization (Chang, 2000). These risk factors will be further discussed in Chapter 9.

C. difficile is not limited to humans, but has been identified in cases of enteric disease in different animals. Consequently, *C. difficile* can be isolated from different environmental sources. A study of 2580 environmental samples, including water, soil and animals, revealed 7% positive for *C. difficile* by spore recovery. Positive samples were found in soil (21%), animal faeces (7%), river (88%) and lake (47%) water samples (Al Saif, 1996). The first animal with *C. difficile* disease was a hamster challenged with *C. difficile* and treated with clindamycin (Bartlett, 1977). Infection has also been shown in horses, ostriches and dogs (Madewell, 1995; Frazier, 1993; Berry, 1986; Weese, 2006). In other studies however, no symptomatic infection has been found in household pets carrying the bacterium (Struble, 1994; Borriello, 1983). *C. difficile* has been recovered from 20-30% of non-diarrhoeal cats, dogs and birds, whereas faecal samples of rabbits, goats and guinea pigs were negative (Borriello, 1983). This suggests that some animal species can be a possible reservoir of *C. difficile*. In Canada, *C. difficile* strains belonging to the emerging PCR ribotype 027 have been recovered from faecal samples of a visiting dog without symptoms,

calves with diarrhoea and from retail ground meat (Lefebvre, 2006; Rodriguez-Palacios, 2006; Rodriguez-Palacios, 2007). This important observation suggests that some forms of CDAD could represent a zoonotic disease.

Isolation of *C. difficile* from soil has been performed, although positive sites were often related to human sewage or to patients with active infection (Hafiz, 1974; Blawat; 1958). Others were unable to detect the bacterium in soil (Kim, 1981; Riley, 1994). In the hospital environment, different studies revealed that *C. difficile* could be recovered from different sources. In a study by Kim *et al.* (Kim, 1981), 9.3% of the hospital surfaces in rooms of patients with CDAD were contaminated with *C. difficile*, whereas only 2.6% of the surfaces in a room without CDAD patients was found to be contaminated. The surfaces that are mostly contaminated are: bedpans, toilets and floors associated with *C. difficile* positive patients (Testore, 1988; Kim, 1981; Mulligan, 1979; Walters, 1982). In addition, health-care workers can carry the bacterium on their hands (Kaatz, 1988; Mulligan, 1979), thereby spreading the bacterium from patient to patient.

C. difficile infection is often associated with hospitalization. However, CDAD is also increasingly recognized as a community-acquired disease, possibly associated with the use of proton pump inhibitors (Dial, 2006; Dial, 2005). Other studies also indicated that cases of CDAD in the community occurs frequently (Terhes, 2004; Noren, 2004; Johal, 2004; Kato, 2001; Kyne, 1998; Riley, 1995; Hirschorn, 1994; Riley, 1991), and carriage without symptoms has been described as well (Viscidi, 1981; Bartlett, 2002). The percentage of carriage of hospital patients can be as high as 16-35% of inpatients (Aslam, 2005). When differentiating between origin and onset of CDAD, the ECDC proposed categories (Kuijper, 2006). A case is health-care associated when symptoms start after at least 48h after admission to the hospital, or onset in the community within 4 weeks after discharge. CDAD is considered as community-acquired, when diarrhoea starts within 48 hours of admission and without previous hospitalization in the last 12 weeks (Fig. 1).





The onset can start either within (healthcare onset) or outside (community-onset) the hospital. In two studies by Riley et al (Riley, 1991; Riley, 1995), rates of 5.5% and 10.7% of community-acquired C. difficile cases were found in patients from a general practice clinic. Of the community-onset cases, 69% had received antibiotics in the last 3 months (Riley, 1991). In a study on healthcare workers, an incidence level of 7.7 per 100000 patient-years was found (community onset), although 65% received antibiotics (health-care associated) within the last 42 days (Hirschhorn, 1994). In a more recent Swedish study, 59 (22%) of 267 patients with a primary episode of CDAD were community-acquired (Noren, 2004). These 59 patients were not hospitalized previously; however, 91% used antibiotics prior to infection. Another recent study included 136 cases of CDAD of which 38 (28%) showed a communityonset of CDAD. However, 87% of these patients were hospitalized in the previous year, and 92% used antibiotics before onset of CDAD (Johal, 2004). Kato et al. studied 1234 healthy adults, including hospital workers, who did not receive antibiotics in the previous 4 weeks, of which 7.6% were positive by culture (Kato, 2001). The latter studies show that it is difficult to differentiate between communityonset and community-acquired CDAD cases.

Since 2002, increasing rates of CDAD with a more severe course, higher mortality (from 4.7% to 13.8%) and more complications (from 7.1% to 18.2%) have been reported in Canada and USA (Pepin, 2004; Loo, 2005; McDonald, 2005). In March 2003, several hospitals in Montreal and Calgary, Canada, had increasing rates of CDAD, with 83 deaths in 18 months, and at least 1400 patients positive for CDAD (Eggertson, 2004). Since the second half of 2002 an increase in incidence of severe

CDAD was noted in a hospital in Quebec. The incidence increased from 35.6 to 156.3 per 100.000 inhabitants from 1991 to 2003. The increase was specifically high for patients over 65 years of age. Cases were more severe: 7% in 1991 to 18% in 2003, with higher 30-days mortality (4.5% vs 13.8%; Pepin, 2004). One specific strain was identified and accounted for at least half of the isolates. This strain belongs to REAgroup BI and PFGE-type NAP1 and was found to belong to toxinotype III, PCRribotype 027 and contained a deletion of 18bp in the tcdC gene. This strain was described as well in a woman with severe pseudomembranous colitis in 1988, known as strain CD196, and was found to contain both binary toxin genes (Popoff, 1988). The 027/BI/NAP1 strain indeed contained these genes as well. A prospective study in 12 Quebec hospitals, with 1703 positive patients, showed that patients with CDAD received more fluoroquinolones (OR 3.9) and cephalosporins (OR 3.8) than patients without CDAD. The most common strain (82%; 129 of 157 patients) was resistant to fluoroquinolones, and 84% (n=132) of isolates contained the binary toxin genes and the deletion in the tcdC gene (Loo, 2005). C. difficile isolates (n=187) from 8 healthcare facilities with outbreaks between 2000 and 2003 in the USA were characterized, associated with a higher morbidity and mortality (McDonald, 2005). Surprising was the fact that the all (100%) of the outbreak-related 027/BI/NAP1 strains were resistant to fluoroquinolones, whereas 42% of non-027/BI/NAP1 strains and none of the historic 027/BI/NAP1 strains found before 2001 were resistant (McDonald, 2005). Typing results indicated that the USA and the Canadian epidemic strains were indistinguishable (Loo, 2005; McDonald, 2005).

Outbreaks with this strain have been found in Europe and Japan since 2004, and some are still ongoing. Countries were the type 027/BI/NAP1 strain has been detected are: the United Kingdom (HPA, CDRweekly, 2005), the Netherlands (see Chapter 8; Van Steenbergen, 2005; Van den Hof, 2006) and Belgium (Joseph, 2005; Delmee, 2006). France (Tachon, 2006; Coignard, 2006), Austria (Indra, 2006), Luxembourg (?), Poland (Hanna Pituch, personal communication) and Japan (Kato, 2007) have also been affected by the same strain. A hospital in the United Kingdom noticed an increase in incidence: between April 2003 and March 2004, 85 positive tests were found, whereas the next 12 months this number increased to 209 cases. Most strains belonged to PCR-ribotype 027 and showed the characteristic deletion in the *tcd*C gene (HPA, CDRweekly, 2005). Other outbreaks in the UK were reported

after this first European report of a type 027/BI/NAP1 outbreak, of which two were associated with a change of antibiotics to moxifloxacin. In July 2005, an outbreak was noticed in Harderwijk, the Netherlands. The incidence of CDAD increased from 4 to 83 per 10.000 admissions, in 2004 and April-July 2005, respectively. Of 33 patients infected by July 2005, 2 patients died due to CDAD and their underlying disease. All strains indeed belonged to type 027/BI/NAP1. Subsequently, all fluoroquinolones were banned. The second cluster was probably related to a transferred patient from Harderwijk (Van Steenbergen, 2005; Chapter 8). A national surveillance study was started. Between February 2005 and November 2006 in 109 health care facilities, 863 patients were tested, of which 218 (25%) were infected with type 027/BI/NAP1 strains. Type 027/BI/NAP1 outbreaks were observed in 10 hospitals and one nursing home. Since 2002, an additional 3 hospitals experienced 027 cases. In total, 21 of 109 (19.3%) hospitals have been affected since 2002, including 11 hospitals without outbreaks. Patients with type 027/BI/NAP1 were significantly older (OR 2.18, 95% C.I. 1.43-3.33) and were treated with significantly more fluoroquinolones (OR 2.88, 1.01-8.20). Clear trends were observed for more severe diarrhoea (OR 1.99, 0.83-4.73), a higher attributable mortality (6.3% vs. 1.2%, OR 3.30, 0.41-26.4) and more recurrences (OR 1.44, 95% CI 0.94-2.20) (Goorhuis, to be published). In the hospital of the second cluster, two outbreaks were detected: 40 patients with type 027/BI/NAP1 and 50 patients with type 017/ttVIII (see below). All CDAD patients in this hospital were treated with vancomycin and showed a difference in recurrences of 37% and 10%, respectively (Goorhuis, to be published; Chapter 8). In September 2005, four patients were found positive for type 027/BI/NAP1 in a Belgian hospital in Leper, with one death due to CDAD and the underlying disease. All patients were female and older than 70 years, with a hospitalization of at least two weeks. Two patients received quinolones as antibiotic therapy. The incidence rose from 10 per 10.000 admissions in January0August 2005 to 33 per 10.000 in September 2005 (Joseph, 2005). A surveillance was started in Belgium from January to September 2006 as well, and showed 168 (50%) of 333 strains with type 027/BI/NAP1, from 23 different health-care facilities. The percentage 027/BI/NAP1 strains increased from 44% in January-March to 67% in July-September. Patients with type 027/BI/NAP1 were older (83.5 years vs. 72 years) than non-027 infected patients, of which most were located at the department of Geriatrics (65% vs. 35%), and more recurrences

were observed among type 027/BI/NAP1 cases (Delmee, 2006). The first cluster of type 027/BI/NAP1 cases in France was reported at the start of 2006. A total of 33 cases of CDAD were observed between January and April 2006. Four of five tested strains were similar to the type 027/BI/NAP1 strain and showed resistance to erythromycin and moxifloxacin (Tachon, 2006). After notification of this first outbreak in France, other clusters were found. A total of 16 healthcare facilities and two nursing homes notified severe CDAD cases. From January 2006 to September 2006, 266 cases have been reported, mainly among older patients. Two facilities accounted for 54% of the cases, and showed 6% (15 cases) of deaths due to CDAD. Of 114 typed strains, 81 (71%) belonged to PCR-ribotype 027, from 11 healthcare facilities and one nursing home (Coignard, 2006). In Austria, one confirmed case of type 027/BI/NAP1 has been noted as well. In March 2006, this patient, a tourist from the UK, was treated with ciprofloxacin and was later found to have PMC by C. difficile . It is believed that the patient acquired the strain in the UK and was infected due to the antibiotic treatment (Indra, 2006). Till now, outside of the US, Canada and Europe only Japan has reported one case of type 027/BI/NAP1. In May 2005, the patient developed a relapse of diarrhoea, after a first episode in March 2005. Strains from both episodes were confirmed to be type 027/BI/NAP1, although they were both sensitive to the newer quinolones, as have been reported before 2001 in the US (see above). Of 150 C. difficile isolates collected between 2003 and 2006, none belonged to type 027/BI/NAP1 (Kato, 2007).

Several studies concluded that exposure to fluoroquinolones is a major risk factor for development of CDAD due to type 027 strains. It has been suggested that the recent acquisition of resistance to the newer fluoroquinolones by this strain was the major reason for its wide dissemination (McDonald, 2005; Loo, 2005), although fluoroquinolone resistance was known to be present in earlier epidemic *C. difficile* strains. The molecular mechanism of resistance to fluoroquinolones has been described in isolates from Ireland (Denise Drudy, Lorraine Kyne, Rebecca O'Mahony, and Séamus Fanning. *gyrA* Mutations in Fluoroquinolone-resistant *Clostridium difficile* PCR-027. Emerg. Infect. Dis . Volume 13, Number 3–March 2007). The most compelling evidence for a role of the fluoroquinolones comes from the study of Pepin *et al.* (Pepin, 2005). They found an adjusted hazard risk (AHR) of 3.44 (2.65-4.47) for development of CDAD. Importantly, the risk was dependent on the duration of

treatment (Pepin, 2005). The use of fluoroquinolones could be a risk factor, as found in different studies (Loo, 2005; Vaessen-submitted).

An increasing number of reports mention severe infections and outbreaks due to strains unable to produce TcdA, yet producing TcdB (TcdA-/TcdB+ strains; Chapter 5; Al Barrak, 1999; Alfa, 2000; Johnson, 2001; Kato, 1998; Kuijper, 2001; Limaye, 2000; Drudy, 2007). Two types of TcdA-/TcdB+ strains have been identified. The first type is characterized, in only one asymptomatic adult, by a large deletion of 5.6 kb in the tcdA gene, and belongs tot toxinotype X (Rupnik, 1997). The representative strain (8864) causes fluid secretion in rabbit intestinal loops and it has been suggested that the production of a variant toxin is associated with its enteropathogenicity (Borriello, 1992; Lyerly, 1992). This variant toxin seems more potent than TcdB and is more similar to C. sordelii lethal toxin (Soehn, 1998). The second type (Fig. 2) is more frequently isolated from human faecal samples and contains a small deletion of 1.8 kb within the repetitive regions of the tcdA gene, belongs to serogroup F, toxinotype VIII and PCR-ribotype 017, and contains the erm(B) gene, coding for resistance to clindamycin (Depitre, 1993). Recently, two additional TcdA-/TcdB+ strains were described, they belong to toxinotypes XVI and XVII, and both contain the binary toxin genes (Rupnik, 2003). One cluster of type 017/VIII strains was shown to have high-level resistance to fluoroquinolones, which is associated with a mutation in the gyrB gene. All other 017/VIII isolates were susceptible to the fluoroquinolones, like the wild-type 017/VIII strain (Drudy, 2006). The occurrence of this resistance change can explain outbreaks in hospitals, as seen in the hospital of the second cluster of 027/BI/NAP1 strains in the Netherlands (Goorhuis, 2007; Chapter 8). The occurrence of these TcdA-/TcdB+ strains implies a more important and TcdA-independent role for TcdB in pathogenesis.

Outbreaks with TcdA-/TcdB+ strains belonging to type 017/VIII have been described. The first occurred in Canada, with 16 cases over a three-month period (Al Barrak, 1999; Alfa, 2000). The second outbreak was located in the Netherlands, with 24 positive patients in 1997-1998. The withdrawal of the use of clindamycin finally controlled the outbreak (Kuijper, 2001). Japan also noted an outbreak, with 10 patients harbouring the type 017/VIII strain (Kato 1998). In a hospital in Ireland, 95% of all isolates of 73 patients belonged to type 017/VIII (Drudy, 2007). In Argentina, type 017/VIII strains replaced TcdA+/TcdB+ strain completely, with 12.5% 017/VIII

(2 of 16) in 2000 to 96% (24 of 25) in 2003 (Goorhuis, to be published; Poster Eccmid 2005).

Figure 2. Schematic view of the deletion in the tcdA gene, resulting in a frameshift mutation and therefore the absence of TcdA production (extracted from Kato *et al.* 1998)



Virulence factors

In 1978, two strains of C. difficile isolated from patients with PMC were found to produce toxin in vitro. When inoculated in hamsters, fatal enterocolitis developed and the toxin was detected in faecal samples (Larson, 1978). To identify the toxins produced by C. difficile, purification and characterization by anionexchange chromatography revealed two toxins (Banno, 1981; Sullivan, 1982). The first eluted toxin was named toxin A (TcdA) and induced fluid accumulation in rabbit ileal loops, was lethal to mice, and increased vascular permeability. The second eluted toxin was named toxin B (TcdB), and showed to be cytotoxic on HeLa cells with only 1pg of toxin (Banno, 1981; Sullivan, 1982). In these first studies on both toxins, an unusual large size (360-600 kDa; Banno, 1981; Sullivan, 1982) of both toxins was reported, as well as the fact that both are a single polypeptide (Barroso, 1990; Dove, 1990). Experiments with TcdA and TcdB on CHO-K1 cells, showed that TcdB was 1000-fold more cytotoxic than TcdA (Sullivan, 1982). Although TcdB was shown to be more cytotoxic than TcdA in the above described studies, TcdA was 100-fold more active against three different (F9, OTF9-63 and P19) epithelial cell lines expressing the tri-saccharide structure Gala1-3GalB1-4GlcNac, compared to the CHO-K1 cell line. Using these cell lines, no difference in effect between TcdA and TcdB could be

demonstrated (Tucker, 1990). The toxic effect of *C. difficile* could be neutralized with a *C. sordellii* antitoxin (George, 1978; Bartlett, 1978; Chang, 1978; Popoff, 1987).

The molecular size of TcdA was established to have a M_r of 308 kDa (Dove, 1990), and a M_r of 270 kDa for TcdB (Barroso, 1990). Both toxins contain three functional domains: the N-terminally located enzymatic domain, the intermediate transmembrane domain and the C-terminally receptor-binding domain. The enzymatic part of the toxin catalyses the glucosylation reaction (Fig. 3) (Hofmann, 1997). The transmembrane region contains a large number of hydrophobic amino acids, therefore it presumably mediates the translocation of the toxin into the cytosol (Moncrief, 1997). The receptor-binding domain is sized about one-third of the molecule and consists of repetitive peptide elements. These repeat elements share homology with other carbohydrate binding regions (Von Eichel-Streiber, 1990). The repeats have a design that possibly functions in primary protein-carbohydrate interactions (Von Eichel-Streiber, 1992).

Figure 3. Structure of *C. difficile* TcdB. The N-terminal 546 amino acids represent the glycosyltransferase domain. The C-terminal domain contains many repetitive sequences likely to be involved in receptor binding. The hydrophobic region in the centre mediates translocation (extracted from Schirmer *et al.* 2004)



The enterotoxin (TcdA) and cytotoxin (TcdB) of *C. difficile*, together with the lethal (LT) and hemorrhagic toxin (HT) of *C. sordellii* and the alpha toxin of *C. novyi* belong to a group called the Large Clostridial Toxins (LCT). These LCTs are grouped together based on their high molecular weight (250-308kDa), making them the largest bacterial protein toxins. Furthermore, the LCT's share functions, in that they all glycosylate small GTP-binding proteins (Von Eichel-Streiber, 1996), and are exclusively found in the Rho and Ras GTPases (Just, 1995; Just, 1996; Popoff, 1996; Just, 1995; Selzer, 1996). Whereas TcdA and B are associated with PMC and antibiotic-associated diarrhoea, *C. sordellii* LT, HT, and *C. novyi* α -T are mainly involved in gas gangrene (Hatheway, 1990). HT is very similar to TcdA, as their

toxins cross-react, and it is as cytotoxic and enterotoxic as TcdA is (Martinez, 1988). However, not much is known on HT. LT is more similar to TcdB, as antisera to LT only neutralize TcdB (Martinez, 1992). LT shares 76% of its amino acid sequence with TcdB, and 47% with TcdA (Green, 1995). TcdB variants appear even more closely related to HT. This was specifically true for the glucosyltransferase domain of strain 8864, a TcdA-, TcdB+ strain (Soehn, 1998). The α -T shares 48% amino acid sequence with both TcdA and TcdB, and is a potent cytotoxin (Ball, 1993).

TcdA has been regarded as the primary virulence factor (Libby, 1982; Lyerly, 1982) and has enterotoxic activity, causing extensive damage to the intestine. First, TcdA forms homodimers, enabling it to bind carbohydrate groups. Subsequently the toxin can be found in coated pits and is then internalised. Both TcdA and TcdB are glucosyltransferases, covalently modifying Rho proteins. Rho proteins are important in the organization of the cytoskeleton, explaining the disrupting effect on the cytoskeleton, after which the cell dies (Just, 1994; Dillon, 1995). TcdB does not damage the intestine (Lyerly, 1982), probably due to the inability to bind receptors on the lining of intestinal cells. Therefore it is believed that TcdA initiates the damage to the intestine, after which TcdB gains access to the underlying tissue, causing the cytotoxic effect. This suggests that both toxins work synergistically (Lyerly, 1985). Comparative sequence analysis of *tcdA* and *tcdB* showed an extensive amino acid sequence identity of 63%. The sequence similarity and the position on the PaLoc suggest that the *tcdA* and *tcdB* genes have a common ancestor and are the result of gene duplication (Von Eichel-Streiber, 1992).

TcdA and TcdB bind to specific receptors on the cell surface. TcdA has been shown to bind to Gal β 1-4GlcNAc carbohydrate structures on rabbit red blood cells, hamster brush border membranes, rat colon and human blood group antigens (Krivan, 1986; Tucker, 1991; Teneberg, 1996; Pothoulakis, 1996). It is not known whether this disaccharide is carried by a membrane protein or lipid. However, TcdA was shown to bind to Lewis X, Y and I antigens and to, although the specific receptors on the human colonic cells have not been determined till now (Tucker, 1991; Pothoulakis, 1996). Although not much is known on the receptor of TcdB, it has been shown that the toxin binds specifically to human colon carcinoma T84 cells and to Don fibroblasts (Chaves-Olarte, 1997). After binding to their specific receptors, both toxins are internalized by endocytosis (Von Eichel-Streiber, 1991).

TcdA and TcdB genes (tcdA and tcdB) are located on a pathogenicity locus (PaLoc; Fig. 4) of 19.6 kb, also encompassing 3 other small open reading frames: tcdD, tcdE and tcdC respectively (Hundsberger, 1997). A high level of TcdC and low levels of the other four transcripts were detected in the early exponential phase, which was inverted in the stationary phase. This suggests a negative influence on transcription of the toxin genes by TcdC. Within the tcdC gene, two variant alleles have been described. Deletions of 18bp and 39bp have been found, which could be of importance since *tcd*C is a putative negative regulator of toxin production (Spigaglia, 2002). Therefore a deletion could result in a non-functional product, which could lead to an increased production of toxins A and B due to the lack of negative regulation. Type 027/BI/NAP1 strains were described to contain the 18bp deletion in the *tcd*C gene, and indeed produce both toxins in higher quantities and at higher rates (Warny, 2005). However, comparative sequencing of the tcdC gene confirmed the 18bp deletion in type 027/BI/NAP1 strains and identified a second, single-base-pair deletion at position 117. This position 117 deletion was identified in all Canadian type 027/BI/NAP1 strains, and in a United Kingdom reference strain. Due to the deletion at position 117, a frameshift in the early portion of the *tcdC* gene was observed, resulting in functional disruption of the negative regulator of the toxin genes (MacCannell, 2006). The increased virulence seen in type 027/BI/NAP1 strains could be explained by this frameshift mutation in the *tcd*C gene.

Figure 4. Location of the open reading frames on the pathogenicity locus (extracted from Rupnik *et al.* 1998).

Sequencing and transcription analysis suggest that TcdD is involved in the positive regulation of TcdA and TcdB expression (Hundsberger, 1997; Hammond, 1997), because of which it was renamed TcdR (Rupnik, 2005). The function of TcdE, located between TcdA and TcdB, was unknown for a long time. However, Tan *et al.* describe the bactericidal effect of TcdE when expressed in Escherichia coli (Tan, 2001). In this same study, the amino acid sequence of TcdE was compared and was

found similar to holins, cytolytic proteins located across the bacterial membrane. Due to the effect on the bacterial cell wall, shown by electron microscopy, TcdE probably supports the release of TcdA and TcdB from the cytoplasm (Tan, 2001). Nontoxigenic, and therefore non-pathogenic, strains of *C. difficile* contain a 127 bp sequence at this locus (Hammond, 1995).

Before the toxins can exert their toxic effects, the germination of spores present in the (hospital) environment and the intestinal tract is required (Kelly, 1998). Spores of C. difficile are resistant to exposure to heat, drying, air, detergents and alcohol, and these spores can persist for at least 5 months in the environment (Department of Health and Public Health Laboratory Service Joint Working Group. Clostridium difficile infection. Prevention and Management. BAPS Health Publication Unit, DSS Distribution Centre, Haywood, Lancashire: 1994; Kaatz, 1988; Struelens, 1991; Fekety, 1981). The sporulation capacity, which may differ between strains, can be a virulence factor associated with spread and persistence of specific strains (Wilcox, 2000). Sporulation and toxin production has been shown to be correlated by Kamiya et al. (Kamiya, 1992). They showed that a sporulation inhibitor resulted in a decrease in both sporulation and toxin production, whereas the number of vegetative cells was not affected. However, another study claims that sporulation and toxin production are inversely correlated (r= 0.66; Akerlund, 2006). The production of spores and toxin were measured at the stationary phase, and may therefore support the hypothesis of an opposite survival strategy for C. difficile entering this phase due to nutrient deficiency.

After germination of the spores entering the human body, the bacterium has to attach to the cells in the colon. One virulence factor associated with this adhesion is the flagellum, encoded by the flagellin gene *fli*C and the flagellar cap gene *fli*D. Although not all strains show flagella in electron microscopy, all strains contain and express *fli*C (Tasteyre, 2000). It was shown that the flagella resulted in a ten times higher adherence to mouse cecum tissue than for unflagellated strains (Tasteyre, 2001). Another adhesion is the surface layer protein (SLP) that surrounds the cell wall of all *C. difficile* strains (Calabi, 2001). This protein is coded by the *slp*A gene, and can vary between strains. Therefore the detection of this gene can be used for typing purposes as well (Karjalainen, 2001). It was shown that SLP adheres to intestinal tissue in both humans and mice, which was blocked by antibodies against the SLP

(Calabi, 2002). The *cwp*66 gene encodes a cell surface-associated protein, and is identified as an adhesin in *C. difficile*. Antibodies against CWP66 inhibit the adherence of *C. difficile* to cultured cells (Waligora, 2001).

Additionally, a recently discovered new binary toxin of C. difficile is currently being studied as a possible additional virulence factor (Perelle, 1997; Stubbs, 2000) and was first detected in a woman with severe pseudomembranous colitis. The strain belonged to PCR-ribotype 027 (Popoff, 1988). This binary toxin, an actin specific adenosine diphosphate-ribosyltransferase (CDT), is related to other clostridial binary toxins, like iota toxin from C. perfringens and C. spiroforme toxin (Popoff, 1988; Perelle, 1997). Genes for the binary toxin are located outside the PaLoc, and encode the *cdtA* gene (the enzymatic component) and the *cdtB* gene (the binding component) (Popoff, 1988). The incidence of binary toxin genes is 6-15.5% in both toxinogenic and non-toxinogenic strains. The incidence was 15.5% in the US (9 of 58 strains), which included 4 (9%) strains of 46 TcdA-/TcdB- strains (Geric, 2003). Another hospital in the US showed 9 (6%) of 153 strains to contain the binary toxin genes, all belonging to variant toxinotypes (Geric, 2004). In France, 22 of 369 strains (6%) harboured both cdtA and cdtB, of which all 22 where TcdA+/TcdB+ (Goncalves, 2004). Terhes et al. found 2 (3%) of 79 toxinogenic strains to contain cdtA and cdtB (Terhes 2004). In Poland, the incidence of binary toxin positive strains was shown to be 12% (5/41) in TcdA+/TcdB+ strains, and in none of the 17 TcdA-/TcdB- strains (Pituch, 2005). Recently, Geric et al. (Geric, 2006) reported that hamsters did not develop disease after challenge with TcdA-/TcdB-, CDT positive C. difficile strains, although these strains did have an effect on rabbit ileal loops. A cytotoxic effect by the binary toxin was detected in a study by Perelle et al. (Perelle, 1997), which was neutralized by anti-Ib (iota toxin binding component) antibodies. These results suggest that binary toxin contributes to the pathogenicity of C. difficile in an adjunctive role to the two toxins TcdA and TcdB, with a correlation with severe diarrhoea.

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Diagnosis of *Clostridium difficile*associated disease (CDAD)

Criteria for selecting patients' faecal samples to test for CDAD

Patients should be considered for diagnosis when the patient is suffering from nosocomial diarrhoea (Bowman, 1988), especially with the presence of fever (Poutanen, 2004). The clinical definition for diarrhoea is at least 3 watery, loose or unformed stools per day for at least 2 days (Johnson, 1998; Lozniewski, 2001). No clear guidelines for selection of faecal samples are available, but different studies come with different specific criteria. In an European survey the three most common criteria applied to select faecal samples for CDAD testing, included all loose or watery stools (40%), all stools from patients with previous antibiotic therapy (46%) and all stools from nosocomial (development of diarrhoea after 3 days of hospitalization) diarrhoea (57%). In 23% of the laboratories, specific departments, i.e. oncology, haematology, intensive care or gastroenterology, were also mentioned as criteria to test for C. difficile (Barbut, 2003). The American Society for Microbiology, and many other studies, also recommended to test all samples from diarrhoeal samples that developed after the third day of hospitalization (Fekety, 1997; Gerding, 1995; Rohner, 1997; Borek, 2005). The other way round, samples from patients hospitalized for at least 72h should not be routinely cultured for Salmonella, Campylobacter, Shigella or Yersinia spp., unless there are specific indications (Bowman, 1992; Fan, 1993; Siegel, 1990; Yannelli, 1988). The upcoming use of computers in laboratories and hospitals created an opportunity for laboratories to set up a Laboratory Information Management System (LIMS), making it easier to apply this 3-days rule for C. difficile testing. The 3 days rule is nowadays common practice in microbiological laboratories in The Netherlands (Chapter 4).

In the absence of clear symptoms, positive tests are still found in approximately 5% of adults without diarrhoea in the hospital (Gerding, 1986; Fekety, 1993). Although there is enough evidence that CDAD can be community-acquired
(Riley, 1991; Chapter 9), not all laboratories choose to test for *C. difficile* in all received samples. These laboratories solely test samples, based on the clinical symptoms and specific requests by the physicians.

One of the main risk factors for *C. difficile* infection is an age over 65 years, specifically when undergoing antibiotic therapy (Delmee, 2001; Brazier, 1998). Therefore, some mandatory surveillance in the UK is focussing on individuals older than 65 years of age. The key elements for a positive *C. difficile* diagnosis are therefore the symptoms and history of the patient, pseudomembranes in the colon, or laboratory evidence of *C. difficile* and its toxins in the faecal sample (Gerding, 1993). Abdominal pain with diarrhoea is another criterion for a likely positive diagnosis, and when used as a selection criterion would result in a 29% decrease in cytotoxin tests (Katz, 1997). A high leukocyte count was also found to be associated with positive test results (Katz, 1997; Jensen, 1994), and was used as one of the criteria in different studies (Bowman, 1984; Bowman, 1988).

Transport and storage of samples

The diagnosis of CDAD requires the detection of toxin producing Clostridium difficile or its products in diarrhoeal faecal specimens. Faecal samples from patients with diarrhoea are preferably fresh and liquid, taking the shape of the container. Samples should be as fresh as possible, since faecal samples stored at room temperature showed a fast decrease in toxin titres, 1.7 log after two days of storage (Bowman, 1986). Complete inactivation of cytotoxic activity was detected in 20% of specimens send by mail (Brazier, 1993). Storage at 4 degrees demonstrated both toxins after 44 days, but lost TcdA detection by EIA after 52 days (Borriello, 1992). Freezing stool samples at -70 degrees showed little effect in the cytotoxicity assay as tested by Manabe et al. (Manabe, 1995). Testing the effects of storage conditions showed that storage at -20 degrees with single and multiple freeze/thaw steps resulted in a 10^4 -fold and 10^5 -fold decrease in cytotoxin titres after 56 days, respectively. C. difficile itself and its spores were not affected. Storage at 4 degrees also showed minimal effects. Buffering of faecal specimens, e.g. with phosphate buffer solution (PBS), may preserve C. difficile viability in transport and storage, preventing the reduction of toxin by ice crystals formed during freezing. Therefore, if faecal samples

need to be stored for longer than 56 days, it is advised to store buffered samples at 4 degrees (Freeman, 2003). Samples send by mail should preferably be delivered by courier or on dry ice and frozen beforehand. For epidemiological research, culture or DNA assays, the storage conditions are not as relevant as for cytotoxin assays (Delmee, 2001). Wilcox summarized some guidelines for diagnosis of CDAD and advised to test only fresh samples or samples stored at 4 degrees. Samples for outbreak investigation sometimes need to be stored for a long time; therefore it was advised to store toxin-positive samples at 4 or -20 degrees for this purpose (Wilcox, 1998).

Diagnostic tests

Macroscopic diagnosis of CDAD, although with a poor sensitivity, can be made by rectoscopy, sigmoidoscopy or colonoscopy, for direct visualisation of the colonic mucosa and detection of pseudomembranes (Delmee, 2001). However, diarrhoea may occur without these pseudomembranes and therefore a negative result does not rule out infection (Poutanen, 2004). Endoscopy is mainly advised when patients are suffering from ileus, who are therefore unable to produce watery or loose faecal samples (Gerding, 1993; Fekety, 1997). Laboratory diagnostic assays (table 1) can be divided into test for the detection of *C. difficile* products, the detection of *C. difficile* toxins, tests for the detection of *C. difficile* genes, or the detection of *C. difficile* itself (figure 1). Although there is no accepted standard, the cell cytotoxicity assay is used most as the 'gold standard' by which other tests are measured (Bartlett, 2002; Delmee, 2001; NCDSG, 2004), although some microbiologists consider toxinogenic culture as the 'gold standard' (Delmee, 2005; Zheng, 2004). The different and most commonly used diagnostic methods will now be described.

Toxin detection: cell cytotoxicity assay

Toxins of *C. difficile* can be detected either by virtue of their biological properties (cell cytotoxicity assay) or by enzyme immuno assays (EIA). The cell cytotoxicity test, the method most often used as the 'gold standard' in comparison studies, is the only useful method detecting the biological properties of toxinogenic *C. difficile* in faecal samples. The method was already found to be useful when faecal



Figure 1. Overview of the most commonly used diagnostic assays for diagnosis of CDAD.

GDH, Glutamate dehydrogenase; EIA, Enzyme Immuno Assay; *tcd*A, TcdA gene; *tcd*B, TcdB gene; *glu*D, GDH gene; *tpi*, triose phosphate isomerase gene; 16S, small ribosomal subunit gene

Table 1. Overview of	of the characteristics	of the most commo	only used diagnost	tic assays for diagnosis of
CDAD.				
Assay	Cell cytotoxicity	(Toxinogenic)	Enzyme	(Real-time) PCR

Assay	Cell cytotoxicity assay	(Toxinogenic) culture	Enzyme Immuno Assays (EIA)	(Real-time) PCR	
Targets	(mainly) TcdB	C. difficile strains	TcdA and TcdB, GDH (glutamate dehydrogenase)	tcdA, tcdB, 16S, gluD, tpi	
Sensitivity	57-100%	high	63-99%	97%	
Specificity	99-100%	low, due to carriers	88-100%	100%	
Duration	48 hours	≥48 hours	20 min - 2 hours	8 hours	
Laborious	yes	yes	no	no	
Hands-on-time	low	low	high	intermediate	
Costs	cheap if available	cheap	expensive	expensive	
Carriers	not detected	detected	not detected	detected	
References	Barbut 1993; Merz 1994; Mylonakis 2001; Delmee 2005	Snell 2004; Zheng 2004	Brazier 1998 (review)	Belanger 2003	
Extra	Cell culture facilities necessary	Also detects nontoxinogenic strains. Necessary for typing and antibiotic tests	None	Laborious DNA extraction	

samples of patients with pseudomembranous colitis were recognized to be toxic to cell lines; this predated the discovery of *C. difficile* itself (Larson, 1977). The cytopathic effect (CPE) was found to be mainly by toxin B, since toxin A is about 1000 times less cytotoxic then toxin B (Rothman, 1984; Riegler, 1995). The first step of the assay consists of the incubation for 24-48 hours of diluted and filtered stool on cultured cell monolayers. The cells are subsequently observed for detection of cytopathic effect, due to disruption of the cell cytoskeleton, resulting in rounding of cells. This effect may be detected in the more severe cases within 6 hours of incubation. The specificity of the assay is confirmed by neutralisation of the cytopathic effect by *C. sordellii* or *C. difficile* antitoxin (Rifkin, 1977). Results can vary due to the use of different dilution factors, the used cell line and the storage of the samples before testing. Vero cell lines are believed to be the most sensitive for the cytotoxicity assay (Brazier, 1998; Delmee, 2001).

