Sylvia Debast

Clostridium difficile Infection:
The role of antibiotics in outbreak control, epidemiology and treatment

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ANTIBIOTICA EN BEZUINIGINGEN ZIJN OORZAAK CLOS

Ziekenhuisziekte

ekenhuizen in Har-
erwijk en Amersfoort
ebben last van een
nieuwe stam van agres-
sieve Clostridiumbacte-
riën. Twee patiënten
overleden. Volgens mi-
crobioloog Ed Kuijper
blijven veel infecties on-
opgemerkt.

Wim Köhler

ZIEKENHUISBACTERIËN zijn
vaak onderdeel van het kenmerkenpa-
nieuwe. Ze zijn er het hele jaar door,
maar halen de krant als de politiek op
zijn. Maar de Clostridi-
nieuwe type 027

snelle diagnostiek met nieuwe mole-
culaire technieken. Bij koos voor Clos-
tridium difficile als een van zijn nieuwe
onderzoeksthema's en ontwikkelde
met promovendus Renate van den
Berg nieuwe testen om de bacterie te
karakteriseren.
De bacterie Clostridium difficile is de be-
langrijkste veroorzaker van diarree
bij mensen die in het ziekenhuis lig-
gen en met antibiotica worden behan-
deld. Ook andere bacteriën van bacte-
riën als Salmonella, Shigella en Cam-
pylobacter. Of van virussen, zoals het
buikgriepvirus, oorspronkelijk

ridiumbacteriën altijd gebruiken. En
medisch microbioloog Sylvia Debast
van het St Jansdal ziekenhuis onderk-
te het haar verbaring veel patiënten.
Vanaf april is stijgend aantal. Ze nam
contact op met Ed Kuijper. Ze nogen
zich over de vraag of dit alleen maar
kwam doordat ze een nieuwe test ge-
bruikten. In dat geval zouden ze naar
een onverwachte, maar normale situ-
atie kijken. Maar er was werkelijk een
Clostridiumuitbraak aan de gang.
"We heb nog nooit eerder gehoord van
een ze grote uitbraak in een relatief
klein ziekenhuis," zegt Ed Kuijper.
"Maar dat wil niet zeggen dat er nooit
zo'n uitbraak is geweest. We hebben
geen gegevens. Er bestaat helaas geen
meldingsplicht voor clostridium-in-
fecties. Je moet het dus hebben van
opletende collega-microbiologen,
zoals in Harderwijk." Cataleze en
Britse zijn artsen inmiddels verplicht
om een uitbraak te melden bij de za-
menheden.

DARMONTSTEKING. In Harder-
wijk zijn in drie maanden tijd 36 pati-
enten met diarree gediagnosticeerd,
bevmet met Clostridium. Dat is veel
meer dan de hoeveel die die ze ze
voorheen per maand zagen. Enkele
patiënten hebben een ernstige darm-
ontsteking opgelopen. Twee personen
zijn overleden. Verwijt ze een infectie
doormaken. Zij waren al ernstig
zieg. Het kennerkende en ernstige
heeld dat clostridium difficile ver-
hefige ontsteking.

Uitleg over bacterie in St Jansdal

Flyers over darmbacterie

← vervolg voorpagina:

door LO VAN DER WAL
HARDERWIJK

Ziekenhuis St Jansdal is gisteren een grootscheepse informatie-campagne begonnen over de darmbacterie. Bij de entreebalie van het ziekenhuis staat sinds gistermorgen een grote stan-daard met flyers, die speciaal zijn bestemd voor bezoekers

Patiënten mijden ziekenhuis

Vrees voor besmetting

door ROEL KLEINE
HARDERWIJK

Patiënten van het St Jansdalziekenhuis in Harderwijk verzoeken afspraken voor onderzoeken uit vrees besmet te raken met de darmbacterie die twee doden heeft geëist. Volgens een woordvoerster van het ziekenhuis zeggen sommige patiënten eerder gemaakte afspraken voor onderzoeken om die reden af of vragen om uitstel. 'Ze vragen zich af of het nog wel veilig is', licht Constantine van der Veen van het St Jansdal toe.

Volgens haar is er nog wel degelijk sprake van onrust in en om het ziekenhuis naar aanleiding van de bacteriebesmetting.
Er zijn sinds afgelopen maandag vier nieuwe besmettingen; ook dekt met de darmbacterie clostri-dium difficile. Het totaal aantal geïnfecteerde patiënten komt hiermee op 33. Twee ervan overleden aan de gevolgen van hun ziekte in combinatie met de besmetting.
Maandag waren er ook vijf besmette patiënten, maar daarvan zijn er

Het beleid voor omgang met patiënten is verder aangescherpt. Zo is de medicatie aangepast. Voor het gebruik van antibiotica is een alternatief gevonden, omdat de antibiotica juist de groei van de bacterie bevorderen.
Ook de voorschriften voor de bezoekers zijn aangescherpt. Ze moeten zich melden bij de verpleegkundigen voor instructies. Zo dienen ze hun handen te wassen en mogen ze geen andere patiënten bezoeken. Ook moeten bezoekers na hun bezoek direct

Sint-Jansdal gaat uit van stabiele situatie

'Mensen hoeven niet bang te zijn om naar het ziekenhuis te komen'

door Alex Gentjens

HARDERWIJK - Ziekenhuis Sint-Jansdal heeft een onrustige week achter de rug. Het Harderwijkse ziekenbekend dat patiënten volgen van darmbacte- in combi-mpt spron- op Sint-

bacterie bij iedereen aanwezig is en dat er een antibioticum is dat ertegen werkt."

Dinsdag werden nog vier patiënten geïsoleerd verpleegd. "Met de genomen maatregelen gaan wij ervan uit dat de situatie stabiel is", zegt Van der Veen. Sint-Jansdal werd maandag overstelpt met telefoontjes en vragen van bezoekers en patiënten. "Mensen die een afspraak hadden gepland voor een darmonderzoek vroegen zich af of ze geen risico liepen", zegt Van der Veen. "En iemand die een vader of moeder op de afdeling had liggen diarree had vroeg zich af of soon geen gevaar liep."

Gezonde mensen worde ziek van de darmbacterie 'cl um difficile'. De bacterie wo leen actief en veroorzaakt diar patiënten met een vermin weerstand die bepaalde so antibiotica krijgen toegediend. Jansdal nam snel maatregelen het aanpassen van het antibio gebruik. "Verpleegkundigen en dici moeten beschermende kled dragen en de handen niet all

sen. Verder zijn er afspraken gemaakt wat verpleegkundigen en artsen moeten doen als bij patiënten diarree optreedt", aldus Van der Veen.

Sint-Jansdal riep de hulp in van een medisch microbioloog van het Leids Universitair Medisch Centrum (LUMC). "Het LUMC doet al jaren onderzoek naar deze bacterie", vertelt Van der Veen. "Er wordt een kweek afgenomen en onderzocht om welke bacterie het

Dodelijke bacterie in Harderwijk

Twee oudere patiënten overleden

door LO VAN DER WAL
HARDERWIJK

In ziekenhuis St. Jansdal in Harderwijk zijn twee patiënten overleden nadat ze er een darminfectie hadden opgelopen. Het gaat om een voor Nederland nieuwe variant van een darmbacterie die diarree veroorzaakt. De patiënten, ouder dan 65 jaar, waren al ernstig ziek.

Vanaf april zijn 29 patiënten besmet geraakt met de darmbacterie 'clostridium difficile'. Daarvan zijn vijf patiënten nog besmet. Zij liggen in het ziekenhuis, maar hun situatie is niet kritiek. Het St. Jansdal noemt de situatie 'stabiel'.
Volgens woordvoerster C. van der Veen gaat het niet om een dodelijke bacterie en is deze goed te behandelen met antibiotica. Alleen ernstig zieke patiënten kunnen in geval van infectie het laatste duwtje in de verkeerde richting krijgen. Dat was ook het geval bij de twee ouderen die zijn overleden. Gevaar voor

besmetting, die diarree veroorzaakt, van mens op m gegaan. 'Zo gaat het ker in een omgeving ren verblijven kan smetting plaatsvind

Canada

Coutinho denkt dat ting uit Engeland men, maar het gramma NOVA wist te melden dat de bac land zou zijn binn via een uit Canada patiënt. 'Dat is goe We reizen veel tege De darmbacterie k

Inspectie pakt bacterie aan

door JELLE BOONSTRA
DEN HAAG/BILTHOVEN

De Inspectie voor de Gezondheidszorg bekijkt of de maatregelen tegen de bestrijding van de 'clostridium difficile' wel afdoende zijn. Door deze besmettelijke darmbacterie bezweken twee bejaarde patiënten in streekziekenhuis St. Jansdal in Harderwijk.

mers verpleegd. Het ziekenhuis ziet nog geen reden om besmette patiënten op de intensive care te behandelen.
De ziekenhuizen in een straal van vijftig kilometer rond Harderwijk reageren tamelijk laconiek op de aanwezigheid van de dodelijke darmbacterie. Ze zeggen geen aanwijzingen te hebben dat de bacterie ook in hun eigen ziekenhuis rondwaart. Er

1270 patiënten aan clostridium. Vorige maand kwamen in Engeland nog twaalf patiënten door de bacterie om het leven.

'Kwade stam'

In normale gevallen is de bacterie onschadelijk. Tachtig procent van alle baby's heeft de bacterie in de darmen en negen procent van de volwassenen loopt ermee rond zonder ziek te wor-

Bacterie fataal voor patiënten

Het Sint Jansdal Ziekenhuis in Harderwijk is getroffen door een besmettelijke darmbacterie, door al twee patiënten op In drie nsen be-acterie. Vijf het ziekenhuis e clostridi-ge diarree en en veroorza-enten lopen

Sylvia Debast

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The role of antibiotics in outbreak control, epidemiology and treatment

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Vita bevis,
ars vero longa,
occasio autem praeceps,
experimentum
periculosum,
iudicium difficile.
Nec solum se ipsum
praestare oportet
oportuna facientem,
sed et aegrum et
assidentes et exteriora.

Hippocrates

Vertaald en besproken in:
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Acta est fabula

Introduction

Chapter 1

General Introduction

Introduction

In 1978, *Clostridium difficile* has been recognized as the agent responsible for most cases of antibiotic-associated pseudomembranous colitis (PMC) [1]. Until then PMC has been regarded as a bothersome, but inevitable and untreatable "side effect" of prolonged hospitalization and use of antibiotics. The detection of an identifiable pathogen for PMC marked a turning point in providing the rationale for research on and developments in laboratory diagnosis, therapeutic options and preventive measures for *C. difficile* infection (CDI).

Over the last decade, CDI has progressively increased in incidence and severity of disease. To date, CDI is considered the leading cause of nosocomial diarrhoea, associated with an increased duration of hospitalization, health-care expenses, morbidity and mortality among patients, especially among the elderly [2-4]. Clinical manifestations of CDI range from asymptomatic carriage to severe diarrhoea and pseudomembranous colitis with toxic megacolon [5]. Since 2003 a significant increase in rates of CDI-associated complications including deaths has been reported in the United States, Canada and Europe. This recent change in epidemiology is at least partly due to the epidemic spread of a novel more virulent ribotype, such as PCR-ribotypes 027 and also due to the emergence of ribotypes 001 and 078 [5-9]. In addition, expansion of CDI is observed in the community and in patients previously considered at low risk [10,11]. The occurrence of CDI is also increasingly recognized in veterinary medicine [12,13].

Clinical disease in humans

C. difficile can be found in the intestinal tract of 1-3% of all healthy adults and in 15-25% of individuals with recent healthcare exposure [14]. Loo and colleagues concluded that more than 50% of hospital patients infected with *C. difficile* might be symptomless carriers. Patients with symptomatic CDI were more likely to be infected with a highly pathogenic strain than were patients with *C. difficile* colonization [15]. Colonization with *C. difficile* and high levels of serum antibody against *C. difficile* toxin A and/or toxin B may provide protection against the development of CDI [15-18] Asymptomatic colonization may occur in 20% or more of patients in acute care hospitals. Increasing length of stay correlates with a greater likelihood of acquisition. From 4% to 20% of long-term care residents may carry the organism [19-21]. Once colonization with *C. difficile* is established several factors favour development of symptomatic CDI. Disruption of bacteria that normally reside in the bowel is the most common, and longer courses and use of multiple antibiotics increase the risk for disease [12,14,15,22]. *C. difficile* has been established as the most common cause of antibiotic-associated diarrhoea, accounting for 15% to 25% of cases [5,12]. Antibiotics that are most frequently related to CDI are: clindamycin, cephalosporins and penicillins, but to date several other antibiotics have been associated with CDI as well *e.g.* fluoroquinolones [22-27].

Fluoroquinolone exposure may be an important risk factor for the development of CDI due to highly fluoroquinolone-resistant PCR-ribotype 027 strains. Aldape *et al.* showed that ciprofloxacin up-regulates toxin gene expression and protein production in BI/NAP1/027 strains [28].

Clinical symptoms of CDI usually appear a few days after beginning antibiotic treatment and may appear up to three months after discontinuation [27]. In a majority of cases, patients with *C. difficile* associated diarrhoea, received antibiotics within 14 days preceding the infection, but in some patients symptoms can occur several months after discontinuation of antibiotic therapy. Olson *et al.* [29] found all patients with antibiotic-associated symptomatic CDI had received an antimicrobial within the previous three months. In a study of cancer patients who were being treated as outpatients, the median interval from hospital discharge to CDI was 20.3 days [30].

The principal risk-factors for the development of (severe) CDI include: antibiotic use [12,27,31], recent hospitalization [27,32], prolonged hospitalization (>3 days) [32-34],

nursing home care [21,32], advanced age [8,15,31,35,36], chronic underlying disease [36,37], impaired host immune response against infections [38,39], gastrointestinal manipulation *e.g.* abdominal surgery, tube-feeding [40], enemas, and use of proton-pump inhibitors [15,35,41]. Colonization pressure quantifies the exposure of a person to a pathogen in terms of the number of infectious contacts and the duration of exposure. *C. difficile* colonization pressure has been shown to be an important exogenous risk factor for CDI at high levels of exposure in an ICU setting [34]. Although these risk factors are also associated with community associated CDI, potential risk factors for community-associated CDI may differ from those associated with nosocomial CDI. Patients with community acquired CDI are on average younger, more likely to be female and less likely to have underlying diseases than patients with healthcare associated CDI [11,32,42]. Importantly, almost half of community acquired CDI cases have not used antibiotics in the month before CDI, two-third of patients has not been hospitalized in the preceding 6 months before infection, and approximately one-third of the patients neither has exposure antibiotics nor recent hospitalization [43-45].

A frequently used case definition for CDI is: diarrhoea (defined as >3 unformed stools in less than 24 hours) and a stool test positive for toxigenic *C. difficile* or its toxins/toxin genes, or colonoscopic/histopathologic findings demonstrating pseudomembranous colitis [46,47]. Physical findings in CDI are variable, depending on the length and severity of disease [5,33]. Signs of dehydration may be present. The abdomen may be tender, and in severe cases, peritoneal signs may be present. Ileus or toxic megacolon may result in abdominal distension [48]. Toxic megacolon is the most serious clinical disease entity of CDI [5], defined as an acute dilatation of the colon (>6 cm) associated with severe colitis and systemic toxicity [49].

The clinical spectrum of symptomatic CDI may be classified by the severity of disease [46,47,50,51]. This classification enables the clinician to make therapeutic decisions and reach prognostic conclusions regarding the care of the patient, although it should be noted that currently there are no prospectively validated severity scores for CDI. Severity of CDI associated colitis can be divided into: mild–moderate or early colitis, severe colitis, and fulminant colitis [52]. Approximately, 4–10% of patients with CDI develop fulminant colitis [53]. Fulminant colitis is a distinct clinical entity in which the release of bacterial toxins results in a systemic inflammatory response and multiorgan dysfunction. Fulminant colitis is characterized by hypotension, rising lactic acid levels, shock and complete ileus or toxic megacolon. Fatality rates

in fulminant colitis may be as high as 33-50%, and it is therefore important to determine a precise time moment for surgical intervention [49,54]. Several clinical risk factors have been identified that are associated with increased postoperative mortality: age of 80 years or older, preoperative shock, preoperative dialysis dependence, chronic obstructive pulmonary disease, wound class III, thrombocytopenia, coagulopathy and renal insufficiency [54-57]. Fulminant colitis is not exclusive for CDI and is encountered in other diseases such as ulcerative colitis as well.

C. difficile genotype has been shown to predict mortality [58], although this finding has been disputed by other investigators who did not detect a ribotype association with CDI case severity [59]. A correlation between excess mortality and genotype-specific changes in biomarkers (neutrophil/white cell counts, C-reactive protein, eosinophil counts and serum albumin) emphasizes the importance of inflammatory pathways as a major influence on the outcome of CDI [58]. Clinical Features and Complications of CDI are summarized in Table 1.

Table 1. Clinical Features and complications of *C. difficile* infection (adapted from Refs. 5, 46, 47, 52 and 60).

Spectrum of Disease	Diarrhoea	Other symptoms	Physical examination	Laboratory findings	Endoscopy* & Imaging
Asympto-matic carrier	None	None	Normal	Normal	Unknown
Mild-to-moderate	Profuse	Nausea, anorexia	Low-grade fever, with or without mild abdominal tenderness	Usually normal	Nonspecific patchy erythema (endoscopy)*
Severe CDI	Profuse	Nausea, malaise, abdominal discomfort	Fever (sometimes high), abdominal tenderness and distension	Leucocytosis (WBC >15 x 10^9 cells/L, with left shift). Serum albumin <3 g/L	Pseudo-membranous colitis: pseudomembranes (endoscopy)*
Severe and complicated colitis	Usually profuse and severe, but may be absent in ileus or toxic megacolon	Nausea, malaise, abdominal discomfort and/or pain	Fever (often high), rigors, abdominal tenderness and significant distention, signs of peritonitis, signs of ileus, hemodynamic instability, end-organ failure, admission to Intensive Care Unit for CDI	Leucocytosis (WBC >35 x 10^9 cells/L, with left shift or <2 x 10^9 cells/L), rise in serum creatinine, elevated serum lactate (>5 mmol/L)	Distension of large intestine, colonic wall thickening, pericolonic fat stranding, perforation, ascites (imaging) Nb. Endoscopy is contraindicated in severely ill patients

**There is insufficient knowledge concerning the correlation of endoscopic findings compatible with CDI, such as oedema, erythema, friability and ulceration, and the severity of disease* [46].

One of the characteristics and main problems of CDI, is the high recurrence rate up to 20-30% after initial successful treatment of CDI with either metronidazole or vancomycin [61-63]. Recurrent CDI is defined as an episode of CDI occurring within eight weeks following an initial response. In daily practice it is impossible to distinguish recurrence due to relapse from recurrence due to reinfection [63]. The risk for recurrence increases with each episode, and may greater than 60% in patients with more than two episodes [63].

Since 2003 the epidemiology and clinical presentation of CDI has changed with the appearance of a new hypervirulent strain: PCR-ribotype 027 [2,65,66]. Large outbreaks of severe CDI in hospitals in Canada, America as well as in Europe were reported, presenting with pseudomembranous colitis and fulminant colitis, and with a higher mortality (3-30% case fatality associated with CDI depending on the methods and definitions used) [4,31,66-68] and recurrence rate (up to 25% within one to three months after treatment is completed) [68-71]. Additionally, the incidence of (severe) CDI in the community and in patients with no known risk factors, such as pregnant women and children has increased significantly as well [12].