The cell cytotoxicity assay has been known as the most specific and very sensitive method (Johnson, 1998), and is therefore considered the 'gold standard'. A limitation is the slow turnaround time of at least 24h to demonstrate cytopathic effect and a further 24 hours to neutralise this effect. Another drawback is that the laboratory requires a supply of cultured cell monolayers, which is time-consuming and expensive. The cell cytotoxicity assay and other toxin detection methods have to be performed on fresh stools, due to the negative effect of storage on the toxin titres. Strains that are TcdA-/TcdB+ can be detected by the cell cytoxicity assay as well. However, these strains will show a different CPE than TcdA+/TcdB+ strains (Blake, 2004; Kato, 1998) and are therefore more difficult to recognize.

The sensitivity of the assay has been tested in different studies. The sensitivity and specificity, evaluated on the basis of a CDAD case definition, were 93% and 100% respectively (Barbut, 1993). Another study found a sensitivity of 87%, where the cytotoxicity and culture assay together were used as the gold standard (Merz, 1994). In these studies, additional 5-10% of cases were detected compared to the used EIAs (Barbut, 1993; Merz, 1994; Whittier, 1993). Sensitivities of 94-100% and a specificity of 99% were noted in a review by Mylonakis *et al.* (Mylonakis, 2001).

Toxin detection: enzyme immuno assay (EIA)

Enzyme immunoassays are easy to perform and provide rapid results. Two types of immunoassay have been developed: conventional enzyme immunoassays and membrane immunochromatography tests. The first description of a conventional enzyme, well-type, immunoassay was in the 1980s (Yolken, 1981; Lyerly, 1983). The sensitivity of the EIA was shown to be 100% when compared with the cytotoxin assay, whereas the specificity was 98%. In addition, the method showed no false-positive results compared to the cytotoxin assay (Yolken, 1981). The first EIA with purified toxin A was able to detect 1ng (5ng/ml) of toxin A, which was more sensitive than the cytotoxicity assay (500ng/ml). In human faecal samples, EIA results were consistently positive with samples with higher cytotoxic titres (Lyerly, 1983). These studies let to the development of many different immunoassays detecting toxin A, and more recently both toxins. The membrane assays were headed by dot immunobinding (Woods, 1990), but nowadays consist of a monoclonal antibody to the toxin, visualizing the precipitation due to the antigen-antibody reaction on a membrane.

The VIDAS assay is the only automated immunoassay to date, and is currently able to detect only toxin A (Shanholtzer, 1992). Other immunoassays detect toxin A or both toxins, some in combination with the detection of glutamate dehydrogenase (GDH). This so-called Triage assay was shown to have a negative predictive value of 99.6% (Barbut, 2000), and can therefore be used as a rapid screening method (Alfa, 2000). There are numerous publications comparing the performance of different kits for enzyme immunoassays, but no meta-analysis has been performed in an attempt to demonstrate the superiority of any particular test. The National C. difficile Standard Group in England recommend the use of EIAs that detect both toxin A and toxin B, because of the increasing awareness of toxin Anegative/toxin B-positive strains (NCDSG, 2004). Studies comparing the well-type EIAs with the membrane assays show contradictory data (Vanpoucke, 2001; O'Connor, 2001). The sensitivities and specificities of commercially available EIA kits compared to the cytotoxicity assay were summarized in a table by Brazier (Brazier, 1998) with sensitivities between 63-99% and specificities between 88-100%. Exceptions were found for the VIDAS assay in one study, with a specificity of only 75% (Whittier, 1993). A new rapid test, the ImmunoCard Toxins A&B (ICTAB, Meridian), has recently been introduced. The ICTAB is a single test enzyme

immunoassay for the detection of toxin A and B in faecal samples within 20 minutes. No sample pre-treatment is required, and an internal procedure control is integrated in each card. The work on this new assay has been described in Chapter 3.

One of the more recent studies showed a comparison of six different EIAs with the cytotoxicity assay, with positive predictive values between 85 and 95%. They advised to only use the Triage or the Immunocard assay, both detecting TcdA and GDH, in combination with culture for an optimal diagnosis, and found a sensitivity of only 70% for VIDAS (Turgeon, 2003). Another study, that evaluated VIDAS as well, tested a total of 38 consecutive cell cytotoxicity positive samples and 33 negative samples (Lipson, 2003). The authors applied a discordant analysis by toxinogenic culture and found a sensitivity, specificity, positive and negative predictive value for the VIDAS assay of 80.6, 96.8, 96.7, and 81.1%, respectively. The concordance with culture was 83%. Although the VIDAS assay displayed a reduced sensitivity compared with the cytotoxicity test, the authors recommend the VIDAS assay as screening method because of the rapid results. O'Connor et al. (O'Connor, 2001) compared four rapid EIAs (Oxoid Toxin A test, ImmunoCard Toxin A test, Techlab Toxin A/B II test, and PTAB) with toxinogenic culture and the cell cytotoxicity assay. With the diagnosis of CDAD as gold standard, the cell cytotoxicity assay had the highest sensitivity (98%) and specificity (99%), whereas the sensitivity and specificity of the Techlab assay and PTAB were EIAs with the best sensitivity and specificity, respectively 79% and 80%, and an 98% specificity. Another study, also including some GDH-assays, concluded that the best strategy is to test all faecal samples on GDH, if positive in an EIA. If this EIA is negative, the cytotoxicity assay should be applied (Snell, 2004).

Culture

The first important development for the culture of *C. difficile* was the use of selective cycloserine-cefoxitin fructose agar (CCFA) (George, 1979). This medium was extended with a prior alcohol-shock treatment of stool specimens, a method which destroys vegetative cells and other bacteria, but allows the survival of spores produced by *C. difficile* (Borriello, 1981; Bartley, 1991). This method is still used in different laboratories nowadays (Barbut, 2003). Enrichment broths for the enhanced isolation of *C. difficile* are used with success as well. The application of enrichment

broths resulted in 16-25% more positive samples (Arroyo, 2005; Buchanan, 1984; Wilson, 1982). There are reports indicating that lysozyme incorporation into culture media enhance germination of C. difficile spores, mainly applied in the environmental detection of C. difficile (Verity, 2001; Wilcox, 2000). The application of enrichment media for culturing C. difficile from faecal samples is considered unnecessary for diagnosis of CDAD, because active disease will yield detectable levels for culture (Brazier, 1998). C. difficile can be recognized as a white-grey colony that might grow irregularly, after at least 48 hours of growth in an anaerobic environment. The colonies produce a characteristic smell close to horse manure. In a Gram-staining C. difficile can be recognized as a Gram-positive rod with large subterminal spores. Confirmation assays can be the use of the detection of the ability to hydrolyze esculin or the detection of the production of proline-aminopeptidase (Fedorko, 1997; Garcia, 1997). In our laboratory, faecal samples were treated with an ethanol shock pretreatment prior to inoculation onto Columbia agar containing colistin and nalidixic acid and onto C. difficile -selective agar with cefoxitin, amphotericin B and cycloserine (CLO; BioMerieux) and incubated in an anaerobic environment at 37 °C for 2 days. CLO medium was also used to inoculate faecal samples that were not pretreated with ethanol. Colonies of Gram-positive rods with subterminal spores were tested for the production of L-proline-aminopeptidase and for hydrolysis of esculine.

Faecal culture takes at least four days before results are available and therefore has no rapid diagnostic value. As non-toxinogenic strains exist, cultured *C. difficile* must be tested for the ability to produce toxins or the availability of free toxin (toxinogenic culture). This latter method is used in different laboratories as the 'gold standard' or as discordant analysis method, mainly due to its high sensitivity (Delmee, 2001; Johnson, 1998). Cultured isolates are also necessary for epidemiological investigations, like typing and susceptibility testing.

The sensitivity of the cytotoxicity assay has been reported to be 70.5% compared to toxinogenic culture, and this has been found in other studies as well (Delmee, 1992; Walker, 1986; Gerding, 1986). In a recent study by Delmee *et al.* (Delmee, 2005), a sensitivity of 57% was seen for the cytotoxicity assay compared to toxinogenic culture. Some other studies published sensitivities of 62% and 74% (Thonnard, 1996; Lozniewski, 2001). The main reason why not to use toxinogenic culture as the 'gold standard' for diagnosis of *C. difficile* infection is the high

detection of asymptomatic carriers (Riley, 1995; Humphreys, 1995). It has been demonstrated that asymptomatic carriership is related to the duration of hospital stay (Kyne, 2002). Culturing *C. difficile* from faecal samples is easy to implement in the routine setting of diagnostic laboratories and should therefore be included in every laboratory. If this cannot be done, the isolates should be stored for eventual future characterization and outbreak or epidemiological studies (Brazier, 1998).

Molecular detection

Molecular detection of genes of C. difficile has the advantage that is a rapid method for diagnosis. The first PCR for detection of C. difficile was performed to discriminate between toxinogenic and nontoxinogenic isolates, using sequenced repeats in the toxin A gene (tcdA), although one C. sordelii sample showed crossreaction (Wren, 1990). Different methods to detect either tcdA or tcdB were consequently developed (Kato, 1991; Wolfhagen, 1993; Alonso, 1997), and showed high sensitivities and specificities comparable to the cell cytotoxicity assay. To avoid the need for culture, the next challenge was to test directly to faecal samples. The detection of C. difficile genes in faecal samples focused first on 16S rRNA gene (Gumerlock, 1991; Kuhl, 1993) However, an important disadvantage of the 16S rRNA approach is that nontoxinogenic as well as toxinogenic strains are detected, and that a confirmation step is needed to verify C. difficile presence. Therefore, more attention was given to the toxin genes of C. difficile and the first successful approaches were published in 1993 (Kato, 1993; Wren, 1993; Gumerlock, 1993). Comparison of conventional PCR with the cell cytotoxicity assay, the 'gold standard', resulted in complete agreement between both methods.

Some recent studies describe different methods and genes for diagnosis using the molecular detection of *C. difficile*. One of the most promising methods is the real-time PCR for the detection of the toxin genes. Real-time PCR is a PCR method using fluorescent probes for direct monitoring of the amplification, with results within one working day. The hands-on time is highly reduced compared to other methods as well, negating the use of post-PCR analysis (20 realtime). Another advantage is the lower risk of carryover contamination compared to conventional PCR. The real-time PCR has been applied by Belanger *et al.* (Belanger, 2003). Compared to the cell cytotoxicity assay a sensitivity, specificity, positive and negative

predictive value of 97, 100, 100 and 96% was found, respectively, on 56 faecal samples. The analytic sensitivity was around 10 genome copies. In Chapter 2, the development of an internally controlled in house real-time PCR is described. The first comparison with other methods than the cell cytotoxicity assay, in a multicenter setup, is described in both Chapter 3 and 4. In another study, a new screening EIA was compared to a sensitive in-house PCR assay for the GDH gene gluD and to toxinogenic culture. The EIA and the PCR on gluD showed comparable results, and performed better than culture (Zheng, 2004). The disadvantage of a PCR on the gluD gene is the fact that it does not discriminate between toxinogenic and nontoxinogenic strains, like 16S PCR, and therefore needs another assay to verify the toxinogenicity. The EIA used in their study (C DIFF CHEK) was preferred above the PCR and culture, also based on costs and laboriousness (Zheng, 2004). However, the advantage of the gluD PCR is that primers are specific for C. difficile, and can therefore be used as either a screening test or used in a multiplex format. This same idea was applied by Lemee et al. (Lemee, 2004), who developed a multiplex PCR targeting the Triose Phosphate Isomerase (tpi) gene, tcdA and tcdB. tpi is a C. difficile housekeeping gene, and can therefore be used the same way as assays detecting the gluD gene. However, this method was not performed on DNA isolated from faecal samples, but on isolates from toxinogenic culture. The advantage is that it combines diagnosis and toxinogenic characterization of the isolates at the same time (Lemee, 2004).

The major disadvantage of the detection of *C. difficile* genes in faecal samples is the elaborate DNA extraction to remove PCR inhibitors. However, new easy-to-use commercial DNA extraction kits are available (Alonso, 1999; Guilbault, 2002), of which one is described in Chapter 2. The use of internal controls is also useful for observation of inhibition, and is applied as described in Chapter 2. Like the EIAs detecting both toxins are preferred above EIAs detecting only toxin A, this same goes for the PCRs detecting both toxin genes, allowing the detection of toxin A negative, toxin B positive strains. However, the detection of the toxin genes does not confirm that toxins have been expressed and the costs for PCR are relatively high. As does culture, PCR has a very high sensitivity and can therefore be used to detect asymptomatic carriers, which could be disadvantageous when using as a diagnostic assay. The main advantages of PCR as a diagnostic tool are the rapidity, the easy-to-use formats and the ability to multiplex different targets for a complete picture for

diagnosis. In addition, the role of asymptomatic carriership among patients and health care workers can be investigated with real-time PCR in more detail.

To reduce the number of tests necessary for diagnosis of C. difficile infection, different studies have been performed to determine the value of repeated testing. Renshaw et al concluded that cytotoxicity assays should not be repeated within seven days from the first sample since only in 1% of cases the repeated testing provided clinically useful information. Introduction of this rule reduced their testing with 36% (Renshaw, 1996). Another group observed 34% repeat samples, and concluded that it is appropriate to reject repeat specimens from patients who are already tested on a recent specimen within a 7-days frame (O'Connor, 2001). The negative predictive value of the first stool specimen was 97% in a study where both EIA and cytotoxicity combined were used as diagnostic method (Manabe, 1995). In a more recent study, only 1 of 78 repeated tests using EIA within 7 days of the first sample became positive after initially being negative. Therefore it was concluded that repeated testing was not clinically justified and economically wasteful (Mohan, 2006). However, if an EIA or cytotoxity test is negative in samples of patients with a strong clinical suspicion for CDAD, additional samples are required (Johnson, 1998; Fekety, 1997). In an outbreak situation, using ICTAB as a diagnostic test for CDAD, 9% of 47 patients were diagnosed on a second or third sample obtained within 7 days. Considering a rapid spread of an epidemic C. difficile strain, repeat stool testing can be of value in the control of an epidemic (Debast, to be published). Testing during or shortly after treatment is not necessary, unless symptoms recur (Johnson, 1998).

Although consecutive samples can become positive within a 7-days frame, samples can remain positive for a period of time. We studied 34 patients with *Clostridium difficile*-associated diarrhoea, who were treated for two weeks with bovine immune whey enriched with polyclonal antibodies against whole cells and toxins of *Clostridium difficile* (MucoMilk). The treatment was started after a conventional treatment with antibiotics. Specific cultures for *C. difficile* were performed before antibiotic treatment and treatment with MucoMilk, one week after start of MucoMilk treatment, 2 weeks after start of MucoMilk treatment, 1 week after completing MucoMilk treatment and 2 weeks after completing MucoMilk treatment. Analysis of the treatment episodes revealed that 47.1% of samples were culture positive 7 days after start of MucoMilk treatment, 54.3% after two weeks, 60.6% one

week after finishing MucoMilk treatment, and 60% 2 weeks after finishing MucoMilk treatment. Free TcdA remained present in 22.2% of faecal samples tested 7 days after start of MucoMilk treatment, in 17.6% after two weeks, in 26.3% one week after finishing MucoMilk treatment, and in 9.5% two weeks after finishing MucoMilk treatment (van den Berg, van der Eijk en Kuijper, data not published).

European and Dutch survey of diagnostic methods

In 2001 a questionnaire on diagnostics of Clostridium difficile-associated infections was send to 12 hospitals in the Netherlands (data not published). The study was part of a European survey to the diagnostic strategies of C. difficile infections in eight different countries, with 212 questionnaires used for analysis (Barbut, 2003). The collaborating countries were: Belgium, Denmark, France, Germany, United Kingdom, Italy, Netherlands and Spain. Of 12 Medical Microbiology-laboratories in the Netherlands who collaborated in this study, one was located in a university hospital, whereas 47.6% of European surveys were from university hospitals. A broad variation in requests for C. difficile tests was seen between laboratories. Figure 2 represents the total number of tests and the number of positive tests per individual responding laboratory in the Netherlands. The European survey reported that 12.3% of laboratories were unable to test for C. difficile themselves, whereas all Dutch hospitals were able to detect C. difficile. Of 12 Dutch hospitals, 7 performed C. difficile investigation only when specifically requested, and 3.3% of European hospitals reported that they never or rarely receive requests for C. difficile. Additional laboratory-based criteria for C. difficile investigation were: diarrhoea during antibiotic treatment, long stays at the intensive care-unit, liquid stools or suspicion for nosocomial diarrhoea. The diagnostic tests were performed on a daily base by 50% of the Dutch laboratories, which was 90.7% in European laboratories. In the European situation, 93% directly tested faecal samples for C. difficile toxins: EIA was used in 79% and cytotoxicity in 17.3%. The cytotoxicity assay was mainly used in the United Kingdom, the Netherlands, France and Belgium, whereas Denmark only performs culture. The EIA was available in 8 (67%) Dutch laboratories. Of the 12 laboratories, 5 (41.7%) used only faecal toxin test, 6 (50%) used toxin tests in combination with culture and 1 (8.3%) used a specific antigen test and culture.

Figure 2. Relative positive tests per hospital in the Netherlands (n=8). Black columns represent the total numbers of requests for *C. difficile* testing. White columns represent the total number of positive *C. difficile* tests.



In the European situation, EIA was mainly used in France, Belgium, Italy and the Netherlands. Of 12 laboratories in The Netherlands, 4 (33.3%) used a cytotoxicity assay, 3 (25%) EIA and 4 (33.3%) another rapid immuno-colorimetric test.

Culturing of *C. difficile* from faecal samples was performed by direct inoculation of faeces on selective media without enrichment or pre-treatment by 4 of 8 Dutch laboratories, compared to 67.7% of European laboratories performing culture (55%). Cycloserine-cefoxitin fructose agar (CCFA) was used by 5 of 8 Dutch laboratories and in 68.6% of European laboratories. Broad variations of identification systems for *C. difficile* were used and 5 of 8 Dutch laboratories also performed antibiotics susceptibility tests of *C. difficile* , whereas only 18.3% of European laboratories used susceptibility testing. France (40%) and the Netherlands (50%) were the countries were susceptibility testing was most common. Barbut *et al.* classified the strategies in three groups. The minimal strategy includes culture only, or antigen detection only, which was only the case for Denmark. The standard strategy includes laboratories detecting only toxins (A or B or both), which was the case quite common in Spain and the United Kingdom, and was employed by 5 Dutch hospitals. More than 50% of the laboratories from Belgium, France and the Netherlands (58.3%) employ the optimal strategy: culture and toxin detection simultaneously or subsequently.

In summary, the results of the 12 responding laboratories in the Netherlands did not differ significantly from other European countries, expect for the relatively high percentage (67%) of laboratories that are still culturing faecal samples for the presence of *C. difficile*. In other European countries a trend is observed to replace culture methods with toxin assays. Although many laboratories use additional criteria for the faecal specimen to be investigated for the presence of *C. difficile* or its toxins, none of the responding laboratories implicated the rule to investigate faecal samples from patients hospitalized longer than 3 days only for *C. difficile* -associated diarrhoea.

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Typing of Clostridium difficile

To study the epidemiology of *Clostridium difficile*, typing methods are the method of choice. Typing methods are also used to determine the role of the environment and patient-to-patient transmission in the cause of infection, and for the investigation of outbreaks. The recurrence rate of CDAD is around 15-20% (Kelly, 1998) and typing methods can be applied to distinguish recurrences in relapse, due to the same strain, or reinfection, due to a new strain. Typing methods can be classified in two large categories, consisting of phenotypic and genotypic methods (Fig. 1). Phenotypic methods differentiate based on products of gene expression, whereas genotyping methods analyze the genetic profile of the strains.



Figure 1. Overview of phenotypic and genotypic methods described in this thesis.

Typing methods can be evaluated based on different properties (Struelens, 1998; Cohen, 2001). One of the most important properties is the discriminatory power, the ability to differentiate epidemiologically unrelated strains from those related to each other. The typeability is the power to identify every single strain without problems. Typing methods also have to be reproducible and stable, the ability to yield the same results after repeatedly testing of the strains on different occasions. The ease of performance is of importance for the applicability of the method in a laboratory, also depending on the ease of learning and understanding the method. This same requirement applies to the ease of interpretation, and the experience needed to interpret data. As for every laboratory method the costs are of importance. With the

upcoming of highly reproducible genotyping methods, the ability to generate libraries for the interlaboratory comparison of results is of interest as well.

Phenotypic methods

These early methods for typing of *C. difficile* strains were mainly used for investigation of hospital outbreaks and local epidemiology. Using these typing techniques, cross-infection of *C. difficile* within hospitals was demonstrated (Wust, 1982; Sell, 1983; Poxton, 1984; Tabaqchali, 1984). The first attempts for typing were made by Nakamura *et al.* (Nakamura, 1981). They used serum agglutination using three different rabbit antisera. This method was later improved, and led to the widely accepted serotyping method by Delmee *et al.* (Delmee, 1985). Antibiotic susceptibility, soluble protein pattern using polyacrylamide gel electrophoresis (SDS-PAGE) and its variant with pyrolysis mass spectroscopy (PyMS), bacteriophage and bacteriocin susceptibility were the earlier methods mainly used for phenotypic typing (Burdon, 1982; Wüst, 1982; Sell, 1983; Cartmill, 1992). The most commonly used methods will be described in further detail.

Serogrouping. The first serogrouping, or serotyping, method used slide agglutination with six rabbit-antisera (Delmee, 1985). Serogroups A, C and D were the only types associated with clinical disease (Delmee, 1985). This method was pursued by Toma *et al.* (Toma, 1988) and resulted in 15 different serogroups, designated A-D, F-I, K, X and S1-S4. In combination with PAGE, subtypes could be described and finally 30 serotypes, designated A1-A11, A13-A17, B, C, D, E6, F, G, H, I, K, S1-S4 and X, can be discriminated nowadays. These reference strains have been used in all our typing studies, see Chapters 5 and 7.

Radio PAGE. SDS-PAGE of [35S] methionine labelled proteins followed by autoradiography (Radio PAGE) was another often used discriminatory method. Nine groups could be identified (A-E, W-Z) of which groups A and D were mainly seen in asymptomatic neonates, and groups E and X were mainly associated with outbreak situations (Tabaqchali, 1984).

Immunoblotting. This phenotypic typing method uses enzymelinked immunoelectrotransfer blotting (Mulligan, 1986). A comparison of the immunoblotting method with both serogrouping and PAGE, showed that immunoblotting is the most valuable method (Mulligan, 1988; Brazier, 1997). In both comparisons, immunoblotting discriminated the most groups. Immunoblotting results were more reproducible then the other two methods as well, although the three methods did show excellent correlation to each other. In another study, antibiogram, pre-formed enzymes analysis, plasmid profiling and analysis of surface proteins by SDS-PAGE and immunoblotting were compared to each other in an outbreak of diarrhea in an orthopaedic ward. The immunochemical method was shown to be the most sensitive and discriminatory, detecting four different types within the outbreak (McKay, 1989).

Although the phenotypic methods showed useful results, none are used widely nowadays. When compared to genotyping methods, phenotypic methods show a low reproducibility, only useful in local outbreak situations. Phenotypic methods are less discriminatory as well, and show sometimes low typeabilities. Therefore, genotyping is the current method of choice.

Genotypic methods

To study the epidemiology of *Clostridium difficile*, a typing method with a high discriminatory power, typeability and reproducibility is required. To reach this goal, methods independent of natural variation are necessary. This can be found in most genotypic typing methods, based on the detection of variation in genes between strains.

Plasmid profiling. Plasmid profiling was the first ever used genotypic typing method, already applied by Arai *et al.* (Arai, 1984). The fact that not all *C. difficile* strains contained these extra-chromosomal elements made the typeability of this method very low. Additionally, strains may lose or acquire plasmids and thereby change in plasmid profile (Arai, 1984; Steinberg, 1987; Clabots, 1988).

REA and RFLP. Restriction enzyme, or endonuclease, analysis (REA) uses the whole genomic DNA. This DNA is digested by rare-cutter restriction enzymes, resulting in restriction fragments readable by PAGE or agarose gel electrophoresis. The first applied REA has been described by Kuijper et al (Kuijper, 1987), using HindIII and XbaI for restriction and agarose gels for analysis of the fragments. They found that the strains detected in two patients were indistinguishable from four samples from the hospital environment, thereby showing the applicability of this method for typing *C. difficile*. They also found that the method was stable after five times subculturing (Kuijper, 1987). Another study described the use of CfoI as the restriction enzyme; however, HindIII is still mostly used (Devlin, 1987; Clabots, 1993). REA has been applied as the standard typing method in North America because of its high discriminatory power and stability (Clabots, 1993), but the interpretation of REA banding patterns is subjective and analysis of isolates has to be performed on the same gel. Due to these reasons, REA data are difficult to exchange between laboratories, which is becoming more important for typing methods.

Restriction fragment length polymorphism (RFLP) is an alternative method for the REA and is not often used due to its low discriminatory power. This method uses subsequent blotting and hybridization with probes for analysis of REA fragments and it was therefore described as a simplified method that was reproducible and easy to perform. The difference between REA and RFLP is very small and are used interchangeable in different studies. The first description of RFLP was by Bowman *et al.*, where restriction enzyme (HindIII) digestion is followed by gel electrophoresis and subsequent Southern blot transfer and hybridization with labelled Escherichia coli rRNA probes (Bowman, 1991). RFLP with an eubacterial 16S rRNA probe was used in a comparison with SDS-PAGE, immunoblotting and REA. The RFLP proved a simple comparison of patterns and yielded good discrimination (Wolfhagen, 1993). Another study compared the RFLP with enhanced chemiluminescence to REA, both with HindIII restriction, and found REA far more discriminatory then RFLP (34 versus 6 types in 116 isolates; O'Neill, 1993).

AP-PCR and RAPD. Arbitrary primed-PCR (AP-PCR) and random amplified polymorphic DNA (RAPD) are two methods based on PCR amplification. The primers used in the PCR have no known homology to the target sequence, because of which low annealing temperatures are used. The difference between AP-PCR and RAPD is the use of a single primer versus the use of two short primers, respectively. The first described AP-PCR used six different arbitrary primers of 10-11 bp and detected six different patterns among six isolates (McMillin, 1992). In an outbreak among eight AIDS patients, the AP-PCR was applied using one arbitrary primer of 10 bp, differing only one nucleotide from one of the primers used by McMillin et al. (Barbut, 1993). Among these eight isolates, seven revealed an identical AP-PCR pattern, whereas four reference strains were discriminated from each other and the outbreak isolates. They concluded that the AP-PCR is simple, rapid and discriminative for typing C. difficile. Another outbreak was confirmed using the same arbitrary primer as the first two studies and this study also describes the lack of reproducibility of the AP-PCR (Wilks, 1994). Compared to the phenotypic immunoblotting method, AP-PCR shows a better typeability and good agreement between both methods (Killgore, 1994). This same good correlation goes for the comparison to REA (Tang, 1995; Samore, 1997; Rafferty, 1998). AP-PCR usually results in 3-12 bands between 450-1300 bp and are therefore simply analysed on agarose gels. The method is cost-effective, but is extremely sensitive to PCR conditions. Therefore, AP-PCR has a low reproducibility and it is difficult to establish interlaboratory comparison for this method (Cohen, 2001). Although not really different in its characteristics, RAPD was first established for C. difficile by Barbut et al. (Barbut, 1993). They showed the discrimination of 10 serogroups and 11 unrelated isolates using one specific set of arbitrary primers for RAPD, and found one predominant strain among two outbreaks and later one strain during an outbreak of CDAD in 15 AIDS patients (Barbut, 1993; Barbut, 1994). In a comparison between RAPD and PFGE (method to be described later), the correlation was high, whereas RAPD was easier to perform, but more difficult to analyze (Chachaty, 1994). The applicability of RAPD in the analysis of relapses versus re-infection in HIV patients was shown by Alonso et al. Relapses were detected in 64% of patients, whereas 32% had a re-infection and 4% had both a relapse and a re-infection (Alonso, 2001). The use of RAPD for subtyping of specific PCR-ribotypes will be described below.

PCR-ribotyping. As a reaction on the publication of an AP-PCR, Gürtler *et al.* wrote about the development of a highly discriminatory new typing method based on the PCR amplification of the 16S-23S rRNA spacer region published earlier (Gürtler, 1993; Gürtler, 1994). This was the first description of the currently widely used PCR-ribotyping. Every bacterial strain contains several rRNA operons, and there is a strain-dependent variation in the size and number of the 16S-23S intergenic spacer regions. Variation in spacer length is also observed between different copies of the rRNA operon in the same genome. Amplification of these regions results in a variety of PCR products whose size and number will vary amongst different strains, which enables the differentiation of these strains. The method by Gürtler et al. detected 14 PCR-ribotypes among 24 strains. However, analysis was performed using radiolabeling and long-rum PAGE. Cartwright et al. was the first to use agarose electrophoresis and found five of six patients with an identical strain in an outbreak and found 41 types among 102 isolates (Cartwright, 1995). In a comparison with the other PCR based typing method AP-PCR, PCR-ribotyping was very discriminatory and showed an agreement of 83% with PFGE (to be discussed later) compared to 60% and 44% for AP-PCR (Collier, 1996). That same year, the primers were modified to obtain smaller fragments for better analysis on agarose gels, by designing the primers closer to the spacer region (O'Neill, 1996). Using these primers, at least 116 types could be discriminated, including nontoxinogenic and environmental strains (Stubbs, 1999). Again, this PCR-ribotyping has been optimised with different primers, more specific for C. difficile based on known sequences of the 16S and 23S genes of C. difficile (Bidet, 1999). A comparison between these latter two PCR-ribotyping methods is described in Chapter 5. No correlation between PCRribotype and disease severity is found (Akerlund, 2006). Although the method by Bidet shows better separation of bands, there is not yet a large library as is the case with the O'Neill method, which is used worldwide (O'Neill, 1996; Stubbs, 1999; Bidet, 1999). PCR-ribotyping has appeared a robust genotyping method, being stable and reproducible (Cartwright, 1995; Collier, 1996; Bidet, 2000). Results can be used for interlaboratory comparison and for the generation of libraries. PCR-ribotyping is currently the preferred typing method in our laboratory.

PFGE. Pulsed field gel electrophoresis (PFGE) consists of the digestion of the whole genomic DNA with an infrequent cutting restriction enzyme (mostly SmaI), after which the restriction pattern is resolved by changing the direction of the current between two electrodes at an angle during electrophoresis. The first application of the PFGE was in a comparison to REA, testing 16 outbreak isolates, 17 unrelated nosocomial isolates and 13 community-acquired isolates (Kristjansson, 1994). In all cases, PFGE showed higher discrimination, although from some isolates the DNA was constantly degraded and thus shows a low typeability. PFGE was also compared to RAPD and both showed good agreement, with 26 and 25 types among 30 isolates,

respectively. Although PFGE was labor-intensive, the easy of interpretation was better (Chachaty, 1994). The application of PFGE in an outbreak situation was shown by Talon et al. (Talon, 1995). The 22 outbreak strains could be divided in five types: 2 types among the serogroup C strains and 3 types among the serogroup K strains. The difference between relapses and re-infections in a 10-year old patient was shown by PFGE. The first two episodes showed an identical strain, whereas both episode three and four showed different types (Kato, 1996). PFGE was compared to both REA and AP-PCR and to PCR-ribotyping. Among 30 isolates from different outbreaks and 15 isolates from sporadic cases, REA and AP-PCR showed respectively 23 and 19 types. However, PFGE was able to discriminate 11 types among only 15 isolates, due to degradation of the DNA of the other 30 isolates (Samore, 1997). Both PCRribotyping (Bidet, 1999) and AP-PCR had a 100% typeability, and PFGE showed 90% typeability. Both PCR-ribotyping and PFGE were 100% reproducible, compared to maximum 88% for AP-PCR. Compared to PFGE was PCR-ribotyping somewhat less discriminatory, although PCR-ribotyping was easier and quicker to perform. The patterns of PCR-ribotyping were more difficult to interpret than PFGE, but the authors designated PCR-ribotyping the method with the best combination of advantages (Bidet, 2000). Another study showed a correlation of 84% for 92 isolates typed by PFGE and PCR-ribotyping, with primers not specific to C. difficile. However, the discriminatory power of PFGE was higher: 28 versus 20 types among 100 isolates, of which PFGE was unable to type 8 isolates (Spigaglia, 2001). From these studies can be concluded that PFGE is the most discriminatory method, but the main drawbacks are the low typeability due to DNA degradation, the long running time and the cost of the equipment (Cohen, 2001). However, different studies tried alternatives for preventing the degradation of DNA due to the DNA isolation on agarose plugs, necessary for PFGE. Alternatives consist of the addition of thiourea, mutanolysion, higher concentrations of lysozyme, proteinase K, and increased incubation times (Corkill, 2000; Fawley, 2002; Alonso, 2005; Gal, 2005).

Toxinotyping. Toxinotyping involves the detection of polymorphisms in the toxin A and B and surrounding regulatory genes, an area of the genome known collectively as the pathogenicity locus or PaLoc. Six PCRs for the amplification of the toxin genes were developed: A1-A3 and B1-B3 (Rupnik, 1997; fig. 2), after which the PCR products are digested by restriction enzymes, like REA. B1 and A3 are considered the most variable and are therefore good markers for detecting most toxinotypes (Rupnik, 2001). Until now, 26 toxinotypes (0-X, XIa, XIb, XII-XIV) can be discriminated among C. difficile strains (Rupnik, 1998; Rupnik, 2001; Rupnik, 2003; http://www.mf.uni-mb.si/mikro/tox), where toxinotype 0 is the most common type and without variants in the toxin genes. Toxinotyping has been compared to serogrouping and PCR-ribotyping, and a good correlation was found. Some toxinotypes were strictly associated with certain serogroups, e.g. toxinotype VIII is always seen in serogroup F strains. However, toxinotyping could further distinguish subgroups within the serogroups (Rupnik, 1998). Compared to PCR-ribotyping, a specific PCR-ribotype was usually associated with similar changes in the toxin genes. Both methods are able to subtype each other, making toxinotyping a good addition to typing schemes (Rupnik, 2001). Barbut et al. (Barbut, 2002) applied the toxinotyping method on toxin A variant strains that were found in 2.7% diarrheal cases in adults and children, and found two variant types, one with a deletion of 600bp, close to toxinotype VII, and one with an insertion of about 200bp, close to toxinotype XIV, by PCR A3. In a study of 153 clinical isolates in an American hospital, 11.1% of strains belonged to toxinotypes other than toxinotype 0. Binary toxin was found only in these variant toxinotype strains, in 9 strains (Geric, 2004). The reproducibility is 100% and the discriminatory power is good, although e.g. PFGE and PCR-ribotyping show more discrimination between strains. The most important advantage of this typing method is that a clear view of the toxin status of C. difficile strains can be acquired.

Figure 2. Representation of five open reading frames, two toxin genes (tcdA and tcdB), and three additional genes (tcdC, tcdD, and tcdE) of the PaLoc in strain VPI (toxinotype 0) (extracted from Rupnik *et al.* 1998).



Recently developed methods. Typing of *C. difficile* is still in development, in search of the most reproducible, discriminatory and typeable method. For this reason, lots of newer methods are being explored, which will be described

here. A PCR on the flagellin gene, *fli*C, was developed by Tasteyre *et al.* (Tasteyre, 2000). A total of 47 isolates belonging to 11 different serogroups were tested, and three profiles could be recognized. Then the method was expanded with RFLP analysis, resulting in nine groups of which three groups corresponded to different serogroups, and the other six belonged to a single serogroup. Although nonflagellated strains were included, they did contain the *fli*C gene. In a study with nine toxin A-/B+ strains, only three strains showed flagella. However, all nine strains belonged to the same type after fliC PCR-RFLP (Pituch, 2002). This method has not been tested in comparison to other methods as of yet.

Another gene studied for typing is the slpA gene, encoding an S-layer precursor protein of C. difficile. The first to describe variation for phenotypic typing using SDS-PAGE and immunoblotting, were McCoubrey et al. (McCoubrey, 2001). Later, seven S-types were found, of which one type accounted for 73% of the clinical cases and 93% of the environmental cases (McCoubrey, 2003). slpA genotyping was subsequently developed and evaluated. Thirty-two strains belonging to 10 serogroups were used for PCR-RFLP and sequencing analysis of the variable region. This RFLPsequence combination led to sequences identical within a given serogroup and differences between serogroups, and was therefore thought of as an alternative typing method for C. difficile (Karjalainen, 2002). The application of this PCR-RFLPsequence method was tested on Japanese outbreak strains and resulted in three subtypes, differing one nucleotide from each other. The method was also applied on 22 faecal samples and gave complete agreement with the cultured strains from these samples. Therefore, *slpA* typing was considered a reproducible method with the advantage of interlaboratory data exchange (Kato, 2005). However, the stability of these types was not tested. slpA typing of 14 different PCR-ribotypes showed nine groups of PCR-ribotypes, where PCR-ribotypes showed completely identical sequence in two cases, and 1-3bp differences within other groups (Eidhin, 2006).

An amplified fragment length polymorphism (AFLP) method, described by Klaassen *et al.* (Klaassen, 2002) was compared with PFGE, and was used due to the bad typeability of PFGE. The AFLP method uses restriction, ligation and selective amplification on the whole genome. Differentiation can be made due to variation per type in restriction sites mutations, mutations in the sequences adjacent to the restriction sites and complementary to the selective primer extensions, and insertions

and deletions within the amplified fragments. AFLP analysis of *C. difficile* strains showed that its discriminatory power was similar to that of PFGE when tested on 30 clinical isolates. The study by Klaassen also showed that the typeability of AFLP was better than PFGE, especially for *C. difficile* isolates for which PFGE showed DNA degradation. In addition, AFLP was considered faster and easy to perform on small quantities of DNA. The reproducibility of AFLP was found similar to PFGE (Klaassen, 2002). The AFLP-method has been compared with the two PCR-ribotyping methods, as can be read in Chapter 5.

One of the newest typing methods for C. difficile is multilocus sequence typing (MLST). MLST consist of DNA sequence analysis of housekeeping genes after PCR amplification, and is mostly used to study genetic relationships and population structures (Lemee, 2004). The MLST developed for C. difficile includes seven housekeeping genes. Among 72 isolates from various origins, 62 PCRribotypes and 34 sequence types could be discriminated. In a dendrogram, three divergent lineages could be recognized of which one strictly contained toxin A-/B+ strains (Lemee, 2004). The method was further expanded by the inclusion of ten virulence-associated genes, among which fliC, slpA, tcdA, tcdB and tcdD (Lemee, 2005). A total of 29 isolates from various origins and selected from the lineages found in their first study were investigated, representing 22 sequence types. The polymorphisms detected in the virulence-associated genes were comparable to those of the housekeeping genes. However, cwp66 and slpA appeared highly polymorphic, although only 11 and 16 alleles could be detected, respectively. Again, toxin A-/B+ strains belonged to a homogenous lineage, and a fourth lineage could be characterized in contrary to the method based on only housekeeping genes (Lemee, 2005). No association was found between the sequence types and the clinical presentation or the source of the isolates (Lemee, 2004; Lemee, 2005). It was concluded that the MLST with the virulence-associated genes included, is more discriminatory then the housekeeping genes alone, although this could depend on the genes chosen. The main advantage is the yield of unambiguous sequence data (Lemee, 2005). No comparisons with other methods have been described to date.

The most recently published new application of a typing method on *C*. *difficile* strains is the multilocus variable-number of tandem repeats (VNTR) analysis (MLVA). This method is based on the amplification of regions with short tandem

repeats. The number of tandem repeats within these loci can differ between strains, and can therefore be used as a typing method. The availability of the complete sequence of the С. difficile genome of strain 630 (http://www.sanger.ac.uk/Projects/C_difficile/; Sebaihia, 2006) provided the opportunity to identify these short tandem repeats. The MLVA developed by Marsh et al. (Marsh, 2006) uses automated sequence detection and subsequent manual determination of the number of tandem repeats per locus. Seven short tandem repeat loci were amplified from 40 isolates from two different sources, and REA was tested on every strain as well. The stability was good, although differences of one repeat could arise. This MLVA clustered outbreak strains of the same REA-type and discriminated different REA-types from each other (Marsh, 2006). In the period this MLVA method was developed and published, we also developed an MLVA method, mainly for subtyping of epidemiologically important strains. Results and development are described in Chapter 7.

Method	Target	Discriminatory power	Typeability	Reproducibility	Performance	Interpretation	Costs	Interlaboratory exchange
plasmid profiling	extrachromosomal plasmid	-	-	+	±	±	+	+
REA	whole genome, restriction	+	+	±	±	-	+	-
RFLP	whole genome, restriction	-	+	±	±	±	+	-
AP- PCR/RAPD	whole genome, random PCR primers	++	+	±	+	+	+	-
PCR-ribotyping	16S-23S intergenic spacer region	++	+	+++	+++	++	+	+++
PFGE	whole genome, restriction	+++	±	+++	±	±	+++	±
Toxinotyping	toxin A and B genes	+	+	+++	++	+	+	++
fliC PCR- RFLP	flagellin gene	±	+	++	++	++	+	++

Subtyping methods

The first subtyping of epidemic strains was described in 1996 by van Dijck *et al.* (van Dijck, 1996). Serogroups A, C and D were the only types associated with clinical disease, of which serogroup C was mainly detected in outbreak situations (Delmee, 1985). Therefore it was considered relevant to subtype these epidemic serogroup C strains (van Dijck, 1996). Using three genotypic methods, 56 isolates belonging to the phenotypic serogroup C were typed. PCR-ribotyping, RAPD and PFGE resulted in 2, 5 and 11 genotypes, respectively, and in 13 general types when combined. Among five outbreaks, three general types could be recognized, whereas 11 general types were found among 14 sporadic isolates. Therefore, it was concluded that the combination of these methods could resolve genetic diversity among strains belonging to serogroup C (van Dijck, 1996).

The importance of subtyping of epidemic PCR ribotypes is high. Therefore, methods that are developed specifically for subtyping will be described below. Typing methods have been developed to differentiate strains belonging to PCR-ribotype 001/serogroup G, such as modified PFGE, RAPD, ribospacer (RS) PCR and repetitive extragenic palindromic PCR (rep-PCR) (Gal, 2005; Fawley, 2005; Northey, 2005; Rahmati, 2005). Since strains belonging to PCR-ribotype 001 are the most common epidemic strains in the UK and are quite common in outbreaks in the United States, the importance of the ability to subtype these strains is high (Cartmill, 1994; Stubbs, 1999; Johnson, 1999; Brazier, 2001). Interesting is the fact that these type 001 strains are the ones that are degradation-susceptible in PFGE typing (Johnson, 1999; Fawley, 2001). The first report that these PCR-ribotype 001 strains could be phenotypically subtyped was by Al-Saif, using PyMS (pyrolysis mass spectrometry). However, PyMS was not robust and reproducible due to batch-to-batch variation (Al-Saif, 1997). The description of both genotypical and phenotypical subtypes was by Fawley et al. (Fawley, 2003). In this study, two groups, AP-PCR Ia and Ib were recognized by both RAPD, ribospacer (RS)-PCR and a modified PFGE, consistent with the absence (Ia) or presence (Ib) of clindamycin resistance (Fawley, 2003; Fawley, 2005). A PFGE method was modified to overcome the DNA-degradation problems, mainly by increasing lysozyme concentration and incubation time, and the addition of mutanolysin. This modified PFGE was able to differentiate 50 type 001 strains in seven subgroups: PF-A to PF-G (Gal, 2005). This author also described the lack of reproducibility of the above described RAPD and the inability of RS-PCR to distinguish these seven PFGE-subtypes. The disadvantage of the (modified) PFGE itself was the low throughput (4-8 days), the complexity and the high costs (Gal, 2005). Repetitive extragenic palindromic PCR (rep-PCR) was explored for typing *C. difficile* by Spigaglia *et al.* (Spigaglia, 2003). A high correspondence between PFGE and rep-PCR patterns was observed, with a higher discriminatory power than PCR-ribotyping (Spigaglia, 2003). Therefore, this rep_PCR was applied by Northey and Rahmati *et al.* (Northey, 2005; Rahmati, 2005) for the subtyping of PCR-ribotype 001 strains. Of 200 isolates from six hospitals, eight subtypes could be detected: rep-PCR 001-008. The main advantage of rep-PCR above PFGE was the easier, cheaper and more rapid application (Rahmati, 2005; Northey, 2005).

Other epidemic strains of interest are the toxin A-/B+ strains (al Barrak, 1999; Alfa, 2000; Johnson, 2001; Kato, 1998; Kuijper, 2001; Limaye, 2000), containing the deletion of 1.8 kb within the repetitive regions of the tcdA gene, belonging to serogroup F/PCR-ribotype 017/toxinotype VIII, or the type 027/BI/NAP1/III strain, increasingly detected in different countries around the world (see General introduction). The PCR-ribotype 017/toxinotype VIII strains could be differentiated in two groups by REA: CF and CG, the latter of which was only detected in asymptomatic children. REA-group CF could be further discriminated in 6 subgroups: CF1-CF6 (Johnson, 2003). The ability of AFLP and PCR-ribotyping to subtype these type 017 strains will be described in Chapter 5. For the subtyping of the type 027/BI/NAP1/III strain, rep-PCR was evaluated, but only showed one predominant type in three different Canadian regions (MacCannell, 2006). The high discriminatory power and the good stability of MLVA was developed (Chapter 7) for this purpose, and was also applied by Marsh et al. (Marsh, 2006). Of the 11 strains belonging to REA-group BI, six different REA-types and nine different MLVA-types were found: BI6 showed 3 MLVA-types and BI9 showed 2 MLVA-types. Therefore, MLVA was shown to be more discriminatory than REA for subtyping of type 027/BI/NAP1/III strains (Marsh, 2006).

Application of typing in recurrences

Patients develop a recurrent *C. difficile* infection in 15-20% of cases after the discontinuation of antibiotic therapy (Kelly, 1998; Wilcox, 1992). Recurrences can be explained by endogenous persistence of *C. difficile* spores (relapse), or by the acquisition of a new strain from an exogenous source (reinfection). Determining if a recurrence is due to a relapse or a reinfection is important for epidemiological studies of *C. difficile*, and therefore, different typing methods have been applied.

Of 11 patients with recurrences, five patients were found to be associated with recurrence due to a new type compared to the first episode. This study, using REA as the typing method, noted that true relapses could have occurred as well. True relapses are presumed to be reinfections with the same strain as previously found, and are suspected when both culture and cytotoxicity assays have been negative in between episodes (Johnson, 1989). O'Neill et al. (O'Neill, 1991) found 75% of ten presumed relapses to be actually reinfections with a new strain using this same REA. However, no difference could be made between relapses due to environmental reinfection or endogenous sources of C. difficile (O'Neill, 1991). As described earlier, Kato et al. tested both immunoblotting and PFGE on four subsequent samples in a 10year old patient, and found the second episode being a relapse after treatment, and the third and fourth episode were both different reinfections (Kato, 1996). Using RAPD, patients were tested of whom multiple fecal samples were tested during hospitalization. For 15 of 27 patients (56%), different RAPD-types could be distinguished, suggesting a reinfection in these cases. The authors pointed out that an endemic clone was present, which could have led to reinfections with the same strain, but from an exogenous source, and therefore could result in a higher reinfection rate (Wilcox, 1998). In a study among 93 hospitalized patients with recurrences over 1994-1997, 48.4% of recurrences were actually reinfections with a different strain, tested by PCR-ribotyping (Barbut, 2000). This study also applied serotyping, but PCR-ribotyping was able to subtype most serogroups found. The median time elapsed between two episodes was 28 and 38 days for relapses and reinfections, respectively, and patients with reinfections were more frequently rehospitalized between episodes. Two patients with both a reinfection and a relapse were described in this study as well

(Barbut, 2000). Recurrences of CDAD in HIV-infected patients was described to be due to reinfections in 32% of cases, and 4% of cases were due to both relapse and reinfection (Alonso, 2001). Tang-Feldman et al. found six of 18 recurrences (33.3%) to be reinfections using RAPD. Relapses were quite common among patients with recurrent CDAD shortly after discontinuation of treatment (Tang-Feldman, 2003). In a prospective study over 2 years time, 90% of 89 PCR-ribotypes patients were considered relapses and 10% reinfections. The median of time between episodes was 28 days for relapses and 41 days for reinfections (Noren, 2004). From this can be concluded that reinfections and relapses occur at the same time in different hospitals, and that reinfection rates are comparable to relapse rates. One of the major risk factors for reinfection is the longer hospitalization of patients, and relapses are mostly seen shortly after the first episode. However, in different studies, the occurrence of two or more strains at the same time has been discussed, due to the observation that sometimes patients show both relapses and reinfections together. Different studies have shown conflicting data concerning this subject (Sharp, 1985; Wilcox, 1998; O'Neill, 1991). Borriello and Honour were the first to describe the presence of both toxinogenic and nontoxinogenic strains in the same stool in six different patients (Borriello, 1983). Using SDS-PAGE with subsequent probing with antisera, Sharp et al. also described two of three fecal samples to contain more than one strain at the same time (Sharp, 1985). However, testing ten colonies from each sample showed only identical REA-patterns in another study, although only ten patients were tested (O'Neill, 1991). In other studies, more colonies have been tested as well, without the detection of simultaneous occurrence of different strains at the same time (Devlin, 1987; Wilcox, 1998). These striking data, and the use of typing methods with a low reproducibility and the implications for the use of single colonies for typing, led us to test some random fecal samples and samples from patients with relapses for this coexistence of strains, as will be described in Chapter 6.

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Chapter 2

Rapid diagnosis of toxinogenic *Clostridium difficile* in faecal samples using internally controlled real-time PCR

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Clinical Microbiology and Infection, 12: 184-186. 2006

Abstract

A real-time PCR assay for *Clostridium difficile* was developed, based on the *tcd*B gene, which detected all known toxinogenic reference strains (n=45), within 30 serogroups and 24 toxinotypes. The analytical sensitivity was $1x10^3$ CFU/ml, and the detection limit in faeces was $1x10^5$ CFU/g. The optimal protocol for DNA-extraction from faecal samples involved use of the MagnaPure system with a Stool Transport and Recovery (S.T.A.R.)-buffer pre-treatment. In a 1-month prospective study of 85 patients with diarrhoea, the sensitivity, specificity and positive and negative predictive values of the assay were 100%, 94%, 55% and 100%, respectively, compared with the standard cell cytotoxicity assay.

Research note

Clostridium difficile has been recognized as the causative agent of antibiotic associated diarrhoea (CDAD) and pseudomembranous colitis (PMC). The enteropathogenicity depends on the production of enterotoxin A (TcdA; 308 kDa) and cytotoxin B (TcdB; 270 kDa) [1,2]. TcdA has been regarded the most important factor causing enteropathogenic disease [3,4], but there have been an increasing number of reports of disease caused by TcdA-negative, TcdB-positive strains [5]. Therefore, the present study designed a real-time PCR assay for *tcdB* to enable rapid diagnosis of CDAD associated with toxinogenic *C. difficile*. An optimal DNA extraction protocol for faecal samples was established, and an internal control was included to verify amplification.

Primers and probe (Table 1) were designed from the non-repeat region of a known *tcd*B sequence (accession no. X53138) using the Primer3 program (http://www.broad.mit.edu/cgi-bin/primer/primer3_www.cgi). Amplification reactions were performed in a 50-µl final volume, containing 25 µl IQ supermix (Bio-Rad, Veenendaal, The Netherlands), 5 pmol forward primer, 10 pmol reverse primer, 4 mM MgCl2, 0.2 µM probe, and 5 µl DNA extract. Following an enzyme activation step for 3 min. at 95°C, amplification comprised 50 cycles of 30 s at 94°C, 30 s at 57°C and 30 s at 72°C in an iCycler IQ real-time detection system (Bio-Rad). The size of the generated fragment is 177 bp. The assay was optimised using *C. difficile* strain ATCC43594, and had an analytical sensitivity in saline 0.9% w/v of 1 colony-forming unit (CFU) per PCR reaction, corresponding to $1x10^3$ CFU/ml saline. In addition, ten-fold dilutions of ATCC43594 ($1x10^7 - 1$ CFU) were spiked into 1 g of pooled *C. difficile* culture-negative faeces to determine the sensitivity of the real-time PCR assay in comparison with culture.

For *C. difficile* culture, faecal samples, with and without an ethanol-shock treatment, were inoculated onto selective media as described previously [6]. Colonies of Gram-positive rods with subterminal spores were tested for L-proline-aminopeptidase and aesculin hydrolysis [7]. Two separate experiments revealed that the sensitivity of the real-time PCR assay with faeces was less than that of culture $(1x10^5 \text{ CFU}/\text{g} \text{ faeces vs. } 1x10^4 - 1x10^5 \text{ CFU/g faeces})$, but was comparable to the sensitivity (5x10⁴ CFU/g faeces) reported for a real-time PCR assay described previously [8].

To define the analytical specificity of the assay, all known C. difficile serogroups (n=30; gift of M. Delmee, University of Louvain, Brussels, Belgium) and toxinotypes (n=24; gift of M. Rupnik, University Goettingen, Goettingen, Germany) were included. All toxinogenic serogroups (n=23) were detected. Of the 24 toxinotypes, 22 tested positive, whereas two toxinotypes (XIa and XIb) do not harbour tcdB, and were therefore not detected by the assay. These results were in contrast with the assay of Belanger et al. [8], who could not detect tcdB in toxinotypes III, IV, and VI. Since this discrepancy was associated with polymorphisms around the 3'-end of these primers, we can conclude that our primers have been designed in a region that is more conserved among all toxinotypes. Nevertheless, after comparison of the primers and probe to the available sequences of TcdA-/TcdB+ strains 1470 and 8864 (accession no. CDTOXBA and CDI011301, respectively), some mismatches were detected. Strain 1470, represented by toxinotype VIII and serogroup F, showed one mismatch with the forward primer, but was still detected by the real-time PCR. Strain 8864, represented by toxinotype X, revealed two mismatches for both the forward primer and the probe, and one mismatch in the reverse primer at the 5'-end. This strain was positive by the real-time PCR as well, but a reduced sensitivity may be expected when analysing clinical samples. The use of degenerate primers could solve this problem, but has not been applied yet as strain 8864 is a naturally occurring isolate that has not been detected in a clinical setting up to now. To further determine the specificity, 9 Clostridium spp., other than C. difficile , were included and tested negative, as did 27 other (including enteropathogenic) bacterial species.

Since effective DNA extraction from faecal samples with removal of inhibitory factors [9] is a key factor for successful application of PCR, we tested polyvinylpolypyrolidone (PVPP) pre-treatment, followed by isolation using the QIAamp DNA blood mini kit (Qiagen, Hilden, Germany), and compared it with a pre-treatment using the "Stool Transport and Recovery" (S.T.A.R.)-buffer, followed by automated extraction with the MagnaPure LC DNA isolation Kit III (Roche, Almere, the Netherlands), in the MagnaPure System. A fixed amount of Phocine Herpes Virus (PhHV), equal to an amount giving a Ct-value of approximately 33-34, was spiked into clinical samples prior to DNA extraction as the internal control [10]. Primers used are listed in Table 1. A standard *C. difficile* solution (6.5x10⁶ CFU/ml) was used

for spiking experiments in *C. difficile* culture-negative faeces. All samples were tested in ten-fold dilution series. No differences in Ct-values could be observed between the two methods in tenfold dilution series ranging from 2.2×10^6 to 22 CFU/g faeces. The internal control was efficiently amplified, indicating absence of inhibition in any sample or procedure. Since no clear differences could be observed in the comparison, the STAR/MP-method was the method of choice for sample preparation for real-time PCR purposes, due to its automated format.

Target	Primers and probes	Nucleotide sequence (5'-3')
C. difficile	398CLDs	GAAAGTCCAAGTTTACGCTCAAT
	399CLDas	GCTGCACCTAAACTTACACCA
	551CLD-tq-FAM	FAM-ACAGATGCAGCCAAAGTTGTTGAATT-TAMRA
PhHV[17]	295PhHVs	GGGCGAATCACAGATTGAATC
	296PhHVas	GCGGTTCCAAACGTACCAA
	531PhHV-tq-CY5	CY5-TTTTTATGTGTCCGCCACCATCTGGATC-BHQ2

Table 1. Primer sequences of primers and probes used for real-time PCR in this study

For clinical evaluation of the assay, faecal samples were obtained from 28 patients with CDAD. Primary diagnosis and selection was made by detection of TcdA using an enzyme-linked fluorescent immunoassay (ELFA): VIDAS CDA2 (BioMerieux, Boxtel, the Netherlands), according to the manufacturer's recommendations. All 28 ELFA-positive faecal samples were positive by real-time PCR and culture for *C. difficile*. In addition, a control group of 43 faecal samples from 43 patients without gastro-intestinal symptoms was included. Three of these 43 samples (7%) tested positive both in real-time PCR and culture, indicating asymptomatic carriage of toxinogenic *C. difficile* [11,12]. These three samples were re-isolated and re-tested by real-time PCR, and were confirmed as positive.

Further clinical validation was achieved in a prospective setting. In October 2003, 85 faecal samples from adult patients with a request for *C. difficile* diagnosis and samples from patients with diarrhoea admitted to the hospital for 3 days or longer were investigated for *C. difficile* using the cytotoxicity assay, ELFA and real-time PCR (Table 2). Faecal samples were initially tested by ELFA, and were subsequently stored at -80°C within 24 hours after arrival, pending further analysis by the cell cytotoxicity assay and real-time PCR. Of the 85 samples, 6 (7%) were positive by the cell cytotoxicity assay (Table 2). Of these 6 samples, 5 were positive by ELFA, and 6

by real-time PCR. Of 79 cytotoxicity negative samples, 1 was positive by ELFA and real-time PCR, and another 5 were positive only by real-time PCR. In total, 11 (13%) of 85 samples were positive by real-time PCR. *C. difficile* was cultured subsequently from these 11 samples. Using the cytotoxicity assay as the 'gold standard', the sensitivity, specificity, PPV and NPV for real-time PCR were 100%, 94%, 55% and 100%, respectively.

Table 2. Prospective application of the cell cytotoxicity assay (indicative for CDAD), ELFA, real-time PCR, and culture on 85 faecal samples from patients with diarrhoea.

Clinical manifestation	No. of patients	No. of positive samples	
		ELFA	Real-time PCR
CDAD	6	5	6
non-CDAD	79	1	5
Total	85	6	11 ^a

a All real-time PCR positives could be confirmed by culture of toxinogenic

In conclusion, detection of the *C. difficile tcd*B gene in faecal samples by real-time PCR, using an automated DNA extraction protocol and an internal control, can be used as a rapid method for diagnosing CDAD and for detecting carriage in asymptomatic patients.

Acknowledgements

This work was supported by a grant from the Foundation Microbiology Leiden. We thank K. Templeton for support in the development of the real-time PCR assay.

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Chapter 3

Prospective multicenter evaluation of a new immunoassay and real-time PCR for rapid diagnosis of *Clostridium difficile*-associated diarrhea in hospitalized patients

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Journal of Clinical Microbiology, 43: 5338-5340. 2005

Abstract

In a prospective multicenter study, 367 fecal samples from 300 patients with diarrhea were tested for *Clostridium difficile*-associated diarrhea (CDAD) with a new immunochromatography assay for toxins A and B (ICTAB), a real-time PCR on the toxin B gene and the cell cytotoxicity assay. Twenty-three (6.2%) of 367 fecal samples were positive in the cell cytotoxicity assay. With the cell cytotoxicity assay as the 'gold standard', the sensitivity, specificity, positive predictive value, and negative predictive value for the ICTAB assay and real-time PCR were 91, 97, 70, 99%, and 87, 96, 57 and 99%, respectively. In conclusion, both the ICTAB and the real-time PCR can be implemented as rapid screening methods for patients suspected of having CDAD.

Introduction

Clostridium difficile-associated diarrhea (CDAD) is the most important infectious cause of nosocomial diarrhea and pseudomembranous colitis (PMC). The enteropathogenicity depends on the production of enterotoxin A (308 kDa) and cytotoxin B (270 kDa). Several authors have suggested that all fecal samples for *C. difficile* from patients with diarrhea hospitalized for more than 72h be investigated (3) irrespective of the physician's request, since length of hospitalization is simple to implement as an inclusion criterion. Conventional diagnostic methods for CDAD are the cell cytotoxicity assay and the enzyme immunoassays (EIA) to detect fecal toxins A (TcdA) and B (TcdB). The cell cytotoxicity assay is considered the 'gold standard'. However, with a turnaround time of more than 48h, this method is laborious and time-consuming. Frequently, EIA are used because of their more rapid turnaround time. Rapid diagnosis of CDAD is important, since it may result in early treatment and prevention of nosocomial transmission.

Material and methods

A new rapid immunochromatography test, the ImmunoCard Toxins A&B (ICTAB, Meridian), has recently been introduced. The ICTAB is a single test enzyme immunoassay for the detection of TcdA and TcdB in fecal samples within 20 min. No sample pre-treatment is required, and an internal procedure control is integrated in each card. The performance of this rapid assay was evaluated in comparison with an in-house developed real-time PCR on *tcd*B and the cell cytotoxicity assay. A positive PCR result for a fecal sample is indicative of the presence of a *C. difficile* strain capable of producing TcdB.

Fecal samples were collected from October 2003 to February 2004 at the Department of Medical Microbiology of three university medical centers in the Netherlands: Erasmus Medical Center Rotterdam (Erasmus MC), Leiden University Medical Center (LUMC), and the VU University Medical Center Amsterdam (VUMC). Fecal samples from adult patients with diarrhea for whom there was a request for *C. difficile* diagnosis and samples from patients hospitalized for more than 72h were included. All samples were stored within six hours after arrival at the laboratory at -20 °C in two individual vials for subsequent testing by the cell

cytotoxicity assay and real-time PCR assays at the LUMC. The ICTAB was performed in the Erasmus MC and the LUMC. All fecal samples were thawed only once for a specific test.

The ICTAB was performed according to the respective manufacturer's instructions. Briefly, enzyme conjugate was added to specimen diluent before the addition of 25 μ l of the fecal sample or the control. After incubation at room temperature for 5 min, the specimen was added to the lower ports of the card. This was again incubated at room temperature for 5 minutes, after which wash reagent was added to the upper ports, followed by substrate addition. Results were read in the upper ports after a 5-min incubation at room temperature.

The cell cytotoxicity assay (1) was performed using Vero-cells in a 24-well format. Fecal samples were diluted 1:4 in Eagle's minimum essential medium (EMEM)-5% FBS and centrifuged. Subsequently, the supernatant was filtered through a 0.45 μ m-pore-size filter. Neutralization of the cytotoxic effect was performed using specific *C. difficile* antitoxin (TechLab, Blacksburg, USA).

For real-time PCR 398CLDs (5'primers GAAAGTCCAAGTTTACGCTCAAT-3') and 399CLDas (5'-GCTGCACCTAAACTTACACCA -3') were designed to amplify 177 bp of the nonrepeat region of the tcdB gene. A specific 6-carboxyfluorescein (FAM)-labeled Taqman-probe (5'- ACAGATGCAGCCAAAGTTGTTGAATT-3') was used as an internal probe (8a). The amplification reactions were performed in a 50-µl final volume, containing 25 µl IQ supermix (Bio-Rad, Veenendaal, The Netherlands), 5 pmol of the forward primer, 10 pmol of the reverse primer, 4 mM MgCl2, 0.2 μ M probe, and 5 µl of DNA. After an enzyme activation step of 3 min at 95°C, the protocol consisted of 50 cycles of 30 s at 94°C for denaturation, 30 s at 57°C for annealing, and 30 s at 72°C for elongation. The iCycler IQ real-time detection system (Bio-Rad) was used for amplification and analysis. DNA-isolation from fecal samples was performed using S.T.A.R.-buffer pretreatment and subsequent automated isolation by use of a MagnaPure LC DNA isolation Kit III (Roche, Almere, the Netherlands) in the MagnaPure System, according to the manufacturer's instructions. An internal control, the Phocine Herpes Virus (PhHV), was included for detection of inhibition in the PCR, as has been described before (6). The sensitivity was 1×10^3 CFU/ml, and in feces the detection limit was 1×10^5 CFU/g.

Results and discussion

In total, 367 samples were included from 300 patients: 183 samples from the Erasmus MC, 65 from the VUMC and 119 from the LUMC. No significant differences were observed for age, gender, department, and the number of hospitalized days of the patients from the three participating centers (data not shown). Forty-three (11.7%) samples of 39 patients were positive in one or more assays and 23 samples (6.3%) of 22 patients were positive by the cell cytotoxicity assay (Table 1). The highest percentage of positive cell cytotoxicity tests (43%) was found in the Erasmus MC, followed by the LUMC (35%) and the VUMC (22%). No inhibitory samples were present in the real-time PCR. The sensitivity, specificity, PPV and NPV were 91, 97, 70 and 99% for the ICTAB assay and 87, 96, 57 and 99% for the real-time PCR, respectively, using the cell cytotoxicity assay as the 'gold standard' (Table 1). The concordance of cell cytotoxicity with ICTAB was 97% and with real-time PCR 95%. No large differences of sensitivity, specificity, NPV and PPV for both tests were observed between the three centers.

Test	Result	No. of samp						
		cytotoxicity assay results		ivity	icity	(%)	(%)	ation
		pos (n=23)	neg (n=344)	sensiti	specif	PPV (NPV (correl: (%)
ICTAB	positive	21	9	91.3	97.4	70.0	99.4	97.0
	negative	2	335					
Real-time	positive	20	15	87	95.6	57.1	99.1	95.1
PCR	negative	3	329					

Table 1. Comparison of ICTAB and real-time PCR to the cell cytotoxicity assay (n=367).

Discrepancy analysis was performed by culture of all samples positive for *C*. *difficile* in one or more assays. Culture is known as the most sensitive method (4) and can therefore be applied for discrepancy analysis. Culture was performed as described previously (10), and all isolated strains were tested by PCR for the presence of *tcdA* and *tcdB* (9). True-positive test results were defined as fecal samples positive for the presence of a toxinogenic *C. difficile* strain. Forty of the 43 samples positive in one or more assays were available for specific culture of toxinogenic *C. difficile*. The results of the discrepancy analysis are presented in Table 2. Real-time PCR showed a

concordance with culture of 80% (32/40). The concordance of the cell cytotoxicity assay and ICTAB with toxinogenic culture was 75% (30/40) for both methods. Using the results of the discrepancy analysis, the recalculated sensitivity, specificity, PPV and NPV were 79, 99, 90 and 98% for the ICTAB assay, 88, 99, 88 and 99% for the real-time PCR, and 70, 100, 100 and 97% for the cell cytotoxicity assay, respectively. The low sensitivity of the cell cytotoxicity assay (70% compared to 79 and 88% for the ICTAB and the real-time PCR respectively) indicates the limitation of the cell cytotoxicity as 'gold standard'. Additionally, it provides an explanation for the low PPV of both ICTAB and real-time PCR in comparison with the cell cytotoxicity assay, also given that the PPV was 20 and 33% higher for ICTAB and real-time PCR in the discrepancy analysis. The relatively low number of positive samples underlines the need for a larger study to verify these results.

Table 2. Discrepancy analysis by culture of toxinogenic *C. difficile* of 40 fecal samples positive in one or more assays. The toxinogenicity of cultured *C. difficile* strains was determined by PCR for the presence of *tcd*A and *tcd*B.

No of fecal	Diagnostic assay fo	Toxinogenic		
samples (n=40)	Cell cytotoxicity	ICTAB	Real-time PCR	culture
19	+	+	+	+
5	-	-	+	+
4	-	+	+	+
2	+	+	-	+
1	+	-	+	+
1	+	-	-	+
1	-	+	-	+
4	-	-	+	-
3	-	+	-	-
Total of positive results	23	29	33	33

Previous results obtained in our laboratory show that the detection limit for culture $(1x10^4 \text{ CFU/gr feces})$ was slightly better than for real-time PCR $(1x10^5 \text{ CFU/gr feces})$. This can offer an explanation for the fact that four of seven samples negative in real-time PCR were positive by toxinogenic culture. The sensitivity for detection of *C. difficile* can be further optimized by inclusion of a target such as the

*glu*D gene, encoding glutamate dehydrogenase (GDH; a moderate, specific enzyme commonly produced by *C. difficile*), or a multiple-copy target (5). A different DNA-extraction method can improve the sensitivity of our real-time PCR on *tcd*B.

Current laboratory diagnosis of CDAD is based on either the cell cytotoxicity assay for its specificity, an enzyme-immunoassay for its fast turnaround time, or toxinogenic culture for its sensitivity (2,4,7). Recently, new rapid EIAs have been evaluated for the detection of GDH in feces (8,11,12). Snell et al. (8) compared two GDH/toxin-assays with toxinogenic culture, with confirmation of toxinogenicity by PCR (on the gluD gene), and the cell cytotoxicity assay. The cell cytotoxicity assay had the highest sensitivity and PPV, but testing in combination with GDH and toxin detection resulted in 100% correct diagnosis of CDAD. In the study by Zheng et al. (12) a new EIA (C DIFF CHEK) for the detection of GDH was described, and was compared to a homemade PCR using gluD and with toxinogenic culture. The PCR outperformed culture, and showed a comparable result to the C DIFF CHEK in sensitivity and specificity. However, the disadvantage of methods based on GDH or gluD is the inability to differentiate between toxin-positive and -negative strains, necessitating subsequent testing by other methods. Despite the excellent test statistics of ICTAB compared to the cell cytotoxicity assay and real-time PCR, a comparison with other rapid EIA's should be performed.

ICTAB results can be obtained within 20 minutes, and results for real-time PCR can be obtained within one working day. We conclude that, based on the excellent sensitivity, NPV and rapidity, the new diagnostic ICTAB assay and in-house real-time PCR can be used as methods for first screening for CDAD.

Acknowledgements

This work was supported by a grant from the Foundation Microbiology Leiden. We thank Hadi Ameen for his technical support and Kate Templeton for her support and assistance with the manuscript.