Pathogenesis

C. difficile is an anaerobic spore-forming bacterium. Spores are shed significantly by infected patients with diarrhoea, which can survive for months in the environment. *C. difficile* can be transmitted via a faecal-oral route from the environment or from the hands of healthcare workers to patients. Ingested spores may transform into the vegetative form, which can then multiply and colonize the bowel [12]. Human colonic microbiota offer protection against bowel infection. However, the mechanisms by which colonic microbiota may mediate colonization resistance against *C. difficile*, how antibiotic disruption of the microbiota can alter this colonization resistance and how antibiotics induce *C. difficile* spore germination and subsequent toxin production, are not yet fully understood [12,72,73]. In recent studies specific anaerobic bacteria (*e.g. Ruminococcaceae*, *Lachnospiraceae* and butyrogenic bacteria) are identified as significantly depleted in *C. difficile* infection and nosocomial diarrhoea [74]. Disruption of this colonic barrier function, *e.g.* by the use of anti-microbial or chemotherapeutic agents, may lead to multiplication and colonization of the bowel with *C. difficile*. However, not all patients colonized with *C. difficile* develop symptomatic disease. Host

and pathogen factors play an important role in the pathogenesis of disease. Only toxin-producing *C. difficile* strains cause disease; toxin-negative strains are considered non-pathogenic. Toxins cause diarrhoea and inflammation of the bowel. Therefore, host production of antitoxin antibody may be protective against development of disease as well as protective against relapsing CDI [16,39,75]. The incubation period from exposure to spores to onset of disease is not yet clear, but is thought to be a median of two days [76].

The main virulence factors of enteropathogenic *C. difficile* strains are two clostridial exotoxins, namely toxin A and toxin B [77]. The toxins are encoded by their genes tcdA and tcdB, which are located, along with surrounding regulatory genes, on a 19.6-kilobase section of chromosomal DNA known as the pathogenicity locus (Paloc) [78,79]. In addition to the major toxin genes, the PaLoc region encodes three accessory genes tcdR and tcdC, which encode proteins involved in regulating the expression of TcdA and TcdB, and tcdE. A schematic overview of the pathogenicity locus is shown in Figure 1.

Secretory diarrhoea and inflammation of the colonic mucosa can largely be explained by the effect of these toxins. Both toxins are cytotoxic, causing disruption of the actin cytoskeleton and tight junctions, and resulting in decreased transepithelial resistance, fluid accumulation, inflammatory response and degradation of the intestinal epithelium. Toxigenic *C. difficile* strains can produce both toxins, or only one of them. Toxin A was thought to be the major virulence factor for many years; however, it has become increasingly evident that toxin B plays a much more important role than anticipated [81,82]. TcdA negative, tcdB positive strains can indeed cause clinical disease in humans [77,83]. The incidence of A- negative B-positive *C. difficile* strains appeared to be increasing worldwide. Patients infected with toxin A-negative, toxin B-positive strains exhibit the full spectrum of symptoms associated with CDI. Some studies also suggest that these isolates are even associated with more severe disease [84]. These strain types now represent a substantial number of *C. difficile* isolates [84,85]. Animal model studies resulted in conflicting results on the importance of toxin A and toxin B [79]. Purified toxin B was shown to be a more potent enterotoxin than toxin A, causing severe damage to the intestinal epithelium and leading to an acute inflammatory response in a mice model [86]. To assess the individual contribution of toxin A and B, recently, research was performed using multiple genetically constructed *C. difficile* tcdA and tcdB toxin mutants [82]. Using these toxin mutants in a hamster model, toxin B was shown to be the major virulence

factor instead of toxin A. In addition, toxin B did not require the presence of toxin A to cause disease. However in a second study using equivalent toxin mutants, contradictory data were reported [77]. In this second study both a toxin A and toxin B mutant caused disease, and the authors concluded that both toxins are important in CDI. From an analysis of both studies [81] it was concluded that it is evident that toxin A is not the major virulence factor, but further experiments are required to accurately determine the relative roles of each toxin in CDI, especially in strains that produce higher levels of toxin, such as PCR-ribotypes 027 or 078.

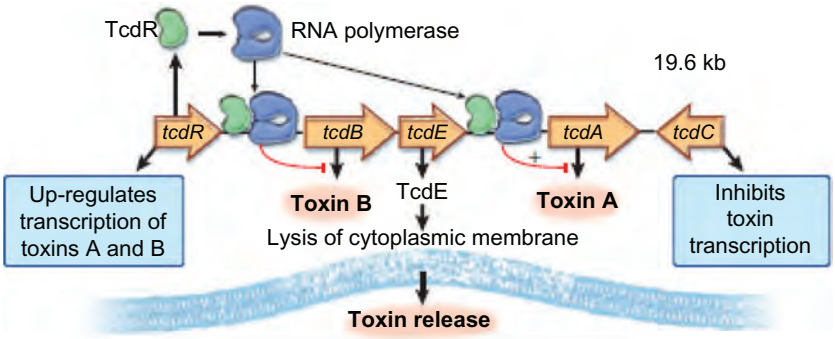

Figure 1. The pathogenicity locus of *C. difficile*: 19.6-kb pathogenicity locus encodes toxin A (tcdA), toxin B (tcdB), a positive regulator of toxin transcription (tcdR), and a putative negative regulator of transcription (tcdC). The function of the tcdE gene product is uncertain but may include the facilitation of toxin release by bacterial membrane lysis. (Adapted from Ref. 80).

C. difficile toxins A and B have no export signature and their secretion is not explainable by cell lysis. Recently, TcdE was found to act as a holin-like protein to facilitate the release of *C. difficile* toxins to the extracellular environment, but unlike the phage holins, does not cause the non-specific release of cytosolic contents. TcdE appears to be the first example of a bacterial protein that releases toxins into the environment by a phage-like system [87].

Some strains produce a third toxin known as CDT or binary toxin [88]. CDT has been suggested to increase the pathogenicity of *C. difficile* strains. CDT ADP-ribosylate actin and inhibits actin polymerization. Indeed a more severe form of disease and a higher case-fatality rate in CDI due to strains

with binary toxin as compared to infections without binary toxins have been described. Binary toxin either is a marker for more virulent *C. difficile* strains or contributes directly to strain virulence [89]. Despite these findings the clinical relevance of binary toxin is not yet well understood.

CDT has been shown not only to depolymerize the actin cytoskeleton but to induce the formation of a novel ribotype of microtubule structures, consisting of long microtubule-based protrusions on the surface of epithelial cells as well, thereby leading to increased adherence of *C. difficile*. Eventually this causes death of target cells [90]. The membrane receptor for CDT uptake by target cells was recently identified by Papatheodorou *et al.* [91]. It has been shown that a related binary toxin (*C. perfringens* iota toxin) enters target cells via this lipolysis-stimulated lipoprotein receptor.

The presence of a naturally occurring mutation in the tcdC has also been associated with the ability of toxigenic strains to become more virulent [79]. The tcdC gene has been reported to down-regulate the expression of tcdA and tcdB. A mutation in the tcdC gene, may therefore lead to increased production of toxin A and toxin B. However, *in vitro* results on the role of tcdC as a major regulator of toxin expression are controversial, and whether increased toxin production also occurs *in vivo* remains unclear [92,93].

An additional regulatory gene on the pathogenicity locus is the tcdR gene. TcdR appears to be a positive regulator for the expression of tcdA an tcdB [94].

C. difficile forms spores that are highly resistant to desiccation, chemicals and extreme temperatures. Spores frequently contaminate the environment around patients with CDI, potentially persisting for months and even years. Before the *C. difficile* toxins can exert their effects, ingestion and germination of spores in the intestinal tract is required [12]. Therefore, it has been postulated that increased sporulation may be associated with hypervirulence and *C. difficile* epidemic strains have been associated with a greater sporulation capacity *in vitro* than non-outbreak strains [95].

In recent years other potential virulence or toxin regulating factors have been identified: *e.g.* hybrid toxins [79], sigma factors TxeR [94] and control proteins CodY and CcPA [96]. Toxin expression may be influenced by specific environmental signals such as the nutritional status of the bacteria. A rapidly metabolizable carbon source such as glucose, inhibits toxin

expression [94]. In addition, general regulatory molecules such as CodY and CcpA are known to influence toxin synthesis [96,97]. CodY was found to repress toxin gene expression in *C. difficile*. CcpA is involved in the glucose-dependent repression of *C. difficile* toxin production. This repression is because of a direct binding of CcpA to the regulatory region of the tcdA and tcdB genes. Unfortunately investigations on the role of environmental factors, toxins and other potential virulence factors are mainly performed in *in vitro* studies or animal models, thereby limiting the clinical implications of these results in human CDI. *In vitro* experiments may not reflect *in vivo* behaviour, and translating *in vitro* or animal derived data into *C. difficile* behaviour in humans is not straightforward. In general it seems likely that multiple factors determine whether a strain is more or less virulent and/or epidemic.

Risk factors for acquisition of *C. difficile* colitis include factors leading to disruption of colonic bacterial flora, such as receipt of antimicrobial agents or chemotherapeutic agents; undergoing solid-organ or bone marrow transplant; inflammatory bowel disease (IBD); factors leading to increased colonization by *C. difficile* spores, such as hospitalization and duration of hospitalization; and factors impacting the host immune system, such as advanced age and immunosuppression [98,99].

Epidemiology

C. difficile is recognized as the primary infectious cause of pseudo-membranous colitis and the principal cause of infectious diarrhoea in hospitalized patients [65]. In recent years incidence, severity, and recurrence rates of CDI have increased dramatically. There has also been a significant increase in severe cases causing admission to a healthcare facility and/or intensive care unit for treatment, in colectomies, and death-related to CDI [2,7,12,65-67]. Additionally, CDI is also emerging in the community and in food-producing animals [6,10,12, 13,100]. Although elderly hospitalized patients receiving antibiotics is still the main group at risk of infection, an increase in CDI in younger populations with no previous contact either with the hospital environment or with antibiotics is noticed [11,31,101,102]

Increases in incidence of CDI have been largely attributed to the emergence of a previously rare and more virulent strain, *C. difficile* BI/NAP1/027 [7]. Increased toxin production and high-level resistance to newer generation of fluoroquinolones made this strain a very successful pathogen in healthcare settings and populations previously thought to be at low risk. However, the underlying reasons for its rapid emergence and the subsequent patterns of global spread remains unknown. To gain more insight into key genetic changes leading to the emergence of this highly pathogenic strain and the subsequent patterns of global spread, whole-genome sequencing and phylogenetic analysis was performed on a global collection of *C. difficile* 027/BI/NAP1 isolated primarily from hospital patients between 1985 and 2010 [103]. It was shown that two, and not one as previously thought, distinct epidemic lineages, FQR1 and FQR2 emerged in North America within a relatively short period after acquiring an identical fluoroquinolone resistance-conferring mutation and a highly related conjugative transposon. The two epidemic lineages showed distinct patterns of global spread, and the FQR2 lineage spread more widely, leading to healthcare-associated outbreaks in the UK, continental Europe and Australia. The data suggested that the acquisition of resistance to commonly used antibiotics to be a major feature of the continued evolution and persistence of *C. difficile* 027/BI/NAP1 in healthcare settings. Furthermore, the ease and rapidity with which the bacterium was transmitted internationally highlighted the interconnectedness of the global healthcare system, which is facilitated by rapid human travel. Human travel was indeed included in a risk assessment framework, which was developed by Clements *et al.* to assess risks of further worldwide spread of this pathogen [104]. The framework the authors present requires identification of potential vehicles of introduction, including international transfers of hospital patients, international tourism and migration, and trade in livestock, associated commodities, and foodstuffs.

Besides *C. difficile* PCR-ribotype 027, several other strains have been associated with outbreaks and severe CDI as well [105,106]. In 2009, Bauer and colleagues [31] performed a hospital-based survey supported by the European Centre for Infectious Disease Prevention and Control, to obtain an overview of CDI in Europe. An incidence of 4.1 per 10,000 patient-days was found. The incidence of CDI and the distribution of causative PCR-ribotypes differed greatly between the European hospitals included in this study. The three most frequently found PCR-ribotypes of toxigenic *C. difficile* strains were 014/020 (16%), 001 (10%) and 078 (8%).

Laboratory diagnosis

CDI is a primarily a clinical diagnosis supported by laboratory or endoscopic evidence. Clinical presentation has been shown to be important when interpreting *C. difficile* diagnostic assays. Specificity of any given *C. difficile* assay for the diagnosis of CDI is increased when clinical symptoms of the patient are included in the reference standard [107]. For this reason only stools from patients with diarrhoea should be tested for *C. difficile*. There are many different approaches that can be used in the laboratory diagnosis of CDI. However, the best standard laboratory test for diagnosis has not been clearly established [60].

Diagnostic tests for CDI include:

(1) Detection of *C. difficile* products: *e.g.* toxins A and B by cell culture cytotoxicity assay (CCA) or EIA, and glutamate dehydrogenase (GDH) by EIA, (2) Culture and detection of toxins produced by the isolate: "toxigenic culture of *Clostridium difficile*", and (3) Molecular diagnostics of *C. difficile* specific targets: 16S RNA, toxin genes, GDH genes. A general overview of diagnostic methods is shown in Figure 2.

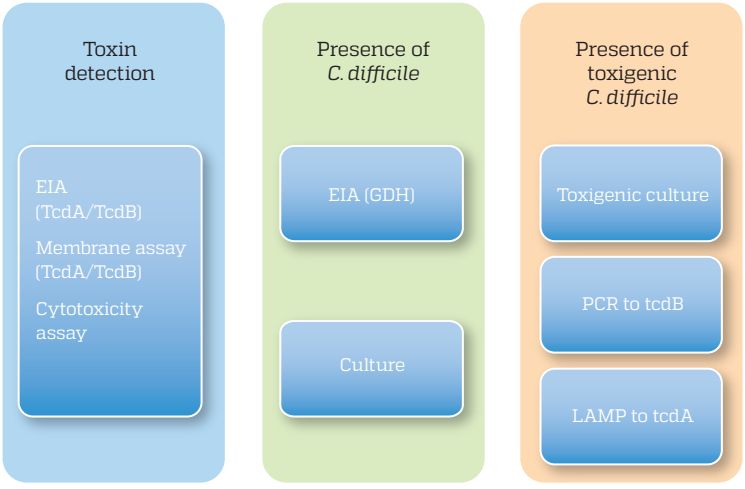

Figure 2. Methods to diagnose CDI can be divided into: the determination of *C. difficile* toxins A and/or B (blue), the presence of *C. difficile* (green) and the presence of toxigenic *C. difficile* (orange).

The detection of neutralizable cell cytotoxicity in stools from patients with antibiotic-associated colitis has led to the discovery that *C. difficile* is the causative agent of this infection [108]. Since then, cell cytotoxicity assay (CCA) has been regarded as the gold standard for the detection of *C. difficile* toxins [47]. CCA is a tissue-culture assay based on the detection of the cytopathic effect of the *C. difficile* toxins present in stool. Using a combination of clinical and laboratory criteria to establish the diagnosis of CDI, the sensitivity of the cytotoxin detection as a single test for the laboratory diagnosis is reported to range from 67% to 100% [109,110]. However, the test is difficult to standardize leading to large variations when performed by different laboratories. Additionally, cell lines may also differ in susceptibility to *C. difficile* toxins, as has recently been detected at the Leiden University Medical Center for Vero cells ATCC CCL-81 and its clone E6, which differ a factor 8 for susceptibility to TcdA (pers. comm. Ing. I.M.J.G. Sanders).

In many laboratories enzyme immunoassays (EIAs) for the detection GDH, Toxin A and/or Toxin B are used, as they are rapid and easy-to-perform assays. Rapid diagnosis of CDI is essential both for improving outcomes of patients with CDI and for reducing horizontal transmission in healthcare facilities. In a systematic review by Crobach *et al.* [111] the diagnostic accuracy of various EIAs (GDH and Toxins A and/or B) and a real-time PCR for *C. difficile* toxin B gene for the diagnosis of CDI, were evaluated and compared with CCA and toxigenic stool culture. EIAs were found to be quite specific, but less sensitive in detecting CDI. Only when these tests are performed in an epidemic situation with a CDI prevalence of 50%, positive predictive values are assumed to be acceptable due to their high specificity. However, in an endemic situation, the prevalence of CDI is expected to range between 5% and 10%. Therefore, it was concluded that EIAs are not suitable as stand-alone tests to diagnose CDI in endemic populations. Because of the lower sensitivity to detect the presence of toxigenic *C. difficile* in stool versus other methods, the Society for Healthcare Epidemiology of America and Infectious Diseases Society of America CDI guidelines state that toxin enzyme immunoassays (EIAs) are a suboptimal approach for the diagnosis of CDI [47].

GDH is an enzyme produced by *C. difficile* in relatively large amounts compared with toxins A and B [112]. Although GDH is sensitive, it is not as specific for CDI, because both toxigenic and non-toxigenic organisms produce this enzyme. The sensitivity of GDH antigen detection (ranging from 75% to >90%) has led to its use as a screening test as part of CDI testing algorithms,

although it should be noted that as many as 10% of patients with toxigenic organisms can be missed by this method [60,111,113-115]. Currently, many laboratories use a combination of a sensitive, but not necessarily highly specific, screening test such as the GDH assay, followed by a more specific test on specimens that test positive to confirm the presence of toxin (*e.g.* an EIA for toxin A and/or B, PRC or toxigenic culture).

Using toxigenic stool culture, *C. difficile* strains are isolated, followed by in toxin detection of the isolate using CCA, EIA or molecular tests to detect TcdA and/or TcdB. Because of the long turnaround time of this method, toxigenic culture is mainly used as a confirmatory test and/or for epidemiological purposes. Disadvantages of CCA and toxigenic culture are that they are expensive, time-consuming and laborious and that interpretation is subjective. Toxigenic stool culture for the detection of *C. difficile* and CCA have been considered the main reference assays for the diagnosis of CDI [110].

More recently, rapid molecular assays such as the real-time polymerase chain reaction (PCR) and technically simpler loop-mediated isothermal amplification (LAMP) have become available for the diagnosis of CDI [60,116-120]. These assays detect conserved regions of toxin A or toxin B genes on the PaLoc of *C. difficile*. Compared to other non-culture-based methods, molecular assays are considered the most sensitive methods available. Evidence suggests that PCR's for toxigenic C. difficile may be good stand-alone tests for toxigenic *C. difficile*. However, clinicians and microbiologists have some concerns regarding their clinical use, because the gene for toxin and not the toxin itself is detected [116]. PCR for the detection of toxigenic *C. difficile* has been shown to have a high sensitivity and excellent negative predictive value. A positive test however cannot differentiate infection from asymptomatic carriage and a second toxin detection test is therefore recommended (see Figure 3).

Currently available Nucleic Acid Amplification Tests (NAAT's), including PCR assays and isothermal amplification tests, which are approved by the Federal Drug Administration (FDA) are: LAMP (Illumigene) [121,122], Xpert *C. difficile* PCR assay (Cepheid, Sunnyvale) [123-126], ProGastro Cd (PG PCR) assay (Prodesse, Waukesha) [127-129], BD GeneOhm™ (BD PCR) assay (Becton Dickinson, San Diego) [127-134], Simplexa-*C. difficile* Universal Direct Test (Quest Diagnostics, Madison), and ribonuclease-mediated isothermal amplification and chip-based detection method test (Great Basin Corp., Salt Lake City) [135].