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Chapter 4

Evaluation of real-time PCR and conventional diagnostic methods for the detection of *Clostridium difficile*associated diarrhoea in a prospective multicentre study

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Journal of Medical Microbiology, 56: 36-42. 2007

Abstract

In this prospective multicentre study, an enzyme-linked fluorescent assay (VIDAS CDA2; BioMerieux), an enzyme-linked assay (Premier Toxins A&B (PTAB); Meridian) and an in-house real-time PCR amplifying the tcdB gene were compared with the cell cytotoxicity assay as the 'gold standard' for diagnosis of Clostridium difficile-associated diarrhoea (CDAD). Faecal samples from patients with a request for C. difficile diagnosis and samples from patients with diarrhoea hospitalized for at least 72h were collected for three consecutive months from four university medical centres in the Netherlands. In total, 547 faecal samples were obtained from 450 patients. Of 540 samples available for all assays, 84 (15.6%) showed a positive result in one or more assays. The cell cytotoxicity assay was positive in 31 samples (5.7%) from 28 patients. A diagnosis of CDAD was not considered by the physician in 5 (23.8%) of 21 patients with CDAD who were hospitalized for at least 72h. Compared with the cell cytotoxicity assay, the sensitivity of VIDAS, PTAB and PCR was 83.9%, 96.8% and 87.1%, respectively. The specificity of VIDAS, PTAB and PCR was 97.1%, 94.3% and 96.5%, respectively. The positive and negative predictive values for VIDAS, PTAB, and PCR were 63.4% and 99.0%, 50.9% and 99.8%, and 60.0% and 99.2%, respectively. Of 61 samples positive in one, two or three assays, 56 were available for discordance analysis. The discordance analysis was performed by culture of toxinogenic strains. The concordance of VIDAS, PTAB and PCR with culture was 53.6% (30/56), 55.4% (31/56) and 71.4% (40/56), respectively. It was concluded that real-time PCR had the highest concordance with toxinogenic culture and is therefore the preferred method for diagnosing CDAD in faecal samples. It was also concluded that diagnosis of patients with diarrhoea whpo have been hospitalized for more than 72h should focus mainly on the detection of C. difficile, irrespective of the physician's request.

Introduction

Clostridium difficile is a Gram-positive, spore-forming rod that grows anaerobically. Strains of *C. difficile* that produce the toxins A (TcdA) and B (TcdB) are known to be involved as the causative agent of *C. difficile* -associated diarrhoea (CDAD) and pseudomembranous colitis (PMC). CDAD is an important nosocomial infection. Various predisposing factors for *C. difficile* infection have been recognised, such as antibiotic use, age, surgical procedures, tube feeding, hospitalization length, use of chemotherapeutic agents and use of acid-suppressive therapy (Brown *et al.*, 1990; Clabots *et al.*, 1992; Kelly & LaMont, 1998). *C. difficile* is usually diagnosed by a cell cytotoxicity assay or by specific culture of toxinogenic isolates. Due to their rapid turnaround time, enzyme immunoassays (EIA) for the detection of TcdA and/or TcdB have been implemented in most microbiological laboratories. As a result of the increasing incidence of strains producing only TcdB (van den Berg *et al.*, 2004; Drudy *et al.*, 2004), EIAs detecting both toxins are preferred. However, despite a turnaround time of >48h, the cell cytotoxicity assay is still considered the 'gold standard' (Johnson & Gerding, 1998; Oldfield, 2004).

The primary aim of this multicentre study was to compare four different diagnostic methods. The performances of the rapid EIA Premier Toxins A&B (PTAB), the VIDAS CDA2 (which detects only TcdA) and a rapid, in-house, realtime PCR assay to amplify the *tcd*B gene were evaluated in comparison with the conventional cell cytotoxicity assay. Discordant samples were analysed further by culture of toxinogenic strains. The second aim of this study was to evaluate the '3 day rule'. When patients hospitalized for at least 72h develop diarrhoea, the advice is to test faecal samples only for *C. difficile* and not for the presence of *Salmonella*, *Campylobacter, Shigella* or *Yersinia* spp. (Bowman *et al.*, 1992; Fan *et al.*, 1993; Siegel *et al.*, 1990; Yannelli *et al.*, 1988). We also tested this diagnostic algorithm in the current study.

Methods

Patient inclusion and faecal samples. Faecal samples from patients with a request for *C. difficile* diagnosis and samples from patients with diarrhoea who had been hospitalized at least 72h were collected for three consecutive months in the

period from October 2003 to February 2004 at the Departments of Medical Microbiology of four university medical centres in the Netherlands: Erasmus Medical Centre Rotterdam (Erasmus MC), Leiden University Medical Centre (LUMC), VU University Medical Centre Amsterdam (VUMC) and the University Medical Centre St. Radboud Nijmegen (UMC St Radboud). A computer algorithm was developed to recognize faecal samples from patients admitted at the hospital for at least 72h. All samples were stored within 6h after arrival at the laboratory at -20 °C in two individual vials. One vial was used in the respective hospitals for their diagnostic methods, and the second vial was used for subsequent testing in the reference centre at the LUMC. All faecal samples were thawed only once for a specific test.

Diagnostic assays. The enzyme-linked fluorescent assay VIDAS CDA2 (BioMérieux), the PTAB assay (Meridian), and an in-house real-time PCR for *tcd*B were used for diagnosing CDAD, and compared with the cell cytotoxicity assay, as the 'gold standard'. All hospitals performed the PTAB assay and conventional culture for *Salmonella*, *Shigella*, *Campylobacter* and *Yersinia* spp. on the faecal samples, whereas real-time PCR was performed only in the LUMC. Erasmus MC, VUMC and LUMC performed the VIDAS assay; the cell cytotoxicity assay was performed in the LUMC. UMC St Radboud performed the cell cytotoxicity assay and their samples were subsequently tested in the LUMC by the VIDAS assay. The VUMC cultured all their samples for the presence of *C. difficile*.

The cell cytotoxicity assay was performed at LUMC using Vero-cells in a 24-wells format. Faecal samples were diluted 1:4 in Eagle's minimum essential medium containing 5% fetal bovine serum and centrifuged. Subsequently, the supernatant was filtered through a 0.45 μ m pore-size filter. Neutralization of the cytotoxic effect was performed using specific *C. difficile* antitoxin (TechLab). At UMC St Radboud, the assay was performed using Vero-cells in a microwell format. Faecal samples were diluted (1:20 to 1:10240) after filtration through a 0.45 μ m pore-size filter. Neutralization was performed using *C. sordelli* antiserum (Techlab).

The VIDAS and PTAB assays were performed according to the instructions of the manufacturers. The interpretation of results of the PTAB assay was the same at Erasmus MC, VUMC and LUMC, with an optical density cut-off value of 1.00 using the spectrophotometric dual wavelength 450/630 nm. UMC St Radboud performed the interpretation visually, with a yellow colour indicating positive samples. Samples with equivocal results for the VIDAS assay (test value threshold \geq 0.40 to < 1.0) were retested with a VIDAS blocking test (VIDAS CDB) according to the manufacturer's recommendations.

For real-time PCR primers 398CLDs (5'-(5'-GAAAGTCCAAGTTTACGCTCAAT-3') and 399CLDas GCTGCACCTAAACTTACACCA-3') were designed to amplify 177 bp of the nonrepeat region of the tcdB gene (van den Berg et al., 2006). A specific 6carboxyfluorescein (FAM)-labelled Taqman-probe (5'-ACAGATGCAGCCAAAGTTGTTGAATT-3') was used as an internal probe. DNA isolation from faecal samples was performed using S.T.A.R.-buffer pre-treatment and subsequent automated isolation using a MagnaPure LC DNA isolation Kit III (Roche) in a MagnaPure System, according to the manufacturer's instructions. Phocid herpesvirus (PhHV) was included as an internal control for detection of inhibition in the PCR.

Discordance analysis. All samples positive for *C. difficile* in one or more assays were cultured for the presence of toxinogenic isolates. Culture was performed as described previously (van den Berg *et al.*, 2005a). Briefly, faecal samples were treated with an ethanol shock pre-treatment prior to inoculation onto Columbia agar containing colistin and nalidixic acid (CNA) and onto *C. difficile* selective agar with cefoxitin, amphotericinB and cycloserin (CLO; BioMérieux) and incubated in an anaerobic environment at 37°C for 2 days. CLO medium was also used to inoculate faecal samples that were not pre-treated with ethanol. DNA was isolated from Gram-positive rods with subterminal spores and a positive proline aminopeptidase reaction (Garcia *et al.*, 1997) using QiaAmp DNA isolation columns (Qiagen) according to the manufacturer's recommendations, including a 10 min incubation at 55°C with proteinase K (Qiagen). These isolated strains were subsequently tested by PCR for the presence of *tcd*A and *tcd*B, as described by Kato *et al.* (Kato *et al.*, 1999; Kato *et al.*, 1998).

Statistical analysis. The statistical software SPSS 11.0 was used. A Chi-square test and the *t*-test for independent samples were used to compare all characteristics between patients hospitalized for at least 72h with and without a request for CDAD diagnosis. The area under the receiver operating characteristic (ROC) curve, a measurement of the accuracy of a test independent of the cut-off values used, was also calculated using the statistical software.

Results

Patients. In total, 547 samples were included from 450 patients: 202 samples from 149 patients from Erasmus MC, 142 from 106 patients from LUMC, 116 from 116 patients from VUMC and 87 from 79 patients from UMC St Radboud. Of these 450 patients, 382 had only one sample, 45 had two samples and 23 patients had three or more samples included in this study.

Diagnostic assays. Only samples with results in all four assays were included in our analysis. Of the total of 547 faecal samples collected, 7 (1.3%) were excluded due to the absence of sufficient material for testing in all assays. Of the remaining 540 samples, 456 samples were negative in all assays. A total of 84 (15.6%) of the 540 samples were positive in one or more assays and 31 (5.7%) samples from 28 patients were positive by the cell cytotoxicity assay (Table 1). The highest percentage of positive cell cytotoxicity tests (9.4%) was found at LUMC, followed by UMC St Radboud (7.6%), EMCR (7.4%) and VUMC (3.4%). Using the cell cytotoxicity assay as the 'gold standard', the highest sensitivity was observed for the PTAB assay (96.8%), compared with 83.9% for the VIDAS assay and 87.1% for the real-time PCR assay. The specificity and positive predictive value (PPV) were comparable for both the VIDAS (97.1% and 63.4%) and real-time PCR (96.5% and 60%) assay and were slightly higher than for the PTAB assay (94.3% and 50.9%). The negative predictive value (NPV) was comparable for all three assays (99-100%; Table 1). Correlation of the VIDAS, PTAB and real-time PCR assays with the cell cytotoxicity assay was 96.3%, 94.4% and 95.9%, respectively. No significant differences in sensitivity, specificity, NPV and PPV for any of the assays were observed between Erasmus MC, VUMC and LUMC (data not shown), compared with Table 1. However, UMC St Radboud showed 100% specificity and PPV for the PTAB assay, with a sensitivity of 83%.

Assay	Result	No. of cell cytotox					_	
		Positive $(n = 31)$	Negative (<i>n</i> = 509)	Sensitivity (%)	Specificity (%)	(%) Vdd	NPV (%)	Correlation (%
VIDAS	Positive	26	15	83.9	97.1	63.4	99.0	96.3
	Negative	5	494	_				
DTAR	Positive	30	29	96.8	94.3	50.9	99.8	94.4
ITAD	Negative	1	480	_				
Real-time	Positive	27	18	87.1	96.5	60.0	99.2	95.9
PCR	Negative	4	491					

Table 1. Results of three different diagnostic assays for diagnosis of CDAD, compared to the cell cytotoxicity assay on 540 faecal samples. Only samples tested in all four assays were included.

The area under the ROC curve was 0.957 (SE=0.016) for VIDAS and 0.993 (SE=0.003) for PTAB compared to the cell cytotoxicity assay as 'gold standard'.

Discordance analysis. Discordance analysis was performed by culture for C. difficile of samples positive in one, two or three of the diagnostic assays. C. difficile isolates were subsequently tested for the presence of tcdA and tcdB to determine the capacity of the isolates to produce TcdA and TcdB. The results of the discordance analysis are presented in Table 2. A total of 56 out of 61 samples were available for specific culture of toxinogenic C. difficile; 22 of these showed a positive culture of a toxinogenic strain. Of 12 samples that were only positive by PCR, five were culture positive. The nine samples that were only positive by the VIDAS assay were culture negative. Of the 19 samples that were only positive by the PTAB assay, three were culture positive. One of these three samples was positive for a TcdA-/TcdB+ strain. The VIDAS assay showed a concordance with culture of 53.6% (30/56), and the PTAB assay had a concordance of 55.4% (31/56). The real-time PCR assay showed a higher concordance with culture of 71.4% (40/56), similar to the concordance of the cell cytotoxicity assay (75%, 42/56). The sensitivity of the cell cytotoxicity assay compared to toxinogenic culture in our discrepancy analysis was 36.4%, although the specificity was 100%.

No. of samples	Assay r	Culture positive			
<u>r</u>	VIDAS	PTAB	Real-time PCR	Cytotoxicity assay	F F
12	-	-	+	-	5
19	-	+	-	-	3 ^b
4	-	+	+	-	3
9	+	-	-	-	0
1	+	-	+	-	1
2	+	+	-	-	1
1	+	+	+	-	1
1	-	-	+	+	1
1	-	+	-	+	1
3	-	+	+	+	3
3	+	+	-	+	3
Total positive $(n = 56)$	<i>n</i> = 16	<i>n</i> = 33	<i>n</i> = 22	<i>n</i> = 8	<i>n</i> = 22

Table 2. Discordance analysis by culturing of toxinogenic *C. difficile*^a of 56 faecal samples positive in one to three of the assays.

^a The toxinogenicity of cultured *C. difficile* strains was determined by PCR for the presence of *tcd*A and *tcd*B. ^b One strain was TcdA-/TcdB+

Determining patient group. Of the 450 patients, 372 were hospitalized for at least 72h when they developed diarrhoea. Of these 372 patients, 251 had a request for *C. difficile* diagnosis, whereas 121 patients had no request (Table 3). The mean age for patients with a request was significantly higher than for the other patients (P=0.005, Table 3). Significant differences were observed for the departments of internal medicine, surgery, neurology and paediatrics between samples where a CDAD diagnosis was requested and for samples without such a request. No significant differences were observed for gender, the number of hospitalized days before onset of diarrhoea or patients with a previous episode of CDAD in the last three months. The data observed in the four participating centres did not differ significantly from each other (data not shown). For five (23.8%) of the 21 patients with diarrhoea and at least 72h of hospitalization who were positive by the cell cytotoxicity assay, the diagnosis CDAD was not considered by the physician. This distribution was not significantly different (Table 3).

	1				
No of patients	Total	CDAD requested	No request for	P-value*	
ito or patients	(n = 372)	(n = 251)	CDAD $(n = 121)$		
Mean age (SD)	52,5 (22.6)	54,8 (21.0)	47,7 (25.0)	0.005^{\dagger}	
Male gender (%)	179/371 48.2%)	128/250 (51.2%)	51/121 (42.1%)	≤ 0.20	
Department:					
Internal Medicine	210	158 (75.2%)	52 (24.8%)	≤ 0.001	
Surgery	52	26 (50%)	26 (50%)	≤ 0.01	
Paediatrics	28	14 (50%)	14 (50%)	≤ 0.05	
Neurology	9	3 (33.3%)	6 (66.6%)	≤ 0.05	
Intensive Care	38	29 (76.3%)	9 (23.7%)	≤ 1	
Other	35	21 (60%)	14 (40%)	≤ 1	
Days of admission before onset mean (SD)	15,5 (15.5)	15,7 (15.4)	14,8 (15.8)	0.603 [†]	
Previous CDAD in last 3 months	6/270 (2.2%)	6/191 (3.1%)	0/79 (0%)	\leq 0.20	
Number of positive cell cytotoxicity tests	21 (5.6%)	16 (6.4%)	5 (4.1%)	≤1	
Request for culture of other enteropathogens	177/370 (47.8%)	86/249 (34.5%)	91 (75.2%)	≤ 0.001	
Salmonella culture positive	0	0	0	1	
Shigella culture positive	1 (0.3%)	0	1 (0.8%)	≤ 0.20	
Campylobacter culture positive	0	0	0	1	
Yersinia culture positive	0	0	0	1	

Table 3. Characteristics of patients (n=372) with diarrhoea, hospitalized for at least 72h, included in this study.

* Analysed by Chi-square test, unless noted otherwise

† Analysed by t-test with independent samples

Discussion

This present study was undertaken to ascertain the diagnostic values of four different assays for the diagnosis of CDAD and to investigate whether patients with diarrhoea hospitalized for at least 72h should be investigated for CDAD, irrespective of the physician's request.

A total of 84 (15.6%) of 540 samples were positive in one or more assays and 31 (5.7%) samples of 28 patients were positive by the cell cytotoxicity assay. Using the cell cytotoxicity assay as the 'gold standard', the PTAB assay showed the highest sensitivity (96.8%), although the positive predictive value (50.9%) was about 10% lower than for real-time PCR and VIDAS assays. Turgeon et al., (Turgeon et al., 2003) compared six different immunoassays to the cell cytotoxicity assay and found PPVs for PTAB and VIDAS of 90.2% and 87.7%, respectively. These data contrast with our results, but may be due to differences in the design of the study. The low PPV in all tests, compared with the cell cytotoxicity assay, probably reflects the low sensitivity of the cell cytotoxicity assay. An explanation could be the storage procedures used in this study. Turgeon tested all faecal samples for cytotoxicity within 24 hours of receipt, whereas the multicentre approach of our study did not allow such a procedure. Faecal samples included in our study were stored within 6 hours after arrival at the laboratories and were thawn once. It has been demonstrated that storage at -20° C and repeat freezing and thawing will decrease the cytotoxic activity of faecal samples containing TcdB of Clostridium difficile, although this has only been tested with artificially contaminated faecal samples (Freeman & Wilcox, 2003). Another study that evaluated the VIDAS assay for the diagnosis of CDAD tested a total of 38 consecutive cell cytotoxicity positive samples and 33 negative samples (Lipson et al., 2003). The authors also applied a discordance analysis by toxinogenic culture and found sensitivity, specificity, PPV and NPV for the VIDAS assay of 80.6, 96.8, 96.7, and 81.1%, respectively. The concordance with culture was 83%. Although the VIDAS assay displayed a reduced sensitivity compared with the cytotoxicity test, the authors recommended the VIDAS assay because of the rapid results. In contrast, we found that the VIDAS assay had a concordance with culture of only 53.6% and we therefore prefer the PTAB assay or the real-time PCR assays as a rapid diagnostic test. O'Connor et al. (O'Connor et al., 2001) compared four rapid immunoassays (Oxoid Toxin A test, ImmunoCard Toxin A test, Techlab Toxin A/B II

test, and the PTAB assay) with toxinogenic culture and the cell cytotoxicity assay. When the final diagnosis of CDAD based on clinical criteria was taken as gold standard, the cell cytotoxicity assay had the highest sensitivity (98%) and specificity (99%), whereas the sensitivity and specificity of the Techlab and PTAB assays were, respectively, 79% and 98% for the Techlab test, and 80% and 98% for the PTAB. Using the cytotoxicity assay as the gold standard, the PTAB test had the best performance. This result is in agreement with our observation, except for the PPV of the PTAB as elucidated above. In contrast to the results of O'Connor et al., we did not perform a retrospective chart review for patients with a positive test for CDAD. All faecal samples submitted to the laboratories participating in our study were derived from patients with diarrhoea. It is therefore impossible to rule out a positive diagnostic test as false positive. A second discrepancy of our results with the results obtained in the study of O'Connor et al., was the low sensitivity (57%) of culture method; however, the authors mentioned a number of factors that may have contributed to the relatively poor performance of culture. There are reports indicating that lysozyme incorporation into culture media enhance germination of C. difficile spores (Verity et al., 2001; Wilcox et al., 2000). Although this results in an increase of isolation of C. difficile from the environment, it is unlikely that more patients will be diagnosed, as vegetative cells are in the majority in faecal specimens. The application of enrichment media for culturing C. difficile from faecal samples is considered unnecessary for the diagnosis of CDAD (Brazier, 1998).

The performance of the diagnostic tests for CDAD for the individual laboratories did not differ with the overall performance of the assays, except for the PTAB. At UMC St Radboud, the PPV for the PTAB assay was 100% compared with 55, 63 and 40% at LUMC, VUMC and Erasmus MC, respectively. UMC St Radboud used visual interpretation of results and all other hospitals applied the procedure as recommended by the manufacturer and used an EIA reader at 450 nm and 630 nm. From this, we conclude that the cut-off values of the PTAB assay need a re-evaluation to improve the PPV and NPV.

In some cases, toxinogenic culture is used as the 'gold standard', instead of the cell cytotoxicity assay (Delmee *et al.*, 2005; Zheng *et al.*, 2004). Therefore, toxinogenic culture was used for discordance analysis on all faecal samples positive in one to three assays. The sensitivity of the real-time PCR was 87.1% and this assay

showed good concordance (71.4%) with toxinogenic culture. In contrast, the PTAB assay had a concordance of 55.4% with culture. Interestingly, all samples positive by the cell cytotoxicity assay were positive by culture and at least one other assay.

A remarkable finding of our study is the high number of samples positive by an immunoassay and negative by cytotoxicity, culture and real-time PCR. Of 19 samples positive only using the PTAB assay in the discordance analysis, 16 samples were negative by toxinogenic culture. Additionally, 9 samples positive only by the VIDAS assay were negative by culture. The results of these immunoassays were therefore considered to be false positive and to contribute to low PPVs. As mentioned previously, another factor responsible for the low PPV of the immunoassays could be the cut-off values used. Compared with the cell cytotoxicity assay, the area under the ROC curve was very good for the VIDAS assay (0.957) and excellent for the PTAB assay (0.993). This means that both tests, independent of the cut-off values used, correlated very well with the 'gold standard'. However, raising the cut-off values to increase the specificity would result in an unacceptable decrease in sensitivity, and would therefore not be helpful to increase the PPV.

For 68 (15.1%) of the 450 patients, more than one sample was tested in this study. Of these 68 patients, 45 had two samples and 23 patients had three or more samples included. In total, 97 of 540 samples (18%) were repeat samples. This is considerably lower than the findings of Renshaw *et al.* and O'Connor *et al.* who observed 36% and 34% repeat samples, respectively. In two cases, a negative cell cytotoxicity assay was followed by a positive result, and in two other cases, a negative result followed a positive one. For these four cases, the switch was detected by all four assays. The time between these switching results was 9 to 17 days. It is therefore appropriate to reject repeat specimens from patients who have already been tested on a recent specimen within a 7 day time frame (O'Connor *et al.*, 2001; Renshaw *et al.*, 1996).

In this study, one faecal sample contained a *C. difficile* strain that lacked part of the toxin A gene (TcdA-) but contained the gene for toxin B (TcdB+). All other cultured isolates from faecal samples were TcdA+/TcdB+. The faecal sample containing the TcdA-/TcdB+ strain was only positive by the PTAB assay and by culture of toxinogenic strains. As the VIDAS only detects TcdA, this result is explainable. The cell cytotoxicity assay and the real-time PCR should have been able to detect this strain, but the load of this bacterium was apparently under the detection level of the cell cytotoxicity and PCR assays. An increasing number of reports mention TcdA-/TcdB+ strains associated with diarrhoea (van den Berg *et al.*, 2004). In some hospitals, TcdA-/TcdB+ strains have completely replaced other types and are now the most prevalent strain (Drudy *et al.*, 2004). Of all *C. difficile* isolates in an Argentinean hospital, the percentage TcdA-/TcdB+ isolates increased from 12.5% in 2000, 58.1% in 2001, 87.9% in 2002 to 96% in 2003 (van den Berg *et al.*, 2005b).

Of 251 patients with diarrhoea and hospitalized for at least 72h, and with a request for CDAD diagnosis, 6.4% had a positive cell cytotoxicity assay. Of the 121 patients without such a request, 4.1% was positive. This indicates that routine testing for CDAD in patients with diarrhoea hospitalized for at least 72h will greatly improve diagnosis of CDAD. In 75.2% of patients at the department of internal medicine with diarrhoea, the physician requested a diagnosis of CDAD. Further comparison of the two groups revealed that physicians from the departments of surgery, neurology and paediatrics considered CDAD less frequently in patients with diarrhoea hospitalized for at least 72h than diarrhoea due to common community-acquired enteropathogens. This is not unexpected, but emphasizes that more education should be given to physicians of these departments to recognize CDAD in order to treat and prevent spread of C. difficile. Only 1 (0.8%) of the 121 patients without a request for CDAD diagnosis, was positive for Shigella spp. This patient was admitted with diarrhoea and the diagnosis of shigellosis was made on a faecal sample submitted on the day of admission. In conclusion, our observation strengthens the suggestion that samples from patients hospitalized for at least 72h should not be routinely cultured for Salmonella, Shigella or Campylobacter spp. unless there are specific indications (Bowman et al., 1992; Fan et al., 1993; Siegel et al., 1990; Yannelli et al., 1988). Two patients with a request for CDAD were positive for Campylobacter spp. They both had a request for culture of the other enteropathogens as well and had been admitted to the hospital for less than 72h. This also strengthens the suggestions made. Implementation of this rule in the hospitals will significantly decrease the costs of culturing faecal samples for community-acquired pathogens.

Comparing the different rapid assays in this study, PTAB shows the highest sensitivity and NPV, whilst the real-time PCR assay shows the highest concordance with toxinogenic culture in the discordance analysis. The VIDAS assay was
outperformed by both PTAB and real-time PCR. Due to the long turnaround time of the cell cytotoxicity assay or toxinogenic culture, rapid assays are advisable in addition. We conclude that real-time PCR is the preferred rapid method for diagnosing CDAD in faecal samples. Additionally, pre-screening using the PTAB method is suggested. Diagnosis of patients with diarrhoea who are hospitalized for more than 72h should be focused mainly on *C. difficile* detection, irrespective of the physician's request.

Acknowledgements

We thank Hendrik-Jan Gerritsen, Hadi Ameen, Irma Schouten, H.W. Weverink, and A.C. Hofman-Langerak for their technical support. This work was supported by a grant from the Foundation Microbiology Leiden.

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Chapter 5

Characterization of toxin A-negative, toxin B-positive *Clostridium difficile* isolates from outbreaks in different countries by Amplified Fragment Length Polymorphism and PCR-ribotyping

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Journal of Clinical Microbiology, 42: 1035-1041. 2003

Abstract

Clinical C. difficile isolates of patients with diarrhea or pseudomembranous colitis usually produce both toxin A and toxin B, but an increasing number of reports mention infections due to toxin A-negative, toxin B-positive (A-/B+) strains. Thirtynine clinical toxin A-/B+ isolates and 12 other unrelated isolates were obtained from Canada, United States, Poland, United Kingdom, France, Japan and the Netherlands. The isolates were investigated by high resolution genetic fingerprinting by use of amplified fragment length polymorphism (AFLP) and two well-described PCR ribotyping methods. Furthermore, the toxin profile and clindamycin resistance were determined. Reference strains of C. difficile representing 30 known serogroups were also included in the analysis. AFLP discriminated 29 types among the reference strains whereas the two PCR ribotyping methods distinguished 25 and 26 types. The discriminatory power of AFLP and PCR ribotyping among 12 different unrelated isolates was similar. Typing of 39 toxin A-/B+ isolates revealed 2 AFLP types and 2 and 3 PCR ribotypes. Of 39 toxin A-/B+ isolates, 37 had PCR ribotype 017/20 and AFLP type 20 (95%). A deletion of 1.8 kb was seen in 38 isolates and 1 had a deletion of approximately 1.7 kb in the tcdA gene, which encodes toxin A. Clindamycin resistance encoded by the erm(B) gene was found in 33 out of 39 toxin A-/B+ isolates, and in 2 of the 12 unrelated isolates (p<0.001, chi-square test). We conclude that clindamycin resistant C. difficile toxin A-/B+ strain (PCR-ribotype 017/20, AFLP type 20, serogroup F) has a clonal worldwide spread.

Introduction

Clostridium difficile has been recognized as a cause of nosocomial diarrhea and pseudomembranous colitis. The enteropathogenicity is associated with the production of enterotoxin A (308 kDa) and cytotoxin B (270 kDa)(6,14). Toxin A is an extremely potent enterotoxin and has been regarded as the primary virulence factor (27,30). The effect of cytotoxin B depends on the tissue damage caused by toxin A, suggesting that both toxins work synergistically (31).

Toxin A (*tcd*A) and B (*tcd*B) genes are located on a pathogenicity island of 19.6 kb also encompassing 3 other small open reading frames (20). Nontoxigenic, and therefore non-pathogenic, strains of *C. difficile* contain a 127 bp sequence at this locus (19). The sequence similarity and the position on the island suggest that the *tcd*A and *tcd*B genes are the result of gene duplication (15).

Clinical isolates from patients with nosocomial diarrhea or pseudomembranous colitis usually produce both toxin A and B, but an increasing number of reports mention severe infections and outbreaks due to toxin A-negative, toxin B-positive (A-/B+) strains (2,3,21,24,26,28). Two types of toxin A-/B+ strains have been identified. The first type is characterized by a large deletion of 5.6 kb in the toxin A gene. The representative strain (8864) causes fluid secretion in rabbit intestinal loops and it has been suggested that the production of a variant toxin is associated with its enteropathogenicity (8,29). This variant toxin seems more potent than toxin B and is more similar to C. sordellii lethal toxin (37). The second type is more frequently isolated from human fecal samples and contains a small deletion of 1.8 kb within the repetitive regions of the *tcdA* gene, and belongs to serogroup F (13).

PCR ribotyping has appeared to be a robust genotyping method. Results can be used for interlab comparison and generation of libraries. However, different primers have been proposed, which raises the question of which are most suited for future studies on the epidemiology of *C. difficile*. The PCR ribotyping by O'Neill as described by Stubbs *et al.* contains a large library which is used worldwide (38). This method has been modified with primers presumed to be more specific for *C. difficile* by Bidet *et al.*(7). Pulsed field gel electrophoresis (PFGE) is considered the 'golden standard' for genotyping, but due to intensive DNA degradation in some strains, other techniques are preferred. Amplified fragment length polymorphism (AFLP) has been applied for molecular typing of a variety of bacterial species (1). Recently, AFLP analysis of *C. difficile* strains showed that its discriminatory power was similar to that of PFGE when tested on 30 clinical isolates. However, reference strains encompassing different reference isolates and toxin A-/B+ strains were not included in the analysis (25). Therefore, in the present study we compared AFLP with two different PCR ribotyping methods. Reference strains of *C. difficile* were included, as were clinical isolates obtained from 7 different countries, with special attention to toxin A-/B+ isolates. Additionally, all strains were characterized for the profiles of the *tcd*A and *tcd*B genes and for clindamycin resistance.

Material and methods

Bacterial strains and culture conditions. Reference strains of *C*. *difficile* encompassing 30 known serogroups were included in this study as control strains: A, A1-A11, A13-A17, B, C, E6, F, G, H, I, K, S1-S4 and X (supplied by M. Delmee, University of Louvain, Brussels, Belgium). Clinical isolates (n=50) were obtained from 7 different countries (Table 1). The biochemical identification of the strains was confirmed on the basis of the morphology in Gram-staining, growth on CDMN agar (*C. difficile* agar with moxalactam, norfloxacin and cystein) (4) and a positive aminopeptidase reaction (18). Strains were stored at -80°C in glycerol broth and subcultured onto sheep-blood agar medium in an anaerobic atmosphere for usage for 48h.

DNA isolation. DNA was extracted from bacterial cultures on solid media using the QIAamp DNA isolation columns (Qiagen, Hilden, Germany) according to the manufacturer's recommendations, including a preceding 10-min incubation at 55°C with proteinase K (Qiagen). The final volume of the DNA extracts was 200 µl.

PCR ribotyping. (i) O'Neill method. The method described by Stubbs *et al.* (38) was followed. The primers used (PRO primers) for amplification are specified in Table 2. Briefly, amplification reactions were performed in 100 μ l final volume, containing 50 pmol of each primer, 2U of Taq DNA polymerase (Pharmacia), and 2.25 mM MgCl2, and 10 μ l of DNA. The final products were separated by electrophoresis on 3% Metaphor agarose (FMC Bioproducts, Rockland, Maine) for 3 hours at 200V. Amplified fragments were visualized by staining the gel for 20 minutes in a 1 μ g/ml ethidium bromide solution. For normalization a molecular size standard (100 bp; Advanced Biotechnologies, Epsom, United Kingdom) was added every five lanes.

Country of origin	Submitting laboratory	No. of isolates	Outbreak isolates	Reference	
Netherlands	Academic Medical Center, Amsterdam	nter, $4 \begin{array}{c} 19 \\ (19) \end{array}$		Kuijper et al.(24)	
	Leiden University Medical Center, Leiden	8	No	-	
Poland	Medical University of Warsaw, Warsaw	12	No	Pituch et al. (31)	
France	Centre Hospitalo-Universitaire Saint-Antoine, Paris	8	No	Barbut <i>et al.</i> (5)	
USA	Northwestern University, Chicago	2	No	Johnson et al.(19)	
Canada	University of Manitoba, Winnipeg	3	Yes (1998)	Alfa <i>et al.</i> (2)	
United Kingdom	University Hospital of Wales, Cardiff	10	No	Brazier et al.(9)	
Japan	Gifu University School of Medicine, Gifu	3	No	Kato et al.(22)	

Table 1. Source of clinical isolates used in this study

(*ii*) *Bidet method.* The method as described by Bidet *et al.* (7) was followed. Primers used for amplification (PRB primers) are specified in Table 2. Briefly, the amplification reactions were performed in a 50 μ l final volume, containing 25 μ l HotStar *Taq* Mastermix (Qiagen), 10 pmol of each primer, and 5 μ l of DNA. After an initial enzyme activation step of 15 min at 95°C, the protocol consisted of 35 cycles of 1 min at 94°C for denaturation, 1 min at 57°C for annealing, and 1 min at 72°C for elongation. The amplified products were separated by electrophoresis on 2% low melting point agarose for 3 hours at 85 V.

AFLP. The conditions for AFLP were as previously described by Klaassen *et al.* (25). Enzymes EcoRI and MseI were used for restriction. For amplification the labeled EcoRI-primer with 6-carboxy-fluorescein was used, and for selective amplification the MseI-primer contained a G residue as the selective nucleotide.

Analysis of fingerprints. The results of fingerprinting by the three genotyping methods were stored as tagged image file format files and imported into the BioNumerics software (Applied Maths, Kortrijk, Belgium) for further analysis, with the Pearson product moment correlation coefficient and the unweighted pair group method with arithmetic mean used for clustering. The clustering level of two duplicates was used to delineate different types.

Table 2. Primersequences of oligonucleotides used for PCR ribotyping and conventional PCR in this study

Primer	Nucleotide sequence (5'-3')	Gene	Fragment length (bp)	
	CTGGGGTGAAGTCGTAACAAGG		variable	
TKO	GCGCCCTTTGTAGCTTGACC	115	variable	
DDBp	GTGCGGCTGGATCACCTCCT		variable	
IKD	CCCTGCACCCTTAATAACTTGACC	115	variable	
282BacS	GAAAARGTACTCAACCAAATA	armB	630	
283BacAS	AGTAACGGTACTTAAATTGTTTAC	ermb	039	
NKV011	TTTTGATCCTATAGAATCTAACTTAGTAAC	tedA	2535	
NK9	CCACCAGCTGCAGCCATA	icuA	2333	
NK104	GTGTAGCAATGAAAGTCCAAGTTTACGC	tedB	204	
NK105	CACTTAGCTCTTTGATTGCTGCACCT	псив	204	

^a PRO, PCR ribotyping by O'Neill.

^b PRB, PCR ribotyping by Bidet.

° ITS, internal spacer region.

Genetic identification of clindamycin resistance. Clindamycin resistance was tested by a PCR targetting the erm(B) gene, which codes for the macrolide-lincosamide- streptogramin B (MLS) resistance, as described previously by Sutcliffe *et al.* (39) for several pathogenic bacterial species. The primers used (282BacS and 283BacAS) (Table 2) also reacted with *C. difficile* according to an alignment in BLAST. The sequence of *C. difficile erm*(B) is more than 97% identical with *erm*(B) genes from other bacterial species (16). Clindamycin-resistant strains were defined as strains with a 639bp amplicon size.

Genetic identification of *tcdA* and *tcdB* profile. All strains were tested for the presence of genes *tcdA* and *tcdB*. For the detection of the *tcdA* gene, primers NKV011 and NK9 (Table 2) were used as described by Kato *et al.* (23). Toxin A-positive strains showed a 2535 bp amplicon size, whereas toxin A-negative strains were defined as strains with a deletion in the *tcdA* gene of 1.8 kb. The *tcdB* profile was verified using primers NK104 and NK105 (Table 2) as described before (24). The presence of a 204 bp fragment was considered as indicative for presence of

the *tcd*B gene. The amplified products were analyzed by separation by agarose gelelectrophoresis. Isolates with the 1.8 kb deletion in the *tcd*A gene and with a *tcd*B positive PCR were included in the toxin A-/B+ group. Isolates with other toxin profiles were included in the so-called 'unrelated' group, since no relation with the toxin A negative isolates existed.

Results

C. difficile reference strains. Twenty-one of 30 reference strains were positive for the *tcd*A and *tcd*B genes (Table 3). Strains of serogroups A7, A9, A10, A11, B, I and X did not contain genes for the toxins, whereas those of serogroups F and S3 harboured only the *tcd*B gene and a variant *tcd*A gene. The reference strain of serogroup F had the 1.8 kb deletion in the *tcd*A amplicon, where the strain of serogroup S3 had a deletion of approximately 0.8 kb.

By AFLP, 29 types were distinguished among the 30 reference strains (Fig.1; Table 3). Strains representing serogroups A7 and A11 were indistinguishable by AFLP. PCR ribotyping by Bidet distinguished 25 genotypes in this group. Strains of serogroups A9 and A10 and of serogroups A8 and S1 were indistinguishable by this PCR ribotyping, but could be differentiated by PCR ribotyping by the O'Neill method. The latter PCR ribotyping was able to identify 26 genotypes among the reference strains. Both PCR ribotyping techniques were not able to separate the reference strain of serogroup H from that of serogroup K and the strain of serogroup A14 from that of serogroup S4 (Table 3). As was the case with AFLP, strains of serogroup A7 and A11 could not be differentiated by the two PCR ribotyping methods.

Clinical isolates of *Clostridium difficile*. In the group of the 50 clinical isolates, one sample (R11092) contained two variant strains. One variant was positive for the tcdA gene and the second strain contained the 1.8 kb deletion. Both strains were used for analysis. A total of 39 isolates contained the deletion of 1.8 kb in the tcdA gene and were positive for the tcdB gene (Table 4). These strains were included in the toxin A-/B+ group. The remaining 12 isolates were included in the unrelated group. Of the unrelated 12 isolates, 9 were toxin A+/B+. Strain Ned1 was the only toxin A-/B- strain, strain 98-15845 had an amplicon size of approximately

1.9 kb, and strain 98-15323 showed an amplicon size of approximately 2.7 kb for the *tcd*A gene.

Table 3. Comparison of AFLP with PCR ribotyping according to Bidet and O'Neill on 30 reference strains of *C. difficile*

Ca	AFLP	PRB ^b	PRO ^c	tcdA	tcdB
Serogroup	type	type	type	deletion	profile ^d
А	1	1	ND^e	None	+
A1	2	2	005	None	+
A2	3	3	002	None	+
A3	4	4	026	None	+
A4	5	5	015	None	+
A5	6	6	063	None	+
A6	7	7	064	None	+
A7	8	8	035	2.5 kb	-
A8	9a	9	018	None	+
A9	10	10	039	2.5 kb	-
A10	11	10	067	2.5 kb	-
A11	8	8	035	2.5 kb	-
A13	12	12	049	None	+
A14	13	13	103	None	+
A15	14	14	045	None	+
A16	15	15	019	None	+
A17	16	16	153	None	+
В	17	17	060	2.5 kb	-
С	18	18	012	None	+
E6	19	19	046	None	+
F	20	20	017	1.8 kb	+
G	21a	21	001	None	+
Н	22	22	014	None	+
Ι	23	23	009	2.5 kb	-
K	24	22	014	None	+
S1	25	9	056	None	+
S2	26	26	050	None	+
S3	27	27	110	0.8 kb	+
S4	28	13	103	None	+
Х	29	29	085	2.5 kb	-

^a Designation of reference strains according to Delmee *et al.*

^b PRB, PCR ribotyping according to Bidet.

° PRO, PCR ribotyping according to O'Neill.

^d PCR for detection of the *tcd*B gene.

e ND, not done.

Figure 1. AFLP on 30 reference strains after BioNumerics analysis. Designations of the strains are located at the right of the lanes. The clustering level of duplicates R7981-1 and -2 was used to delineate different types (see black line).



Nine genotypes were differentiated among the 12 unrelated isolates by PCR ribotyping of Bidet, while the PCR ribotyping of O'Neill discriminated 8 genotypes (Table 4). With AFLP, the same nine genotypes were distinguished as with Bidet's method. In contrast with AFLP and PCR ribotyping of Bidet, PCR ribotyping of O'Neill did not discriminate between isolates Ned3 and 98-15323.

AFLP recognized 2 genotypes among 39 toxin A-/B+ isolates. The PCR ribotyping methods of Bidet and O'Neill differentiated 2 and 3 genotypes among toxin A-/B+ isolates, respectively. Isolate R10542 was recognized as a unique type by PCR ribotyping of O'Neill, but not by PCR ribotyping of Bidet and AFLP. The 2 other genotypes found were comparable to isolate 8864 and the serogroup F-like

strains. The toxin A-/B+ isolates from the Netherlands, USA, Canada, Poland and Japan belonged all to one genotype irrespective of the method used. The French isolates harboured 2 genotypes according to all 3 methods, and those from Wales 2 types according to the O'Neill method, but the AFLP method and the Bidet method could not distinguish between these two.

Of three erm(B) positive strains also tested in our study, the presence of the erm(B) gene resulted in a clindamycin MIC of $\geq 256 \mu g/ml$ by the E-test (AB Biodisk, Sweden) (26). Clindamycin resistance was found in 33 of 39 toxin A-/B+ isolates, and in 2 of the 12 unrelated isolates (p<0.001, chi-square test) (Table 4).

Discussion

In this study, the discriminatory power and typeability of AFLP and two different PCR ribotyping methods were compared using 30 reference strains and 51 clinical isolates of *C. difficile*. The strains were also characterized for their toxin profile and susceptibility to clindamycin. The AFLP had the highest discriminatory power for differentiation of the reference strains. Concerning the 39 toxin A-/B+ isolates and 12 unrelated strains, the typeability was 100% for all 3 genotyping methods with a similar discriminatory power.

The AFLP method uses restriction, ligation and selective amplification on the whole genome. Differentiation can be made due to variation per type in restriction sites mutations, mutations in the sequences adjacent to the restriction sites and complementary to the selective primer extensions, and insertions and deletions within the amplified fragments. AFLP for *C. difficile* has been compared with PFGE by Klaassen *et al.* (25). Previously, PFGE was found to be more discriminatory than random amplified polymorphic DNA (RAPD) and PCR ribotyping (41). The study by Klaassen also showed that the typeability of AFLP was better than PFGE, especially for *C. difficile* isolates for which PFGE showed DNA degradation. In addition, AFLP was considered faster and easy to perform on small quantities of DNA. The reproducibility of AFLP was found similar to PFGE (25), which has a much higher reproducibility than restriction enzyme analysis (REA), arbitrary primed PCR (AP-PCR) and RAPD (10). PFGE data are readily exchangeable between laboratories and

Table 4. Comparison of AFLP with PCR ribotyping according to Bidet and to O'Neill on 39 toxin A-/B+ isolates of *Clostridium difficile* and on 12 unrelated isolates.

Toxin profile	Isolate	Submitting	AFLP	PRB	PRO type	tcdA dalation	tcdB	ermB
T 1 (D)	designation	laboratory	type	type	017	deletion	prome	prome
Ioxin A-/B+	Canl	Winnipeg	20	20	017	1.8 kb	+	+
	Can3	Winnipeg	20	20	017	1.8 KD	+	-
	Can5	Winnipeg	20	20	017	1.8 kb	+	+
	60	Paris	20	20	017	1.8 kb	+	+
	8864	Paris	30	30	036	1.7 kb	+	-
	98-15632	Paris	20	20	017	1.8 kb	+	+
	98-16948	Paris	20	20	017	1.8 kb	+	+
	98-4318	Paris	20	20	017	1.8 kb	+	+
	99-3050	Paris	20	20	017	1.8 kb	+	+
	Jap1	Gifu	20	20	017	1.8 kb	+	+
	Jap2	Gifu	20	20	017	1.8 kb	+	-
	Jap3	Gifu	20	20	017	1.8 kb	+	+
	CD15	Amsterdam	20	20	017	1.8 kb	+	+
	CD16	Amsterdam	20	20	017	1.8 kb	+	+
	CD17	Amsterdam	20	20	017	1.8 kb	+	+
	Ned6	Leiden	20	20	017	1.8 kb	+	+
	Ned7	Leiden	20	20	017	1.8 kb	+	-
	1110/98	Warsaw	20	20	017	1.8 kb	+	-
	1745/00	Warsaw	20	20	017	1.8 kb	+	+
	205/99	Warsaw	20	20	017	1.8 kb	+	+
	2233/98	Warsaw	20	20	017	1.8 kb	+	+
	2428/95	Warsaw	20	20	017	1.8 kb	_	
	2420/95	Warsaw	20	20	017	1.0 KU	+ +	- T
	2785/07	Warsow	20	20	017	1.0 KU		т -
	2887/07	Warsaw	20	20	017	1.0 KU	+	+
	200/08	Warsaw	20	20	017	1.0 KU	+	+
	599/98	Warsaw	20	20	017	1.0 KD	+	+
	592/98	Warsaw	20	20	017	1.8 KD	+	+
	P250/00	warsaw	20	20	017	1.8 KD	+	+
	P268/00	Warsaw	20	20	017	1.8 kb	+	+
	CF2	Chicago	20	20	017	1.8 kb	+	-
	CF4	Chicago	20	20	017	1.8 kb	+	+
	R10205	Cardiff	20	20	017	1.8 kb	+	+
	R10430	Cardiff	20	20	017	1.8 kb	+	+
	R10542	Cardiff	20	20	047	1.8 kb	+	+
	R11092-2	Cardiff	20b	20	017	1.8 kb	+	+
	R12878	Cardiff	20	20	017	1.8 kb	+	+
	R13044	Cardiff	20	20	017	1.8 kb	+	+
	R13167	Cardiff	20	20	017	1.8 kb	+	+
	R2140	Cardiff	20	20	017	1.8 kb	+	+
Toxin A+/B+	98-15323	Paris	32	32	014	-0.2 kb	+	-
	98-15845	Paris	31	31	045	0.6 kb	+	-
	CD21	Amsterdam	33	33	023	None	+	-
	Ned2	Leiden	34	34	005	None	+	-
	Ned3	Leiden	24	22	014	None	+	-
	Ned4	Leiden	9b	9	018	None	+	+
	Ned5	Leiden	9h	9	018	None	+	+
	Ned8	Leiden	21b	21	001	None	+	
	R11002.1	Cardiff	210	21	110	None	т 	-
	R7771	Cardiff	27	27	110	None	т	-
	R7081	Cardiff	27	27	110	None	т	-
Toxin A /P	No.41	Laidan	25	25	0.005	2.5.14	Ŧ	-
10AIII A-/D-	mean	Leiden		33	085	∠.J KD	-	-

^a PCR for detection of the *erm*B gene.

it might be expected that future standardization of techniques for AFLP will also allow interlaboratory comparisons (40).

PCR ribotyping is based on the amplification of the intergenic spacer region between the 16S and the 23S rRNA genes. Every bacterial strain contains several rRNA operons, and there is a strain-dependent variation in the size and number of the 16S-23S intergenic spacer regions. Variation in spacer length is also observed between different copies of the operons in the same genome. Amplification of these regions results in a variety of PCR products whose size and number will vary amongst different strains, which enables differentiation of these strains. The PCR ribotyping by O'Neill and Stubbs was applied on 2030 strains, including 1631 clinical isolates and 133 reference strains, and differentiated 116 genotypes (38). Nineteen serogroups were tested and yielded different banding patterns. This method has been modified with different primers specific for C. difficile by Bidet et al. (7). The latter method tested and discriminated 20 serogroups, but was not tested for further discrimination of different strains. Although this PCR ribotyping is a rapid method, there is not yet a large library as is the case with the O'Neill method which is used worldwide. In our study, the discriminative power of the PCR ribotyping of O'Neill on reference strains was higher than the method of Bidet; the main difference was the ability of O'Neill's method to differentiate between strains representing serogroups A9 and A10 and A8 and S1.

The toxin profile of the reference strains of the present study was identical to that of previous studies (12,34), except for 3 reference strains (A9, A11 and S3). No background information was found on the toxin profile of reference strains A17 and S2. Reference strains A9 and A11 were found negative for the *tcd*A and *tcd*B genes, although the presence of both genes has been reported by Rupnik *et al.* (34). The deletion of approximately 0.8 kb in the *tcd*A amplicon of the reference strain for serogroup S3 was in contrast with their results as well. In their study, strains from one serogroup sometimes belonged to 2 or more different toxinotypes. Our discrepant results obtained with these 3 isolates may be explained by lack of an association of serotyping with toxinotyping. Genotyping of the reference strains showed a higher discriminatory power for AFLP than for both PCR ribotyping methods. The AFLP method was not able to differentiate reference strains A7 from A11, whereas the two

PCR ribotyping methods were not able to differentiate between these and other reference strains.

The findings on the toxin profiles of the clinical isolates in our study were in agreement with previously described results (2,3,5,21,24,26,33). Two French isolates had variant *tcd*A genes: 98-15323 and 98-15845. Both isolates exhibited a positive result for toxin A detection by EIA, according to the original observation. Based on the original observation, isolate 98-15323 belonged to serogroup H and had an insertion of 200 bp in the amplified fragment using primers NK11 and NK9, whereas isolate 98-15845 was found to have a 600 bp deletion(5). Two variants of *C. difficile* strain R11092 were isolated from the stored culture. The two variants differed in PCR ribotype and AFLP pattern, and therefore were probably derived from a mixed infection.

For 12 unrelated isolates, the PCR ribotyping by Bidet and AFLP were able to discriminate nine types, whereas the PCR ribotyping by O'Neill could not discriminate isolate Ned3 (serogroup K) from 98-15323 (serogroup H). PCR ribotyping is unable to discriminate between these two serogroups.

No major difference in discriminatory power of the 3 genotyping techniques was observed for toxin A-/B+ isolates. Since a large number of these isolates were apparently clonal related, the discriminatory properties of the various typing methods could not be evaluated using these isolates. Both AFLP and PCR ribotyping by Bidet distinguished 2 types and PCR ribotyping by O'Neill 3 types. Remarkably, isolate R10542 was recognized as a unique type by PCR ribotyping of O'Neill, but not by PCR ribotyping of Bidet and AFLP. Isolate R10542 was isolated from a patient in Birmingham, UK, and showed no differences to other toxin A-/B+ isolates in toxin profile or toxinotype (38). Of special interest is the occurrence of one type among 12 Polish toxin A-/B+ isolates. The incidence of toxin A-/B+ strains in Poland has been reported as high as 11% among 159 C. difficile strains isolated from patients with antibiotic-associated diarrhea (33). Molecular typing of these isolates by PCR ribotyping revealed that 8 of 17 toxin A-/B+ strains had distinct patterns. The 9 others belonged to one PCR ribotype and were included in our comparison together with 3 new isolates of the same PCR ribotype from Poland. All these isolates however showed identical AFLP patterns suggesting a clonal spread. Typing of 23 toxin A-/B+ strains isolates from the UK, USA and Belgium revealed that a specific toxin A-/B+

clone (PCR type 017, serogroup F, REA type CF4) is widely distributed in Europe and Northern America (22). PCR ribotype 017 was also the most prevalent type in our study, since 37 of 39 toxin A-/B+ isolates (95%) represented this type. This PCR ribotype was not found among the 12 unrelated isolates. The conclusion that further subtyping of toxin A-/B+ strains is possible is not supported by the genotyping results from this study. However, it would be interesting to compare AFLP typing with toxinotyping. Recently Rupnik *et al.* found two new toxinotypes (XVI and XVII) among 56 toxin A-/B+ strains (36) using the toxinotyping technique. Interestingly, most toxin A-/B+ strains belonged to toxinotype VIII and PCR ribotype 017 and did not contain the binary toxin *cdt*B gene (35). However, the 2 new toxinotypes XVI and XVII both did(36).

A remarkable high percentage of 85% (33 of 39) of toxin A-/B+ *C. difficile* isolates showed resistance to clindamycin due to MLS-resistance by the presence of erm(B) gene. This high percentage of clindamycin resistance among toxin A-/B+ isolates compared to the unrelated isolates is noteworthy. Resistance to clindamycin increases the risk of *C. difficile* disease (11). The MLS-resistance encoded by the erm(B) gene is found to be incorporated at a site homologous with the *C. difficile* tcdA gene, which suggests an association between MLS-resistance and pathogenicity of toxin A-/B+ strains (32). Evidence is provided by Farrow *et al.* that the *erm(B)* gene resides on a transposon, and is therefore likely to be transferred between *C. difficile* isolates (17). The absence of this transposon could lead to clindamycin susceptibility. Another interesting explanation is the recent observation of Johnson *et al.* (22), who detected two different restriction enzyme patterns (CF2 and CF4) within one PCR ribotype (017). In our study, CF2 and CF4 isolates differed in their susceptibility for clindamycin. This suggests that REA typing could discriminate clindamycin susceptible and resistant strains within PCR ribotype 017.

The present study shows a better discriminatory power of reference strains for the AFLP technique than for the PCR ribotyping methods. However, for toxin A-/B+ *C. difficile* isolates, the AFLP technique has a discriminatory power similar to that of PCR ribotyping. It can be concluded that clindamycin resistant *C. difficile* toxin A-/B+ strains of PCR ribotype 017/20, AFLP type 20, and serogroup F have a clonal worldwide spread.

Acknowledgements

We thank, in alphabetical order, Michelle Alfa (University of Manitoba, Winnipeg, Canada), Frederic Barbut (Centre Hospitalo-Universitaire Saint-Antoine, Paris, France), Stu Johnson (Northwestern University, Chicago, Ill.), Haru Kato (Gifu University School of Medicine, Gifu, Japan) and Hanna Pituch (Medical University of Warsaw, Warsaw, Poland) for kindly providing the isolates used in this study.

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Chapter 6

Coexistence of multiple PCR-ribotype strains of *Clostridium difficile* in faecal samples limits epidemiological studies

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Journal of Medical Microbiology, 54: 173-179. 2005

Abstract

Clostridium difficile is an important cause of antibiotic-associated diarrhoea (CDAD). The simultaneous presence of different strains in individual faecal samples is unknown, but is important for epidemiological studies. Recurrences of CDAD are observed in 15-20% of patients and have been reported as relapse, or reinfection with a new strain.

In a period of one year, 28 faecal samples from 23 patients with a first episode of CDAD, were collected at the Leiden University Medical Centre. In addition, 52 faecal samples of 23 patients, from three different hospitals, with one (n=19), two (n=2) or three (n=2) recurrences were studied. PCR-ribotyping was applied as the standard typing method for the isolates. The toxinogenic and clindamycin-resistance profile of the isolates was determined by PCR. Of 23 patients with a first episode of CDAD, two (8.7%) harboured two different types within one faecal sample, with no difference in toxinogenicity or clindamycin resistance. One of the 23 patients showed two types in three faecal samples from the same episode. Of 23 patients with recurrences, six (26%) showed a different type strain isolated in a recurrent episode. The presence of multiple *C. difficile* strains in faecal samples from the number of different strains present in recurrent episodes (chi-square test, $p \le 0.2$). This observation limits the application of typing methods for studying the epidemiology of CDAD.

Introduction

Clostridium difficile has been recognized as a cause of nosocomial diarrhoea and pseudomembranous colitis. The enteropathogenicity is associated with the production of enterotoxin A (308 kDa) and cytotoxin B (270 kDa) (Barroso *et al.*, 1990; Dove *et al.*, 1990). Clinical isolates from patients with nosocomial diarrhoea or pseudomembranous colitis usually produce both TcdA and TcdB, but an increasing number of reports mention severe infections and outbreaks due to TcdA negative, TcdB positive strains (al-Barrak *et al.*, 1999; Alfa *et al.*, 2000; Kuijper *et al.*, 2001). It has been reported previously that clindamycin resistance is high among these strains, in contrast with TcdA and TcdB positive strains (van den Berg *et al.*, 2004).

Patients often develop a recurrent *C. difficile* infection (15-20%) after discontinuation of antibiotic therapy (Wilcox & Spencer, 1992). Recurrences can be explained by endogenous persistence of *C. difficile* spores (relapse), or by the acquisition of a new strain from an exogenous source (reinfection). Determining if a recurrence is due to a relapse or a reinfection is important for epidemiological studies of *C. difficile*. There are conflicting data of studies to the simultaneous presence of different strains in individual faecal samples using molecular typing methods and immunochemical assays (Wilcox *et al.*, 1998; O'Neill *et al.*, 1991; Devlin *et al.*, 1987; Borriello & Honour, 1983; Sharp & Poxton, 1985).

PCR-ribotyping has been described as a robust method for genotyping of *C*. *difficile* strains, although restriction enzyme analysis (REA) is also used frequently applied. REA is able to subgroup PCR-ribotypes (Johnson *et al.*, 2003), but is a difficult method to interpret and lacks objective interpretation (Cohen *et al.*, 2001). Stubbs *et al.* (1999) applied the PCR-ribotyping method on 2030 strains and differentiated 116 genotypes. All known serogroups could be differentiated by this method as well (Stubbs *et al.*, 1999; van den Berg *et al.*, 2004). Therefore, this PCR-ribotyping method was used to investigate the occurrence of different *C. difficile* isolates in faecal samples of patients with one or more episodes of CDAD. Additionally, all isolates were characterized by PCR for the exact profile of *tcdA* and *tcdB*, and for clindamycin resistance (*erm*(B)).

Table 1. Characteristics of the patients and PCR-ribotyping results of the isolates from 23 patients with a first episode of CDAD.

*Designation of PCR-ribotypes, for this study assigned codes, \dagger PCR for detection of the *tcdA* gene, \ddagger PCR for detection of the *tcdB* gene, \$PCR for detection of the *erm*(B) gene, ink, not known

Patient/ sample no.	Gender	Age	Department	CDAD	Relapse	No. of isolates studied	PCR- ribotype [*]	<i>tcdA</i> profile [†]	<i>tcdB</i> profile [‡]	<i>erm</i> (B) profile [§]
1	m	46	Nephrology	mild	-	5	A1	+	+	-
2	f	76	Outpatient	nk¦	-	5	A2	+	+	-
3	m	71	Internal Medicine	severe	-	5	A3	+	+	-
4	m	50	Internal Medicine	severe	+	5	A4	+	+	+
5	m	61	Outpatient	mild	-	5	A5	+	+	-
6	f	48	Haematology	mild	-	5	A6	+	+	-
7a	f	69	Outpatient	severe	-	2	A7	+	+	-
7b						5	A7	+	+	-
8	f	65	Outpatient	mild	-	3	A8	+	+	-
9	m	62	Gastroenterology	mild	-	5	A2	+	+	-
10	m	75	Infectious Disease	severe	-	2	A9	+	+	-
						3	A10	+	+	-
11	f	60	Gastroenterology	mild	+	5	A11	+	+	-
12a	f	48	Nephrology	severe	-	5	A8	+	+	-
12b						5	A8	+	+	-
13	f	13	Paediatrics	severe	-	5	A12	+	+	-
14	f	51	Outpatient	mild	-	5	A6	+	+	-
15	f	40	Gastroenterology	mild	-	5	A3	+	+	-
16	m	77	Urology	mild	+	5	A13	+	+	-
17a	m	13	Paediatric surgery	mild	+	5	A14	+	+	-
17b						4	A14	+	+	-
17c						5	A15	+	+	-
18	m	56	Outpatient	severe	-	4	A15	+	+	-
19	m	75	Intensive Care	mild	-	5	A15	+	+	-
20	m	77	Outpatient	mild	-	5	A15	+	+	-
21a	f	73	Outpatient	mild	+	4	A16	+	+	-
						1	A15	+	+	-
21b						5	A16	+	+	
22	f	79	Neurology	mild	+	5	A17	+	+	-
23	m	75	Intensive Care	mild	+	4	A18	+	+	-

Methods

Patients included in this study. In 2002, all faecal samples positive by both Enzyme-Linked Fluorescence Assay (VIDAS CDA2) and culture for *C. difficile* at the bacteriological laboratory in the Leiden University Medical Centre were stored in glycerol broth (50% w/v) at -80°C. A total of 28 faecal samples from 23 patients with a first episode of CDAD were available for the current study. Of four patients (7, 12, 17 and 21, Table 1) more than one faecal sample from the same diarrhoeal episode was included. For comparison, *C. difficile* strains of 23 patients with recurrent *C. difficile* infection were obtained from three different hospitals (Table 2). The Academic Medical Centre, Amsterdam (hospital I) provided *C. difficile* isolates from 14 patients with recurrences, collected over a period of 11 years (1989-2000). *C. difficile* strains cultured from five patients with CDAD recurrences in a period of seven months (May to November 2003) at The VU University Medical Centre, Amsterdam (hospital II) were also available for this study. The remaining four patients with CDAD recurrences were obtained at the Leiden University Medical Centre (hospital III) from June 2002 to April 2003.

Faecal culture. Faecal samples treated with an ethanol shock pretreatment prior to inoculation were plated onto Columbia agar containing colistin and nalidixic acid (CNA) and onto *C. difficile* agar with moxalactam, norfloxacin and cystein (CDMN), and were incubated in an anaerobic environment at 37°C for 2 days (Aspinall & Hutchinson, 1992). CDMN media were also used to inoculate faecal samples not pre-treated with ethanol. Colonies of Gram-positive rods with subterminal spores were tested for the production of L-proline-aminopeptidase and for hydrolysis of esculin (Garcia *et al.*, 1997). DNA was isolated from subcultures of individual colonies. A total of five colonies from each faecal sample were picked for DNA isolation: three colonies from the two culture plates after ethanol-shock treatment (CDMN or CNA plate), and two from the CDMN plate inoculated with untreated faecal sample.

DNA isolation. DNA was isolated from colonies of *C. difficile* by QiaAmp DNA isolation columns (Qiagen, Hilden, Germany) according to the manufacturers recommendations, including a 10 minutes incubation at 55°C with proteinase K (Qiagen, Hilden, Germany). Final volume of the DNA extracts was 200 μ l.

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Hospital-	Age	Gender	Department	No. of	PCR-	tcdA	tcdB	erm(B)
patient no.				episodes	ribotype	profile	profile*	profile
1-1	41	m	Internal Medicine	2	B1	+	+	-
					B2	+	+	-
I-2	1	m	Paediatrics	2	B3	-	-	-
					B4	+	+	+
I-3	61	m	Outpatient	2	B5	+	+	-
I-4	76	f	Intensive Care	2	B3	-	-	-
I-5	37	m	Internal Medicine	2	B6	+	+	-
I-6	78	m	Intensive Care	2	B3	-	-	+
I-7	66	m	Surgery	2	B1	+	+	-
I-8	60	m	Internal Medicine	2	B7	+	+	-
I-9	20	f	Intensive Care	2	B1	+	+	-
I-10	81	m	Intensive Care	3	B10	+	+	-
					B11	+	+	-
I-11	62	f	Surgery	2	B12	+	+	-
I-12	5	m	Paediatrics	2	B3	-	-	+
-					B13	-	-	-
I-13	75	f	Outpatient	4	B7	+	+	-
					B8	-	-	-
					B9	+	+	-
I-14	52	m	Intensive Care	2	B14	-	-	-
II-1	68	m	Internal Medi- cine/Oncology	2	B7	+	+	-
П-2	70	m	Vascular surgery	2	B17	+	+	-
II-3	83	m	Cardiochirurgy	2	B15	+	+	-
II-4	66	m	Intensive Care Surgery	2	B16	+	+	-
II-5	78	f	Vascular surgery	2	B7	+	+	-
III-1	78	m	Outpatient	4	B18	+	+	-
III-2	60	f	Gastroenterology	2	B10	+	+	-
III-3	49	f	Outpatient	2	B7	+	+	-
III-4	13	m	Paediatric surgery	3	B19	+	+	-
					B20	+	+	-

Table 2. Characteristics of the patients and PCR-ribotyping results of the isolates from 23 patients with recurrent CDAD.

*Designation of PCR-ribotypes, for this study assigned codes

[†]PCR for detection of the tcdA gene

‡PCR for detection of the tcdB gene

§PCR for detection of the erm(B) gene

PCR-ribotyping. The method described by Bidet et al. (1999) was used.

The template DNA was amplified with the PRB-primers, as described in Table 3. The amplification reactions were performed in a 50 μ l final volume, containing 25 μ l HotStar Taq Mastermix (Qiagen, Hilden, Germany), 10 pmol of each primer, and 5 μ l of DNA. After an initial enzyme activation step of 15 minutes at 95°C, the protocol

consisted of 35 cycles of 1 min. at 94°C for denaturation, 1 min. at 57°C for annealing, and 1 min. at 72°C for elongation. The amplified products were analysed by agarose gel electrophoresis. Codes for the PCR-ribotyping assigned for the two different patient groups were numbered sequentially.

Genetic identification of *tcdA* and *tcdB* profiles. All isolates were tested for the presence of genes *tcdA* and *tcdB*. For the detection of *tcdA*, primers NKV011 and NK9 (Table 3) were used as described by Kato *et al.* (1999). TcdA-positive strains showed a 2535 bp amplicon size. The *tcdB* profile was verified using primers NK104 and NK105 (Table 3) as described before (Kato *et al.*, 1998). The presence of a 204 bp fragment was considered as indicative for presence of *tcdB*. The amplified products were analysed by separation by electrophoresis on agarose gels.

Genetic identification of clindamycin resistance. Clindamycin resistance was tested by PCR. The target was the *erm*(B) gene, coding for the macrolide-lincosamide- streptogramin (MLS) resistance, as described previously by Sutcliffe *et al.* (1996). The primers used are described in Table 3. Clindamycin resistant strains were defined as strains with a 639 bp amplicon size. The amplified products were analysed by separation by electrophoresis on agarose gels.

Table 3. Primer sequences of oligonucleotides used for PCR-ribotyping and conventional PCR in this study.

Primer	Nucleotide sequence (5'-3')	Gene	Fragment length (bp)	Reference
PRBs [*]	GTGCGGCTGGATCACCTCCT	ITS^{\dagger}	variable	Bidet
PRBas	CCCTGCACCCTTAATAACTTGACC			
NKV011	TTTTGATCCTATAGAATCTAACTTAGTAAC	tcdA	2535	Kato
NK9	CCACCAGCTGCAGCCATA			
NK104	GTGTAGCAATGAAAGTCCAAGTTTACGC	tcdB	204	Kato
NK105	CACTTAGCTCTTTGATTGCTGCACCT			
282BacS	GAAAARGTACTCAACCAAATA	erm(B)	639	Sutcliffe
283BacAS	AGTAACGGTACTTAAATTGTTTAC			

*PRB, PCR-ribotyping by Bidet, †ITS, internal spacer region

Results

Typing of *C. difficile* isolates from 28 faecal samples of 23 patients with a first episode of CDAD. Cultures for *C. difficile* were performed of 28 faecal samples from 23 patients with a first episode of CDAD. Of the 23 patients, 52% were male and 48% female. The median age was 59.1 years (13-79). Of 23 episodes, 35% were diagnosed in outpatients, 13% diagnosed in patients at the Gastroenterology department, 9% in patients at the Nephrology department, and 9% in patients at the Internal Medicine department. Severe cases of CDAD were seen in seven of 23 patients (30%), and mild cases in 16 patients (70%) (Table 1). Severe cases were defined as bloody diarrhoea with high fever, hypovolumia, peripheral blood leukocytosis and hypoalbuminemia or with pseudomembranous lesions by endoscopy. In a follow-up observation period of two years, seven patients (Table 1: 4, 11, 16, 17, 21, 22 and 23) showed a recurrence of a *C. difficile* infection. Faecal samples from recurrent episodes of patients 11, 16 and 17 were available for further study and therefore also included in the group of patients with recurrent CDAD (patients III-2, III-1 and III-4, Table 2).

For PCR-ribotyping, five isolates per sample were tested if possible. Of five faecal samples, only four (n=3), three (n=1), or two isolates (n=1) were acquired (Table 1). In total, 132 isolates were available for typing studies. Among 132 isolates, 18 different PCR-ribotypes were observed. PCR-ribotype A15 was found in 5 (18%) of 28 faecal samples. PCR-ribotypes A2, A3, A6, and A8 were isolated from more than one patient, and were all from different hospital departments. Of the 23 patients with a first episode of CDAD, two (7%) patients (10 and 21a, Table 1) contained two different PCR-ribotypes in the same faecal sample (Fig. 1). From four patients (Table 1: 7, 12, 17 and 21) more than one faecal sample from the same diarrhoeal period was obtained. Two faecal samples of patient 17 showed isolates that were PCR-ribotype A14, whereas the isolate of the third sample (17c, Table 1) was identified as PCR-ribotype A15. The other three patients had identical PCR-ribotypes in consecutive faecal samples. All 132 isolates were *tcdA*-positive and *tcdB*-positive and only patient 4, with PCR-ribotype A4, carried an isolate resistant to clindamycin (Table 1).

Figure 1. PCR-ribotyping results of two patients with multiple PCR-ribotypes in one faecal sample. Lanes 1-2: two isolates from patient 10 belonging to PCR-ribotype A9; lane M: marker 500-400-300 bp; lanes 3-5: 3 isolates from patient 10 belonging to PCR-ribotype A10; Lanes 6,8-10: 4 isolates from patient 21a belonging to PCR-ribotype A16; Lane 7: 1 isolate from patient 21a belonging to PCR-ribotype A15.