The diagnostic accuracy of real-time polymerase chain reaction in detection of *C. difficile* in the stool samples of patients with suspected CDI was evaluated in a meta-analysis performed by Deshpande *et al.* [119]. The analysis included 19 diagnostic accuracy studies comparing PCR with cell culture cytotoxicity neutralization assay or toxigenic culture of *C. difficile* [119]. Three commercial PCR assays were investigated: GeneOhm Cdiff Assay (BD Diagnostics GeneOhm, San Diego); Xpert *C. difficile* Test (Cepheid); and ProGastro Cd Assay (Gen-Probe, San Diego). The investigators concluded that real-time PCR has a high sensitivity (90%) and specificity (93%) to confirm CDI. More importantly however, test accuracy depended on the prevalence of *C. difficile* and not on the reference test used: with a low *C. difficile* prevalence of 10%, the positive predictive value was only 71%, and with a high prevalence of >20% it was 93%. Real-time PCR may therefore be an adequate diagnostic assay in epidemic conditions with higher *C. difficile* prevalence but might not be the best diagnostic test in endemic situations with low *C. difficile* prevalence. In endemic situations PCR may serve as a screening test with emphasis on a negative test result.

Peterson *et al.* recently evaluated ten diagnostic tests (including one commercial PCR: BD Diagnostics Cdiff PCR test (Becton Dickinson) for the detection of toxigenic *C. difficile* compared with toxigenic culture. The authors concluded PCR for toxigenic *C. difficile* and GDH testing to be the most sensitive assays for detection of *C. difficile* in stool specimens. GDH and PCR were statistically more sensitive than various toxin A and B EIAs and cell-cytotoxicity assay [133].

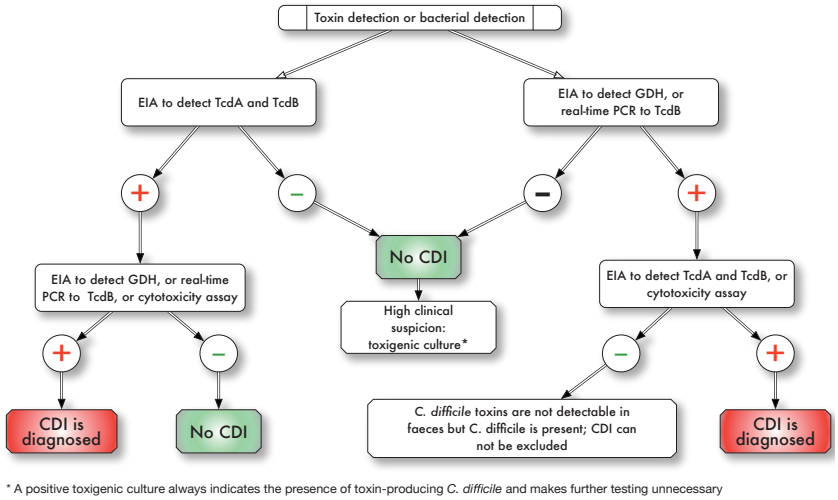

Figure 3. A two-step algorithm to diagnose CDI [111].

De Boer *et al.* developed two real-time PCR assays for the detection of *C. difficile*, and subsequent identification of a tcdC mutation at nucleotide 117 directly in stool specimens [136]. The authors concluded that this assay was a rapid method to identify all toxigenic strains and stool samples containing the epidemic 027/NAP1 strain. The mutation has also been applied as a rapid identification method for PCR-ribotype 027 in the GeneOhm Cdiff Assay (BD Diagnostics GeneOhm) and Xpert *C. difficile* Test (Cepheid, Sunnyvale). However, the mutation is not specific for PCR-ribotype 027, as the same single-base-pair tcdC nucleotide 117 deletion was also demonstrated in other PCR-ribotypes such as PCR-ribotype 076 [137].

Limitations in sensitivity and specificity of common rapid diagnostic tests, have led to the development of several diagnostic algorithms that combine two and sometimes three tests to improve diagnostic accuracy *e.g.* screening with the GDH antigen test and confirmatory testing with toxigenic culture and/or PCR [60,111,113,115,117,118,138]. A two-step approach, with a second test or a reference method in case of a first positive test to diagnose CDI is proposed by Crobach *et al.* [111] (Figure 3).

Antibiotics and CDI

Antimicrobial therapy, often given for treatment of other infectious diseases, can render the patient susceptible to CDI if the patient is exposed to a toxigenic strain of the organism. When CDI was first reported, prior use of clindamycin was established as a significant risk factor [14,49,139,140]. However, in the years thereafter several other antibiotics were found to be associated with a risk of CDI [15,22,27,31,32,36,141]. Cephalosporins and fluoroquinolones replaced clindamycin as the major risk factor, but almost all antibiotics carry some risk. Fluoroquinolones have been linked to CDI and to severe epidemics, particularly those caused by PCR-ribotype 027 [61,141-143]. *C. difficile* strains that are resistant to multiple antimicrobial agents may thrive in an environment where other commensal flora are suppressed in the presence of these antibiotics [22,144-149]. In addition reduced susceptibility or resistance to common treatment agent *e.g.* metronidazole and vancomycin, may have clinical implications [150-153].

Tenover *et al.* [146] investigated the prevalence of antimicrobial resistant strains in 316 toxigenic clinical isolates of *C. difficile* from seven hospitals in the United States and Canada (Quebec) during 2008-2009. Multidrug resistance (*i.e.* resistance to clindamycin, moxifloxacin and rifampicin) was present in 22 of 80 (27.5%) *C. difficile* PCR-ribotype 027 isolates from the United States and Canada but was unusual among other ribotypes. In several studies high rates of clindamycin resistance have been demonstrated in a variety of ribotypes, including ribotype PCR-ribotypes 001, 014, 017, and 027 worldwide [145-149].

Resistance to the antimicrobial agents most commonly used to treat CDIs, *i.e.* metronidazole is reported rarely in the literature and the clinical impact has not yet been assessed [153,154]. Vancomycin resistance has not yet been documented.

Therapeutic options

Currently three guidance documents are available for the treatment of CDI: a guideline supported by the European Society of Clinical Microbiology and Infection (ESCMID) [46], a second guideline including recommendations of the Australian Society for Infectious Diseases (ASID) [115] and Clinical Practice Guidelines for *CDI* in adults published by the Society for Healthcare Epide-

miology of America (SHEA) and the Infectious Diseases Society of America (IDSA) [47]. A Cochrane systematic review has also been published recently [155].

One of the main problems in the treatment of CDI is the occurrence of (sometimes multiple) relapse rates in patients after successful initial therapy is completed, ranging up to 25% and thereby increasing the infectious burden in patients significantly [69-71]. Recommendation of medical treatment options for CDI, are often subdivided in: the first episode of CDI, severe/complicated infection, recurrent infection and prevention of (recurrent) disease.

An overview of treatment options is given in Table 2. Data in this table have been collected from the current ESCMID guideline for the treatment of CDI [46].

Table 2. Overview of therapeutic options for *C. difficile* infection (CDI) and recommendations by the ESCMID as of 2009 (marked in green) [46].

Therapeutic options	Treatment	Treatment guideline ESCMID 2009*	
		Recommendation	Indication
Antibiotic	Oral antibiotic	Metronidazole Vancomycin	Non-severe CDI: • Initial infection • First recurrence Severe CDI Recurrent CDI (>1)
	Parenteral antibiotic	Metronidazole iv Metronidazole iv + vancomycin intracolonic	Non-severe CDI Severe CDI
Non-antibiotic **(in combination with antibiotics)**	Probiotics	Not recommended	–
	Toxin binding resins and polymers	Not recommended	–
	Immunotherapy	Not recommended	–
	Faecal transplant	Not recommended	–
	Surgery	Colectomy	Complicated disease: • perforation of the colon • deteriorating clinical condition despite antibiotic therapy

The first step in CDI treatment is the discontinuation of the antimicrobial therapy if possible. The rate of spontaneous resolution of CDI is unknown. In one study a spontaneous recovery rate in hospitalized patients with

diarrhoea and a positive toxin assay who did not undergo endoscopy or had no pseudomembranous colitis on colonoscopy of 33% was found [1]. Except for very mild CDI, which is clearly induced by antibiotic usage, antibiotic treatment is advised. The (initial) antibiotics used for the treatment of CDI in various European countries, generally include oral vancomycin and metronidazole [46]. However, in severe CDI and recurrent infection antibiotic treatment may fail [63,156]. The last five years several new antibiotic agents (*e.g.* fidaxomicin and rifaximin) for CDI have been developed and limitations of the currently recommended treatment options of CDI are at discussion [69,70,157]. In addition new treatment modalities other than antibiotics have become available, such as donor faeces installation and use of monoclonal antibodies against toxins A and B [158-161]. Recently, the first randomized controlled trial comparing a standard of vancomycin versus duodenal infusion of donor faeces has been published. Infusion of donor faeces was significantly more effective for the treatment of recurrent CDI than the use of vancomycin [158].

Infection Control

Various infection control measures, including barrier precautions (contact isolation), hand hygiene, environmental cleaning, use of single-use rectal thermometers, endoscope disinfection, and limited use of select antibiotics, have been described in CDI guidelines [47,115,162].

Environmental cleaning with sodium hypochlorite (bleach) solutions (concentration of at least 1000 ppm available chlorine) decreases *C. difficile* surface contamination and has been associated with a significant reduction in the transmission risk of CDI [95, 162,163]. However, cleaning is required prior to disinfection with chlorine-based solutions, as they have poor activity in dirty conditions [164]. Alcohol-based hand sanitizers are thought to be ineffective in controlling CDI transmission, because they have poor activity against CD spores. Therefore hand-washing with water and soap is advised [162, 165].

As some hypervirulent strains (*e.g.* PCR-ribotype 027) are resistant to fluoroquinolones, increased use of these antimicrobial agents is proposed to contribute to the emergence of epidemics. For this implementation of an antimicrobial management program including a reduction in the use of antibacterials may be essential in outbreak control [106,162,166].

Increased toxin production and hypersporulation are suggested to facilitate environmental contamination and contribute to outbreaks of infection as well. Since 2000, outbreak investigation has guided the sequential introduction of control measures and the development of a comprehensive CDI control "bundle approach" in which several outbreak measures are taken simultaneously [47,106,162,167-169]. An overview of recommended measures for the prevention and control of CDI recommended by the ESCMID is given in Table 3.

Table 3. Overview of recommended measures for the prevention and control of *C. difficile* infection (CDI): "bundle approach" [162].

Interventions for the prevention and control of *C. difficile* infection	
Diagnosis	Early, rapid and reliable diagnostics
Awareness	Education and communication
Surveillance	Monitor: incidence of CDI, distribution of PCR-ribotypes, clinical outcome
Hygiene	Hand hygiene Protective clothing Medical equipment: single use, disinfection, disposables Environmental cleaning and disinfection
Barrier precautions	Contact isolation in a single room Cohort isolation (outbreaks)
Antibiotics	Stop antibiotics in case of CDI Good antibiotic stewardship

Monitoring the epidemiology of CDI (prevalence and incidence) is important for assessing risk factors and outcome of disease for planning prevention programs and focusing antibiotic stewardship efforts [170]. Access to *C. difficile* ribotyping in national surveillance programs to measure the distribution of PCR-ribotypes was associated with significant control of epidemic strains, especially of PCR-ribotype 027 [171,172]. Changes in prevalence of epidemic strains coincided with markedly reduced CDI incidence and related mortality [172].

Economics and CDI

The economic burden associated with healthcare associated CDI is high for primary and recurrent infection [173-175]. Healthcare-associated cases of CDI are associated with significantly higher mean cost and longer length of hospital stay [3,176,177]. Recently, Wiegand *et al.* reviewed all studies published in the English language between 2000 and 2010 to determine the clinical and economic burden associated with CDI acquired and treated in European healthcare facilities [4]. CDI mortality at 30 days ranged from 2% (France) up to 42% (UK) and median length of hospital stay due to CDI ranged from eight days (Belgium) to 27 days (UK). The incremental cost of a CDI case was estimated £ 4,577 in Ireland and £ 8,843 in Germany. The high economical burden of CDI was also confirmed by a recent study by McGlone *et al.* [178], in which a computer simulation model was developed to determine the costs attributable to healthcare acquired CDI.

In 2009, a range of estimates for the annual direct hospital cost of treating healthcare-associated infections in the United States was reported by the CDC using results from the published medical and economic literature [179]. The number of *C. difficile* cases in this analysis was derived from a study by McDonald *et al.* [180] and the estimated cost of hospital-associated CDI from a study by Dubberke *et al.* [175]. The estimated number of healthcare-associated CDI was 178,000 annually. The estimated average attributable per patient costs of healthcare-associated CDI ranges from $ 6,408 to $ 9,124. The estimated total annual costs associated with healthcare-associated CDI in U.S. hospitals ranges from $1.01 to 1.62 billion *per annum*.

Further research is required to establish the costs and effectiveness of possible infection control interventions for CDI in order to estimate the benefits of them on medical cost savings. However, considering the estimated economical burden of CDI the benefits (or savings) of prevention and surveillance programs on direct medical cost of preventable healthcare associated with CDI are considered to be significant [3,172,173,176,179,181].

Outline of this thesis

An important question at the start of this research was if PCR-ribotype specific risk factors for the development of CDI could be recognized, and subsequently if specific measures could be identified and applied to control hospital outbreaks. Fluoroquinolones appeared to play a part in the global emergence of the PCR-ribotype 027 strains. In contrast to other PCR-ribotypes, the 027 strain was found to be resistant to the newer generation of fluoroquinolones and an increase in the incidence of CDI due to this ribotype was assumed to be associated with (an increased) exposure to this antibiotic in healthcare facilities [25,141,142, 182]. There was a need to further elucidate the role of antibiotic stewardship as part of outbreak control protocols for CDI.

This thesis contains the first report on a hospital outbreak of severe CDI with PCR-ribotype 027 in the Netherlands. Rapid laboratory diagnostics used in this hospital outbreak, specific risk factors associated with *C. difficile* PCR-ribotype 027 and applied measures for outbreak control were analysed. During a second outbreak in the same hospital with two PCR-ribotypes (027 and 017) occurring simultaneously, PCR-ribotype-specific risk factors as well as outcome parameters were investigated. Though infections with *C. difficile* PCR-ribotype 027 only occur in hospitals, other PCR-ribotypes reveal a different behaviour.

With an increase in the incidence of CDI, early recognition of CDI patients has become of prime importance to prevent spread of the bacterium, especially in the context of outbreak control. Because standard 'reference' tests (cell culture cytotoxic assay and toxigenic culture) are slow and labour-intensive, and require specialised facilities and expertise, novel rapid diagnostic methods were developed. A major advance in the diagnosis of CDI has been the development of rapid enzyme immunoassays (EIA) for detection of GDH and/or toxins A and B in stool samples. In recent years EIAs for the detection of Toxins A an B have become a widely used diagnostic method for CDI because of their rapid turnaround time, low cost, and simplicity to perform. However, EIAs for toxins A and B are known to have low sensitivity (60%–80%) compared with toxigenic stool culture [183,184]. One of the questions in this thesis was if testing sequential stool samples could enhance the diagnostic yield of EIA for toxins A and B in an epidemic situation.

Besides hospital acquired CDI, studies in the United States [182] and Europe [7] suggested that the incidence in community-associated CDI is also increasing [11,102]. This increase in community-associated CDI has led to the investigation of other potential vehicles for the transmission of CDI. Several studies suggested the role of animals in human CDI [12,100]. To investigate the relatedness of *C. difficile* strains found in humans and livestock, there was a need for further pheno- and genotypically characterization and comparison of strains.

Soon after the decrease of PCR-ribotype 027, a new ribotype (078) emerged which was also found in patients with community-acquired CDI and in animals. We studied the significance of PCR-ribotype 078 in animals and established the molecular relatedness of isolates obtained from animals and humans with CDI.

Antibiotics used to treat CDI are usually vancomycin or metronidazole. Metronidazole has been the drug of first choice for mild infections, whereas vancomycin is recommended for the treatment of severe infections [46]. With a change in PCR-ribotype distribution, there has been increasing concern about changes in the antibiotic susceptibility of endemic and epidemic *C. difficile* strains for metronidazole, vancomycin, and novel agents such as fidaxomicin. Given the potential implications of antibiotic resistance for CDI therapy, there was a need for surveillance of antibiotic susceptibility of *C. difficile* isolates, in order to develop up-to-date guidelines for the treatment of CDI.

In this thesis we analysed the antimicrobial susceptibility of *C. difficile* in Europe to the most frequently used agents and also tested two new agents (LFF-571 and fidaxomicin). Finally, we updated the CDI European treatment guideline from 2009 supported by the European Society of Clinical Microbiology and Infection (ESCMID) and an international team of experts from 11 European countries.

The studies described in this thesis were organised in the following way:

Outbreak control

Chapter 2 describes the first hospital outbreak of CDI due to the hypervirulent PCR-ribotype 027 in the Netherlands. Risk factors, clinical outcome and outbreak control measures were investigated.

Chapter 3 describes the laboratory diagnosis during hospital outbreaks of *C. difficile* PCR-ribotypes 027 and 017. In this study, the value of sequential analyses of stools on the diagnostic yield was investigated using a rapid membrane immunoassay for the detection of *C. difficile* toxins A and B in faeces followed by classic selective culturing.

Chapter 4 describes an outbreak with two virulent strains of *C. difficile* (PCR-ribotypes 027 and 017) that simultaneously occurred in one hospital in the Netherlands. Ribotype-specific risk factors for clinical disease and clinical outcome were studied.

Epidemiology

Chapter 5 describes the emergence of *C. difficile* PCR-ribotype 078 as a pathogen in human and animal disease. To gain epidemiological insight in the possible transmission from symptomless or diseased animals to humans through direct contact, food or through the environment, as a zoonotic disease, *C. difficile* isolates from Dutch food-producing pigs were characterized and compared to human strains. Using multiple-locus variable-number tandem-repeat analysis (MLVA) a genetically relationship between *porcine* and human isolated *C. difficile* PCR-ribotype 078 strains was studied.

Treatment

Chapter 6 describes ribotype-specific susceptibility patterns of *C. difficile* to therapeutic agents. *C. difficile* isolates obtained from a European hospital-based survey were investigated to compare antimicrobial susceptibility patterns of common PCR-ribotypes across Europe.

Chapter 7 describes the therapeutic options for CDI. In this study the currently available evidence concerning treatment of CDI is evaluated and recommendations for treatment are formulated. Aim was to develop an up-to-

date / state-of-the-art European treatment guidance document supported by the European Society of Clinical Microbiology and Infectious Diseases.

Aim of the studies

This thesis focuses on antibiotics in the outbreak control, epidemiology and treatment of infections with toxigenic *C. difficile*.

The objectives were to i) investigate the importance of antibiotic stewardship as part of the infection control measures in hospital outbreaks with CDI, and ii) discover risk factors for the development of an infection with specific PCR-ribotypes. This was done with the purpose to gain more insight into ribotype-specific antibiotic risk factors, so that preventive and outbreak control measures can be improved further. The reservoir for pathogenic *C. difficile* is largely unknown. A potential source and thus risk for CDI may be in the environment of humans. It is known for longer time that animals can suffer from CDI. Another aim of this thesis was therefore to investigate whether this animal-borne CDI could clarify for a part the emergence of specific PCR-ribotypes in animals and humans. Studies were also intended to inspect the antibiotic susceptibility of *C. difficile* within Europe, with the purpose to up-date and optimize European guidelines for the antibiotic treatment of CDI.