Typing of C. difficile isolates from 52 faecal samples of 23 patients with recurrent CDAD. Of 23 patients with recurrent episodes of CDAD, 19 patients had two episodes, two patients had three episodes, and two patients had four episodes (Table 4). The mean age of patients with recurrent CDAD was 55.7, varying between 1 and 83 years of age. Thirty percent was female, and 70% was male (Table 4); this differed not significantly from the 23 patients with a first episode of CDAD. Of the 19 patients with one recurrence, two were outpatients, whereas no outpatients were present among the two patients with two recurrences. The two patients with three recurrences were both outpatients. Symptom free intervals varied between an average of 6.5 to 13.5 weeks. The second symptom free interval was longer than the first symptom free interval, when comparing the groups of patients with different number of recurrences (Table 4). In total, 20 different PCRribotypes were observed (Table 2). The most common PCR-ribotypes in faecal samples of 23 patients with recurrent CDAD were PCR-ribotype B7 and B3, present in faecal samples of 5 (22%) and 4 (17%) patients, respectively (Table 2). In hospital I, 14 types were found among 31 C. difficile isolates. The most common PCRribotype was type B3 in 4 of the 14 (29%) patients. However, the tcdA and tcdB negative PCR-ribotype B3 was found erm(B) negative in patients I-2 and I-4, whereas patients I-6 and I-12 had erm(B) positive isolates (Table 2). In hospital II, five different PCR-ribotypes were found. Of these five patients, two had PCR-ribotype B7 in their faecal samples. C. difficile isolates from four patients in hospital III belonged to four different PCR-ribotypes.

	first	second	third
	recurrence	recurrence	recurrence
total number of patients	19	2	2
mean age	54.4	47	76.5
gender m/f	13/6	2/0	1/1
inpatients	17	2	0
outpatients	2	0	2
1st symptom free interval (wk)	6.2	7.5	8.5
2nd symptom free interval (wk)	-	13.5	13.5
3rd symptom free interval (wk)	-	-	10
no. of patients with different PCR-ribotypes (no. of types)	3	2 (2)	1 (3)
no. of patients with isolates with different toxin profiles	2	0	1
no. of patients with erm(B)-positive strains	2	0	0

Table 4. Characteristics of 23 patients with recurrent episodes of CDAD

Of 23 patients with recurrent CDAD, six (26 %) showed a different PCRribotype isolate in a recurrent episode (Table 2). This is not significantly different from 2 of 23 patients with a first episode of CDAD (chi-square test, $p\leq0.2$). Of these six patients, patient I-13 harboured three different genotypes. Patient III-4 carried two toxinogenic strains, and is the same patient as patient 7 from Table 1. A total of three PCR-ribotypes were found in this patient: A14 (the same type as B20), A15 and B19. All three strains were toxinogenic (Table 1 & 2). Patients I-2 and I-13 carried both toxinogenic and a nontoxinogenic isolates, whereas patients I-4, I-6, I-12 and I-14 had only nontoxinogenic isolates (Table 2) in their faecal samples. Clindamycin resistance was found in three of the 23 patients with recurrences (patients I-2, I-6 and I-12, Table 2).

Discussion

Using PCR-ribotyping, toxinogenicity and clindamycin resistance, multiple types of *C. difficile* were found in two of 23 (8.7%) patients with a first episode of CDAD. Additionally, six of 23 (26%) patients with recurrent *C. difficile* infection had different types in their consecutive episodes. No significant difference was found in the presence of multiple types within one faecal sample and the occurrence of multiple types in recurrent CDAD.

The finding of multiple types of C. difficile to be present in faecal samples in 8.7% of 23 patients with a diarrhoeal episode is not in agreement with the findings of three previous reports, using REA and randomly amplified polymorphic DNA (RAPD) fingerprinting (Wilcox et al., 1998; O'Neill et al., 1991; Devlin et al., 1987). This difference could be due to the fact that three different plates and a combination of untreated and ethanol-treated faecal samples were applied in our study for selection of colonies. In contrast, colonies were only selected from a primary selective culture plate without an ethanol pre-treatment of faecal samples in other studies (O'Neill et al., 1991; Devlin et al., 1987). Two other studies were in agreement with our observations. Sharp & Poxton (1985) reported that two of three selected faecal samples contained different strains of C. difficile by immunochemical fingerprinting of C. difficile surface antigens. This observation was probably associated with the high number of different colonies (n=8) investigated from each faecal sample. Borriello & Honour (1983) showed the concomitance of a cytotoxigenic and a noncytotoxigenic C. difficile strain in 7 faecal specimens of patients with clinical symptoms of CDAD, which were at first diagnosed as non-cytotoxinogenic by cytotoxicity assays. In our study, all isolates of the 23 patients with a first episode of CDAD were tcdA and tcdB positive, but only faecal samples with a positive toxin test were included.

Of 23 patients with recurrences, 6 (26%) had culture positive episodes with tcdA and tcdB negative isolates (Table 2). Moreover, one patient (I-12) had two episodes with tcdA and tcdB negative *C. difficile*. One explanation could be that these strains are capable to produce another toxin. In addition to the two large clostridial toxins (TcdA and TcdB), some strains of *C. difficile* also produce an actin-specific ADP-ribosyltransferase, called binary toxin CDT. The frequency of binary toxin genes among *C. difficile* strains that do not produce large clostridial toxins was reported to be 15.5% in one case (Geric 2003). Binary toxin has cytotoxic effects on Vero cells, and may act as an additional virulence factor together with the large clostridial cytotoxins. Another possibility is that the six patients were simultaneously infected with a toxin producing strain that was not cultured. We favour this explanation, since TcdA was detected in the recurrent episodes by an enzyme immuno-assay. It also confirms the findings of the study by Borriello & Honour

(1983) that concomitance of a cytotoxigenic and a non-cytotoxigenic *C. difficile* strains frequently occurs.

Recurrences of CDAD occur in 15-20% of cases after discontinuation of treatment (Wilcox & Spencer, 1992). In our study encompassing an observation period of 2 years, a recurrency rate of 30% was found among the 23 patients with a first episode of CDAD. Once recurrent episodes develop, 45-60% continue to have repeated episodes (McFarland et al., 2002). Using PCR-ribotyping, our reinfection rate could be estimated as 26%. This is lower than shown in other studies, where the percentage of reinfection was found to be between 33 and 75% (Tang-Feldman et al., 2003; Barbut et al., 2000; O'Neill et al., 1991). Relapses can be due to the persistence of spores, not completely eradicated by therapy. Discrimination between reinfections and relapses is difficult, if a particular strain is widespread present in the environment and reinfects patients. Wilcox & Spencer (1992) showed that 56% of recurrences were reinfections, using the random amplified polymorphic DNA method to fingerprint strains from 27 patients from six different hospitals. They also found, however, that an endemic clone of C. difficile accounted for 53% of all isolates, and they hypothesized that the frequency of reinfections was probably underestimated because of the reacquisition of the same strain from the hospital environment. We included patients with recurrent CDAD from 3 different hopsitals and found no endemic clone. In addition, patients can also contaminate their own environment by shedding the strain of the first episode, and subsequently become reinfected with the same strain. Finally, from the results of our current study we conclude that a differentiation between reinfection and relapse on microbiological grounds is also difficult to determine, since patients may have been infected simultaneously with multiple types. It depends on the culture methods and number of colonies selected from different culture media for further typing studies, if this will be recognized.

No significant differences of age, gender or in- and outpatient numbers were observed among 16 patients with a single episode of CDAD in comparison with 30 patients with recurrent CDAD. This is in contrast with previous studies (Young *et al.*, 1986; Fekety *et al.*, 1997; McFarland *et al.*, 1999; Do *et al.*, 1998). Young *et al.* (1986) investigated 35 patients and found a significant difference in age and a history of recent abdominal surgery. Fekety *et al.* (1997) and McFarland *et al.* (1999) performed a retrospective analysis of risk factors for CDAD, and a prospective analysis during a 2-month study. Female gender, an onset of the initial episode in spring, the number of previous episodes, and antibiotic treatment for another infection short after a CDAD episode, were significantly associated with recurrent CDAD (Fekety *et al.*, 1997). Two other risk factors predictive for recurrent CDAD were increasing age and a decreased quality-of-life score at inclusion (McFarland *et al.*, 1999). Chronic renal insufficiency, a high white blood cell count and community-acquired diarrhoea of the first episode have also been significantly associated with recurrent CDAD (Do *et al.*, 2000). This discrepancy with our findings may be due to the fact that we compared patients with a first episode diagnosed at one hospital in 2002 with patients suffering from recurrent episodes who were diagnosed in a period of 15 years at three different hospitals.

In summary, the simultaneous presence of multiple *C. difficile* PCRribotypes in faecal samples from patients with a first episode and recurrent CDAD did not differ significantly. This observation limits the application of typing methods for studying the exogenous or endogenous source of recurrences.

Acknowledgements

We thank the skillful technical support of Anneke Oei and Rob Weyts. This work was supported by a grant from the Foundation Microbiology Leiden.
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Chapter 7

Typing and subtyping of *Clostridium difficile* isolates using Multiple Locus Variable Number of Tandem Repeats Analysis (MLVA)

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Journal of Clinical Microbiology, 45: 1024-1028. 2007

Abstract

Using the genomic sequence of *Clostridium difficile* strain 630, we developed Multi-Locus Variable number of tandem repeat Analysis (MLVA) with automated fragment analysis and multi-colored capillary electrophoresis as typing method for *C. difficile*. All reference strains representing 31 serogroups, 25 toxinotypes, and 7 known subtypes strains of PCR-ribotype 001 could be discriminated from each other. Application of MLVA to 29 isolates from 7 outbreaks due to the emerging hypervirulent PCR-ribotype 027/PFGE-type NAP1 resulted in recognition of 13 clusters. Additionally, 29 toxin A-/B+ isolates belonging to PCR ribotype 017 from 8 different countries, revealed 8 country specific clusters. MLVA is a highly discriminatory genotyping method, and is an new tool for subtyping of new emerging variants of *C. difficile*.

Introduction

To study the epidemiology of Clostridium difficile, a typing method is necessary with a higher discriminatory power, typeability and reproducibility than currently available methods is required. Multi-locus variable number of tandem repeats analysis (MLVA) is a new candidate technique, that has already been applied successfully on a number of bacterial and fungal species (5,10). Recently, MLVA has been developed for C. difficile using automated sequence detection and subsequent manual determination of the number of repeat loci (12). For a faster and easier application of the MLVA for C. difficile, we developed a MLVA method using smaller short tandem repeats (2-9bp) to facilitate automated fragment analysis with multi-colored capillary electrophoresis instead of sequencing. Subsequently, we applied MLVA on 7 subtypes of a common PCR ribotype 001 and two emerging other PCR ribotypes of C. difficile. Since 2004, a new toxin hyperproducing C. difficile strain characterized as PCR-ribotype 027, toxinotype III, PFGE type NAP1 and REA group BI has been recognised in Canada, USA, United Kingdom, The Netherlands, Belgium and France as an important cause of hospital outbreaks {Anonymous, 2005 51 /id;Joseph, 2005 50 /id;Kuijper, 2006 37 /id;Loo, 2005 49 /id;Pepin, 2004 48 /id}. Additionally, an increasing number of reports mention severe infections and outbreaks due to toxin A-negative, toxin B-positive (A-/B+) isolates (1,2,7,14). These toxin A-/B+ isolates belong to PCR-ribotype 017, REA group CF and toxinotype VIII and were first recognized as a cause of an outbreak in 1999 in Canada as well (1,2).

Methods

Bacterial strains. Isolates included in the analysis were 57 reference strains, all seven subtypes of PCR-ribotype 001, 27 toxin A-/B+ isolates belonging to PCR-ribotype 017 from 8 different countries, and 29 isolates belonging to PCRribotype 027 from The Netherlands (Table 1) and United Kingdom. Of these 29 PCRribotype 027 strains, 28 strains were outbreak related from six different hospitals in the Netherlands and one in the UK, and one strain was a sporadic isolate from 2003 (8). The UK strain was obtained from dr. Jon Brazier (Anaerobe Reference Laboratory, NPHS Microbiology Cardiff, Cardiff). The outbreak strains of each hospital were at randomly selected. DNA was isolated from colonies of *C. difficile* by QiaAmp DNA isolation columns (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. PCR ribotyping was performed as described previously and the method of Rupnik *et al.* was used for toxinotyping (4,15).

Table 1. Isolates included in this study (n=120)

Strains tested	Number of strains	Identification for strains	Source	Place and country
Serogroups	31	A-I, K, X, A1-A11, A13- A17, S1-S4	Reference	Belgium
Toxinotypes	25	I-XXII	Reference	Slovenia
Strain 630	1	630	Reference	Rome
Subtypes of PCR-ribotype 001, by REP-PCR* (2)	7	001-1 to 001-7	Endemic	United Kingdom
	12	Arg28, 31, 32, 36-38, 77, 126, 127, 134, 143, 152	Outbreak	Argentina
	2	CD16, CD17	Outbreak	Amsterdam, Netherlands
	2	Can1, Can3	Outbreak	Canada
	1	123825R	Endemic	Leiden, Netherlands
Toxin A-/B+ strains, PCR- ribotype 017	2	1110/98, 1745/00	Endemic	Poland
	2	60, 99-3050	Endemic	France
	2	CF2, CF4	Endemic	USA
	2	R10205, R10430	Endemic	United Kingdom
	2	GAI95601, GAI95602	Endemic	Japan
	5	AF1-5	Outbreak	Amersfoort, Netherlands
	7	AMC1-7	Outbreak	Amsterdam-1, Netherlands
	1	VUMC	Endemic	Amsterdam-2, Netherlands
Strains belonging to PCR-	3	SV1-3	Outbreak	Amsterdam-3, Netherlands
ribotype 027 (3)	4	HL1-4	Outbreak	Haarlem, Netherlands
	5	HW1-5	Outbreak	Harderwijk, Netherlands
	3	UMC1-3	Outbreak	Utrecht, Netherlands
	1	UK027	Outbreak	United Kingdom

MLVA. Seven regions with short tandem repeats spread over the genome, named as markers MLVA CdA6, CdB7, CdC6, CdE7, CdF3, CdG8 and CdH9, were identified using Tandem Repeat Finder v3.21 on the genome of C. difficile strain 630 (http://www.sanger.ac.uk/Projects/C_difficile/) (16). Four of them, MLVA CdA6, CdB7, CdE7 and CdG8, were identical to CDR4, CDR49, CDR48 and CDR9, respectively, in the assay described recently by Marsh et al. (12). Primers were designed on the flanking sequences of the repeats using the Primer3 program (http://www.broad.mit.edu/cgi-bin/primer/primer3_www.cgi). Three separate duplex PCRs (MLVACd A6/H9, B7/F3 and C6/E7) and one singleplex PCR (MLVACd G8) were developed (Table 2). Amplification of the repeats was performed using a single PCR-protocol. The amplification reactions were performed in a 50 µl final volume, containing 25 µl HotStar Taq Mastermix (Qiagen, Hilden, Germany), 1 µM of each primer, 3 mM magnesium chloride and 5 µl of DNA. After an initial enzyme activation step of 15 minutes at 95°C, the protocol consisted of 35 cycles of 30 sec. at 94°C for denaturation, 30 sec. at 51°C for annealing, and 30 sec. at 72°C for elongation. A final elongation step was performed for 10 min. at 72°C. The forward primers of each PCR were labelled at the 5'-end with carboxyfluorescein (FAM), hexachlorofluorescein (HEX), 2'-chloro-7'-phenyl-1,4-dichloro-6-carboxyfluorescein (VIC) or 2'-chloro-5'-fluoro-7',8'-fused phenyl-1,4-dichloro-6-carboxyfluoresccin (NED). PCR-fragments were analysed using multi-coloured capillary electrophoresis on an ABI3100, with a ROX500-marker as internal marker for each sample. The size of each marker was determined by the Genescan software (Applied Biosystems). Markers from a selected number of isolates were sequenced to verify accurate assignment of repeat numbers. All sequence results were equal to the results from the fragment analysis by the ABI system and to the calculated repeat numbers. The repeat numbers were analyzed using BioNumerics, version 3.5, software (Applied Maths, Kortrijk, Belgium) and the unweighted pair group method (UPGMA) with arithmetic averages with the multistate categorical similarity coefficient (MCSC). All markers were given an equal weight, irrespective of the number of repeats. The percentages in the dendrogram reflect the percentage of homology between between the specific markers. Subsequently, if two strains have an equal number of repeats in six of seven markers, they are 86% identical.

		Location ^a	Forward primer	Reverse primer
Marker	Repeat motif		sequence (label- 5'-3')	sequence (5'-3')
			FAM-	
<i>Cd</i> A6	AAGAGC	755721	TTAATTGAGGGAGAA	AAATACTTTTCCCAC
			TGTTAAA	TTTCATAA
			FAM-	
<i>Cd</i> B7	ATCTTCT	3688632	CTTAATACTAAACTA	TTATATTTTATGGGC
			ACTCTAACCAGTAA	ATGTTAAA
			HEX-	
<i>Cd</i> C6	TATTGC	3239736	GTTTAGAATCTACAG	ATTGGAATTGAATGT
			CATTATTTGA	AACAAAA
			FAM-	
<i>Cd</i> E7	ATAGATT	167124	TGGAGCTATGGAAAT	CAAATACATCTTGCA
			TGATAA	TTAATTCTT
			HEX-	
<i>Cd</i> F3	TTA	1954915	TTTTTGAAACTGAAC	ACAAAAGACTGTGCA
			CAACATA	AATATACTAA
			NED-	
<i>Cd</i> G8	TAAAAGAG	664660	TGTATGAAGCAAGCT	AATCCAGCAATCTAA
			TTTTATT	TAATCCA
			VIC-	
<i>Cd</i> H9	TCTTCTTCC	4116072	GTTTTGAGGAAACAA	GATGAGGAAATAGA
			ACCTATC	AGAGTTCAA

Table 2. Characteristics of markers and primers.

^a Location on the genomic sequence of strain 630 (36)

Results and discussion

C. difficile control strain 630 revealed identical results in five different experiments using both separate cultures and DNA extractions. The stability of the repeat numbers of the different markers was tested in duplicate after a total of 10 and 30 times of subculturing of isolates 014 and 027. The repeats from the isolate belonging to type 014 were stable in all experiments. An expansion of 1 repeat unit in marker *Cd*A6 was observed in one duplicate sample of the type 027 isolate after 10 times of subculturing, which subsequently returned to the original number of repeats after 30 times of subculturing for this isolate. Based on the stability tests, we concluded that a difference of one repeat unit between strains should not be

interpreted as indicative for separate types or subtypes. This conclusion is in complete concordance with the study of the stability of C. difficile MLVA loci by Marsh et al. (12). They found three pairs of serial isolates from individual patients to have a single locus variation of only one tandem repeat, and one pair of isolates with a double locus variation of both one tandem repeat. Therefore, they concluded that isolates with a summed tandem-repeat difference of ≤2 are genetically related. MLVA discriminated between isolates belonging to all 31 serogroups, the 7 subtypes of PCR-ribotype 001 and all 25 toxinotypes, except for toxinotypes XII, XIII, and XIV. An isolate belonging to serogroup A15 was completely identical (100%) to toxinotype V, as has been observed previously (15). Toxinotypes XII, XIII, and XIV were clustered into one MLVA-type with 100% similarity, indicating that toxinotyping is a method that merely reflects the status of the toxin genes (15). With one marker difference, toxinotype XIb was comparable to the 100% cluster of toxinotypes XII-XIV. The similarity of isolate 630 to serogroup C (closest match) was only 43% (3 of the 7 markers), although marker CdB7 and CdE7 differed only one repeat. PCR-ribotype 001 isolates were quite stable in markers CdE7 (5-7 repeats), CdF3 (5 repeats), CdG8 (6-8 repeats) and CdH9 (2 repeats). Using these characteristics, type 001 isolates can be discriminated from most serogroups and toxinotypes (6). Subsequently, MLVA is capable to replace PCR ribotyping and PFGE to identify and recognize subtypes of 001. Until recently, strains belonging to PCR-ribotype 001 were the most common in the UK, and the importance of the ability to subtype these strains is high. A recent Health Protection Agency Report indicated that PCR ribotypes 106 and 027 are the most common in the UK, followed by 001 in still around 25% (available at http://www.hpa.org.uk/).

Among the isolates belonging to PCR-ribotype 027 (n=29) a 100% similarity (Fig. 1) was detected for isolate AF4 with HW3, for isolate SV1 with SV2, for isolate AF5 with AMC4, HW1, HW2 and HW5, and for isolates AMC3 with AMC6 and AMC7. With 86% similarity, 14 clusters were detected among the 29 isolates. Hospital-specific clusters were seen for SV, HW, HL and AMC (fig. 1). The sporadic endemic isolate from the VUMC 2003 was only 53% similar to the outbreak isolates and 71% similar to isolate HL3. The UK isolate was only 40% identical to all Dutch outbreak isolates. For all type 027 isolates, markers CdE7, CdF3 and CdH9

were complete identical except for the UK isolate which had 6 repeats for marker CdE7 (Fig. 1).

Toxin A-/B+ isolates (including the two reference strains belonging to serogroup F and toxinotype VIII) (n=29), belonging to PCR-ribotype 017 and toxinotype VIII, could be divided in eight clusters at a similarity of 86% (6 markers identical) (table 3, clusters G-N). Six clusters with 100% homology were recognized (table 3, clusters A-F). All isolates with 100% similarity were country-specific (cluster A-F), as were clusters H and I. Toxin A-/B+ isolate could be differentiated from all other types using the combination of markers *Cd*A6 (2 repeats), *Cd*F3 (5 repeats), *Cd*G8 (fragment size >400bp) and *Cd*H9 (2 repeats). For marker *Cd*G8, all PCR-ribotype 017 isolates showed the previously described larger fragment size exceeding the 400bp detectable by our system. MLVA discriminated toxin A-/B+ isolates better than Amplified Fragment Length Polymorphism (AFLP, 18).

Figure 1. Dendrogram based on profiles of seven markers for all PCR-ribotype 027 isolates (n=29) tested in this study. The numbers represent the number of repeats for the specified marker.



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	Origin	No. of repeats detected for the following							
Isolate					marke	er			Cluster(s) ^a
Isolate		CdA6	CdB7	CdC6	CdE7	CdF3	CdG8	CdH9	
Arg32	Argentina	2	7	18	7	5	>400	2	G
Arg38	Argentina	2	7	17	7	5	>400	2	G
CF2	USA	2	7	31	7	5	>400	2	G
Can3	Canada	2	9	20	7	5	>400	2	Н
1110/98	Poland	2	10	23	7	5	>400	2	Ι
1745/00	Poland	2	10	15	7	5	>400	2	Ι
R10205	United Kingdom	2	8	26	8	5	>400	2	A, J
R10430	United Kingdom	2	8	26	8	5	>400	2	A, J
Can1	Canada	2	8	22	8	5	>400	2	J
CF4	USA	2	8	30	8	5	>400	2	J
F	Reference strain	2	13	22	8	5	>400	2	Κ
CD16	Amsterdam	2	10	30	8	5	>400	2	B, L
CD17	Amsterdam	2	10	30	8	5	>400	2	B, L
123825R	Leiden	2	10	32	8	5	>400	2	L
GAI95601	Japan	2	10	43	8	5	>400	2	L
GAI95602	Japan	2	10	40	8	5	>400	2	L
Arg77	Argentina	2	7	20	8	5	>400	2	С, М
Arg134	Argentina	2	7	20	8	5	>400	2	С, М
Arg152	Argentina	2	7	19	8	5	>400	2	D, M
Arg37	Argentina	2	7	19	8	5	>400	2	D, M
Arg127	Argentina	2	7	19	8	5	>400	2	D, M
Arg31	Argentina	2	7	18	8	5	>400	2	Е, М
Arg126	Argentina	2	7	18	8	5	>400	2	E, M
Arg28	Argentina	2	7	21	8	5	>400	2	F, M
Arg36	Argentina	2	7	21	8	5	>400	2	F, M
Arg143	Argentina	2	7	22	8	5	>400	2	М
60 (fr)	France	2	7	28	8	5	>400	2	М
99-3050	France	2	7	24	8	5	>400	2	М
VIII	Reference strain	2	9	21	8	5	>400	2	Ν

Table 3. MLVA results and cluster information for toxin A-/B+ isolates belonging to PCR-ribotype 017 (n=29) isolates tested in this study

Application of MLVA on C. difficile isolates was easy-to-perform and consisted of four separate PCR mixes and a single PCR protocol. Although MLVA has yet to show its value in longer-term epidemiology or phylogeny studies, MLVA

can be widely applied in outbreak situations. Therefore, MLVA is an important new tool to study the epidemiology of the newly worldwide emerging toxin A-/B+/PCR-ribotype 017 and the PCR-ribotype 027/PFGE NAP1/REA BI isolates. MLVA is a highly discriminatory genotyping method for *C. difficile* and is able to discriminate between isolates with identical PCR-ribotypes belonging to types 001, 017 and 027. MLVA also clearly differentiated these PCR-ribotypes from other ribotypes included in this study. Future studies should be performed on all currently available PCR ribotypes to explore this in more detail.

Acknowledgements

This work was supported by a grant from the Foundation Microbiology Leiden. We thank, in alphabetical order, Michelle Alfa (University of Manitoba, Winnipeg, Canada), Frederic Barbut (Centre Hospitalo-Universitaire Saint-Antoine, Paris, France), Jon Brazier (University Hospital of Wales, U.K), Stu Johnson (Northwestern University, Chicago, Ill.), Haru Kato (Gifu University School of Medicine, Gifu, Japan), Cristina Legaria (Hospital Tornú, Buenos Aires, Argentina), Paola Mastrantonio (Instituto Superiore di Sanita, Rome), Hanna Pituch (Medical University of Warsaw, Warsaw, Poland), Maja Rupnik (University of Maribor, Slovenia) for kindly providing the isolates used in this study.

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Chapter 8

Clostridium difficile ribotype 027, toxinotype III in the Netherlands

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Emerging Infectious Diseases, 12: 827-830. 2006

Abstract

Outbreaks due to *Clostridium difficile* polymerase chain reaction (PCR) ribotype 027, toxinotype III, were detected in 7 hospitals in the Netherlands from April 2005 to February 2006. One hospital experienced at the same time a second outbreak due to a toxin A-negative *C. difficile* PCR ribotype 017 toxinotype VIII strain. The outbreaks are difficult to control.

Introduction

Since March 2003, outbreaks of severe cases of Clostridium difficileassociated disease (CDAD) were reported in hospitals in Montreal and Quebec (1,2). Increased virulence was suspected, since the proportion of patients with CDAD who died within 30 days after diagnosis rose from 4.7% in 1991-1992 to 13.8% in 2003 (1). In addition, the Centers for Disease Control and Prevention reported a growing threat of CDAD in US hospitals and found the strain to be associated with high illness and death rates during hospital outbreaks in 11 states (3). The increased virulence was considered to be associated with the production of a binary toxin and an increased production of toxins A and B (4). Further characterization of this strain showed that it belonged to toxinotype III, pulsed-field gel electrophoresis (PFGE) type NAP1, restriction endonuclease analysis group BI, and polymerase chain reaction (PCR) ribotype 027 (2,3). Toxinotyping involves detecting polymorphisms in the toxin A and B and surrounding regulatory genes, an area of the genome known collectively as the pathogenicity locus or PaLoc (5). By toxinotyping, 24 different types can be recognized, whereas the library of PCR ribotypes comprises 116 distinct types of C. difficile identified on the basis of differences in amplification profiles generated (6). The PCR ribotype 027, toxinotype III, strain is resistant to ciprofloxacin and the newer generation of fluoroquinolones, such as gatifloxacin, levofloxacin, and moxifloxacin (3). Exposure of patients to fluoroquinolones and cephalosporins is recognized as a risk factor for CDAD caused by 027 (2,3). Increasing use of fluoroquinolones in US healthcare facilities may have provided a selective advantage for this epidemic strain and promoted its widespread emergence.

Methods and results

In July 2005, the medical microbiologic laboratory at the Leiden University Medical Center was requested to type *C. difficile* strains from an outbreak in a hospital (hospital l) in Harderwijk (Fig. 1, Table 1). The incidence of CDAD in the hospital had increased from 4 per 10,000 patient admissions in 2004 to 83 per 10,000 admissions from April through July 2005.

Figure 1. Location of the hospitals with outbreaks of Clostridium difficile –associated diarrhea in the Netherlands



Table 1. Characteristics of 9 hospitals with patients with *Clostridium difficile*-associated diarrhea due to PCR ribotype 027, toxinotype III

		Admiss	ions					
Hospital no. and setting	No. beds	Incidence/10.00 0 before outbreak	Max incidence/mo/ 10.000 during outbreak ^a	Date of outbreak onset	Total no. CDAD patients in given period, 2005	Deaths/30d ^b	No. strains studied	No. PCR ribotype 027/toxinotype III strains
1. Harderwijk	341	4	83	Apr 2005	51, Apr-Nov	3	30	19
2. Amersfoort	600	11	87	May 2005	85, Jan–Dec	19	50	15
3. Utrecht	1,013	16	-	No outbreak	37, Jun-Dec	nk	17	6
4. Nieuwegein	584	11	-	No outbreak	13, Jan-Dec	nk	4	1
5. Amsterdam	1,002	38	52	June 2005	68, Jan–Oct	1	28	12
6. Amsterdam	310	10	66	Apr-May 2005	42, Jan-Oct	nk	34	16
7. Haarlem	744	7	27	2004	66, Jan-Dec	nk	9	7
8. Hoofddorp	455	3	76	jan-05	73, Jan–Dec	nk	8	8
9. Beverwijk	383	4	47	2002	24, Jan-Dec	nk	4	3

^a Timeframe 2-4 months

^b nk, not known

Cultured isolates were subsequently identified as toxinotype III and PCR ribotype 027 (7). The strain also had the binary toxin genes and contained an 18-bp deletion in a toxin regulator gene (tcdC). As determined by E test (AB Biodisk, Solna Sweden), the isolates were resistant to erythromycin (MIC >256 mg/L) and ciprofloxacin (MIC >32 mg/L) and susceptible to clindamycin (MIC 2 mg/L) and metronidazole (MIC 0.19 mg/mL). Measures taken by the hospital included isolating

all patients with diarrhea until 2 tests were negative for *C. difficile* toxin, cohorting all *C. difficile* –infected patients on a separate ward, banning all fluoroquinolone use, and limiting use of cephalosporins and clindamycin. A case-control study is being performed in the hospital to determine risk factors for acquiring this strain, and a follow-up study will determine the rate of complications and relapses. As of January 2006, the situation appears to be under control since the number of patients per month with positive test results has decreased. All 9 CDAD cases from September 2005 to January 2006 were caused by non-027 ribotypes. Therefore, cohort isolation and the limitation on antimicrobial agents have been stopped.

A second epidemic occurred in another hospital 30 km from the first hospital (hospital 2, Amersfoort) and was probably related to the outbreak in hospital 1 through a transferred patient with CDAD. Isolates obtained from patients were indistinguishable from the Harderwijk isolates. After the index patient was transferred, the incidence of CDAD, which had been 2–3 cases per month for the last 2 years, rose to an average of 15 cases per month during May, June, and July. From August to December, the number of CDAD patients per month was 7, 7, 8, 14, and 10, respectively. Of the 85 CDAD patients found through December 2005, 19 (22%) patients died, and 16 (19%) had relapses. Of 50 strains characterized at the reference laboratory, 15 belonged to PCR ribotype 027, and 14 belonged to PCR ribotype 017, toxinotype VIII. The 017 strain had a deletion of the toxin A gene, did not contain genes for binary toxin production, and had a normal *tcdC* gene.

In response to the outbreaks in the Netherlands, the Centre for Infectious Disease Control at the National Institute for Public Health and the Environment in Bilthoven organized a meeting with experts in the fields of microbiology, infectious diseases, infection control, and epidemiology. The team agreed to combine parts of existing national hospital guidelines relevant for infection control of CDAD and to use national and international experience in drawing up specific CDAD guidelines for infection control and treatment separate for hospitals and nursing homes. Diagnostic facilities were increased and made accessible for all microbiology laboratories in the Netherlands. Relevant professionals were informed through different communication channels, including various scientific societies (7). Plans were made to register and monitor new outbreaks. Laboratories were encouraged to send patient isolates or fecal samples for typing to the reference laboratory in Leiden when an outbreak was

suspected on the basis of an increase in monthly incidence or a rapid spread of clinically suspected cases.

Subsequently, 3 hospitals in the western part of the country (hospitals 7–9) also reported an increase in incidence of severe CDAD. In 2005, the public health laboratory serving these 3 hospitals diagnosed CDAD in 163 patients. Of 21 strains sent to the reference laboratory, 18 were identified as PCR ribotype 027, toxinotype III (Table). Retrospectively, an increase of CDAD was first evident in July 2004 for hospital 7 and in 2002 for hospital 9. The public health laboratory diagnosed CDAD in 120 patients in 2004, in 58 in 2003, and in 47 in 2002. No strains or fecal samples before 2005 were available for typing. A nursing home in the same region was also found to have patients with CDAD due to PCR ribotype 027, with evidence of spread within the facility. No epidemiologic relationship could be established between this region ad that of the first 2 outbreaks.

Two hospitals in the center of the Netherlands (hospitals 3 and 4) did not notice an increase in the incidence of patients with CDAD but submitted strains to the reference laboratory for typing. Type 027 was found in 6 (35%) of 17 and 1 (25%) of 4 isolates tested, respectively. None of the 7 patients with CDAD due to type 027 had severe disease.

A cluster of 12 patients with CDAD by PCR ribotype 027, toxinotype III, was reported in July and August in a large teaching hospital in Amsterdam (hospital 5). One patient died from consequences of CDAD, and severe complications developed in 2 other patients. Another hospital in Amsterdam (hospital 6) also reported an increase of severe cases of CDAD in July 2005 in geriatric patients. Strains cultured from fecal samples of 7 patients in August 2005 showed PCR ribotype 027, toxinotype III.

Discussion

Shortly after the reports in June 2005 of the detection of *C. difficile* PCR ribotype 027, toxinotype III, in English hospitals, this more virulent type was detected in the Netherlands (7,8). More recently, the reference laboratory at Leiden University Medical Center also detected this strain in samples from Belgium as a causative agent of outbreaks of CDAD (9). The virulence factors of this emerging strain are not well

understood. It contains a binary toxin, but the importance of binary toxin as a virulence factor in *C. difficile* has not been established. The binary toxin, an actin-specific adenosine diphosphate–ribosyltransferase, is encoded by the *cdtA* gene (the enzymatic component) and the *cdtB* gene (the binding component), which are not located within the pathogenicity locus (10,11). Nonpathogenic strains that contain *cdtA* and *cdtB* genes but lack the pathogenicity locus are also capable of producing binary toxin. The binary toxin is present in $\approx 6\%$ of all *C. difficile* isolates, irrespective of the toxinotype (10,11). We therefore consider it likely that the binary toxin in PCR ribotype 027, toxinotype III, strains merely reflects clonal spread of a restricted number of strains.

The importance of the 18-bp deletion in tcdC of the PCR ribotype 027, toxinotype III, strains is also unknown. tcdC is considered a negative regulator of the production of toxins A and B, but whether this 18-bp deletion results in a nonfunctional product is unknown (3). A recent report, however, indicates that toxinotype III isolates produce toxins A and B in considerably greater quantities in vitro than toxinotype 0 isolates (4). On the other hand, deletions in tcdC are frequently present in toxinogenic isolates. Of 32 toxinogenic strains studied in 2002, 8 belonged to toxinotypes 0, V, and VI and contained deletions in tcdC of 18 bp or 39 bp, although this deletion was not associated with severity of disease (12).

The PCR ribotype 027, toxinotype III, strain has a characteristic antimicrobial susceptibility pattern, since it is resistant to the newer fluoroquinolones and erythromycin but susceptible to clindamycin. Macrolide, lincosamide, and streptogramin B (MLSB) resistance is usually due to an erm(B) gene, but PCR ribotype 027 and toxinotype III strain did not contain an erm(B) gene. All current PCR ribotype 027 and toxinotype III strains but no historical isolates (obtained before 2001) were resistant to gatifloxacin and moxifloxacin (3). The resistance for ciprofloxacin and newer fluoroquinolones is not specific for the new virulent strains, since it has also been found in other common PCR ribotypes in the United Kingdom (13).

The observation that outbreaks due to different strains can occur simultaneously emphasizes that microbiologic monitoring is important for epidemiologic studies of CDAD. PCR ribotype 017 strain lacks a part of the toxin A gene and was first recognized as a cause of an outbreak in Canada in 1999 (14).

Subsequently, toxin A-negative, toxin B-positive strains caused outbreaks of CDAD in Ireland (D. Drudy, pers. comm.), Argentina (M.C. Legaria, *et al.*, unpub. data), and the Netherlands (*15*).

The outbreaks in the Netherlands are difficult to control. In the Harderwijk epidemic, using rapid diagnostic tests for CDAD and cohort isolation in combination with restricting use of fluoroquinolones and cephalosporins appeared to be successful. Outbreaks in the other hospitals are still not completely under control.

Acknowledgements

We thank R. Countinho and M. Tersmette for their contributions to this study.

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Chapter 9

Characteristics and incidence of *Clostridium difficile*-associated disease in The Netherlands, 2005

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Clinical Microbiology and Infection, in press.

Abstract

During a two months period in 2005, 13 laboratories participated to a surveillance study of the incidence of Clostridium difficile-associated disease (CDAD) in 17 hospitals in the Netherlands. The median incidence rate of CDAD was 16/10 000 patient admissions (2.2/10 000 patient-days) and varied from 1 to 46/10 000 per hospital. In total, 81 patients with CDAD were reported; 49 (61%) patients had nosocomial CDAD, and 29 (36%) patients were admitted to the hospital when already suffering from diarrhoea. Two (2%) deaths were attributable to CDAD; both were admitted with severe community-onset CDAD and were aged >80 years. Among 64 toxinogenic isolates, ten (16%) belonged to PCR ribotype 027 and ten (16%) to PCR ribotype 014. Type 027 was identified in ten patients from one hospital during an unrecognized outbreak. Toxinotyping of the 64 isolates revealed the presence of six different toxinogenic types, with 41 (64%) isolates of toxinotype 0, ten (16%) isolates of toxinotype III, and nine (14%) isolates of toxinotype V. Of the 64 toxinogenic isolates, seven (11%) had a 39bp deletion in the tcdC-gene, 11 (17%) had an 18bp deletion, and one (1%) had a deletion of c. 44bp. Genes for binary toxin were present in 21 (33%) of the 64 toxinogenic isolates, mainly associated with toxinotypes III and V. It was concluded that the median CDAD incidence rate of 16/10 000 patient admissions in The Netherlands is considerably lower than that in Canada and the USA, but that the emerging 027 type can spread unnoticed. The high proportion (36%) of CDAD cases with a community onset has important implications for future studies of the epidemiology of CDAD.

Introduction

Since the recognition of *Clostridium difficile* as the causative agent of pseudo-membranous colitis in 1978, this anaerobic spore-forming bacterium has emerged as an important enteropathogen. The spectrum of *C. difficile* -associated disease (CDAD) varies from mild diarrhea to severe colitis, and it may lead to toxic megacolon, perforation, sepsis and death (1). Antibiotic exposure is considered to be the major risk factor for CDAD, but other predisposing factors for CDAD have also been recognised (2,3). Pathogenic strains of *C. difficile* release enterotoxin A (TcdA; 308kDa) and cytotoxin B (TcdB; 270 kDa), which ultimately mediate diarrhoea and colitis. Some *C. difficile* isolates have been reported to produce an actin-specific ADP-ribosyltransferase (binary toxin), but the significance of this is unclear (4). For epidemiological purposes, *C. difficile* can be divided in >150 PCR ribotypes and 25 toxinotypes (5-8).

Since 2002, the rate and severity of CDAD has been increasing in the USA, Canada and Europe because of the spread of one specific strain that belongs to restriction enzyme analyze (REA) group BI, pulsed-field gel electrophoresis (PFGE) type NAP1, PCR ribotype 027 and toxinotype III (9-14). In 2005, a European study was performed to characterise isolates from nosocomial cases of CDAD in hospitals of 14 different countries, with three hospitals per country. The present study described an extension of this investigation in The Netherlands to 17 hospitals, with the inclusion of all diagnosed forms of CDAD, irrespective of whether the disease had a community or hospital onset.

Patients and methods

Definitions. A CDAD case was defined as a patient with diarrhoea and a positive laboratory assay for *C. difficile* toxin A and/or B in stools (9). A CDAD case was classified as severe if a patient fulfilled at least one of the following criteria: (i) polynuclear neutrophil count \geq 20,000/mm³; or (ii) serum albumin concentration <35 g/l. Patients were classified into three categories, based on the severity of the underlying disease, according to the McCabe score: no fatal disease (A); fatal disease in the following 5 years (B); or fatal disease in the following year (C) (15). A case was considered to be nosocomial if diarrhoea started \geq 48h after admission (9).

Community-onset CDAD was defined as a patient admitted with diarrhea, or if the diarrhoea started within two days of admission. Only one episode/patient was included in the survey. An episode was designated as a recurrence when it occurred within 8 weeks of the onset of a previous episode (9).

Design of the study. In January 2005, microbiologists in The Netherlands were invited to participate in a prospective laboratory-based study of the incidence of CDAD in the period 1 May to 1 July 2005. Thirteen microbiologists employed by 17 hospitals (six small hospitals included) agreed to participate and informed their associated clinicians and infection control practitioners. Laboratories applied their own algorithms and no recommendations were given concerning specific toxin faeces tests. All patients diagnosed with CDAD during the study period were included. Information was recorded concerning the number of bed, patient-days and number of admissions, and the laboratory tests used to diagnose CDAD. Each participating microbiologist used a standardised questionnaire to collect information concerning each patient's age and gender, the ward in which CDAD was diagnosed, the duration of diarrhoea and consistency of faeces, the presence of abdominal pain or fever (≥38°C), maximal white blood cell count, serum albumin concentration, risk factors, antibiotic treatment in the month preceding a positive test, and treatment outcome. Faecal samples of patients with CDAD were stored at -20°C before being sent to the Reference Laboratory at Leiden University Medical Center for culture and strain characterisation.

Culture and identification of *C. difficile* isolates. Culture of faecal samples for *C. difficile* was performed at the Reference Laboratory using *C. difficile* selective agar (CLO-medium; bioMérieux, Marcy I'Etoile, France) with and without ethanol shock pretreatment (16). *C. difficile* was identified phenotypically by production of L-proline-aminopeptidase and hydrolysis of aesculin (17). Isolates of *C. difficile* were characterised further by PCR.

Strain characterization. All isolates were identified genetically as *C*. *difficile* by an in-house PCR for the presence of the *gluD* gene, encoding the glutamate dehydrogenase specific for *C*. *difficile*, using forward primer: 5'-GTCTTGGATGGTTGATGAGTAC-3' and reverse primer: 5'-TTCCTAATTTAGCAGCAGCTTC-3'. For detection of *tcdA*, primers NKV011 and

NK9 were used as described by Kato *et al.* (18). The *tcd*B profile was verified with primers NK104 and NK105 (19). The presence of deletions in *tcd*C was investigated by an in-house PCR with forward primer: 5'- CATATCCTTTCTTCTCCTCTTC-3' and reverse primer: 5'-AATTGTCTGATGCTGAACC-3', yielding an expected amplicon size (without a deletion) of 159 bp (20). The presence of the genes for the binary toxin, *cdt*A and *cdt*B, was investigated as described by Stubbs *et al.* (21). PCR ribotyping was performed according to the method described by Bidet *et al.* (6). Toxinotyping was performed as described by Rupnik *et al.* (8), using the data for two fragments, B1 and A3.

Results

Participating hospitals. The participating hospitals were distributed equally throughout The Netherlands. Of 17 participating hospitals, six were university-affiliated centers (Table 1, laboratories 1-5 and 13) and 11 were community hospitals (Table 1, laboratories 6-12). The laboratories of two participating hospitals also functioned as public health laboratories (laboratories 6 and 11). Two district laboratories provided microbiological services for several small community hospitals, covering 600 beds in two hospitals and 1730 beds in four hospitals, respectively (laboratories 8 and 9).

Diagnostic methods. Laboratories using direct assays to detect the presence of *C. difficile* toxins in faecal samples used either cytotoxicity assays (n = 6; laboratories 3, 4, 6, and 9-11) or enzyme immunoassays (n = 7; laboratories 1, 2, 5, 7, 9, 12, and 13). One laboratory performed both types of assay (laboratory 9). Of the 13 laboratories, six cultured *C. difficile* from faecal samples routinely (laboratories 1, 2, 5, 8, 10, and 13). Assays applied to cultured isolates to recognise toxin production included cytotoxicity test (laboratory 10), enzyme immunoassays (laboratories 1, 2, and 8) or PCR (laboratory 13).

Incidence of CDAD. The number of CDAD cases during the study period varied from 1 to 13 cases/laboratory. The overall incidence (median) rate of CDAD was 16/10 000 patient admissions, and varied from 1 to 46/10 000 (Table 1). There was no correlation between the incidence of CDAD and the number of hospital beds.

Laboratory no.	No. of hospital beds	Incidence/10. 000 admissions	Incidence/10. 000 bed-days	No. of patients diagnosed with CDAD	No. of different ribotypes	No. of different toxinotypes
1	653	46	6	11	5	2 (0, V)
2	1002	37	4	13	5	3 (0, III, VI)
3	662	16	2	3	3	1 (0)
4	815	17	3	6	5	3 (0,V,XII)
5	1300	14	2	4	3	2 (0, V)
6	1070	13	3	7	4	1 (0)
7	653	14	2	3	3	2 (0, V)
8 ^a	600	3	<1	1	1	1 (0)
9 ^b	1730	2	<1	4	4	1 (0)
10	1368	4	1	3	3	1 (V)
11	697	5	1	2	2	1 (0)
12	1100	14	2	6	5	3 (0, IV, V)
13	882	12	2	4	4	2 (0, XII)

Table 1. Incidence of *Clostridium difficile*-associated disease (CDAD) in 13 participating laboratories and general characteristics of the *C.difficile* isolates

^a Provided microbiological services for two small hospitals.

^b Provided microbiological services for four small hospitals.

Characteristics of patients with CDAD. In total, 91 completed questionnaires were obtained. Ten patients were excluded from the analysis: three patients were considered to be asymptomatic carriers; incomplete data were obtained from one patient; and six patients yielded non-toxinogenic isolates from toxin-negative faeces (hospital A). The patient characteristics are depicted in Table 2. The highest incidence of CDAD (58%) was found in patients aged >65 years. An estimated fatal underlying disease was present in 40 (50%) of all patients with CDAD (McCabe B/C; Table 2). Of 81 patients with CDAD, 20% experienced it as a recurrence. Of these 81 patients, 29 (36%) were admitted to the hospital with

Characteristic		No. of patients (%)
Gender	male	38 (47)
Gender	female	43 (53)
	≤35	10 (12)
Age (vears)	36-50	9 (11)
Age (years)	51-65	15 (19)
	>65	47 (58)
	Α	38 (47)
McCabe score ^a	В	24 (30)
Weeabe score	С	16 (20)
	Unknown	3 (3)
Davalonment of	Nosocomial	49 (61)
diarrhoea ^b	Community onset	29 (36)
uluinotu	Unknown	3 (3)
	<2 days	14 (17)
Length of diarrhoea	2-7 days	28 (35)
Length of tharmoea	>7 days	33 (41)
	Unknown	6 (7)
	<10	26 (32)
Maximal white blood	10-19	23 (29)
cells count $(10^9/l)$	≥20	15 (19)
	Unknown	17 (21)
	<35	40 (50)
Albumin	35-49	6 (7)
concentration (g/l)	≥50	1 (1)
	Unknown	34 (42)
	Surgery	31 (37)
	Previous hospitalization (1 mo)	39 (48)
Predisposing factors	Cancer	10 (12)
record for the second second	Nasogastric tube	15 (19)
	Aids	1 (1)
	Previous antibiotic use	61 (75)

Table 2. Characteristics of 81 patients with Clostridium difficile-associated disease

^a A, no fatal disease; B, fatal disease in the following 5 years; C fatal disease in the following year (17)

community-onset diarrhoea. Of this group, 13 (45%) had been hospitalized in the previous month and five (46%) of these experienced CDAD as a recurrence. Data for the remaining 3% patients were not available.

The majority (53%) of patients with CDAD were hospitalized in a general medical ward, with 26% in a surgical department and 10% in an intensive care unit. Patients with nosocomial CDAD in different departments did not differ in terms of age, McCabe score or duration of hospitalisation (data not shown). The most common clinical presentation of CDAD was as a diarrhoeal disease with liquid or loose faeces for 2-7 days (35%) or >7 days (41%), without abdominal pain (50%) and without fever (67%). A severe course of CDAD was observed in 12 (15%) patients.

Of all CDAD patients, 48% had been hospitalised in the previous month. Antibiotic use in the preceding month was recorded for 61 (75%) of the 81 patients. The most frequently prescribed antibiotics were ß-lactam antibiotics (47% of treated patients) and fluoroquinolones (12% of treated patients).

Treatment and outcome. Of 75 patients with CDAD for whom treatment information was available, 60 (69%) required specific treatment: 51 were treated with metronidazole alone; five received metronidazole followed by vancomycin; and four were treated with vancomycin alone. Seven (8%) patients died during the study period, with CDAD considered to be the cause of death for two patients. One of these was a male aged 83 years with diarrhoea, which was present for more than a month before admission. The patient was not treated with antibiotics during the previous month and had none of the known risk factors. He was not treated specifically for CDAD and died 5 days after admission because of respiratory insufficiency. Typing of the *C.difficile* isolate revealed that it belonged to toxin producing PCR ribotype 001, toxinotype 0, with an 18bp deletion in the *tcd*C gene. The second death was of a female, aged 86 years, with diarrhoea for >7 days before admission. This patient died within a week of admission because of cardiac insufficiency. Typing of the *C. difficile* isolate revealed that it belonged to the toxin producing PCR ribotype 078, toxinotype 0, with a 39bp deletion in the *tcd*C gene.

C. difficile isolates. Faecal samples of 81 patients were included in the analysis. Of these, 67 contained *C. difficile*, as determined by a positive PCR for the presence of *glu*D. Of 67 *C. difficile* isolates, 64 were positive for *tcd*A and *tcd*B (Table 3); of these, 45 (70%) harboured an intact *tcd*C gene, seven (11%) had a 39bp

deletion in the *tcd*C gene, and 11 (17%) had an 18bp deletion, with ten of the latter isolates belonging to PCR ribotype 027/toxinotype III (Table 3). One (2%) ioslate had a deletion of *c*. 44 bp, which has not been described previously (toxinotype 0; Table 3). Of the 64 isolates, 19 (30%) contained both binary toxin genes, whereas two (3%) contained only the *cdt*A binary toxin gene (Table 1, laboratories 4 and 12).

PCR-ribotyping and toxinotyping. Among the 67 isolates, 28 different PCR ribotypes were recognised (Table 1). Among the 64 toxinogenic isolates, 41 (64%) were of toxinotype 0 (Table 3). The most frequently occurring PCR ribotypes were type 027 (16%) and 014 (16%). Ten (48%) of the 21 binary toxin-positive isolates belonged to PCR ribotype 027/toxinotype III and nine (43%) belonged to toxinotype V (Table 3). The latter isolates were obtained from six different hospitals (Table 1).

Table 3. Characteristics of 64 toxinogenic Clostridium difficile isolates

	es	/pes	<i>tcd</i> A and	es with genes	No. of isolates with deletions in <i>tcd</i> C gene (bp)			
Toxinotype	No. of isolat	No. of riboty	Presence of <i>i</i> <i>tcd</i> B	No. of isolat binary toxin (<i>cdtA/cdt</i> B)	Δ 0 bp	Δ 18 bp	Δ 39 bp	∆ >39 bp
0	41	21	41	1 <i>cdt</i> A and <i>cdt</i> B	37	1	2	1
III	10	1	10	10 cdtA and cdtB	0	10	0	0
IV	1	1	1	1 <i>cdt</i> A and <i>cdt</i> B	1	0	0	0
v	9	3	9	7 <i>cdt</i> A and <i>cdt</i> B 2 <i>cdt</i> A	4	0	5	0
VI	1	1	1	0	1	0	0	0
XII	2	2	2	0	2	0	0	0

Discussion

This study found a mean incidence of CDAD in the Netherlands of 16/10 000 patient admissions (2.2/10 000 bed-days), which is comparable with the results of a European study performed in 2002 (22). The incidence rate in the participating hospitals was comparable with the pre-outbreak incidence in eight hospitals in The Netherlands that were affected by PCR ribotype 027 during 2006. Interestingly, one

outbreak hospital (no. 2) also participated in the present surveillance study and had a higher incidence level than the other hospitals (14).

The incidence of CDAD as a nosocomial disease was $13/10\ 000$ patient admissions. This low incidence does not reflect the use of inadequate diagnostic tests, since all participating laboratories used a toxin assay, and 46% applied the cytotoxin test to faecal samples. The observation that 16% of positive faecal samples were culture negative reflects the low concordance of enzyme immunoassays with culture of *c*. of 60% (23). Recent data from studies in the USA and Canada have demonstrated a much higher incidence of CDAD among hospitalised patients of 100-450/10 000 admissions, which has been associated with emergence of infections caused by the hypervirulent PCR ribotype 027 strain (10-13). In Quebec, Canada, where type 027 is endemic, a survey of 39 hospitals revealed a mean CDAD incidence rate of 19/10 000 bed-days, which is nearly ten-fold higher than the CDAD incidence in The Netherlands (24).

C. difficile is often defined as a nosocomial pathogen. However, it is widely distributed in the environment, and has been isolated from soil, water and the faeces of many wild, domestic and farmed animals (5). Surprisingly, 31 (36%) of all patients with CDAD were admitted to the hospital with diarrhea, or developed diarrhoea within 2 days of admission. However, 13 (33%) patients had been hospitalised in the previous month, indicating a community onset with hospital association. Two (6%) of these 31 patients died, with CDAD as an attributable cause of death. These two cases represent community-acquired CDAD with a fatal outcome. This observation is in agreement with reports in the USA from the CDC that mention severe community-onset CDAD in populations considered previously to be at low risk (25).

Since the finding of outbreaks caused by type 027 in at least 20 healthcare facilities in The Netherlands, several recommendations and guidelines have been published by the Dutch Centre for Infectious Disease Control (National Institute for Public Health and the Environment, Bilthoven) for use by clinicians and microbiologists. Most laboratories apply the 3-day rule and use the definitions suggested by the European CDC (9) to recognise and differentiate hospital-acquired and community-acquired CDAD. During a 3-month study using this algorithm at four university laboratories, a 30% increase in the number of CDAD patients diagnosed was recorded (23).

In the present study, 28 different PCR ribotypes were found in 17 hospitals. This represents 15% of the 109 hospitals in The Netherlands. The PCR ribotypes were distributed equally among all hospitals, except for PCR ribotype 027, which was restricted to a single hospital. The commonest PCR ribotype was 014 (16%), found in six different hospitals. These results agree with the results of a study performed during 2005 in Hungary, in which 24.8% of isolates belonged to PCR ribotype 014 (26). However, in 2001, PCR ribotype 087 accounted for 39% of 165 *C. difficile* isolates in Hungary (27), demonstrating clearly that the incidence of PCR ribotypes can change over time.

All isolates in the present surveillance study contained genes encoding toxins A and B, and no toxinotype VIII isolates were found. Although A'/B^+ isolates were not detected, an outbreak was recognized in February 2006 in a hospital in The Netherlands, which simultaneously experienced an outbreak caused by PCR-ribotype 027 (14).

In the present study, one hospital experienced an outbreak caused by PCR ribotype 027, toxinotype III. C. difficile type 027 isolates have an 18bp deletion in the tcdC gene. This gene is thought to be a negative regulator of TcdA and TcdB production, and it has been reported that these type 027 isolates produce TcdA and TcdB in considerably greater quantities than toxinotype 0 isolates (28). Deletions in tcdC are frequently present in toxinogenic isolates (20), and the present study found that 30% of toxinogenic isolates had a deletion of 18bp or 39bp, with one strain having a previously unreported deletion of 44 bp in tcdC. It is possible that different deletions in *tcd*C cause different functionality and affect toxin production differently. Interestingly, the isolates from the two patients who died did not belong to type 027, but contained deletions in the toxin regulator gene tcdC. In this study, toxinotype 0 was the most frequently isolated type (46%), followed by III, IV, V, VI and XII, which is similar to the results of previous studies encompassing isolates from collections in Europe and Asia (8). Binary toxin genes were detected in 33% of toxinogenic isolates, mainly in association with toxinotypes III and V. This percentage is higher than that reported previously and can be explained by differences in the geographical distribution of different clones (29).

In summary, the incidence of CDAD in the Netherlands varied considerably in individual hospitals, but is considerably lower than the incidences reported in the
USA and Canada, independent of the occurrence of PCR-ribotype 027. The finding that 36% of all patients diagnosed with CDAD have a community onset or community association merits further studies to determine its significance.

Acknowledgements

We thank all medical microbiologists, Dr. A. Troelstra, Dr. T. Schulin, Prof Dr. J.E. Degener, Dr. M. Peeters, Dr. A. Demeulmeester, Dr. C.H.W. Klaassen, Dr. A. Horrevorts, Dr. A. Möller, Dr. P. van Keulen, Dr. A. van Griethuysen and Dr. P.C. Wever, from the participating hospitals for their contribution to the surveillance study.

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Chapter 10

General discussion

Clostridium difficile was first discovered in 1935, but it was not until 1977 that this bacterium was found to be associated with pseudomembranous colitis. The disease was considered to be caused by the production of two *C. difficile* toxins, toxins A and B (TcdA and TcdB). TcdA was shown to exhibit enterotoxic effects on the intestine, whereas TcdB was shown to be more cytotoxic. Strains of *C. difficile* that do not produce these toxins are known to be non-toxinogenic and therefore non-pathogenic. It was suggested that both toxins worked synergistically. However, outbreaks and more severe infections due to strains producing only TcdB have been described. These strains can be recognized by a deletion in the *tcd*A gene, resulting in the inability to produce TcdA.

To diagnose *C. difficile* -associated disease, a method with a high sensitivity and specificity is necessary. This thesis describes the application of methods based on the molecular detection of the pathogen in comparison with conventional microbiological methods. After isolation of the pathogen, the epidemiology of *C. difficile* -associated disease can be studied using molecular typing methods on the isolates. These typing methods need to be highly discriminatory, depending on the epidemiological data to be studied, and methods need to be stable en reproducible, with the possibility to exchange data between different laboratories all over the world.

Diagnosis of *Clostridium difficile*-associated disease (CDAD)

As described in Table 1 in the General introduction (**Chapter 1**), several diagnostic methods have been developed for the detection of *C. difficile* and its products. The production of toxins by *C. difficile* can be detected by the cell cytotoxicity assay and the enzyme immunoassay (EIA). *C. difficile* itself can also be cultured, or (toxin) genes can be detected by PCR.

Conventional diagnostic methods

'*Gold standard*'. The cell cytotoxicity assay detects the cytopathic effect of TcdB on tissue culture cells. Due to the high specificity and the good sensitivity of this assay, it was considered the 'gold standard' (Johnson, 1998). However, the cell

cytotoxicity assay has some drawbacks. The long turnaround time and the need for cultured monolayers, but also the effect of storage of faecal samples on the toxin titres, make a re-evaluation as a 'gold standard' necessary. Another method, recommended as 'gold standard', is the toxinogenic culture method, where cultured isolates are further investigated for their toxin production (Delmee, 2001; Delmee, 2005; Zheng, 2004). Although culture is often considered a highly sensitive method, it also detects asymptomatic carriership, which can increase to 15% during a stay in a hospital where *C. difficile* is endemic (Samore, 1994). Of the 496 admissions, 11% of cases in this study showed *C. difficile* by culture within 72h after admission. In two other studies, the percentage of carriage of healthy adults was shown to be as high as 7.6%-12.9% (Kato, 2001; Ozaki, 2004). Walker *et al.* found 16 (7%) of 225 asymptomatic patients positive by toxinogenic culture. This was significantly associated with the use of antibiotics (Walker, 1993). Another drawback of toxinogenic culture as the 'gold standard' is the long turn-around time (at least 48h) and the necessity to verify the toxinogenicity of strains by other assays.

In conclusion, although both the cytotoxicity assay and toxinogenic culture require a long turn-around time, the cytotoxicity assay is used as the 'gold standard' (**Chapters 2-4**).

Cytotoxicity assay versus enzyme immuno assays (EIA). EIAs have been evaluated for use as a gold standard, since an EIA easy to perform and provides rapid results. EIAs can detect TcdA and/or TcdB by an enzyme-bound antibody against the toxin(s). In general, the sensitivities and specificities of commercially available EIA kits compared to the cytotoxicity assay vary between 63-99% and between 88-100%, respectively (Brazier, 1998). It is advised to use an EIA detecting both TcdA and TcdB, since TcdA-/TcdB+ strains do occur. In the period from October 2001 to October 2002, 2 TcdA-/TcdB+ of 40 isolated strains (5%) were found within our hospital (unpublished data). VIDAS is the only automated enzyme assay, but detects only TcdA (**Chapters 2 and 4**), and would therefore have missed the two strains mentioned above.

A new generation of rapid diagnostic tests has been developed, such as the membrane-associated immuno assay: ImmunoCard TcdA and TcdB (ICTAB), as described in **Chapter 3**. In this chapter, the sensitivity and PPV of ICTAB was 91.3% and 70% in comparison with the cell cytotoxicity assay, respectively. Of 344

cytotoxicity negative samples (93.7%), 9 (2.6%) were positive by ICTAB. In Chapter 4, the enzyme assays VIDAS and Premier TcdA and TcdB (PTAB) were compared against the cell cytotoxicity assay, resulting in sensitivities of 83.9% and 96.8%, respectively. The PPVs were 63.4% and 50.9%, respectively. Of the 509 cytotoxicity negative samples (94.3%), 15 (2.9%) were positive by VIDAS, and 29 (5.7%) were positive by PTAB. In Chapters 3 and 4 it has been described that ICTAB, VIDAS and PTAB show samples solely positive by EIA, but negative by the cytotoxicity assay (EIA+/cyto-). Since the discrepant samples resulted in 3 of 8, 10 of 13, and 21 of 26 EIA+/cyto- samples to be negative by culture for ICTAB, VIDAS and PTAB, respectively, these results suggest false positive EIA results. This may also explain the low PPVs detected for the EIAs. The use of low cut-off values can result in false-positives, accepting more samples to show positive results, although the cut-off values used were suggested by the manufacturer. In other studies, EIAs have been shown to result in slightly lower sensitivities when compared to the cytotoxicity assay as well (O'Connor, 2001; Turgeon, 2003). In these studies, the sensitivities of VIDAS, ICTAB and PTAB, were 70, 54-56% and 80%, respectively. The VIDAS assay was outperformed in all comparisons by the other EIAs (Turgeon 2003, Chapter 4). However, both PTAB and ICTAB are still of interest due to their rapid turnaround time, with results known within one day. Furthermore, membrane immunoassays (ICTAB; Chapter 3) are faster than the well-type assays, although they are more expensive. Another advantage of EIAs above the cytotoxicity assay is that no specific laboratory requirements are needed and that the methods are easy to perform.

In conclusion, the sensitivity of EIAs has been shown to be lower than that of the cytotoxicity assay, with low PPVs as well. The low PPVs can be explained by false positives in the EIAs. Due to the rapid and easy-to-perform use of the EIAs, they are still of interest as rapid screening methods, although it should not be used as the sole laboratory test available. However, a critical evaluation of the cut-off values of EIAs needs to be performed to reduce the number of false-positives. Furthermore, more sensitive rapid diagnostic assays should be developed.

Molecular diagnostic methods

A promising new rapid diagnostic method for diagnosis of CDAD is (realtime) PCR. Using PCR, both toxin genes (*tcd*A and *tcd*B) or other common genes (e.g. *glu*D) can be detected. Real-time PCR includes a fluorescent probe, which allows monitoring of PCR-amplification in 'real-time' and quantification of PCRproducts. PCR has been applied to detect *C. difficile* in different studies (Wren, 1990; Kato, 1998; Kato, 1999; Belanger, 2003). The advantage of real-time PCR over conventional PCR is the reduced hands-on time, due to the absence of post-PCR analysis (Mackay, 2004).

Cytotoxicity assay versus real-time PCR. The sensitivity of our inhouse real-time PCR compared to the cell cytotoxicity assay was 100%, 87% and 87.1% in **Chapters 2, 3 and 4** respectively. Belanger *et al.* also describe a real-time PCR assay for diagnosis of CDAD in comparison with the cytotoxicity assay. Although only 56 faecal samples were tested in this study, the sensitivity was 97% (Belanger, 2003). They concluded that the real-time PCR was rapid, sensitive and specific and could therefore be used for diagnosis of CDAD. The real-time PCR can be performed within one day. However, the sensitivity of our real-time PCR was lower than for ICTAB (91.3%) and PTAB (96.8%), in respectively **Chapters 3 and 4**, although this could be explained by the occurrence of false positives in the EIAs (as discussed above).

In **Chapter 2**, real-time PCR showed a PPV of 55% compared to the cell cytotoxicity assay. In **Chapters 3 and 4**, the PPV was 57.1% and 60%, respectively. Although all cell cytotoxicity positive samples in **Chapter 2** (n=6; 7%) were also positive by real-time PCR, 5 (6%) of the 79 cell cytotoxicity negative samples were positive by real-time PCR and culture. In **Chapters 3 and 4**, 9 of 13, and 10 of 18 faecal samples positive by real-time PCR and negative by the cytotoxicity assay, were positive by culture. This could imply that both PCR and culture detected *C. difficile* in faecal samples, representing asymptomatic carriage. However, another explanation for the low PPV could be a low sensitivity of the cell cytotoxicity assay. Storage and transport conditions could have resulted in low cytotoxin titres, with little or no effect on real-time PCR and culture detection. This will be discussed below.

It can be concluded that real-time PCR is a rapid method, allowing the detection of *C. difficile* genes directly from faeces. The detection of asymptomatic carriers is another application of interest. However, the sensitivity can be increased, as will be discussed below.

Real-time PCR development. For the development of a (real-time) PCR method for the diagnosis of CDAD, DNA isolation is of importance. In the case of C. difficile, faecal samples are the samples for DNA isolation, which can result in inappropriate PCR amplification, due to inhibitory faecal residues. Another problem could be a poor DNA recovery or ineffective release of DNA, for example from spores present in faeces. To overcome these problems for isolation of DNA from faecal samples, pre-incubation steps have been advised (Monteiro, 1997; Morgan, 1998). The boiling of faecal samples removes some of these inhibitory substrates and makes lysis of the cells more effective. In the study by Morgan et al (1998), polyvinylpyrolidone (PVPP) was used to remove inhibitory substances from faecal samples. Therefore two pre-treatments were evaluated in Chapter 2. The method using the column-based extraction with PVPP pre-incubation was shown to be as sensitive as the automated extraction with STAR-buffer pre-treatment. However, due to the advantages of an automated method, this latter method was chosen as the method of choice.

Also, the number of bacteria present in the faeces influences the sensitivity. In **Chapter 2**, we observed a higher analytic sensitivity of culture in comparison with real-time PCR. The sensitivity of culture $(1x10^4 \text{ CFU/g faeces})$ was shown a factor 10 higher than the sensitivity of real-time PCR $(1x10^5 \text{ CFU/g faeces})$. Therefore, the sensitivity of the real-time PCR should improve before we can implement this method in routine diagnostics of CDAD. One option for optimisation is a new or different extraction method, compared to the two methods described above.

To control for inhibition and the efficient amplification of the PCR products, an internal control is advised (Barkham, 2004; Hoorfar, 2003). An internal control is preferably universal, regardless of the target. By addition of a standard concentration of the internal control, the effect on the expected Ct-values can be observed. In this thesis, Phocine Herpes Virus (PhHV) was used as the internal control (**Chapter 2**). The internal control is amplified in a multiplex format, containing the target of interest and the internal control. Since the target is different from *C. difficile* itself, it is hard to control for complete lysis of the bacterium. However, the internal control does control for PCR-inhibitory factors. The exploration of a modified *C. difficile* target as an internal control can result in control of lysis is therefore advised.

Multiplexing is one of the main advantages of real-time PCR over conventional PCR, and can be used for optimisation of the sensitivity due to the use of other targets. The real-time system is able to detect four different fluorophores, and can therefore detect up to three targets and an internal control at the same time. A multiplex assay can therefore be used to detect different micro-organisms, or different genes from the same micro-organism. As will be discussed below, diarrhoea starting after three days of hospitalization leads to investigation for C. difficile and excludes the detection of other diarrhoea causing bacteria. Viral and parasitic agents however may be other causes of hospital acquired diarrhoea and could be included in a multiplex PCR. However, a combination of different C. difficile genes should be explored first, to optimise the real-time assay. An option for a gene to expand the tcdB real-time PCR, is gluD, as discussed in Chapter 2. Although this target will not differentiate between toxinogenic and nontoxinogenic strains, it is present in all C. difficile isolates and it uses primers that are specific to C. difficile , and do not detect other anaerobes that produce glutamate dehydrogenase. A possible third target is the tcdA gene, although deletions are known within this gene. Another approach, based on the known genome sequences of the toxin genes, is the use of identical targets within both *tcd*A and *tcd*B to obtain a 'multicopy' effect.

A possible restriction to the (real-time) PCR is the opportunity to detect all known genotypes of the target. The first described real-time PCR for *C. difficile tcd*A and *tcd*B (Belanger, 2003) was unable to detect *tcd*B in toxinotypes III, IV and VI. The detection of toxinotype III is of specific importance, due to the occurrence of type 027/BI/NAP1 strains belonging to this toxinotype. As has been described in **Chapter 2**, our real-time assay is able to detect all types, although the TcdA-/TcdB+ strains show some mismatches and can therefore result in reduced sensitivity of the assay. Therefore, a target that is constant in all types (e.g. the *glu*D gene) should be explored to establish the detection of all types with the same sensitivity.

The use of real-time PCR to quantitate the bacterial load has to be investigated as well. The inclusion of a standard dilution series may yield semiquantitative results, due to the properties of faeces, which vary from patient to patient. To establish a semi-quantitative analysis, the DNA isolation from faecal samples needs to be standardized as well, with a known amount of faeces. That is hard to establish due to the differences in liquidity of the faeces. Not only does this standardization need further attention in the future, the implication of high or low concentrations must be known as well. This is specifically of interest for detection of the differentiation between carriage and disease. The carriage of *C. difficile* and the detection by real-time PCR has been described in **Chapter 2**. In this chapter, 3 of 43 patients (7%) without symptoms were positive by both culture and real-time PCR, but negative by the toxin detection assays, indicating asymptomatic carriage.

In conclusion, for the application of the real-time PCR as the sole diagnostic assay for CDAD, DNA isolation should be optimised. The automation of this isolation is of importance as well, reducing the hands-on time. Optimisation of DNA isolation methods can increase sensitivity of the assay, and can yield the opportunity to semiquantitate the bacterial load, taken into account that the whole assay should be standardized. However, an increased sensitivity could also lead to the detection of carriage of *C. difficile*. Therefore, the implication of different concentrations of *C. difficile* in faeces should be further investigated. For control of lysis and PCR-inhibition, an internal control is of importance, preferably using a modified *C. difficile* target. Furthermore, a multiplex assay with multiple targets of *C. difficile* could increase the diagnostic yield, specifically when a target is included that is detected with the same sensitivity in all type strains.

Criteria for sampling

Faecal samples and storage. For detection of *C. difficile* in the intestinal tract, faecal samples and rectal swabs can be used. Rectal swabs have been shown to be more sensitive for culture than swabs taken from faecal samples (McFarland, 1987; Arroyo 2005). The consistency of the faecal samples would ideally be watery, loose or unformed, and solid samples should be refused. It is advised to test all faecal samples within 24h of receipt (Turgeon, 2003). If the laboratory is unable to process samples within this timeframe, samples for testing by the cell cytotoxicity assay should be stored for a maximum of 56 days at 4 °C. Samples can be stored at -20 °C without multiple freezing/thawing steps, for at least 28 days.