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Outbreak control

2

Chapter 2

Successful combat of an outbreak due to *Clostridium difficile* PCR-ribotype 027 and recognition of specific risk factors

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Abstract

In the period April–September 2005, an outbreak of *Clostridium difficile* infection (CDI) due to PCR-ribotype 027 occurred among 50 patients in a 341-bed community hospital in Harderwijk, The Netherlands. A retrospective case–control study was performed to identify risk factors specific for CDI, using a group of patients with CDI (n = 45), a group of randomly selected control patients without diarrhoea (n = 90), and a group of patients with non infectious diarrhoea (n = 109). Risk factors for CDI and for non-CDI diarrhoea were identified using multiple logistic regression analysis. Independent risk factors for CDI were: age above 65 years (OR 2.6; 95% CI 1.0–5.7), duration of hospitalization (OR 1.04 per additional day; 95% CI 1.0–1.1), and antibiotic use (OR 12.5; 95% CI 3.2–48.1). Of the antibiotics used, cephalosporins and fluoroquinolones were identified as the major risk factors for development of CDI. The risk of developing CDI was particularly high in people receiving a combination of a cephalosporin and a fluoroquinolone (OR 57.5; 95% CI 6.8–483.6). The main factors affecting the risk of non-CDI diarrhoea were proton-pump inhibitors, immunosuppressive drugs, underlying digestive system disease, previous surgery, and gastric tube feeding. The outbreak ended only after implementation of restricted use of cephalosporins and a complete ban on fluoroquinolones, in addition to general hygienic measures, cohorting of patients in a separate ward, education of staff, and intensified environmental cleaning. The results of this study support the importance of appropriate antimicrobial stewardship in the control of hospital outbreaks with *C. difficile* PCR-ribotype 027.

Introduction

Clostridium difficile infection (CDI) is one of the most common hospital-acquired infections, and is a frequent cause of morbidity and mortality among elderly hospitalized patients [1]. Recent reports indicate an increasing occurrence and severity of CDI [2-5]. This change in epidemiology and clinical presentation can, to a certain extent, be explained by the spread of a new, potentially more virulent isolate, referred to as PCR-ribotype 027/toxinotype III/pulsed-field gel electrophoresis type NAPI/REA group BI (027/III/NAPI/BI), which has caused outbreaks in North America and Europe [6-12].

The most important risk factor for CDI is prior antibiotic use. Other risk factors are: increasing age, severe underlying disease, prolonged duration of hospitalization, CDI pressure (defined as the sum of a patient's daily exposure to patients with CDI who share the same unit or ward divided by the length of stay of the patient at risk [13,14]), gastrointestinal surgery, and enteral tube feeding [15-19]. During the recent outbreaks caused by *C difficile* PCR-ribotype 027, several new putative risk factors have been reported, *e.g.* the use of proton-pump inhibitors [20-22], of non-steroidal anti-inflammatory drugs [22], and of fluoroquinolones [23-25].

Given the high *a priori* chance of non-infectious diarrhoea developing in hospitalized patients, it is often difficult to distinguish between risk factors specific for CDI and risk factors for diarrhoea due to other causes in the setting of an epidemic of CDI.

To unravel the risk factors specific for CDI, we performed a case-control study using a group of patients with CDI and a group of patients with non-infectious diarrhoea, both diagnosed during an outbreak of *C difficile* PCR-ribotype 027 in a community hospital.

Materials and Methods

Study population and definition of CDI cases

This study was conducted during an epidemic of CDI caused by *C. difficile* PCR-ribotype 027 in St Jansdal Hospital, a 341-bed community hospital in

Harderwijk, The Netherlands. CDI was defined by the presence of diarrhoea (two or more loose bowel movements per day) and a positive *C. difficile* toxin assay result from a stool sample. All faecal samples were tested within 1-18 h after arrival at the laboratory, using a rapid enzyme immunoassay (ImmunoCard Toxin A and B (ICTAB); Meridian, Boxtel, The Netherlands). In patients with diarrhoea and a negative rapid immunoassay result, a second faecal sample was tested after 24-48 h. When two tests gave negative results, CDI was considered to be unlikely.

Characterization of C. *difficile* isolates

Toxin-positive faecal samples were cultured for the presence of *C difficile,* using non-selective and selective agar supplemented with cefoxitin, amphotericin B, and cycloserin (CLO-medium; Biomérieux), with and without ethanol shock pre-treatment. After incubation in an anaerobic environment at 37°C for 48 h, colonies of Gram-positive rods with sub-terminal spores were tested for the production of L-proline aminopeptidase and for the hydrolysis of esculine. All culture-positive strains isolated from faecal samples were identified as *C. difficile* using a PCR for the presence of the *gluD* gene encoding the glutamate dehydrogenase specific for *C. difficile* [9]. *C. difficile* isolates were tested for the presence of the tcdA and tcdB binary toxin genes and deletions in *tcdC,* as described previously [9]. PCR ribotyping and toxinotyping were performed as described previously [26,27]. For all isolates, Etest (AB Biodisk, Solna, Sweden) was used to determine susceptibility to erythromycin, ciprofloxacin, clindamycin and metronidazole.

Case-control study

To identify risk factors specific for CDI, patients were assigned to three different study groups during the peak of the outbreak in St Jansdal Hospital (Table I). Study group I consisted of 45 patients diagnosed with CDI as described above. Study group II consisted of 109 patients diagnosed with non-CDI diarrhoea, *i.e.* patients with diarrhoea who tested negative in the *C. difficile* toxin assay of two faecal samples collected at least 24 h apart. Study group III consisted of 90 randomly selected control patients without diarrhoea. Patients with non-CDI diarrhoea (study group II) and control patients (study group III) were randomly selected from among all patients residing at the same time and in the same ward as the patient newly diagnosed with CDI.

A standardized questionnaire was used to collect clinical and demographic data from hospital records. Data were collected concerning each participant's age and gender, time of onset and duration of diarrhoea, duration of hospital stay, previous hospitalization, co-morbidity, and level of care prior to the development of diarrhoea. Comorbidity was defined according to the International Classification of Disease, version 10 (ICD-10). For study groups I and II, the duration of hospital stay was defined as the number of days from admission to the development of diarrhoea; for study group III, it was defined as the number of days from admission to discharge. Information on the use of antibiotics or other medication within the preceding 3 months was extracted from an electronic pharmacy database. This database contained information on all medications prescribed both within and outside the hospital for every participant in this study. All medications used were categorized according to the latest international ATC code [28]. The defined daily dose of antibiotics was established according to the WHO Collaborating Centre for Drug Statistics Methodology guidelines for ATC classification and defined daily dose assignment [28].

For each patient diagnosed with CDI, additional information was collected concerning severity of disease, treatment regimen, disease recurrence, and 30-day mortality. Recurrent disease was defined as a second episode of diarrhoea within 30 days of diagnosis of CDI following initial clinical improvement, combined with a positive *C. difficile* toxin assay result from a stool sample.

Statistical analysis

The distributions of risk factors in study group I and study group II were compared to the distribution in the control group (study group III). Continuous data were compared among groups using analyses of variance. A Yates-corrected chi-square test was used for the analysis of proportions. If a cell value was less than five in the two-by-two table, Fisher's exact test was used. A multiple logistic regression model was used to study the association of putative risk factors with CDI and non-CDI diarrhoea. Relative risks were estimated as ORs and presented with a 95% CI. Both crude ORs and ORs after adjustment for the possible confounder's age, duration of hospital stay, comorbidity (ICD-10 category), level of care and co-medication are presented in Table 2. All p-values were two-sided. Finally, for both cephalosporin therapy and fluoroquinolone therapy, the

population-attributable risk percentage (PAR%) was calculated as previously described [29]. All analyses were performed using SPSS for Windows, version 13.0.

Results

Description of the outbreak

The background incidence of CDI in St Jansdal Hospital was 3.8 patients per 10,000 admissions in 2004. In 2005, a more than ten-fold increase in the incidence of CDI was observed (Fig. I). In this study, we included the first 45 patients diagnosed with CDI in 2005. In total, 50 patients with CDI were diagnosed during the outbreak. Faeces were cultured, and *C. difficile* isolates were identified as toxinotype III and PCR-ribotype 027. In addition, the strain had the binary toxin genes and contained an 18-bp deletion in the toxin regulator gene *tcdC*. The isolates were resistant to erythromycin (MIC >256 mg/L) and ciprofloxacin (MIC >32 mg/L), and susceptible to clindamycin (MIC 2 ml/L) and metronidazole (MIC 0.19 mg/L).

A multidisciplinary hospital outbreak management team (OMT) was formed to coordinate measures to control the epidemic. Special folders informed medical personnel in the hospital. In addition, all clinicians were informed personally. The medical microbiologist and infection control practitioner organized special meetings on the involved wards with the nursing staff. The cleaning team received special instructions for intensified cleaning procedures from the infection control practitioner. All measures were described in a CDI hospital guideline by the OMT.

Measures taken by the OMT to control the epidemic (from I May 2005 onwards) included isolation of all patients with diarrhoea (until two tests, 24 h apart, gave negative results for *C. difficile* toxin), hand washing with water and soap, use of chlorine-containing disinfectant (0.1% sodium hypochlorite), and cohorting of all *C. difficile*-infected patients on a separate ward. In addition, from 7 July 2005 until 14 September 2005, a complete ban on all fluoroquinolones was established, and the use of cephalosporins and clindamycin was limited.

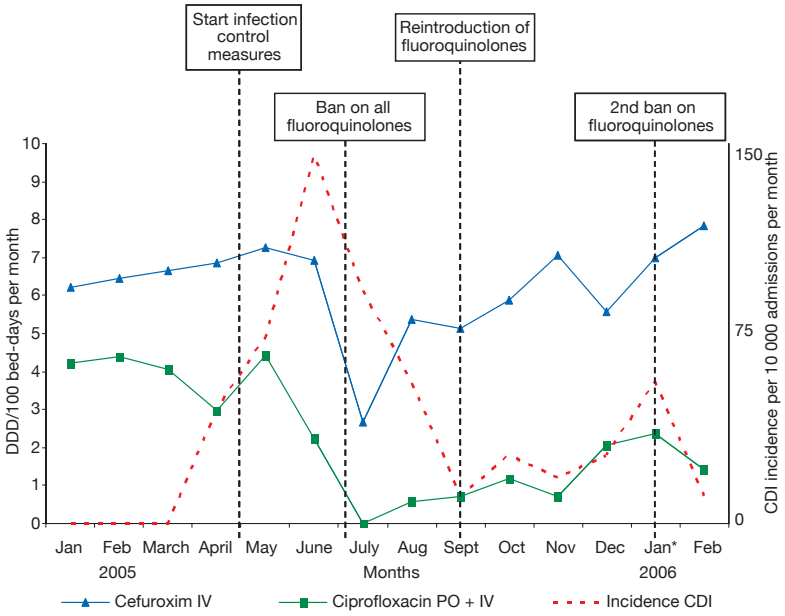

Figure 1. Course of the epidemic and dynamics of antibiotic use in St Jansdal Hospital. DDD, defined daily dose; PO, oral administration; IV, intravenous administration.

The course of the epidemic, including the time-scheme of all infection control measures taken and the use of antibiotics in the hospital, are depicted in Figure 1. The outbreak came to an end in September 2005. After the re-introduction of fluoroquinolones, however, a temporary increase in CDI was noticed.

Description of *C. difficile*-associated disease cases

From April 2005 until the end of August 2005, a total of 45 patients met the case definition of CDI. Clinical characteristics of the CDI cases are given in Table 1. Thirty-five patients developed diarrhoea during their stay in the hospital (mean duration of hospital stay prior to development of symptoms was 13 days). Of the ten patients admitted with diarrhoea, nine patients had healthcare-associated CDI, as they had been hospitalized in the same

hospital within the preceding 3 months. The only patient who had not been hospitalized before was suffering from ulcerative colitis and was known to have frequent periods of diarrhoea.

The symptoms and signs most frequently observed within the first 2 weeks following onset of diarrhoea were fever (53.3%), abdominal pain (20%), high white blood cell count in the first 2 weeks after onset of diarrhoea (mean 1.6 X 10^{10} cells/mL; >2.0 X 10^{10} cells/mL in 23.7% of cases), high erythrocyte sedimentation rate (mean, 48.2 mm/h), high serum creatinine level (mean, 0.149 mmol/L; >0.200 mmol/L in 17.5% of cases), and low serum albumin level (mean, 28.6 g/L). Bloody stools were noticed in only three patients (6.7%). All but two patients were treated with vancomycin or metronidazole or a combination of both. Recurrence of diarrhoea following initial improvement was observed in ten patients (22%). In nine of these patients, a positive *C. difficile* toxin assay result was obtained from a stool sample. Recurrence of CDI was more often seen in patients with a peak white blood cell count >2.0 X 10^{10} cells/mL (p 0.002; OR 16, and 95% CI 2.8-90.4) or a peak serum creatinine level >0.200 mmol/L (p 0.03; OR 7.1, and 95% CI 1.3-40.2). Nine patients (20%) with CDI died within 30 days after diagnosis, three (7%) as a direct result of CDI. A peak white blood cell count >2.0 X 10^{10} cells/mL within the first 2 weeks following onset of diarrhoea was a strong predictor of mortality (p 0.01; OR 7.8, and 95% CI 1.5-39.1).

Case-control study

Table 1 presents the characteristics of the participants in the case-control study. Table 2 summarises the risk of CDI and non-CDI diarrhoea. Both crude ORs (univariate analysis) and adjusted ORs (multivariate analysis) are given (only characteristics that were significantly different among study groups in the univariate analysis are shown). After adjustment for differences in comorbidity, level of care, and co-medication, the independent risk factors for CDI were age above 65 years (OR 2.6), duration of hospitalization (OR 1.04 per additional day), and antibiotic use (OR 12.5). Independent risk factors for non-CDI diarrhoea were underlying digestive system disease (OR 3.1) and previous surgery (OR 2.1). Although immunosuppressive agents and proton-pump inhibitors were not associated with CDI, patients with non-CDI diarrhoea were less often treated with these. Finally, nasogastric tube feeding appeared to be a general risk factor for diarrhoea, being associated both with CDI (OR 3.6) and with non-CDI diarrhoea (OR 4.8).

Antibiotic use was exclusively associated with CDI. Of all antibiotics, cephalosporins, macrolides and fluoroquinolones were associated with CDI in the univariate analysis (Table 2). After correction for differences in comorbidity, level of care, co-medication, and the use of multiple antibiotics, the association of CDI with macrolides was no longer significant. Even with the small numbers in our study, we could demonstrate a statistically significant interaction between cephalosporin and fluoroquinolone use in the multivariate analysis (OR for the interaction factor, 13.6; p 0.006). To study this interaction in more detail, we analysed the risk of CDI for different treatment schemes (Fig. 2). In this analysis, cephalosporin monotherapy (OR 7.8, 95% CI 2.9-20.9) and fluoroquinolone monotherapy (OR 28.8, 95% CI 2.6-319.2) were shown to be independent risk factors for CDI. Patients who used a combination of both antibiotics in the preceding 3 months had the highest risk of developing CDI (OR 57.5, 95% CI 6.8-483.6). The PAR%, *i.e.* the proportion of CDI cases in the study population that was attributable to the use of cephalosporin or fluoroquinolone therapy, was calculated as 56% and 33%, respectively.

Table 1. Baseline characteristics of participants in the case-control study.

Characteristic	CDI	Non-CDI	Controls
n	45	109	90
Gender			
Male	19 (42.2)	36 (33.0)	42 (46.7)
Female	26 (57.8)	73 (67.0)	48 (53.3)
Age, years			
18–64	8 (17.8)	41 (37.7)	30 (33.3)
≥65	37 (82.2)*	68 (62.3)	60 (66.7)
Main comorbidity (ICD-10 classification)			
Neoplasm	12 (26.7)	28 (25.7)	23 (25.6)
Endocrine disease	16 (35.6)	30 (27.5)	18 (20.0)
Cardiovascular disease	28 (62.2)**	52 (47.7)	34 (37.8)
Respiratory system disease	16 (35.6)*	14 (12.8)	17 (18.9)
Digestive system disease	11 (24.4)	32 (29.4)*	14 (15.6)
Musculoskeletal disease	6 (13.3)	14 (12.8)	10 (11.1)
Genitourinary disease	13 (28.9)	22 (20.2)	20 (22.2)
Duration of stay in hospital (prior to diarrhoea), in days: median (range)	7 (0–77)*	4 (0–97)	4 (0–63)
Level of care			
Intensive-care unit stay	9 (20.0)	19 (17.4)	8 (8.9)
Surgery	7 (15.6)	42 (38.5)**	20 (22.2)
Endoscopy prior to CDI	6 (13.3)	9 (8.3)	11 (12.2)
Nasogastric tube	10 (23.3)**	24 (22.9)**	7 (7.8)
Antibiotics received in the preceding 3 months			
Any antibiotic	42 (93.3)***	53 (50.5)	42 (46.7)
Penicillins	10 (22.2)	22 (20.2)	21 (23.3)
Cephalosporins	33 (73.3)***	18 (16.5)*	25 (27.8)
Tetracycline	3 (6.7)	0	0
Aminoglycosides	2 (4.4)	4 (3.7)	2 (2.2)
Macrolides	16 (35.6)***	4 (3.7)	9 (10.0)
Clindamycin	1 (2.2)	5 (4.6)	5 (5.6)
Quinolones	13 (28.9)***	7 (6.4)	3 (3.3)
Other	12 (26.7)	13 (11.9)	14 (15.6)
Other drugs received in the preceding 3 months			
Proton-pump inhibitors	21 (46.7)	27 (24.8)	31 (34.4)
H2 blockers	2 (4.4)	0	2 (2.2)
Drugs used in diabetes	7 (15.6)	10 (9.2)	11 (12.2)
Antithrombotic agents	30 (66.7)**	55 (50.5)	40 (44.4)
Cardiovascular system, all agents			
Digoxin	31 (68.9)*	37 (33.9)*	43 (47.8)
Diuretics	11 (24.4)***	2 (1.8)	5 (5.6)
β-Blocking agents	18 (40.0)	21 (19.3)	26 (28.9)
Calcium channel	8 (17.8)	17 (15.6)	15 (16.7)
Blockers	8 (17.8)	9 (8.3)	10 (11.1)
Renin–angiotensin	17 (37.8)**	17 (15.6)	17 (18.9)
Modifying agents	7 (15.6)	8 (7.3)	10 (11.1)
Lipid-modifying agents	28 (62.2)**	17 (15.6)**	30 (33.3)
Respiratory medication	17 (37.8)	7 (6.4)***	22 (24.4)
Immunosuppressive agents, NSAIDs	24 (53.3)	48 (44.0)	43 (47.8)

Data are no. (%) of patients, unless otherwise indicated.
n, number of patients; CDI, *Clostridium difficile* infection; non-CDI, diarrhoea due to another cause; NSAIDs, non-steroidal anti-inflammatory drugs.
Significantly different from control group (*p <0.05, **p <0.01, ***p <0.001).

Table 2. Crude and adjusted ORs for development of diarrhoea, according to demographic, clinical and pharmaceutical characteristics

	CDI				Non-CDI			
	Crude (95% CI)	OR	Adjusted (95% CI)[a]	OR	Crude (95% CI)	OR	Adjusted (95% CI)[a]	OR
Age, years								
18–64	1 (reference)		1		1		1	
≥65	2.6 (1.0–5.7)*		2.6 (1.0–5.7)*		0.8 (0.5–1.5)		0.8 (0.5–1.5)	
Duration of stay in hospital	1.03 (1.0–1.1)*		1.04 (1.0–1.1)*		1.0 (0.9–1.0)		1.0 (0.9–1.0)	
Cardiovascular disease	2.7 (1.3–5.6)**		1.9 (0.8–5.2)		1.5 (0.8–2.6)		1.9 (0.9–3.8)	
Respiratory system disease	2.3 (1.0–5.2)*		2.7 (0.9–7.9)		0.6 (0.3–1.4)		1.3 (0.5–3.7)	
Digestive system disease	1.7 (0.7–4.2)		2.7 (0.9–8.9)		2.2 (1.1–4.5)*		3.1 (1.2–7.8)*	
Surgery	0.6 (0.3–1.6)		0.7 (0.2–2.4)		2.2 (1.2–4.1)*		2.1 (1.0–4.3)*	
Nasogastric tube	3.4 (1.2–9.5)*		3.6 (1.0–14.3)*		3.3 (1.4–8.1)**		4.8 (1.7–13.6)**	
Antibiotics								
Any antibiotic	15.3 (4.4–53.2)**		12.5 (3.2–48.1)**		1.1 (0.6–2.0)		1.8 (0.9–3.7)	
Cephalosporins	7.0 (3.1–15.7)**		5.7 (1.8–18.6)[b]**		0.5 (0.3–1.0)		0.9 (0.3–2.1)	
Macrolides	4.9 (2.0–12.3)**		2.4 (0.7–8.7)[c]		0.3 (0.1–1.2)		0.3 (0.1–1.6)	
Quinolones	11.6 (3.1–43.6)**		15.3 (2.7–84.6)[d]**		2.0 (0.5–7.8)		2.2 (0.5–10.6)	
Other drugs								
Proton-pump inhibitors	1.6 (0.8–3.4)		1.1 (0.5–2.6)		0.6 (0.3–1.0)*		0.9 (0.5–1.7)	
Antithrombotic agents	2.5 (1.2–5.2)*		1.2 (0.5–2.9)		1.1 (0.7–2.2)		1.7 (0.8–3.6)	
Cardiovascular agents, all								
Digoxin	2.4 (1.1–5.0)*		1.3 (0.4–4.9)		0.6 (0.3–1.0)*		0.7 (0.4–1.5)	
Renin–angiotensin	5.4 (1.8–16.8)**		2.3 (0.6–12.1)		0.3 (0.1–1.7)		0.5 (0.1–3.2)	
Modifying agents	2.6 (1.2–5.7)*		2.2 (0.7–7.0)		0.8 (0.4–1.6)		0.9 (0.4–2.2)	
Respiratory medication	3.2 (1.5–6.8)**		1.1 (0.4–3.2)		0.4 (0.2–0.7)**		0.6 (0.2–1.2)	
Immunosuppressive agents	1.8 (0.9–4.0)		0.9 (0.3–2.5)		0.2 (0.1–0.5)**		0.4 (0.1–1.0)*	
NSAIDs	1.2 (0.6–2.5)		1.0 (0.4–2.4)		0.8 (0.5–1.5)		1.0 (0.5–1.9)	

CDI, *Clostridium difficile* infection; non-CDI, diarrhoea due to another cause.
[a]Adjusted for differences in age, duration of hospital stay, comorbidity (ICD-10), level of care, and comedication.
[b]Additional adjustment for concomitant use of macrolides and quinolones.
[c]Additional adjustment for concomitant use of cephalosporins and quinolones.
[d]Additional adjustment for concomitant use of cephalosporins and macrolides.
*p <0.05; **p <0.01; ***p <0.001.

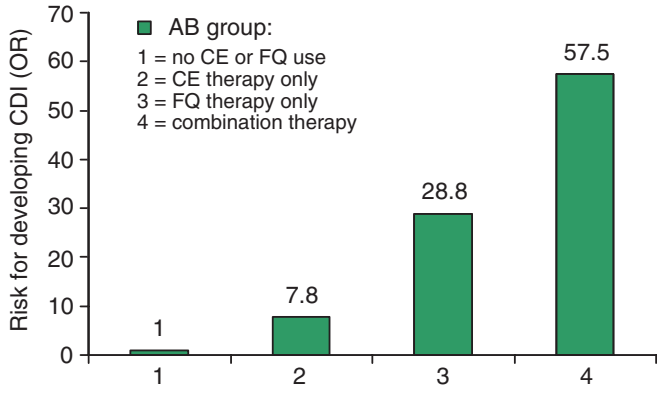

Figure 2. Risk for development of *Clostridium difficile* infection (CDI), stratified by cephalosporin (CE) and fluoroquinolone (FQ) therapy within the preceding 3 months.

Discussion

In 2005, the first outbreak of CDI due to *C. difficile* 027/111/ NAPI/BI occurred in a medium-size hospital in The Netherlands. As was also observed during the recent epidemics in Europe and North America, the outbreak was very difficult to control, and came to an end only after implementation of measures in addition to general measures of hygiene, *i.e.* cohorting of all *C. difficile* infected patients on a separate ward, education of staff, intensified cleaning of the environment, and strong limitations on antibiotic use. These measures have also been described as an effective comprehensive 'bundle' approach to combat CDI outbreaks in the USA [30,31].

The use of cephalosporins is a well-documented risk factor for the development of CDI [15-17]. In this study, fluoroquinolone therapy, especially in combination with cephalosporin therapy, was identified as another major risk factor for the development of CDI. Ciprofloxacin is still the main fluoroquinolone used in The Netherlands. In our study population, 22 patients used ciprofloxacin and only one patient used moxifloxacin. Although fluor-oquinolones account only for a small proportion of all antibiotics used in St Jansdal Hospital (9.5% of all antibiotics prescribed, in contrast to 31% for cephalosporins), the proportion of CDI cases in the study population that was attributable to the use of fluoroquinolones was as high as 33%. This finding is in line with results reported by Pepin et *al.* [23], who calculated a PAR.% of 35.9% for fluoroquinolones during a large outbreak of nosocomial CDI in Canada.

Associations between CDI and fluoroquinolones, including ciprofloxacin, have been described previously [23-25,32-36]. A recent study, which included 'CDI pressure' as a risk factor for the development of CDI, found ciprofloxacin to be an independent factor [14]. In The Netherlands, ciprofloxacin has been recognized as a risk factor for acquisition of CDI, particularly infection due to PCR-ribotype 027 [12]. However, the exact role of fluoroquinolones in the aetiology of CDI is still unclear. An important factor might be the increasing fluoroquinolone resistance of *C. difficile,* which has been observed worldwide [37,38], coupled with an increasing use of fluoroquinolones, leading to more efficient proliferation of resistant clones following disruption of colonic flora. Until 2000, no relationship between CDI and the use of ciprofloxacin and ofloxacin had been reported. Interestingly, two historical isolates of *C. difficile* from 1987, which were also typed as 027/III/NAPI/BI, were susceptible to fluoroquinolones [6]. Therefore, we consider it very likely that the acquisition of fluoroquinolone resistance contributed to the increased spread of this hypervirulent strain. Recently, several authors have underlined the importance of the improved anti-anaerobe spectrum of the newer fluoroquinolones in the aetiology of CDI [25,37]. However, this does not apply to ciprofloxacin, which possesses poor *in vitro* activity against anaerobic bacteria.

As correctly stated by Wilcox et *al.* [1], the duration of treatment and antibiotic polypharmacy affect the incidence of CDI, and may confound risk analyses for antimicrobial agents. Pepin et *al.* [23] suggested that long duration of fluoroquinolone therapy, in particular, enhances the risk of CDI. Unfortunately, we did not have sufficient data to assess the possible effect of duration of treatment on the risk of CDI in our study. With respect to polypharmacy, it must be noted that, in The Netherlands, fluoroquinolones are often administered together with cephalosporins, *e.g.* in empirical therapy of severe community-acquired pneumonia. In a separate analysis, after correcting for differences in co-medication and the use of multiple antibiotics, we could demonstrate that patients who had received fluoroquinolone monotherapy within the preceding 3 months were also at very high risk of developing CDI. This clearly demonstrates that fluoroquinolones represent an independent risk factor for CDI in our population. Surprisingly, the risk of developing CDI was extremely high in people receiving a combination of cephalosporins and fluoroquinolones. The fact that the OR in these subjects was much higher (57.5) than could be explained by simply summing the ORs for the separate antibiotics (7.8 and

28.8, respectively) could suggest a synergistic effect of cephalosporins and fluoroquinolones in the aetiology of CDI.

In addition to antibiotic use, several other risk factors have been associated with the development of CDI [15-25]. Analysing three different study populations, we were able to demonstrate that underlying digestive system disease, previous surgery and gastric tube feeding are not specifically associated with CDI, but are general risk factors for (non-infectious) diarrhoea. In addition, we demonstrated that although proton-pump inhibitors and immunosuppressive medication were not associated with CDI, subjects with non-infectious diarrhoea less frequently used these drugs. This observation indicates that differences in selection of control subjects may underlie the inconsistency among studies regarding the role of proton-pump inhibitors and immunosuppressive medication in the aetiology of CDI. Unfortunately, we were unable to determine the role of 'CDI pressure' as a risk factor [13,14].

Most experts emphasize that antimicrobial intervention alone is unlikely to result in successful control of all CDI outbreaks. Issues related to the environment, education and infection control should also be addressed [30]. A recently published ECDC-supported guideline emphasizes the importance of antimicrobial stewardship in conjunction with proper environmental disinfection, hand hygiene compliance, protective clothing, education of staff, and single-room isolation or cohorting of CDI patients [39]. The outbreak described here ended only after the formation of a multidisciplinary hospital OMT to coordinate measures to control the epidemic, the enhancement of case-finding and compliance by continuous education, isolation of all patients with diarrhoea until CDI was excluded, increasing the rapidity of microbiological diagnosis by using repeated stool ICTAB testing, the implementation of specific hygiene measures (including hand washing with water and soap and intensified environmental cleaning procedures), the cohorting of all CDI patients on a separate ward, and the implementation of an antimicrobial stewardship programme. The value of implementation of a CDI control 'bundle', including early identification, coupled with appropriate control measures, in reducing the rate of CDI and the frequency of adverse events in a university hospital was shown recently by Muto *et al.* [31]. The importance of appropriate antimicrobial stewardship has recently been illustrated by a report from Canada. Valiquette *et al.* [40] reported that no change in CDI incidence was noted after strengthening of infection control procedures, but that implementation of the antimicrobial stewardship

programme was followed by a marked reduction in incidence. These observations are very similar to those made in this study, as an effective outbreak control was only obtained after strong restrictions on the use of cephalosporins and a complete ban on the use of ciprofloxacin. The decline in CDI cases following restriction of cephalosporin use and a complete ban on the use of fluoroquinolones in our hospital, followed by an increase in CDI cases following the reintroduction of fluoroquinolones, underline the importance of these antibiotics in the development of CDI.

In conclusion, cephalosporin therapy and fluoroquinolone therapy were identified as important risk factors for the development of CDI during an outbreak of *C. difficile* PCR-ribotype 027 in The Netherlands. The risk of developing CDI was particularly high in people receiving a combination of cephalosporins and fluoroquinolones. Our data indicate the importance of good antimicrobial stewardship, in relation with other measures, to control outbreaks of *C. difficile* PCR-ribotype 027.

Transparency Declaration

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3

Chapter 3

Effect on diagnostic yield of repeated stool testing during outbreaks of *Clostridium difficile*-associated disease

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Abstract

The effect on diagnostic yield of testing sequential stools was assessed during two hospital epidemics of *Clostridium difficile*. Using a rapid immuno-assay, *C. difficile*-associated disease was diagnosed in 237 diarrhoeal patients, of whom 204 (86%) were diagnosed from the first faeces sample and 12 (5%) were diagnosed from follow-up samples obtained within 1 week. The remaining 21 (9%) patients yielded a positive test from stools obtained >1 week after the initial negative sample. It was concluded that repeated testing of stools for *C. difficile* toxin is of value in controlling outbreaks of *C. difficile* infection.

Research note

Clostridium difficile-associated disease (CDAD) is one of the most common hospital-acquired infections [1]. Early recognition of CDAD patients is of prime importance to prevent spread and to enable rapid implementation of adequate isolation and hygiene procedures and the initiation of CDAD-specific therapy. For rapid diagnosis, a fast, one-step immunoassay (ICTAB; Meridian Bioscience Europe, Boxtel, The Netherlands) is available for the detection of *C. difficile* toxins A and B in faeces samples. Using the cell cytotoxicity test as a reference standard, the relative sensitivity and specificity, and positive and negative predictive values of the ICTAB assay were 91%, 97%, 70% and 99%, respectively [2]; similarly, Diederen *et al.* [3] reported a relative sensitivity of 88.6% compared with the cytotoxicity test.

Current guidelines for the diagnosis of CDAD recommend analysis of additional samples for *C. difficile* toxin when the first sample is negative and clinical suspicion is high [4,5]. This recommendation has been disputed in two published studies [6,7]; however, both of these studies were performed in an endemic situation. The purpose of the present study was to assess the effect of sequential analysis of stools on diagnostic yield when using the ICTAB immunoassay as an alternative to the cytotoxicity test in CDAD outbreaks caused by *C. difficile* strains belonging to PCR-ribotypes 027 and 017.

A CDAD epidemic caused by *C. difficile* PCR-ribotype 027/toxinotype III occurred in hospital A between April and September 2005, with the incidence of CDAD increasing rapidly from 3.8 to 58.4/10,000 admissions. At a distance of 35 km, a second epidemic occurred in hospital B between May 2005 and October 2006, caused by *C. difficile* PCR-ribotype 027/toxinotype III and PCR-ribotype 017/toxinotype VIII. Physicians were instructed to collect stools from all diarrhoeal patients who were hospitalized for >3 days and/or who were clinically suspected of CDAD. Samples were tested within 24 h of arrival at the laboratory because of possible toxin degradation. The ICTAB immunoassay was performed at least twice daily for as long as the epidemics continued. Following a negative result, the responsible clinicians were requested to resample diarrhoeal patients, preferably within 48 h. When both tests were negative, CDAD was considered unlikely, and a new test was requested and the corresponding sample was cultured only

if clinical suspicion remained. Toxin-positive faeces were cultured for the presence of *C. difficile* and isolates were identified as described previously [8]. PCR-ribotyping was also performed as described previously [9].

During the epidemic in hospital A, 50 patients eventually yielded an ICTAB-positive sample, with 43 (86%) patients being ICTAB-positive on initial testing (Table 1). Within 7 days, a second sample was collected from 131 patients who were initially ICTAB-negative, of whom three (2%) were positive with the second sample; thus, 46 (92%) patients were diagnosed correctly with CDAD following two sequential samples. One additional patient was ICTAB-positive with a third sample, also obtained within 7 days, and three (2%) patients were positive with samples taken within, on average, 24 days of the first sample. Considering the interval between samples, this suggested a new infection. The final four samples mentioned above were confirmed by specific culture of *C. difficile*. Of the ICTAB-positive samples, 37 were available for culture, with 33 (90%) yielding *C. difficile*. Twenty-five (76%) isolates were identified as *C. difficile* PCR-ribotype 027. The remaining eight isolates belonged to various other PCR-ribotypes. A comparison of patients with CDAD caused by PCR-ribotype 027 and other PCR-ribotypes revealed no differences in the test results.

In hospital B, 187 patients were diagnosed with CDAD, of whom 161 (86%) were found to be ICTAB-positive on initial testing (Table 1). Following a negative first test, 15 patients were resampled within 1 week, of whom eight were positive. Thus, CDAD was diagnosed in <1 week in 169 (90%) of 187 patients. In addition, two patients were found to be ICTAB-positive with a second sample obtained 10 days after the first negative sample. The remaining 16 patients were diagnosed as positive with samples taken >14 days after the initial sample. Of the total of 187 ICTAB-positive samples, 165 were cultured for the presence of *C. difficile,* with 149 being culture-positive. Isolates from 147 samples were available for further typing (Table 1). The epidemic strains isolated from patients in hospital B were identified as PCR-ribotypes 017 (toxinotype VIII; $n = 47$) and 027 (toxinotype III; $n = 40$). The remaining 60 isolates belonged to a range of PCR-ribotypes.

Thus, overall, 12 (5%) of 237 diarrhoeal patients from hospitals A and B were diagnosed following the analysis of one or more additional samples within a week of the initial negative result. An additional 21 (9%) samples became positive within, on average, 45 days of the initial sample, which

probably reflects the development of CDAD in diarrhoeal patients after the observation period of 1 week. Of 202 positive samples from hospitals A and B, 20 (10%) were negative by culture for *C. difficile*. Importantly, in both hospitals, all retested and subsequently cultured (n = 9) ICTAB-positive samples that were taken within 1 week of the first negative sample yielded a positive culture for *C. difficile*.

In conclusion, testing of multiple stool samples, collected at an interval of a few days, for *C. difficile* toxin appears to be of value for combating outbreaks of toxin-producing *C. difficile*. In particular, when highly epidemic strains are involved, the additional costs of repeated testing may be rapidly offset by the benefits associated with prevention of spread of the disease, including preventing closure of wards and expensive treatment of patients.

Table 1. Value of repeated testing with Immunocard toxins A and B (ICTAB) for patients with *Clostridium difficile*-associated disease.

	Hospital A	Hospital B			
			Subdivided by PCR-ribotype[b]		
Patient characteristics	**All ribotypes[a]**	**All ribotypes**	**027**	**017**	**Other ribotypes**
Number of CDAD patients	50	187	40	47	60
ICTAB-positive with first sample (% of all positive patients)	43 (86%)	161 (86%)	36 (90%)	40 (85%)	51 (85%)
ICTAB-positive with repeated sample ≤1 week (cumulative % of all positive patients)	4 (94%)	8 (90%)	1 (93%)	3 (91%)	1 (87%)
ICTAB-positive with repeated sample >1 week (cumulative % of all positive patients)	3 (100%)	18 (100%)	3 (100%)	4 (100%)	8 (100%)

[a]PCR-ribotyping was only performed for ribotype 027 in hospital A (25 isolates, 76%).
[b]Not all isolates were available for typing.

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4

Chapter 4

PCR-ribotype-specific risk factors and outcome in an outbreak with 2 different *Clostridium difficile* Types simultaneously in one hospital

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Abstract

Background. *Clostridium difficile* infection (CDI) due to polymerase chain reaction (PCR) ribotype 027 has been described worldwide. In some countries, an increase was reported of toxin A-negative PCR-ribotype 017. We encountered an outbreak due to these 2 types occurring simultaneously in a 980-bed teaching hospital in the Netherlands.

Methods. In a case-control study from May 2005 through January 2007, we investigated general and PCR-ribotype-specific risk factors as well as outcome parameters for CDI due to ribotype 027 or 017. Clonal dissemination was investigated by multilocus variable number of tandem repeat analysis (MLVA).

Results. We identified 168 CDI patients: 57 (34%) with ribotype 017, 46 (27%) with ribotype 027, and 65 (39%) with 1 of 36 different other types. As controls, we included 77 non-CDI diarrheal patients and 162 patients without diarrhoea. Risk factors for CDI were nasogastric intubation, recent hospitalization, and use of cephalosporins and clindamycin. PCR-ribotype-specific risk factors were older age for both types 017 and 027, use of clindamycin and immunosuppressive agents for ribotype 017, and use of fluoroquinolones for ribotype 027. At day 30 of follow-up, the overall mortality among patients with types 017, 027, other types, non-CDI diarrheal patients, and non-diarrheal patients was 23%, 26%, 3%, 2%, and 6%, respectively. MLVA showed persistent clonal dissemination of types 017 and 027, despite appropriate infection control measures.

Conclusions. Patients with CDI have PCR-ribotype-specific risk factors and mortality rates, with prolonged clonal spread of ribotype 027 or 017.

Introduction

Clostridium difficile infection (CDI) due to polymerase chain reaction (PCR) ribotype 027 has been described worldwide [1-4]. This strain harbours the toxin genes *tcdA* and *tcdB* as well as binary toxin genes, and has a deletion at position 117 in the toxin regulatory gene *tcdC,* which is associated with increased virulence [5]. *C. difficile* strains lacking toxin A (A-/B+) are also increasingly found to cause outbreaks, especially in some Eastern European countries, South America, and Asia [6-12]. The most commonly found A-/B+ strain belongs to PCR-ribotype 017 [7]. Although several outbreaks of CDI due to ribotype 017 have been reported, it is unclear whether the clinical characteristics, spread, response to therapy, and outcome differ from outbreaks due to other *C. difficile* types [6, 11].