However, multiple freezing/thawing steps have a detrimental effect. Culture has been shown to be unaffected by storage temperature (4 °C or -20 °C) for at least 56 days, or multiple freezing/thawing steps (Weese, 2000; Freeman, 2003). No effects of storage on EIA toxin titres have been shown; therefore it is advised to store them at a maximum of 56 days at 4 °C. The optimal storage condition of faecal samples for PCR analysis is unknown. The effect of storage and transport was shown to be a problem in our multicenter studies (**Chapters 2-4**). In these studies, a low PPV compared to the cell cytotoxicity assay was observed for the toxin detection assays (VIDAS, ICTAB, PTAB) and the real-time PCR. Although the detection of false-positives by the toxin detection assays, another explanation could be a low sensitivity of the cell cytotoxicity assay. The lower sensitivity of the cell cytotoxicity assay is probably associated with storage at -20 degrees for at least one month before processing, which is in agreement with the study of Freeman *et al.* (Freeman, 2003). In following studies, samples should therefore be tested at the time of arrival, or should be stored at 4 °C.

Three-day rule. When hospitalized patients are admitted for at least 72h and subsequently develop diarrhoea, it is advised to test only for *C. difficile* and not for *Salmonella, Campylobacter, Shigella* and *Yersinia* (Sack, 1980; Fan 1993, **Chapter 4**). We evaluated this three-day rule in **Chapter 4**. Of 121 patients without a request for CDAD diagnosis, 5 patients (4.1%) were positive by the cell cytotoxicity assay. The implementation of this rule resulted in a 23.8% higher yield of CDAD positive patients. Since the finding of outbreaks due to PCR-ribotype 027 in at least 20 healthcare-facilities in The Netherlands, several recommendations and guidelines have been published by the Centre for Infectious Disease Control at the National Institute for Public Health and the Environment for clinicians and microbiologists. One of these recommendations is the application of the three-day rule and most of the laboratories in the Netherlands have implemented this.

CDAD in the community. CDAD in the community can be differentiated in CDAD with a community onset but with a hospital association, or CDAD with a community onset and community association (Kuijper, 2006). Of all CDAD patients with community-onset described in **Chapter 9**, 13 of the 31 patients were previously admitted to the hospital. Two of these community-onset patients died, in which CDAD was the attributable cause of death. This is in agreement with a report of the CDC, which showed an increase in severe community-acquired CDAD in a population with a low risk of infection (Chernakl, 2005). The incidence of CDAD in the community is largely unknown and should be investigated further.

Repeated testing. Another aspect of diagnosing CDAD is the number of samples needed for testing. Using a 7-days timeframe and a cell cytotoxicity assay or an EIA for both toxins, it has been described that repeat samples will yield extra information for diagnosis in 1.3%-1.6% of cases (Renshaw, 1996; Mohan, 2006). The consequence of this observation is that a high reduction of test samples can be achieved by refusing to test repeat faecal samples. A reduction of 34-36% has been found (Renshaw, 1996; O'Connor, 2001). In Chapter 4, two or more samples per patient were tested in 68 of 450 (15.1%) patients, and 97 of 540 (18%) of samples were repeat samples. Two of 97 repeat samples became positive in a second sample after a first negative test, and two other cases became negative in a second sample, within a timeframe of 9-17 days. From this it was concluded that repeat samples within 7 days can be rejected for testing, reducing the amount of samples by 18% (97 of 540 samples). In a study by Debast et al. (manuscript in preparation) in the Netherlands, the value of repeat samples was evaluated in an outbreak situation due to the C. difficile PCR-ribotype 027 and 017 strain. Of 50 patients positive by ICTAB in a type 027/BI/NAP1 outbreak situation, 4 patients (8%) were positive by ICTAB within 7 days after a first negative sample. In another hospital with both a type 027/BI/NAP1 and a type 017/VIII outbreak at the same time, 10 of 166 (6%) ICTABpositive samples were positive within 7 days after the first negative sample. The value of repeated testing may be type-specific. Therefore it was concluded that repeated testing within 48h in the case of an epidemic can control the rapid spread of epidemic strains, specifically for PCR-ribotypes 017 and 027, since these epidemics are difficult to control.

Concluding remarks

With respect to the increasing number of newly developed diagnostic tests, the need for a better 'gold standard' than the cell cytotoxicity assay is growing. To identify a new gold standard, a well designed prospective study with the use of appropriate clinical definitions of CDAD and standardization of sampling is necessary. Although culture and real-time PCR are the most sensitive methods, both are also detecting carriage, which needs to be differentiated from CDAD. Ideally, real-time PCR should be used as a screening method, followed by a rapid faecal toxin test, but improvement of the currently available assays is necessary.

Genotyping of Clostridium difficile

Typing methods can be used to detect an outbreak (**Chapter 8**; Hernandez, 2004; Mohr, 2004; Noren, 2004). Typing methods also allow us to learn more about the different types circulating in the (hospital) environment, or can be used to distinguish between re-infection or relapse within the same patient. In the case of an outbreak situation, samples need to be types as soon as possible. However, for long-term epidemiology hospitals collect all strains for typing afterwards. The specific characteristics of a typing method for these purposes will be discussed below. Since genotyping methods outperform the (older) phenotyping methods, only genotyping will be discussed. In **Chapters 5-7**, different genotyping methods have been applied: PCR-ribotyping, amplified fragment length polymorphism (AFLP), toxinotyping and MLVA.

Typing methods for early detection of an outbreak

For the detection of outbreaks, a typing method has to be highly discriminatory, rapid and preferably cheap, easy-to-perform, and easy-to-interpret. The most frequent applied methods to investigate an outbreak situation are: restriction enzyme analysis (REA), arbitrarily primed-PCR (AP-PCR)/random amplified polymorphic DNA (RAPD) and PCR-ribotyping. REA has a high discriminatory power but is extremely difficult to automate. It remains a highly subjective typing method, but is still used in North America as the standard typing method (Marsh, 2006; Johnson, 2003). Compared to REA, restriction fragment length polymorphism (RFLP) shows a lower discriminatory power, detecting 34 versus 6 types in 116 isolates (O'Neill, 1993) and is therefore less useful in typing analysis. Due to the often subjective analysis and poor reproducibility, REA is only useful in the analysis of small outbreaks. AP-PCR/RAPD has a low reproducibility (Wilks, 1994), but is a

simple, rapid and discriminative method that is cost-effective, and can therefore be applied in local outbreak situations. PCR-ribotyping is fast (results within one day), discriminatory, stable and reproducible and has the advantage that results can be exchanged between laboratories. Because of this interlaboratory exchange, PCR-ribotyping seems also suitable for the detection of world-wide epidemics, as is currently seen for TcdA-/TcdB+ strains and strains belonging to type 027/BI/NAP1. A disadvantage is that certain common circulating PCR-ribotype strains can be further discriminated in subtypes by other methods, such as REA, pulse-field gel electrophoresis (PFGE), and multi-locus variable number of tandem repeats analysis (MLVA) (**Chapter 7**), as will be described below.

Toxinotyping is a genotyping method able to discriminate 25 types among *C. difficile* strains, based on PCR-RFLP, but this method is laborious and 62% to 80% of all strains belong to toxinotype 0 (Rupnik, 2003; Geric, 2004). Toxinotyping is considered a fingerprinting method of the PaLoc and represents the virulence characteristics of the isolates. However, other virulence factors outside of the PaLoc are considered as well, specifically for the epidemic PCR-ribotype 027 strain. In **Chapter 9** we found that 41 of 64 (64%) strains collected from May 1st to July 1st 2005 in the Netherlands to belong to toxinotype 0. This is in accordance with the above mentioned percentages. Toxinotyping recognizes TcdA-/TcdB+ strains and PCR-ribotype 027/BI/NAP1 strains as toxinotype VIII and III, respectively (Rupnik, 1998; Pepin, 2005). Other PCR-based methods, like multi-locus sequence typing (MLST) and MLVA (**Chapter 7**) can be used as well due to their high reproducibility. Although results of these latter assays are interchangeable, they are more laborious then PCR-ribotyping, but the detection of subtypes of specific types like type 027/BI/NAP1 at the same time is one of the main advantages.

Typing methods for long-term epidemiology

For studying long-term epidemiology, methods with a high discriminatory power, reproducibility and stability are necessary. This is specifically of interest for the interlaboratory exchange of typing data for world-wide epidemiology of strains. Methods applied in long-term epidemiology are AFLP, PFGE, REA and PCRribotyping. Although AFLP has not been studied intensively, it did show a higher discriminatory power than two PCR-ribotyping methods, as has been described in **Chapter 5**. Of the 81 strains tested, AFLP was able to detect 40 types, whereas the PCR-ribotyping methods by Bidet *et al.* (Bidet, 1999) and O'Neill et al (Stubbs, 1999) detected 36 and 37 types. Therefore, AFLP and the subsequent cluster analysis can discriminate many types based on the whole genome and can therefore be used for epidemiological purposes. However, AFLP is sensitive to different DNA extraction circumstances, sometimes leading to a lower typeability of strains, although re-extraction of DNA solves this problem. AFLP also has the disadvantage that the reproducibility is lower than 100%, unless samples are tested within a single assay. Due to these disadvantages, the interlaboratory exchange of AFLP-data and the implementation for long-term epidemiology are difficult.

Other methods known to have high discriminatory powers are PFGE and REA. REA has already been discussed above. However, PFGE has been shown to have a higher discriminatory power compared to PCR-ribotyping (Bidet, 2000). PFGE discriminated 28 types among 100 isolates, compared to 20 types for a PCR-ribotyping method (Spigaglia, 2001). Although PFGE is known as the most discriminatory method, the low typeability (due to DNA degradation), the long running time and the expensive equipment remain drawbacks of this method (Cohen, 2001).

PCR-ribotyping, although slightly less discriminatory than AFLP, PFGE and REA, as previous mentioned, remains stable and reproducible. Therefore, PCRribotyping has a large library available for world-wide epidemiology data, and is therefore the preferred method for long-term epidemiology.

Subtyping

Subtyping of endemic and epidemic strains could lead to the detection of the infectious route of *C. difficile* from one patient to another, or the detection of the infectious (mostly environmental) source. For this purpose, methods are needed that are able to subtype PCR-ribotype strains that are common in the world, the country or the hospital, and the reproducibility, stability and interchangeability need to be high. Subtyping has been applied on epidemic strains belonging to PCR-ribotype 001, most common in the UK and the USA, the TcdA-/TcdB+ strains and the type 027/BI/NAP1 strains.

PCR-ribotype 001 strains. A modified PFGE method was able to differentiate 50 PCR-ribotype 001 strains in seven subgroups: PF-A to PF-G (Gal, 2005). Another study showed eight subtypes: rep-PCR 1-8, among 200 isolates of PCR-ribotype 001 (Northey, 2005; Rahmati, 2005). We developed an automated MLVA (**Chapter 7**) with the purpose to subtype different PCR-ribotype strains, including the rep-PCR subtypes of PCR-ribotype 001. MLVA was indeed able to discriminate 7 of the 7 received rep-PCR 001 subtypes.

PCR-ribotype 017, TcdA-/TcdB+ strains. The TcdA-/TcdB+ strains, belonging to PCR-ribotype 017/toxinotype VIII, could be differentiated in two groups by REA: CF and CG, the latter of which was only detected in asymptomatic children. REA-group CF could be further discriminated in 6 subgroups: CF1-CF6 (Johnson, 2003). In **Chapter 5** is described that AFLP was able to subtype PCR-ribotype 017 in 2 groups, whereas two PCR-ribotyping methods showed 2 and 3 types. The MLVA, as described in **Chapter 7**, is able to discriminate 8 country-specific clusters among 29 type 017/VIII strains from 8 different countries. We concluded from these results that MLVA is able to subtype PCR-ribotype 017 strains, specifically from different countries, and is able to differentiate these strains from all other type strains.

PCR-ribotype 027, BI/NAP1 strains. The type 027/BI/NAP1 strains could not be further subtyped by rep-PCR, the method that was able to subtype PCRribotype 001 strains, as tested in type 027/BI/NAP1 strains from three different Canadian regions (MacCannell, 2006). However, strains belong to type 027/BI/NAP1 can be subtyped by PFGE (NAP1a and NAP1b) and REA (BI, with 6 subtypes). Marsh et al (Marsh, 2006), who developed an MLVA as well, found 11 strains belonging to REA-group BI, and six different REA BI-types. Using MLVA, they were able to detect nine different MLVA-types: BI6 showed 3 MLVA-types and BI9 showed 2 MLVA-types. In **Chapter 7** it was shown that 13 clusters could be detected among 28 strains from 7 outbreaks. Clusters tended to be hospital-specific, with the largest difference between the outbreak strain from the UK and the Dutch outbreaks. Therefore it can be concluded that MLVA is a new method that shows high expectations in the ability to subtypes important strains, and the discrimination from other type strains.

In an international typing study (manuscript in preparation), 22 type 027/BI/NAP1 strains from four different countries were typed by 8 different

genotyping methods: REA, PFGE, MLVA, MLST, slpA, AFLP and two different PCR-ribotyping methods (Table 1). MLST, both PCR-ribotyping methods and AFLP were unable to differentiate the type 027/BI/NAP1 strains, whereas REA and MLVA are the methods with the highest discriminatory and subtyping power.

Table 1. Overview of the typing results of 8 different genotyping methods on 22 type 027/BI/NAP1 strains from four different countries.

Sample No.	REA	PFGE	MLVA	MLST	Ribo US	Ribo UK	slpA	AFLP
CA2	BI 8	NAP1a	B8	35	2	027	gc8-1	VUCDO3
CA3	BI 8	NAP1	B8	35	2	027	gc8-1	VUCDO3
CA4	BI 17	NAP1	B10	35	2	027	gc8-2	VUCDO3
CA5	BI 8	NAP1	B1	35	2	027	gc8-1	VUCDO3
CA6	BI 8	NAP1a	B10	35	2	027	gc8-2	VUCDO3
NL1	BI 17	NAP1a	B4	35	2	027	gc8-1	VUCDO3
NL2	BI 17	NAP1a	B4	35	2	027	gc8-1	VUCDO3
NL3	BI 17	NAP1a	B10	35	2	027	gc8-1	VUCDO3
NL4	BI 17	NAP1a	B6	35	2	027	gc8-1	VUCDO3
NL5	BI 17	NAP1a	B10	35	2	027	gc8-1	VUCDO3
UK6	BI 21	NAP1	B7	35	2	027	gc8-1	VUCDO3
UK7	BI 17	NAP1a	B11	35	2	027	gc8-1	VUCDO3
UK8	BI 17	NAP1a	B5	35	2	027	gc8-1	VUCDO3
UK9	BI 21	NAP1	B10	35	2	027	gc8-1	VUCDO3
UK10	BI 23	NAP1	B7	35	2	027	gc8-1	VUCDO3
US31	BI 8	NAP1a	B9	35	2	027	gc8-1	VUCDO3
US32	BI 8	NAP1a	B10	35	2	027	gc8-3	VUCDO3
US35	BI 8	NAP1a	B13	35	2	027	gc8-3	VUCDO3
US38	BI 12	NAP1a	B2	35	2	027	gc8-1	VUCDO3
US39	BI 13	NAP1a	B1	35	2	027	gc8-3	VUCDO3
US41	BI9	NAP1c	B5	35	2	027	gc8-3	VUCDO3
US42	BI 8	NAP1a	B1	35	2	027	gc8-1	VUCDO3

*slp*A, the surface layer protein A gene

The main advantage of MLVA over other methods is the high discriminatory power, allowing subtyping of epidemiologically important PCRribotype strains. Another favourable characteristic is the automated analysis, reducing the hands-on time needed for analysis. Due to the high reproducibility and stability of the MLVA, the interchangeability of data is definitely of interest for worldwide implementation of this method.

Recurrences

The last application of typing methods is the differentiation between patients with relapses and patients with re-infections. Recurrences, which could be either due to relapse or due to re-infections, are detected in 15-20% of patients with CDAD (Wilcox, 1992). This recurrence rate was shown to be 21% in the incidence study, tested in 13 hospitals in the Netherlands, as described in Chapter 9. Of the recurrence, re-infection rates of 10% to 75% were found in different studies (Johnson, 1989; O'Neill, 1991; Wilcox, 1998; Barbut, 2000; Alonso, 2001; Tang-Feldman, 2003; Noren, 2004). However, patients with both relapses and re-infections in different episodes have been described as well (Kato, 1996; Barbut, 2000; Alonso, 2001). Other studies describe that patients can harbour two or more strains at the same time (Borriello, 1983; Sharp, 1985; Devlin, 1987; Wilcox, 1998). Therefore, we performed a study observing two groups of patients: 28 faecal samples from 23 patients with a first episode of diarrhoea, and 52 faecal samples from 23 patients with recurrent CDAD (Chapter 6). The method used for analysis was PCR-ribotyping. We found that 8.7% of patients with a first episode to harbour two different types within one faecal sample. Among patients with recurrent CDAD, 26% showed a different type strain in a recurrent episode. This observation limits the application of typing methods for studying the epidemiology of CDAD. On the other hand, methods able to subtype C. difficile strains, like PFGE, rep-PCR and MLVA, could result in data of the evolution and modification of strains within a patient.

Concluding remarks and future recommendations

New genotyping methods are constantly in development, leading to methods with a higher discriminatory power, high reproducibility and stability, and methods able to subtyping strains. For the early and rapid detection of outbreak situations, rapid, cheap and easy methods like REA, AP-PCR and PCR-ribotyping are used. However, PCR-ribotyping has a higher reproducibility and stability, and is therefore preferred in the detection of outbreaks. For long-term epidemiology, AFLP, PFGE, REA and PCR-ribotyping are of interest. These methods all show a high discriminatory power, although PCR-ribotyping is the method with the highest reproducibility. Due to the large library that contains world-wide strains, PCR-ribotyping is preferably applied for long-term epidemiology purposes as well. However, subtyping methods like modified-PFGE, REA, rep-PCR and MLVA, have been shown to be able to subtype specific PCR-ribotype strains. For subtyping, the method with the highest discriminatory power and the highest reproducibility and stability is preferred. Therefore, MLVA seems a promising new genotyping method. Although typing methods have been used in recurrences to determine the difference between reinfection and a relapse, the application of typing seems limited due to the ability to carry two strains at the same time. To be able to use typing methods for recurrences, it is advised to test at least five colonies of the cultures from the faecal samples of the first and subsequent episodes.

MLVA is method of choice for subtyping of PCR-ribotype strains and for the investigation of infection routes. However, the stability of the repeats detected in this assay should be re-evaluated, including the effect of sporulation and subsequent germination. This may lead to insights in the patient-to-patient transmission via spores. Furthermore, a general MLVA has to be developed, which can be introduced all over the world, including the introduction of a world-wide library.

Some typing methods that have been described in the general introduction (plasmid profiling, RAPD) are not discussed here. This is mainly due to their low discriminatory power, low reproducibility and the inability to create libraries or interchangeable data. The newer methods, like the PCR-based detection of the flagellin gene (*fliC*), *slpA* and MLST, need to be further evaluated, preferably in comparison to PFGE, REA, PCR-ribotyping and MLVA.

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Samenvatting

De bacterie *Clostridium difficile* werd voor het eerst ontdekt in 1935, maar werd pas in 1977 geassocieerd met pseudomembraneuse colitis. Al vrij snel ontdekte men dat de bacterie twee toxines produceert, die toxine A en B werden genoemd (TcdA en TcdB). Stammen van de bacterie die deze toxines niet produceren zijn niet ziekteverwekkend. TcdA heeft een enterotoxische werking, terwijl het cytotoxine TcdB juist toxisch is voor cellen. Er werd aangenomen dat de toxines synergistisch werken, waarbij TcdA het belangrijkste toxine zou zijn. Recent zijn er echter ook stammen gevonden die alleen TcdB produceren en toch ziekte veroorzaken. Deze stammen bevatten wel een deel van het *tcd*A gen, maar door de deletie in het gen kan er geen functioneel toxine meer geproduceerd worden.

Voor de diagnose van C. difficile-geassocieerde ziekte zijn methodes nodig die een hoge gevoeligheid en specificiteit hebben. Bestaande, conventionele methoden, zoals de cel cytotoxiciteit test en kweek, hebben minimaal twee dagen nodig hebben voor een uitslag en zijn arbeidsintensief, terwijl andere bestaande methoden, zoals de enzym immunoassays, wel snel zijn, maar vaak een lage gevoeligheid vertonen. In dit proefschrift wordt een methode beschreven, gebaseerd op moleculaire detectie, welke snel is en een hoge gevoeligheid heeft. Met dit doel voor ogen werd voor de moleculaire detectie van C. difficile een real-time polymerase chain reaction (PCR) methode ontwikkeld. De conventionele methoden detecteren één of beide toxines of de bacterie zelf, waar de moleculaire testen juist het toxine gen detecteren. Door middel van PCR kan het DNA van de bacterie snel vermeerderd en daardoor gedetecteerd worden. Real-time PCR kenmerkt zich juist door het gebruik fluorescentie tijdens de PCR methode, waardoor er achteraf geen van detectiemethoden meer nodig zijn. Doordat de analyse achteraf niet meer nodig is, reduceert men ook de kans op kruisbesmetting.

In de **hoofdstukken 2 tot en met 4** wordt ingegaan op de ontwikkeling van de real-time PCR methode en de vergelijking met standaard diagnostische methoden.

In **hoofdstuk 2** wordt de ontwikkeling van de real-time PCR beschreven. Voordat een methode in klinische setting kan worden toegepast, zal de test eerst analytisch gevalideerd moeten worden. Het target van de PCR is het *tcd*B gen, omdat elke toxine producerende stam van *C. difficile* dit gen bij zich draagt. Analytisch bekeken was de real-time PCR in staat om alle verschillende toxineproducerende referentiestammen te detecteren met een gevoeligheid van 1x10³ kolonie vormende units per milliliter. De optimale DNA isolatie methode uit feces bleek de STARbuffer voorbehandeling, gevolgd door automatische extractie door de Magna Pure apparatuur. Ook werd een interne controle geïncludeerd, voor controle van remming in de PCR. In een groep van 85 patiënten met en zonder CDAD, bepaald door de cel cytotoxiciteit test, gaf de real-time PCR een gevoeligheid van 100% en een specificiteit van 94%. Ook werd de real-time PCR positief bij drie patiënten zonder symptomen, wat ook werd bevestigd met kweek.

In een prospectieve studie, uitgevoerd in meerdere universitaire centra, werd de nieuw ontwikkelde real-time PCR samen met een nieuwe immunochromatografie test vergeleken met de standaard methode, de cel cytotoxiciteit test. De immunochromatografie methode is in staat om beide toxines te detecteren, maar maakt geen onderscheid. In **hoofdstuk 3** bleek de positief voorspellende waarde van beide testen erg laag (57-70%), mede door een lage gevoeligheid van de cytotoxiciteit test. Toch kon, op basis van de hoge gevoeligheid (87-91%), de goede specificiteit (96-97%), de hoge negatief voorspellende waarde (99%) en de snelheid van de testen, geconcludeerd worden dat zowel de immunochromatografie test als de real-time PCR geschikt zijn als eerste screening methode voor CDAD diagnose.

In een uitgebreide prospectieve evaluatie in vier universitaire verschillende centra, werd de real-time PCR vergeleken met een aantal conventionele diagnostische methoden. Dit wordt beschreven in **hoofdstuk 4**. De real-time PCR, een geautomatiseerde enzym immunoassay voor detectie van TcdA en een enzym immunoassay voor beide toxines werden vergeleken met de cytotoxiciteit test, waarbij de resultaten van kweek in een discrepantie analyse werd gebruikt. Ook wordt in dit hoofdstuk de drie-dagen regel geëvalueerd. Bij patiënten die langer dan drie dagen in het ziekenhuis zijn opgenomen en diarree ontwikkelen, werd via een laboratorium algoritme automatisch de feces onderzocht op *C. difficile*. Dit leverde 4% meer positieve monsters op dan wanneer alleen de clinicus een gerichte aanvraag deed. De enzymtest die beide toxines detecteerde had de hoogste gevoeligheid (97%), maar de laagste positief voorspellende waarde (51%). De discrepantie analyse toonde echter

een concordantie tussen de resultaten van de real-time PCR en de kweek aan van 71%, hoger dan de enzym immunoassays (54-55%). Om die reden werd geconcludeerd dat de real-time PCR verkozen wordt boven de enzym immunoassays, hoewel de test voor beide toxines als een goede eerste screening methode ingezet kan worden.

Nadat het pathogeen gekweekt is uit feces, kan er gekeken worden naar de epidemiologie van CDAD door het gebruik van moleculaire typeringsmethoden. Voor deze typeringsmethoden is het belangrijk dat er genoeg onderscheid gemaakt kan worden tussen de verschillende types van *C. difficile* en dat de technieken stabiele en reproduceerbare resultaten geven. Ook heeft het de voorkeur als de resultaten uitwisselbaar zijn tussen verschillende laboratoria. Ondertussen zijn vele feno- en genotyperingsmethoden ontwikkeld. In de **hoofdstukken 5 tot en met 7** worden enkele genotyperingsmethoden vergeleken.

De Amplified Fragment Length Polymorfism (AFLP) methode wordt vergeleken met twee verschillende PCR-ribotyperingsmethoden in **hoofdstuk 5**. Naast deze vergelijking, heeft het hoofdstuk ook het doel om te onderzoeken of de verschillende TcdA-negatieve stammen, die over de hele wereld verspreid geïsoleerd zijn, ook daadwerkelijk tot één kloon behoren. Het onderscheidend vermogen van de AFLP was iets beter dan dat van beide PCR-ribotyperingsmethoden. Alle methoden waren in staat alle geteste stammen te typeren, waardoor hun typeerbaarheid 100% is. In de groep van TcdA-negatieve stammen werden twee verschillende deleties in het *tcd*A gen gevonden, wat ook opgemerkt werd door de AFLP en de PCR-ribotypering. Eén van beide PCR-ribotyperingsmethoden was in staat nog een derde type te herkennen. Er kon dus geconcludeerd worden dat er inderdaad sprake is van een wereldwijde klonale verspreiding van de TcdA-negatieve stam.

In de helft van de patiënten met een recidief van CDAD, wordt hetzelfde type als bij de eerste episode gevonden. Bij de andere helft wordt een ander type geïsoleerd, waarbij er wordt verondersteld dat er een nieuwe infectie is opgetreden. Omdat dit onderscheid belangrijk is voor de behandeling van de patiënt, wordt in **hoofdstuk 6** een studie beschreven waarin wordt gekeken of patiënten in staat zijn meerdere PCR-ribotypen tegelijk bij zich te dragen. Dit werd vergeleken met patiënten met meerdere episodes van CDAD. Twee van de 23 patiënten bleken twee types in hetzelfde fecesmonster te hebben en er werd nog één patiënt gevonden met meerdere types in meerdere monsters binnen dezelfde episode. Zes van de 23 patiënten met meerdere episodes hadden een ander type in vergelijking tot hun eerste episode. De observatie die hier beschreven wordt, geeft aan dat het testen van een enkele kolonie na kweek niet garandeert dat het gevonden type inderdaad de veroorzaker is van de ziekte. Hierdoor wordt het verschil tussen een recidief of een nieuwe infectie moeilijk te bepalen, tenzij er meerdere kolonies uit één fecesmonster getypeerd worden.

Omdat het onderscheidend vermogen van de boven beschreven technieken nog steeds niet optimaal is, werd een nieuwe methode ontwikkeld die voor vele andere bacteriën en schimmels al toegepast wordt. Deze methode is de multiple-locus variabel aantal in tandem-repeats analyse (MLVA). Zeven PCRs op targets met tandem repeats werden ontwikkeld, waarbij stammen worden getypeerd op basis van de variatie in het aantal repeats. Dit wordt beschreven in **hoofdstuk 7**. De MLVA was in staat alle 56 referentiestammen van elkaar te onderscheiden, evenals zeven subtypes van PCR-ribotype 001. De mogelijkheid tot het subtyperen werd getest op zeven verschillende uitbraken van het PCR-ribotype 027 en op het PCR-ribotype 017. In beide PCR-ribotypes konden met MLVA respectievelijk 13 en 8 subtypes onderscheiden worden. Ook door de hoge reproduceerbaarheid en stabiliteit van de methode kan geconcludeerd worden dat MLVA zeer geschikt is als nieuwe typeringsmethode voor het bestuderen van de epidemiologie van *C. difficile*.

Typeringsmethoden worden veel gebruikt voor het monitoren van de epidemiologie van *C. difficile*. Een belangrijk voorbeeld daarvan is de recente uitbraak van het PCR-ribotype 027, toxinotype III in verschillende landen in de wereld. De Nederlandse situatie wordt nader toegelicht in **hoofdstuk 8**. De eerste uitbraak werd in Canada ontdekt. Evenals in Canada, Amerika en Engeland, steeg de incidentie van CDAD gevallen flink en waren de stammen resistent voor fluoroquinolonen. Door cohortisolatie van CDAD patiënten en het verbod op fluoroquinolonengebruik in het ziekenhuis, werd de uitbraak in Harderwijk tot een

halt gebracht. In een ander ziekenhuis werd ook het PCR-ribotype 027 gevonden, tegelijkertijd met een uitbraak met het TcdA-negatieve PCR-ribotype 017, toxinotype VIII. Uiteindelijk worden in dit hoofdstuk zeven ziekenhuizen beschreven met patiënten met PCR-ribotype 027.

Voordat de eerste uitbraak met type 027 werd herkend, vond een surveillance studie plaats naar de incidentie van CDAD in 17 ziekenhuizen in Nederland, zoals wordt beschreven in **hoofdstuk 9**. Ook werd de PCR-ribotypering en de toxinotypering methode toegepast, om een beeld te krijgen van de verspreiding van verschillende types in het land. De gemiddelde incidentie was 16 per 10 000 patiënten opnames. In één ziekenhuis werd het PCR-ribotype 027 aangetroffen. PCR-ribotype 014 was het meest algemeen voorkomende type in meerdere ziekenhuizen. Toxinotypes 0, III en V waren het meest voorkomend qua toxinotypering. Vier verschillende deleties in het *tcd*C gen, een negatieve regulator voor toxine productie, werden gevonden. Tevens presenteerde 36% van de patiënten zich met CDAD buiten het ziekenhuis en kan CDAD daarom niet alleen als een nosocomiale infectie worden beschouwd.

Conclusie

De verkregen resultaten tonen aan dat in de diagnostiek en typering van *Clostridium difficile* moleculaire methoden een belangrijke plaats hebben gekregen. De ontwikkelde real-time PCR voor detectie van het *tcd*B gen bleek een goede eerste screeningsmethode voor de diagnose CDAD, hoewel de gevoeligheid hoger zal moeten worden dan de kweek, voordat deze test in de routine kan worden opgenomen. Verder is de real-time PCR een snelle methode, waardoor binnen een dag een uitslag gegeven zou kunnen worden. Voor de typering van uitbraken van CDAD, blijkt de PCR-ribotypering zeer geschikt voor het herkennen van uitbraken en de verspreiding van types. AFLP, pulsed-field gel electroforese (PFGE), restrictie-enzym analyse (REA) en PCR-ribotypering zijn zeer geschikt als typeringsmethode, al zijn de data lastig wereldwijd te standaardiseren. Wil men echter een beter beeld krijgen over de verspreiding van bepaalde klonen, dan is subtypering noodzakelijk. Meerdere methoden, zoals aangepaste PFGE, REA, rep-PCR en MLVA, zijn in staat PCR-ribotypes te subtyperen, maar MLVA lijkt de methode met het beste discriminerend

vermogen, de hoogste reproduceerbaarheid en de hoogste stabiliteit. Voor het gebruik van typeringsmethoden voor het onderscheiden van herinfecties met andere types of recidieven met hetzelfde type, wordt geadviseerd minimaal vijf kolonies van kweek te testen. In de toekomst is het belangrijk dat de stabiliteit van de repeats, gedetecteerd met MLVA, goed geëvalueerd worden, mede onder invloed van sporulatie en de daarop volgende ontkieming. Dit kan leiden tot een beter inzicht van de patiënt-patiënt verspreiding. MLVA kan zo ontwikkeld worden, dat er een wereldwijde databank opgezet kan worden.

Curriculum Vitae

Renate Johanna van den Berg werd op 27 april 1978 geboren te Velsen. Ze behaalde het VWO diploma aan het Meridiaan College, Het Nieuwe Eemland te Amersfoort in 1996. Daarop starte zij in datzelfde jaar met de opleiding Medische Biologie en in 1999 met de opleiding Geneeskunde, beide aan de Vrije Universiteit te Amsterdam. Tijdens deze studies liep ze stage bij de afdelingen Medische Chemie en Gastrointestinale Immunogenetica aan de Vrije Universiteit te Amsterdam. In 2000 behaalde zij het doctoraaldiploma Medische Biologie en het propedeusediploma Geneeskunde. Zij studeerde hierna nog een jaar Geneeskunde, waarna werd gestart met het promotie-onderzoek. Van september 2001 tot en met augustus 2006 was zij promovendus bij de afdeling Medische Microbiologie van het Leids Universitair Medisch Centrum. Vanaf september 2006 werd dit onderzoek de van onderzoeker binnen voorgezet in vorm het referentielaboratorium voor Clostridium difficile in het Leids Universitair Medisch Centrum, maar voornamelijk is ze sinds die datum werkzaam als onderwijscoordinator bij de afdeling Moleculaire Celbiologie en Immunologie van het Vrije Universiteit Medisch Centrum te Amsterdam.

Acknowledgements
Een boekje zoals u dat nu voor zich ziet, is natuurlijk niet zomaar gemaakt. Om het in deze vorm aan u te kunnen presenteren waren vele mensen nodig. Ik wil dan ook iedereen bedanken die hier enige hand in heeft gehad.

Ten eerste wil ik alle medewerkers van de afdeling Medische Microbiologie bedanken voor hun hulp en gezelligheid. Sommigen bedank ik graag nog apart. Hadi, bedankt voor je hulp bij de feceskweken. Kate, Els and Jolanda, thanks for helping me getting started in the laboratory and for your ideas in the later years. Jayant, bedankt dat ik met alle promotievragen bij je terecht kon. Celine, bedankt voor de fijne samenwerking.

Kamergenoten, bedankt voor de gezelligheid: de gehele Moleculaire Diagnostiek, Servi, Sitha en Thirza. Lesla, veel succes met het afronden van je eigen promotie. Nathalie, bedankt dat je mijn paranimf wilt zijn.

Ook al mijn stagiaires wil ik bedanken, om de hoeveelheden werk die ze hebben verricht en om de zeer goede en bruikbare resultaten: Duddy Oyib, Takahiro Furusawa, Anna de Breij, Inge Schaap en Rosanna Guseinova.

Marjolein van der Eijk, Corné Klaassen en alle artsen en analisten van de inzendende afdelingen speelden een belangrijke rol voor mijn onderzoek. Bedankt voor jullie samenwerking.

Biomeiden, Jeske, Ruth, Marleen, Yvonne, Irene en Nienke, bedankt voor de gezellige avonden. Jeske, bedankt voor onze gezellige dagjes uit. Ruth, veel succes met je eigen laatste loodjes voor je promotie.

Familie, bedankt voor jullie enthousiasme en interesse.

Mijn ouders, paps en mams, bedankt voor alle steun en enthousiasme in de beslissingen die ik heb genomen, ik kon altijd bij jullie terecht

Lieve Jenno, paranimf, je was continu geïnteresseerd en je hebt me altijd aangespoord om de zaken af te ronden. Bedankt voor je relativerend vermogen. Nu op naar ons gezamenlijke feestje! List of publications

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