We encountered a unique CDI outbreak due to ribotype 027 and ribotype 017 occurring simultaneously in a 980-bed teaching hospital in the Netherlands. In response, we performed a case-control study to investigate PCR-ribotype-specific risk factors and outcome of CDI patients, compared with control patients without diarrhoea. Risk factors for diarrhoea in general were also analysed by inclusion of a control group of diarrheal patients without CDI. Finally, we studied clonal dissemination using multilocus variable number tandem repeat analysis (MLVA).

Methods

Study Design

The medical ethics committee and the institutional board of the hospital approved the study. We included all consecutively diagnosed CDI patients with a positive faeces toxin test and culture of *C. difficile* from May 2005 through January 2007. For every CDI patient, we randomly selected a control patient without diarrhoea, matched for ward, age, sex, admission period, and duration of hospitalization. We also included a group of control patients with non-CDI diarrhoea, as determined by a negative *C difficile* toxin assay. We matched these patients for ward and date of toxin testing, but because of an insufficient number of available controls, not for age, sex, or duration of hospitalization.

Microbiological Analysis

We tested diarrheal faecal samples from hospitalized patients with a rapid enzyme immunoassay (ImmunoCard Toxin A and B [ICTAB]; Meridian). This test was selected because of its easy use and good performance in comparison with cell cytotoxicity and real-time PCR [14]. All toxin test-positive stool samples were cultured for the presence of *C. difficile* using previously described methods, and isolates were further investigated by PCR ribotyping [15,16]. A randomly selected number of isolates were tested for antimicrobial susceptibility to ciprofloxacin, moxifloxacin, erythromycin and clindamycin, using E tests. We defined resistance to all 4 antibiotics at > 4 mg/1 [17]. Molecular genotyping was performed by multilocus variable-number tandem-repeat analysis (MLVA) and minimum spanning tree (MST) analysis was used to determine the genetic distance between isolates [18]. We used the number of differing loci and the summed tandem-repeat difference (STRD) between MLVA types as coefficients for the genetic distance, using the BioNu-merics software program (version 4.6, Applied Maths). Genetically related complexes were defined by a STRD < 10 and clonal complexes by a STRD < 2 [8, 19].

Clinical Analysis

CDI was defined as diarrhoea in combination with a positive laboratory assay for *C. difficile* toxin A or B in stools. Diarrhoea was considered as severe when it occurred in combination with 1 or more of the following: bloody stools, hypovolemia, hypo-albuminemia (< 20 g/L), fever (T > 38.0°C), leukocytosis (white blood cell count > 12 X 10^9 cells/L), and pseudomembranous colitis. For each death, two physicians (A. G. and J. C. D.) reached consensus about whether CDI was the direct cause of death (attributable mortality), contributed (contributable mortality) to the death, or was not related to death.

We collected patient information on age, sex, ward of acquisition, disease severity, mortality and Charlson comorbidity index on admission [13]. Data were collected on procedures (endoscopy, abdominal surgery), previous admissions, and use of antibiotics and medications during the 3 months prior to the first CDI episode. This period was determined by calculating backward from a reference date. For CDI and non-CDI diarrheal patients, this reference date was defined as the day on which the diarrhoea started. For

non-diarrheal control patients, we determined the reference date by adding the hospitalized period of the matched CDI patient (time between admission and start of diarrhoea) to the admission date of the control patient.

We assessed comorbidity using the International Classification of Diseases 10 (ICD-10) classification. For each prescribed antibiotic, defined daily dose (DDD) was calculated according to the World Health Organization (WHO) recommendation *(http://www.escmid.org/research_projects /study_ groups/ esgap/abc_calc/)*. Low exposure to antibiotics was defined as the use of < 3 DDDs of a certain antibiotic, and high exposure to antibiotics as the use of > 3 DDDs.

Statistical Analysis

To compare risk factors for CDI with risk factors for diarrhoea in general, we compared the distribution of risk factors among patients with CDI diarrhoea and with non-CDI diarrhoea with the distribution among non-diarrheal control patients. Relative risk was expressed as an odds ratio (OR) with a 95% confidence interval (95% CI).

Because non-diarrheal control patients were matched to case patients for potential risk factors, a conditional logistic regression analysis was performed that took this matching fully into account. The comparisons between non-CDI diarrheal patients and non-diarrheal control patients, as well as comparisons of CDI caused by different PCR-ribotypes were analysed by unconditional logistic regression analysis.

When a patient died who had lived within the community boundaries, the hospital received notification from the community council. For this subgroup of patients, the vital status was certain at the end of follow-up. To determine the overall 30-day mortality, we therefore only included this subgroup of patients.

In the multivariable model, we always adjusted for age, sex, and ward, except in the matched analysis between CDI patients and non-diarrheal control patients where these factors were taken into account by the matching. For the analysis of the effect of antibiotics and other medications, we addition-

ally adjusted for comorbidity and use of co-medication. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) for Windows software, version 16.0.

Results

In July 2005, CDI occurred among 20 patients, and a CDI outbreak was recognized, with an increased incidence of 101 cases per 10,000 admissions. Predominantly affected were the departments of haematology, nephrology, and general surgery. Although implementation of infection control measures (disinfection, isolation, cohort nursing, antibiotic stewardship) resulted in a decrease in incidence, several new peaks were noticed following the release of these measures, forcing their reimplementation. After introducing a restriction on the use of fluoroquinolones and cephalosporins in June 2006, the incidence finally decreased to around 30 cases per 10,000 admissions in early 2007.

Microbiology

Isolates from 168 of 223 patients with CDI (75%) were available for PCR ribotyping. Of these, 57 patients (34%) had ribotype 017, 46 (27%) had ribotype 027, and 65 (39%) had a ribotype other than 017 or 027. Within this last group, the following types were found: 014 (14 patients), 001 (6), 078 (5), 015 (3), 070 (3), 002 (2), 045 (2), 122 (2), 016 (1), 029 (1), 056 (1), 064 (1), 077 (1), 081 (1), 117(1), 126(1), 135(1), 164(1), and unknown types (18).

We performed susceptibility testing on a random selection of 19 ribotype 027 isolates and 19 ribotype 017 isolates. All ribotype 017 and ribotype 027 isolates were resistant to ciprofloxacin (minimum inhibitory concentration [MIC] > 32 mg/L). All ribotype 017 isolates and 18 ribotype 027 isolates (94.7%) were resistant to erythromycin (MIC > 256 mg/L). Resistance to moxifloxacin was found among 17 (89.4%) of both the ribotype 017 and ribotype 027 isolates. Resistance to clindamycin was found among 18 (94.7%) ribotype 017 isolates (MIC > 256 mg/L), whereas all ribotype 027 isolates had MICs <4 mg/L for clindamycin.

In total, 108 isolates of 168 CDI patients (64%) were available for investigation by MLVA: 33 ribotype 027 isolates, 42 ribotype 017 isolates, and 33 isolates that belonged to other types. MST analysis of the ribotype 017 and ribotype 027 isolates is depicted in Figure 1. Of the 33 ribotype 027 isolates, 32 (97%) were genetically related (STRD < 10), and among these isolates, 3 clonal complexes (STRD < 2) were found (boxed clusters CC-A through CC-C). In total, 25 (76%) of the ribotype 027 isolates belonged to a clonal complex. Similarly, 41 of the 42 ribotype 017 isolates (98%) were genetically related and 4 clonal complexes (CC-D through CC-G) were found, comprising 37 of the ribotype 017 isolates (88%). In contrast, no clonal complexes were found among 10 ribotype 014 isolates and only 3 (30%) were genetically related (not shown in Figure 1). Among 23 isolates that belonged to types other than 014, 017, or 027, 1 clonal complex was found, comprising 2 isolates belonging to ribotype 070 (not shown in Figure 1). Clonal spread of types 027 and 017 was predominant on the wards of geriatrics (CC-B), internal medicine, and Surgery (CC-A, CC-D, CC-E, CC-F). Clonal complexes persisted on these wards for a maximum of 12 months (CC-D) and persisted throughout the hospital for a maximum of 18 months (CC-A).

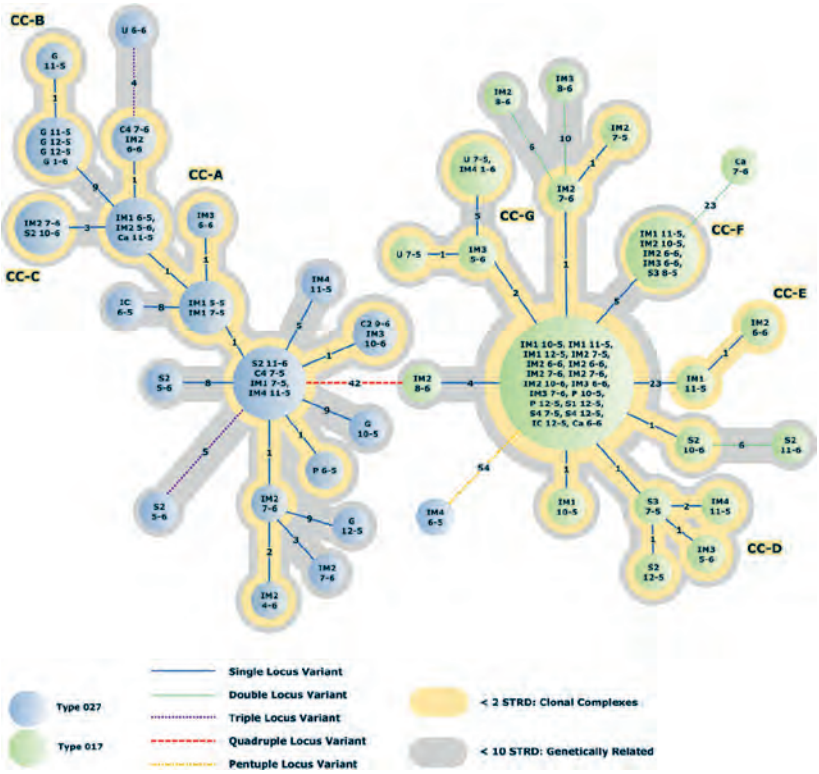

Figure 1. Minimum spanning tree analysis of *Clostridium difficile* isolates typed by multilocus variable-number tandem-repeat analysis (MLVA): 33 ribotype 027 isolates (in blue circles) and 42 ribotype 017 isolates (in green circles). Each circle represents either a unique isolate or more isolates that are 100% homologous. The numbers between the circles represent the summed tandem-repeat difference (STRD) between MLVA types. Within the spanning tree, genetically related complexes (STRD < 10) are marked in grey. Clonal complexes (CC-A to CC-G) with a STRD < 2 are marked in yellow. Isolates are marked according to the ward where CDI was diagnosed and date of diagnosis (mm-yy). Abbreviations: IM1, internal medicine, ward 1 (department of haematology); IM2, internal medicine, ward 2 (department of nephrology); IM3, internal medicine, ward 3; IM4, internal medicine, ward 4; S1-4, surgery, wards 1-4; Ca, cardiology; IC, intensive care; G, geriatrics; U, urology; P, pulmonology. Example: *IM1 6-5* stands for "internal medicine, ward 1, June 2005".

Clinical Analysis Risk Factors

General risk factors are shown in Table 1, whereas Table 2 depicts odds ratios (ORs) for specific antibiotics.

Patients With CDI Versus Non-diarrheal Control Patients

Significant crude risk factors were discharge from the hospital in the month before the current admission, colonic diseases, abdominal surgery, complications of surgical care, nasogastric intubation, and any use of antibiotics. In the adjusted model, the association with recent discharge became weaker, whereas the association with use of antibiotics became stronger (increase in OR from 8.33 to 12.6). Specific antibiotics exposures associated with an increased risk were low and high exposure to second-generation cephalosporins and high exposure to clindamycin. In the adjusted model, the association with penicillins disappeared, whereas the association with high exposure to second-generation cephalosporins and clindamycin remained statistically significant (the OR for clindamycin increased from 5.17 to 8.79). Finally, control patients had significantly higher Charlson comorbidity indices than those of CDI patients (matching effect; Charlson scores not shown in Table 1).

Patients With Non-CDI Diarrhoea Versus Non-diarrheal Control Patients

Significant risk factors, in both crude and adjusted models, were inflammatory bowel disease and abdominal surgery. Use of any antibiotics was also a significant risk factor in the adjusted model (increase in OR from 1.86 to 2.56). Specifically, low exposure to first-generation cephalosporins was associated with and increased risk, both in the crude and in the adjusted model.

Patients with non-CDI diarrhoea were significantly younger (19% vs 36% older than 80 years) and had a lower Charlson comorbidity index.

Patients With PCR-ribotype-027 CDI Versus Patients With CDI Due to Other Types (Non-027/Non-017)

Significant risk factors, in both crude and adjusted models, were older age and haematological malignancy (Table 3). High exposure to ciprofloxacin was specifically associated with ribotype 027 CDI (Table 4).

Patients With PCR-ribotype-017 CDI Versus Patients With CDI Due to Other Types (Non-027/Non-017)

Significant crude risk factors were male sex, higher Charlson comorbidity score, and use of immunosuppressive agents. All these factors, except male sex, remained statistically significant in the adjusted model. Haematological malignancy (increase in OR from 3.83 to 4.78) was also a significant risk factor in the adjusted model. Regarding antibiotic exposure, high exposure to clindamycin was specifically associated with ribotype 017 CDI, which remained statistically significant in the adjusted model, with an increased OR from 2.24 to 3.56.

Clinical Course and Outcome

Compared with non-CDI diarrheal patients, patients with CDI more often had severe diarrhoea: 47.8% versus 25.7% (adjusted OR 3.06; 95% CI 1.58-5.92).

As shown in Table 5, patients with CDI had a higher 30-day mortality than both non-CDI diarrheal patients and non-diarrheal control patients. As shown in table 6, the attributable in-hospital mortality rates among patients with CDI types 027 and 017 were higher than the rate among patients with other types (not significant). Patients with ribotype 027 or 017 had similar overall mortality rates after 30 days, which were significantly higher than the mortality rate observed among patients with CDI due to other types.

Table 1. Risk factors among *Clostridium difficile* infection cases and *non-Clostridium difficile* infection diarrheal patients compared with non-diarrheal control patients.

	Patient groups						Case vs control		Non-CDI vs control	
	Case[a] (n = 168)		Non-CDI[b] (n = 77)		Control[c] (n = 162)		OR (95% CI)		OR (95% CI)	
	n	(%)	n	(%)	n	(%)	Crude	Adjusted	Crude	Adjusted
Age, years 18–64	39	(23.2)	22	(28.6)	38	(23.6)	matched	matched	1 (ref. group)	1 (ref. group)
65–80	69	(41.1)	40	(51.9)	65	(40.4)	matched	matched	1.06 (.55–2.05)	1.09 (.56–2.11)
80+	60	(35.7)	15	(19.5)	58	(36.0)	matched	matched	.45 (.21–.97)*	.43 (.20–.95)*
Recent discharge	76	(45.8)	16	(22.9)	46	(30.1)	1.83 (1.11—3.01)*	1.56 (.88–2.78)	.69 (.36–1.33)	.72 (.36–1.42)
<1 week before current admission	24	(14.5)	4	(5.7)	8	(5.2)	4.06 (1.57–10.5)*	2.34 (.80–6.84)	.99 (.29–3.44)	.98 (.27–3.52)
<1 month before current admission	33	(19.9)	8	(11.4)	18	(11.8)	2.10 (1.00–4.41)*	1.60 (.68–3.81)	.88 (.36–2.15)	.89 (.35–2.26)
Hematological malignancy	22	(13.3)	8	(11.3)	11	(6.8)	2.50 (.97–6.44)	3.20 (1.16–8.81)*	1.73 (.66–4.51)	2.17 (.77–6.11)
Diseases of the digestive system	58	(34.9)	27	(38.0)	43	(26.9)	1.49 (.90–2.41)	1.74 (1.02–2.98)*	1.67 (.92–3.02)	1.59 (.81–3.14)
Inflammatory bowel disease	6	(3.6)	7	(9.9)	1	(0.6)	6.00 (.72–49.8)	7.07 (.79–63.6)	17.4 (2.10–144)*	22.9 (2.19–238)*
Other colonic diseases	23	(13.9)	8	(11.3)	12	(7.5)	2.32 (1.01–5.34)*	2.74 (1.09–6.84)*	1.58 (.61–4.04)	1.07 (.33–3.52)
Abdominal surgery	34	(20.4)	17	(22.1)	19	(11.7)	2.62 (1.16–5.93)*	2.67 (1.18–6.03)*	2.13 (1.04–4.38)*	2.19 (.99–4.83)
Complications of surgical care	19	(11.7)	4	(5.7)	7	(4.3)	3.75 (1.25–11.3)*	3.99 (1.31–12.2)*	1.33 (.38–4.71)	1.67 (.42–6.61)
Nasogastric intubation	40	(26.5)	7	(9.9)	20	(12.6)	2.73 (1.27–5.88)*	2.73 (1.07–6.99)*	.76 (.31–1.89)	.56 (.20–1.58)
Any antibiotic	155	(92.8)	60	(77.9)	106	(65.4)	8.33 (3.57–19.4)*	12.6 (3.74–42.4)*	1.86 (.99–3.50)	2.56 (1.23–5.31)*
Immunosuppressive agents	53	(31.7)	17	(22.1)	48	(29.6)	1.05 (.61–1.80)	.65 (.32–1.30)	.67 (.36–1.27)	.73 (.35–1.52)

Abbreviations: CDI, *Clostridium difficile* infection; CI, confidence interval; OR, odds ratio; ref., reference.

An asterisk indicates statistical significance.

[a] The number of patients of whom information was available varied between 151 and 168.

[b] Between 70 and 77 patients.

[c] Between 153 and 162 patients.

Table 2. Antibiotic use among cases and *non-Clostridium difficile* infection diarrheal patients compared with non-diarrheal controls.

		Patient groups					Case vs control		Non-CDI vs control	
		Case[a] (n = 168)		Non-CDI[b] (n = 77)		Control[c] (n = 162)	OR (95% CI)		OR (95% CI)	
Antibiotics		n	(%)	n	(%)	n	(%)	Crude	Adjusted	Crude	Adjusted
Penicillins	DDD < 3	9	(5.7)	3	(4.2)	6	(4.0)	1.74 (.54–5.63)	.63 (.11–3.54)	1.21 (.29–5.09)	.70 (.12–4.02)
	DDD ≥ 3	82	(52.2)	30	(42.3)	53	(35.1)	2.32 (1.36–3.95)*	1.36 (.62–2.94)	1.37 (.76–2.46)	1.06 (.45–2.51)
Cephalosporins	DDD < 3	13	(8.6)	11	(15.5)	9	(5.8)	2.74 (1.03–7.28)*	3.53 (.82–15.2) *	3.40 (1.31–8.87)*	4.66 (1.62–13.4)*
	DDD ≥ 3	81	(53.6)	23	(32.4)	44	(28.2)	5.44 (2.57–11.5)*	4.15 (1.56–11.0) *	1.46 (.78–2.73)	1.33 (.61–2.90)
1st generation	DDD < 3	18	(11.1)	13	(17.8)	13	(8.1)	1.70 (.67–4.32)	.77 (.14–4.18)	2.57 (1.12–5.89)*	3.30 (1.29–8.44)*
	DDD ≥ 3	3	(1.9)	4	(5.5)	4	(2.5)	.81 (.18–3.64)	.45 (.05–4.21)	2.57 (.62–10.6)	3.22 (.73–14.3)
2nd generation	DDD < 3	6	(3.8)	1	(1.3)	3	(1.9)	4.91 (1.06–22.8)*	2.88 (.34–24.5)	.71 (.07–6.94)	.70 (.07–7.28)
	DDD ≥ 3	65	(41.1)	16	(21.3)	33	(20.8)	4.26 (2.12–8.53)*	3.19 (1.39–7.31) *	1.03 (.52–2.02)	.86 (.39–1.89)
3rd generation	DDD < 3	5	(3.1)	1	(1.3)	1	(0.6)	6.12 (.69–54.6)	11.3 (.80–161)	2.19 (.14–35.6)	3.12 (.17–57.4)
	DDD ≥ 3	20	(12.4)	6	(8.0)	10	(6.3)	2.59 (.97–6.91)	1.48 (.48–4.54)	1.31 (.46–3.76)	1.21 (.38–3.81)
Clindamycin	DDD < 3	3	(1.9)	0	(0.0)	1	(0.6)	3.00 (.31–28.8)	3.38 (.29–39.3)	No OR	No OR
	DDD ≥ 3	32	(19.9)	3	(3.9)	6	(3.8)	5.17 (2.16–12.4)*	8.79 (2.46–31.4) *	1.05 (.25–4.31)	1.58 (.34–7.34)
Ciprofloxacin	DDD < 3	6	(3.7)	1	(1.4)	2	(1.3)	3.22 (.64–16.1)	.95 (.15–6.18)	1.08 (.10–12.1)	1.01 (.07–13.7)
	DDD ≥ 3	34	(21.0)	11	(14.9)	22	(13.9)	1.67 (.83–3.36)	.44 (.16–1.17)	1.08 (.49–2.37)	.88 (.35–2.21)

Abbreviations: CDI, *Clostridium difficile* infection; CI, confidence interval; OR, odds ratio; DDD, daily designated dose.

An asterisk indicates statistical significance.

[a] Per risk factor, the number of patients of whom information was available varied between 151 and 168.

[b] Between 71 and 76 patients.

[c] Between 151 and 162 patients.

Table 3. Risk factors among patients with ribotype 017, ribotype 027 and other (non-017/non-027) ribotypes.

	CDI caused by different PCR ribotypes						Type 027 vs other types (non-027/non-017)		Type 017 vs other types (non-027/non-017)	
	Type 027[a] (n = 46)		Type 017[b] (n = 57)		Other type[c] (n = 65)		OR (95% CI)		OR (95% CI)	
	n	(%)	n	(%)	n	(%)	Crude	Adjusted	Crude	Adjusted
Male sex	20	(43.5)	23	(57.1)	22	(34.3)	1.47 (.67–3.20)	1.89 (.81–4.39)	2.54 (1.22–5.32)*	2.08 (.95–4.55)
Age, years 18–64	6	(13.0)	14	(24.6)	19	(29.2)	1 (ref. group)	1 (ref. group)	1 (ref. group)	1 (ref. group)
65–80	23	(50.0)	26	(45.6)	20	(30.8)	3.64 (1.22–10.9)*	4.56 (1.36–15.4)*	1.76 (.71–4.35)	1.95 (.74–5.12)
80+	17	(37.0)	17	(29.8)	26	(40.0)	2.07 (.69–6.24)	2.07 (.62–6.95)	.89 (.35–2.23)	1.11 (.40–3.09)
Hematological malignancy	10	(21.7)	9	(16.1)	3	(4.8)	5.56 (1.43–21.5)*	8.68 (1.89–40.0)*	3.83 (.98–14.9)	4.78 (1.03–22.2)*
Diseases of the digestive system	20	(43.5)	18	(31.6)	20	(31.7)	1.65 (.75–3.64)	1.90 (.75–4.86)	.99 (.46–2.14)	1.36 (.52–3.56)
Immunosuppressive agents	12	(26.1)	30	(52.6)	11	(17.2)	1.73 (.66–4.53)	1.87 (.65–5.42)	5.35 (2.33–12.3)*	5.00 (1.89–13.2)*

Abbreviations: CDI, *Clostridium difficile* infection; PCR, polymerase chain reaction; CI, confidence interval; OR, odds ratio; ref., reference.

An asterisk indicates statistical significance.

[a] The number of patients of whom information was available varied between 43 and 46.

[b] Between 55 and 57 patients.

[c] Between 60 and 64 patients.

Table 4. Antibiotic use among patients with PCR-ribotype 027 or PCR-ribotype 017 versus patients with other (non-27/non-017) *Clostridium difficile* infection ribotypes.

		CDI caused by different PCR ribotypes						Type 027 vs other types (non-027/non-017)		Type 017 vs other types (non-027/non-017)	
		Type 027[a] (n = 46)		Type 017[b] (n = 57)		Other type[c] (n = 65)		OR (95% CI)		OR (95% CI)	
Antibiotics		n	(%)	n	(%)	n	(%)	Crude	Adjusted	Crude	Adjusted
Clindamycin	DDD <3	1	(2.2)	0	(0.0)	2	(3.3)	.61 (.05–6.97)	.62 (0.04–8.71)	No OR	No OR
	DDD ≥3	4	(8.7)	20	(36.4)	8	(13.3)	.61 (.17–2.17)	.48 (0.13–1.87)	2.24 (1.01–4.98)*	3.56 (1.25–10.2)*
Ciprofloxacin	DDD <3	0	(0.0)	2	(3.6)	4	(6.5)	No OR	No OR	.64 (.11–3.66)	.62 (.10–3.96)
	DDD ≥3	14	(31.1)	13	(23.6)	7	(11.3)	3.29 (1.20–9.04)*	3.47 (1.10–10.9)*	2.37 (.86–6.49)	1.50 (.48–4.72)

Antibiotic use is divided in low exposure: <3 DDDs and high exposure use: ≥3 DDDs.

Abbreviations: CDI, *Clostridium difficile* infection; CI, confidence interval; OR, odds ratio; DDD, daily designated dose.

An asterisk indicates statistical significance.

[a] Per risk factor, the number of case patients of whom information was available varied between 43 and 46.

[b] Between 55 and 57 patients.

[c] Between 60 and 64 patients.

Table 5. Clinical outcome among cases (all types combined), *non-Clostridium difficile* infection diarrheal patients, and non-diarrheal controls.

	Patient groups						Case vs non-CDI	Case vs control
	Case (n = 168)		Non-CDI (n = 77)		Control (n = 162)			
Outcome	n	(%)	n	(%)	n	(%)	OR (95% CI)	OR (95% CI)
Overall 30-day mortality[a]	16/93	(17.2)	1/51	(2.0)	6/95	(6.3)	10.4 (1.34–80.8)*	3.08 (1.15–8.27)*
Overall in-hospital mortality	29	(17.3)	5	(6.5)	14	(8.6)	3.13 (1.16–8.41)*	2.30 (1.70–4.52)*
Attributable mortality	8	(4.8)						
Contributable mortality	14	(8.4)						

Abbreviations: CDI, *Clostridium difficile* infection; CI, confidence interval; OR, odds ratio.

An asterisk indicates statistical significance.

[a] Data only from patients who had lived within the community boundaries (of these patients, the hospital received a notification of death from the community council, so the vital status was certain at the end of follow-up). The two numbers that are shown in the n columns reflect the number of patients that died on the total number of patients that lived within the community boundaries (numerator/denominator).

Table 6. Clinical outcome among patients with *C. difficile* infection due to different PCR-ribotypes.

	CDI caused by different PCR ribotypes						Type 027 vs other types (non-027/non-017)	Type 017 vs other types (non-027/non-017)
	Type 027 (n = 46)		Type 017 (n = 57)		Other Type (n = 65)			
Outcome	n	(%)	n	(%)	n	(%)	OR (95% CI)	OR (95% CI)
Overall 30-day mortality[a]	7/27	(25.9)	8/35	(22.9)	1/31	(3.2)	10.5 (1.20–92.0)*	8.89 (1.04–75.8)*
Overall in-hospital mortality	9	(19.6)	10	(17.5)	11	(16.9)	1.19 (.45–3.17)	1.04 (.41–2.68)
Attributable mortality	3	(6.5)	4	(7.0)	1	(1.6)	4.40 (.44–43.7)	4.75 (.52–43.8)
Contributable mortality	6	(13.0)	5	(8.8)	3	(4.7)	3.05 (.72–12.9)	1.96 (.45–8.57)

Abbreviations: CDI, *Clostridium difficile* infection; CI, confidence interval; OR, odds ratio.

An asterisk indicates statistical significance.

[a] Data only from patients who had lived within the community boundaries (of these patients, the hospital received a notification of death from the community council, so the vital status was certain at the end of follow-up). The two numbers that are shown in the n columns reflect the number of patients that died on the total number of patients that lived within the community boundaries (numerator/denominator).

Discussion

We experienced an outbreak with 2 different *C. difficile* PCR-ribotypes (types 017 and 027), which occurred simultaneously at the departments of internal medicine, geriatrics, and general surgery in 1 hospital in the Netherlands. Using MLVA, we discerned a pattern of clonal dissemination of types 027 and 017. Transmission occurred despite appropriate infection control measures, with prolonged presence of clones on wards and throughout the hospital for >1 year.

Several factors may have contributed to failure to control the outbreak. Per ward, only a limited number of single rooms were available. Until patients with CDI were sequestered on a separate *C. difficile* ward, CDI patients who sojourned in 2- or 4-patient rooms were placed in contact isolation in single rooms, often on another ward. This exchange of CDI patients may explain the observation of repeated small outbreaks on several wards. Second, use of clindamycin was not restricted, which may have affected the incidence of *C. difficile* ribotype 017. During the study period, we observed no significant increase in the overall incidence of hospital-acquired infections due to vancomycin-resistant *Enterococcus* (only 3-9 clinical isolates per year), norovirus, or extended-spectrum β-lactamase-producing Gram-negative bacteria. In the first months of 2005, an outbreak due to methicillin-resistant *Staphylococcus aureus* (MRSA) occurred in the geriatric department, which was related to an outbreak in an adjacent nursing home. The outbreak was controlled soon after implementation of intensive MRSA outbreak control measures.

The setting of this outbreak provided a unique opportunity to study PCR-ribotype-specific risk factors, clinical presentation, and outcome of CDI. The inclusion of a non-CDI diarrheal control group enabled us to discriminate between risk factors for CDI and for diarrhoea in general, which may be of importance in outbreaks (when specific infection control measures are considered) and in epidemiological studies. Risk factors for CDI included increased comorbidity, haematological malignancy, nasogastric intubation and use of antibiotics, especially high exposure to cephalosporins and clindamycin. These factors have previously been recognized, although studies lacked appropriate control groups of non-CDI diarrheal patients [20-23]. Risk factors for diarrhoea were prior abdominal surgery, coexisting diseases of the digestive system, and low exposure to first-generation cephalosporins (generally prescribed as perioperative prophylaxis).

In this study, risk factors for ribotype 027 CDI differed from risk factors for ribotype 017 CDI, although patients were nursed on similar departments and did not differ in age or comorbidity. Interestingly, high exposure to fluoroquinolones was a specific risk factor for ribotype 027, whereas high exposure to clindamycin was a specific risk factor for ribotype 017. In contrast to 027 isolates, 95% of the 017 isolates were resistant to clindamycin, supporting the hypothesis that differential susceptibility correlates with exposure rates. This is not applicable for the association of ribotype 027 with fluoroquinolones, because strains of both ribotype 027 and ribotype 017 were resistant. Possibly, to a lesser extent, fluoroquinolones also increase the risk to develop CDI due to ribotype 017 (adjusted OR was 1.5), but our study was too small to reach statistical significance. Other possible explanations are that fluoroquinolones influence the host defence against ribotype 027 by specific changes of the microbiota or increase spread of ribotype 027 by enhanced sporulation.

An interesting finding in this study was the high 30-day mortality rate among patients with CDI due to types 017 and 027 (23% and 26%, respectively); the rate for CDI caused by other types was only 3% and comparable to the 30-day mortality for non-CDI diarrheal patients and non-diarrheal control patients. The first explanation for this large difference is that infections with PCR-ribotypes 027 and 017 are markers of underlying disease severity. Although Charlson comorbidity indices at baseline did not differ, we were not informed about the severity of underlying disease during and after admission, which is not taken into account by this score. A second possible explanation is that mortality depends on the involved PCR-ribotype, as was also recently described by Miller *et al.*, who found that among patients aged 60-90 years, those with ribotype 027 CDI were twice as likely to die as those with non-ribotype 027 CDI [24]. By contrast, in 2 studies, ribotype 027 was not associated with adverse outcome; however, one described an endemic setting with ribotype 027 CDI, and the second study did not compare CDIs caused by different types [25, 26].

In another recent study, the independent impact of hospital acquired CDI on in-hospital mortality was investigated, after adjusting for the time-varying nature of CDI and baseline mortality risk at hospital [27]. On average, patients with CDI had a 3-fold increased risk of death. In this study, the strain-ribotype that caused CDI was not taken into account. However the results

of this study match those that we found for the outbreak strains, types 017 and 027, which suggests that our findings are probably not unique for this hospital.

In this outbreak setting, ribotype 017 was associated with similar clinical presentation and outcomes as ribotype 027. This is surprising, because ribotype 017 lacks toxin A gene and contains none of the proposed virulence markers typical of ribotype 027. We hypothesize that yet unknown virulence markers might be involved, such as variants of *TcdB,* or non-toxin-related virulence factors. In a very recent study applying comparative genome analysis of 14 sequences strains, we found SNPs that were found in 2 candidate genes with yet-unknown functions were associated with severe CDI [28]. Interestingly, these SNPs were found to be present among ribotype 027 strains, but also among ribotype 017 strains that lacked toxin A.

Limitations of our study are firstly that we had to perform a matched analysis in the comparison between patients with CDI and non-diarrheal control patients to take possible confounding into account introduced by the matching [29]. The resulting loss of power may have obscured other significant associations. Second, non-CDI diarrheal patients could have falsely tested negative for CDI, due to lack of sensitivity of the applied diagnostic test. However, almost all these patients were repeatedly tested negative and none developed CDI at a later stage. Finally, we could assess mortality only in a limited number of patients and although the observed differences were statistically significant, the confidence intervals were wide. The high 30-day mortality among CDI patients therefore warrants more detailed investigation, specifically aimed at attributable and contributable mortality at longer-term follow-up.

Notes

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Potential conflicts of interest

All authors: No reported conflicts.

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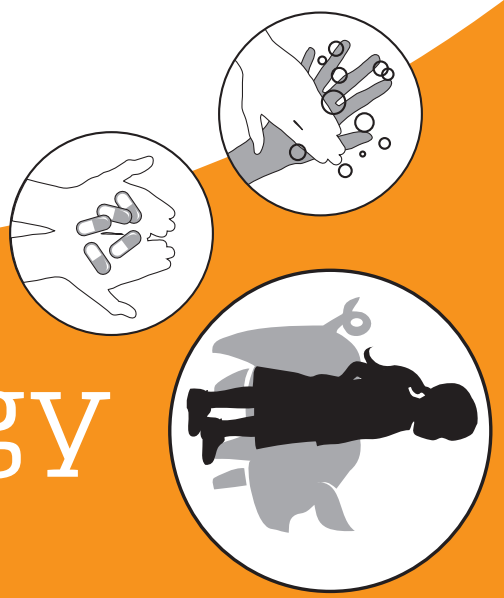

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Chapter 5

Human *Clostridium difficile*-associated disease PCR-ribotype 078 toxinotype V identified in Dutch food-producing swine

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Abstract

In diseased piglets from two Dutch pig-breeding farms with neonatal diarrhoea for more than a year, culture and PCR analyses identified the involved microorganism as *C. difficile* PCR-ribotype 078 harbouring toxin A (tcdA) and B (tcdB), and binary toxin genes. Isolated strains showed a 39 bp deletion in the tcdC gene and they were ermB gene-negative. A number of 11 porcine and 21 human isolated *C. difficile* PCR-ribotype 078 toxinotype V strains were found genetically related by multiple-locus variable-number tandem-repeat analysis (MLVA). Moreover, a clonal complex was identified, containing both porcine and human isolates. The porcine isolates showed an antimicrobial susceptibility profile overlapping that of isolates from Dutch human patients. On the basis of these pheno- and genotypical analyses results, it was concluded that the strains from affected piglets were indistinguishable from increasingly encountered *C. difficile* PCR-ribotype 078 strains of human *C. difficile* infections in the Dutch population and that a common origin of animal and humans strains should be considered.

Introduction

Recent reports suggest an increase in occurrence and severity of human *Clostridium difficile* infections [1]. These changes in epidemiological and clinical presentation can to a certain extent be explained by the emergence of epidemic hypervirulent *C. difficile* strains capable to produce increased amounts of entero-toxins (encoded by the genes *tcdA* and *tcdB)* due to a defect in a toxin-regulating gene, and the presence of a so-called binary toxin [1]. The resulting protein toxins A and B are especially associated with *C. difficile's* pathogenicity [1]. The detection of these toxins is used in screening assays for early diagnosis of *C. difficile* infections (CDI) by virulent *C. difficile* in humans.

The relationship of CDI in humans and animals has been subject of ongoing discussions [2-4]. The disease and the microorganism have been reported in pigs, calves, dogs, horses, ostriches and elephants [5-11]. Early typing comparisons did not identify animals as an important source for human infection. Recent reports, however, showed overlap between *C. difficile* isolates from animals and humans. For example, the increasing proportion of binary toxin-positive strains in the human population may have an animal origin, as such strains have a relative high prevalence among animals, such as horses, piglets and cattle [4]. The role of animals in human CDI was further suggested by the isolation of highly virulent *C. difficile* ribotype 027 from a dog following a hospital visit [2]. In calves, two predominant human outbreak types, 017 and 027, were found [10]. Recently, *C. difficile* ribotype 078 was isolated from pigs and calves in the USA as the most prominent ribotype [3]. The discovery of *C. difficile* in retail meat samples hints a possible transmission route from food to humans [7,12]. So far, CDI has been diagnosed microbiologically only occasionally in animal populations [3].

The major objective of this study was to characterize *C. difficile*-suspected pig isolates pheno- and genotypically to investigate their relatedness with human isolates.

Results and discussion

Escherichia coli and *Clostridium perfringens* are the most common causes of neonatal diarrhoea in piglets [13,14]. Through vaccination of sows by using commercial vaccines that contain pilus antigens F4, F5, F6 and F41, *E. coli*-

induced neonatal diarrhoea can be effectively prevented. The Dutch available vaccines against *C. perfringens* type C, the most common cause of clostridial diarrhoea in piglets, only contain the toxoid of β1 toxin. As these vaccines are not effective against prevailing β2 toxin-containing strains, clostridial diarrhoea may occur in neonates despite vaccination [14]. This explains why preventive treatment of newborn piglets with amoxicillin is practiced.

Diarrhoeal piglets from two herds with a long history of neonatal diarrhoea, despite preventive use of amoxicillin, were examined pathomorphologically. The piglets showed exudative fibrino-haemorrhagic colitis, but no necrotic lesions in the mucosa of the small intestine characteristic for *C. perfringens* type C [13] were present. All affected piglets excreted the same characteristic yellow to orange and pasty to watery diarrhoea reported for *porcine* neonatal CDI [13,15]. As no pathogenic *C. perfringens* was isolated from the autopsied piglets, cases were suggestive for neonatal CDI.

The detection of *C. difficile*-specific toxins A and B is usually the primary CDI diagnostic test. Detection of these toxins through their cytotoxicity for Chinese hamster ovary (CHO) cells is the reference method. Recently, the applicability of two alternative methods for detection of porcine CDI was investigated [16]. These enzyme immunoassays (EIA) were developed originally for the diagnosis of human CDI and were tested with 115 samples from neonatal pigs with the CHO cell cytotoxicity test as the gold standard. A sensitivity of 91% and 39%, and a specificity of 86% and 100% were obtained for these Tox A/B™ (Techlab) and Gastro-tect *C. difficile* Toxin A + B (Medical Chemical Corporation) assays respectively [16].

Here, a commercially available one-strip test [Immuno-Card toxin A and B (ICTAB)] developed for the screening of human patients was used as an alternative diagnostic assay for porcine samples. In human diagnosis and using the cell-cytotoxicity test as the standard, this assay scored a sensitivity, specificity, positive predictive value, and negative predictive value of 91%, 97%, 70% and 99%, respectively [17].

Pooled piglet faecal samples were ICTAB tested. When at least one sample was positive, the corresponding litter was considered positive. In this way, one out of six litters (Farm 1) and three out of six litters (Farm 2) were found *C. difficile* toxin A- and/or B-positive. In addition, all faecal samples were cultured specifically. Colonies that were considered characteristic for

C. difficile were picked for PCR analysis, which confirmed all ICTAB-positive samples as *tcdA*- and *tcdB*-positive *C. difficile*. Two ICTAB-negative litter samples from Farm 2, however, were culture-positive and were confirmed as *tcdA*- and *tcdB*-positive *C. difficile* strains.

Following a time interval of 9 months, Farms 1 and 2 showed continued problems with diarrhoea, although the animal population had changed considerably, as usual on a pig farm, and antibiotic consumption was more restricted to therapeutic instead of routinely preventive use. Toxin screening (ICTAB) and culturing of 31 faecal samples from 31 affected piglets revealed the chronic character of CDI in pigs and the persistence of the pathogenic microorganism on these farms. PCR analysis confirmed the identity of *C. difficile* ribotype 078.

Using this limited number of 31 samples, relative sensitivity, specificity and accuracy for the ICTAB compared with specific culture were estimated as 83%, 68% and 74%, respectively. Accordingly, in a human epidemic CDI situation, not all sampled patients showed a positive ICTAB test in a first sample as well [18]. Here, the farm can be considered as a single entity. Farms harbour relatively homogenetic animal populations. The contact structure between environment and other animals is relatively simple and all pigs receive identical care, including veterinary drug treatment. So, despite the relatively low accuracy, rapid toxin testing of multiple samples from a swine population with diarrhoea during an epidemic situation in a single farm can predict an involvement of *C. difficile* as the causing agent.

The spore-forming bacterium was determined by culture only in a part of the sampled litters, whereas all sampled piglets presented identical symptoms. It is therefore challenging to accept that no *C. difficile* was involved in the negative-cultured diarrhoeal piglets, as other causative agents were excluded carefully by specific examinations (results not shown). The outcome of apparently negative litters following specific culturing may be explained by, *e.g.* the overgrowing of *C. difficile* in the culture. Some studies included a time-consuming bacterial enrichment step before culturing of *C. difficile* [3,10]. Despite this enrichment, *C. difficile*-positive samples were missed in these studies as well [10].

To assess the occurrence of this pathogenic microorganism in non-diseased animals, 272 healthy weaned piglets were sampled on seven farms with

no recent history of gastrointestinal diseases. These seven farms were considered to be a reflection of the approximately 800 large pig-breeding facilities in the Netherlands. Faecal samples were screened for the presence of toxins A and B using the ICTAB assay and cultured specifically for *C. difficile*. Suspected isolates were analysed finally by PCR. Despite that none of the samples was found suspected by the ICTAB assay, 12 out of 68 pooled samples indicated growth of Clostridia. None of the picked colonies, however, could be recognized as *C. difficile* by PCR analysis. It is of importance for the understanding of, for example, the possibility of pig-borne CDI in humans that although symptomless *C. difficile* toxin-positive piglets have been reported [15], no *C. difficile* carriers could be identified here among healthy piglets on farms without diarrhoeal problems.

Intriguingly, CDI is exclusively reported in neonatal piglets [15]. Also in this study, none of the mother sows (n = 20) at either of the two affected farms showed diarrhoea. Sampled sows were all ICTAB- and/or culture-negative. Whether this finding reflects symptomless carriership of undetected low levels of (concealed) bacteria among adult pigs or rejection of the pathogen by the animals is hitherto not clear. This phenomenon seems to be in accordance with results of neonatal *C. difficile* carriers and absence of maternal carriers in the human population [19]. It must be noted that this study did not include small farms. In general, management structures, hygiene standards, protocols and measures are different on these farms and may give more or better possibilities for the bacterium to colonize and/or survive in these animal housings.

PCR analysis of the piglet-derived suspected isolates revealed the occurrence of *C. difficile* ribotype 078 on both sampled problem farms. PCR-ribotype 078 was also described in calves in Canada with a 39 bp deletion in the tcdC gene [10] and in calves in the USA [3] accounting for 23% and 94%, respectively, of all typed *C. difficile* isolates. This ribotype has also been detected in 83% of the swine isolates in the USA [3]. Recently, the first finding of a *C. difficile* ribotype 078 toxinotype V in pigs on the European continent in Slovenia has been presented [20]. Our isolates were also typed as toxinotype V.

Further inspection showed a 39 bp deletion in the *tcdC* gene, which has not been reported earlier in swine, in all isolates. In addition, the cells harboured toxin A and B, and were binary toxin gene-positive but *ermB* gene-negative. It must be noted that these apparently identical strains were

isolated from distant and independent farms. Of high and particular interest for the outcome of this study is the isolation of *C. difficile* PCR-ribotype 078 toxinotype V from Dutch hospitalized patients showing identical characteristics with our animal isolates [21]. The human isolates also contained *tcdA*, *tcdB* and binary toxin-positive genes and a 39 bp deletion in the *tcdC* gene. To substantiate this match of the *porcine* with human ribotype 078 isolates, colonies of 11 isolates were analysed by multiple-locus variable-number tandem-repeat analysis (MLVA) and results were compared with 21 Dutch patient isolates. Figure 1 shows the minimum spanning tree (MST) analysis of these 32 isolates. All were genetically related [summed tandem repeat difference (STRD) < 10] and four clonal complexes (CC) with an STRD < 2 were recognized (boxed CC-A to CC-D). Of these, CC-A contained both human (n = 4) and *porcine* (n = 4) isolates. Two *porcine* isolates in CC-A were 100% homologous to one and two human isolates respectively.

Table 1. Antimicrobial drug resistance (MIC in mg/ml) of *C. difficile* isolated from diseased piglets determined by E-test on Mueller-Hinton agars after 48 h incubation[a].

C. difficile infected litter (No. of isolates tested)	Ciprofloxacin	Clindamycin	Erythromycin	Metronidazol	Moxifloxacin	Penicillin	Vancomycin
Farm 1 (1)	> 32	0.5	> 32	0.02	0.38	1.5	0.38
Farm 2 (4)	> 32	2.0	> 32	0.06	0.5	1.5	0.38

a. Breakpoints were as described in *Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria* (Clinical and Laboratory Standards Institute, 2007).

The antimicrobial susceptibility of the isolated porcine strains (Table 1) towards metronidazole and vancomycin are consistent with the results in human strains. Here, in accordance with human strains, the isolated *porcine C. difficile* PCR-ribotype 078 strains were resistant towards ciprofloxacin. The susceptibility patterns to erythromycin, clindamycin and moxifloxacin were comparable between human and animal strains.

A *C. difficile* strain identical to that isolated here from Dutch pigs has attributed to the death of a patient in the Netherlands [22]. Such findings raise the question whether *C. difficile* strains are exchanged between species, including humans.

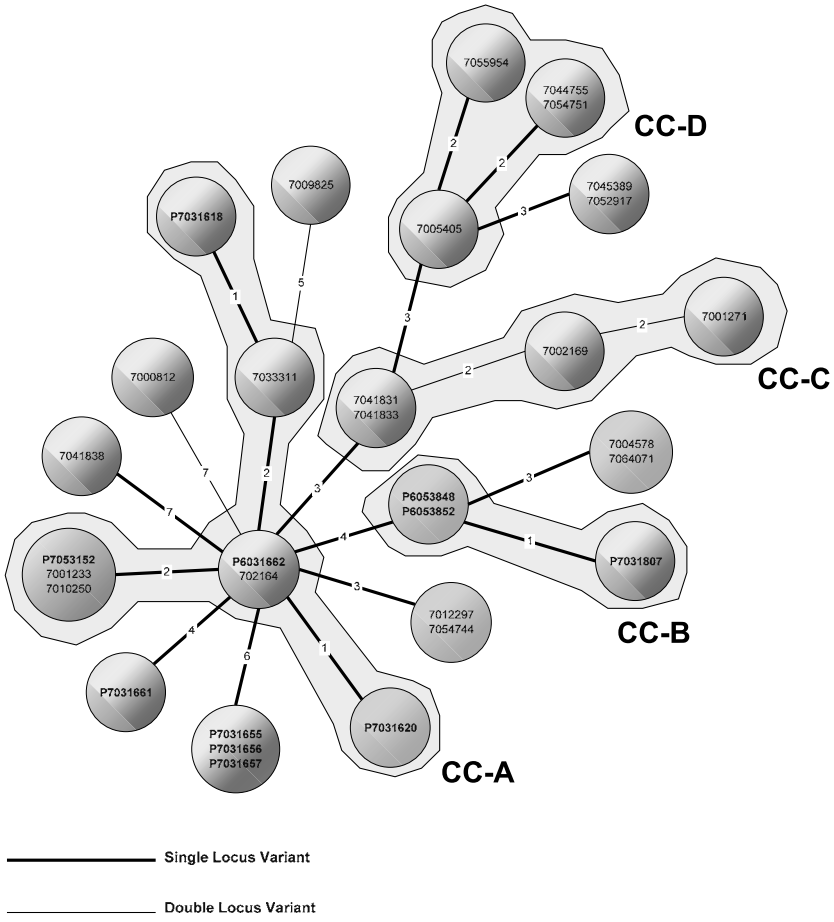

Figure 1. Minimum spanning tree (MST) analysis of 32 *Clostridium difficile* PCR-ribotype 078 strains: 11 *porcine* and 21 human isolates. *Porcine* isolates are printed bold with the first letter P. Each circle represents either a unique isolate or more isolates that are 100% homologous. The numbers between the circles represent the summed tandem repeat difference (STRD) between MLVA types. Thick lines represent single-locus variants, and thin lines represent double-locus variants. Within the spanning tree, four boxed clonal complexes (CC-A to CC-D) with an STRD < 2 are depicted.

Knowledge is greatly lacking worldwide with respect to the reservoir of *C. difficile*. The contribution of swine-born *C. difficile* PCR-ribotype 078 to community-acquired *C. difficile* diseases is not clear. At the *C. difficile*-infected pig farms in this study, none of the farm-workers and none of the family members of the farm-owner replied to have suffered from any recent gastrointestinal tract problem. It is difficult to assess the meaning of this observation with respect to the potentiality of *C. difficile* as a newly recognized zoonotic agent. Infections with *C. difficile* only affect certain patients at risk, such as elderly patients with an underlying disease who recently used antibiotics. The interviewed persons have no underlying disease and may have acquired an immune status protecting them from *C. difficile*-associated disease. Furthermore, hygiene standards at a modern pig farm are relatively high, *i.e.* environmental exchange of organisms is limited and the farmer is obliged to shower and clean clothes when entering and leaving the animal housing. This might have prevented transmission among animal caretakers and their family.

Conclusion

Clostridium difficile PCR-ribotype 078 is increasingly found as a human pathogen in nosocomial and, in particular, community-associated disease [23,24]. In fact, PCR-ribotype 078 was the third common isolated type in 2005 in the Netherlands [22]. Here, *C. difficile* strains have been isolated from diseased Dutch food-producing pigs, which were indistinguishable to those isolated from Dutch patients in terms of genetic identity, toxin production and antimicrobial susceptibility.

The Netherlands is relatively highly populated with humans and animals. It is therefore of eminent public health importance to gain epidemiological insight in the onset and possible transmission patterns of hypervirulent CDI from symptomless or diseased animals in large as well as in small herds to humans through direct contact, food or through the environment, as a zoonotic disease. On the other hand, we are not informed on the occurrence of ribotype 078 in other animals, in the environment and in the (animal) food chain. Our data could merely indicate that the pig strains and human strains may have derived from a common source. The transmission between humans and animals (anthropozoonosis) or from humans to animals only (reversed zoonosis), however, is also an intriguing possibility to investigate.

Experimental procedures

Farms and animals

Neonatal piglets (n = 48), 1-4 days of age, suffering from diarrhoea were sampled in two herds of 240 (Farm 1) and 520 (Farm 2) sows respectively. The yellowish to orange diarrhoea varied from pasty, slimy to watery. The live births ratio was 12.2 piglets per sow at Farm 1, which delivered 10.7 weaned piglets per sow. At Farm 2, these numbers were 13.0 and 11.5 respectively. The incidence of diarrhoea was high among litters (50-90%) and within litters (> 90%). The piglets were stained yellow to orange by their diarrhoea, whereas the mother sows showed no diarrhoeal problems. The sow herds had a long history of neonatal diarrhoea that was caused by *C. perfringens* type A (α and β2), whereas *C. perfringens* type C (α and β), *E. coli*, *Isospora suis*, rotavirus were excluded as causal organisms. Commercial vaccines, used in sows to prevent *C. perfringens* type C diarrhoea in their offspring, proved to be ineffective in preventing neonatal diarrhoea. Periparturient medication of sows with trimethoprim-sulfadiazine (600 mg of trimethoprim and 3 g of sodium sulfadiazine for 8 days) and consecutive preventive treatment of all neonatal piglets with amoxicillin (50 mg of amoxicillin trihydrate) at the first day of life did not prevent the neonatal diarrhoea problem and the veterinary faculty was consulted. The diarrhoeal disease was characterized by a high morbidity (up to 80% of all born piglets) but low overall mortality (approximately 12%, which is within the range of non-problem herds). Growth rates of the piglets during suckling period were severely affected by the neonatal diarrhoea. Dissection of acute diseased piglets (n = 6) from both herds revealed enteritis of the large intestine (colitis), but no enteritis of the jejunum or ileum. All piglets had mesocolonic oedema and an exudative colitis with fibrino-haemorrhagic exudate. The colon content varied from pasty orange stained faeces to watery stool. Examination of the native content, revealed polymorphonuclear cells and high numbers of Gram-positive spore-forming rods.

In addition, 272 piglets from seven large Dutch pig farms were sampled as controls. These seven farms, which are members of the European Pig Producers Association, are spread over the provinces Flevoland, Noord-Brabant and Gelderland (central-east and south-east regions of the

Netherlands). The selected farms reflected together over 10,000 sows and produced together approximately 300,000 piglets annually. At these farms, the averaged live births ratio was 12.4 piglets and 10.9 weaned piglets per sow respectively. They represent the approximately 800 relative large pig farms in the Netherlands. These farms have implemented formal working methods in addition to IKB (Integrated Chain Control) quality assurance system, including standard operating procedures for drug treatment of pigs.

Sampling

Diarrhoeal animals. At the problem farms, faecal samples were taken from 1- to 4-day-old piglets with diarrhoea. All piglets in a litter with diarrhoea were sampled as well as the sow. Faecal samples were taken using cotton swabs from the rectum of the piglets or by gently pressing their abdomen. Faeces of two piglets were combined to a single sample, and in the case that an insufficient amount of faeces was harvested, samples of up to four piglets were pooled. Six litters were sampled at each farm and from each litter at least two pooled samples were analysed. Rectal samples were also taken from the sow of each sampled litter. The farms were re-visited 9 months later, and 31 affected piglets were sampled from which a satisfactory amount of faeces could be collected. These samples were not pooled but processed individually.

Healthy animals. From seven farms, 68 pooled faecal samples representing 272 piglets were collected from apparently healthy 4- to 5-week-old weaned piglets. Faecal samples were collected in vials following the stimulation of defecation by massaging the inside of the rectum. Gloves were changed to avoid cross-contamination of collected samples between pens. Four pigs of each pen with 12-14 pigs were sampled and pooled. Samples were stored between 2°C and 8°C and processed for ImmunoCard toxins A and B analysis and culturing the next day.

Detection of *C. difficile* toxins A and B in faecal samples

ICTAB (Meridian, Boxtel, the Netherlands) was used for the detection of *C. difficile* toxins in porcine faecal samples. The ICTAB is an immunoaffinity assay based on a so-called one-strip test or lateral flow device for the

detection of *C. difficile* toxins A and B. Hitherto it was applied exclusively for early diagnosis of human CDI. The test was performed according to the instructions of the manufacturer using pig faeces.

Culturing and identification of *C. difficile*

Collected samples were all cultured for presence of *C. difficile* using selective agar supplemented with cefoxitine, amphotericine B and cycloserine (CLO-medium, Biomérieux), with and without ethanol shock pre-treatment as described [25,26]. After incubation in an anaerobic environment at 37°C for 48 h, colonies of Gram-positive rods with sub-terminal spores were tested for the production of L-proline-aminopeptidase and for the hydrolysis of esculine [26].

PCR analyses

Colonies were picked and examined genetically using an in-house PCR method to determine the presence of the *gluD* gene encoding glutamate dehydrogenase specific for *C. difficile* [22,27]. The PCR-confirmed *C. difficile* clones were then PCR-ribotyped [28] and toxinotyped [29]. The presence of *ermB, tcdA, tcdB,* and binary toxin genes was investigated according to standardized techniques [30-33]. Deletions in *tcdC* were determined by PCR using in-house designed primers [22,25].

Multiple-locus variable-number tandem-repeat analysis (MLVA)

Molecular genotyping by MLVA was performed on a random selection of ribotype 078 strains as described previously [34], with a minor modification: a new reverse primer was used for marker CdG8 reflecting 5'-ACCAAAAATTTCTAACCCAAC-3'. Minimum spanning tree analysis of MLVA types was performed to determine the genetic distance between isolates, using the number of differing loci and the STRD as coefficients for the genetic distance in the BioNumerics software program (version 4.6, Applied Maths, Belgium) [34-36]. Isolates with an STRD < 10 were defined as genetically related. Clonal complexes were defined by an STRD < 2 [35,36].

Antimicrobial susceptibility testing

Minimal inhibitory concentrations (MIC) for ciprofloxacin, clindamycin, erythromycin, metronidazole, moxifloxacin, penicillin and vancomycin

were determined by using the E-test method (AB Biodisk, Solna, Sweden). A suspension of *C. difficile* colonies was placed on Mueller-Hinton blood agar plates for an 48 h incubation in an anaerobic environment at 37°C as described [37].

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Treatment

6

Chapter 6

Antimicrobial Activity of LFF571 and three treatment agents against *Clostridium difficile* isolates collected at a pan-European survey in 2008. Clinical and Therapeutic Implications

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Abstract

Objectives. In November 2008, a study was performed with support from the European Centre for Disease Prevention and Control (ECDC) to obtain an overview of CDI in European hospitals. A collection of 398 *C. difficile* isolates obtained from this hospital-based survey was utilized to identify antimicrobial susceptibility patterns of common *C. difficile* PCR-ribotypes across Europe.

Methods. The MICs of three approved therapeutic agents (vancomycin, metronidazole and fidaxomicin) and LFF571 (a novel semi-synthetic thiopeptide antibiotic) were determined by the agar dilution method.

Results. MICs of fidaxomicin and LFF571 were in general 2–4-fold lower than those of vancomycin and metronidazole. Isolates belonging to clade 2, including the hypervirulent ribotype 027, had one-dilution higher MIC50 and MIC90 values for fidaxomicin and metronidazole, whereas similar MIC values were observed for vancomycin and LFF571. Isolates belonging to *C. difficile* PCR-ribotype 001 were more susceptible to fidaxomicin than other frequently found PCR-ribotypes 014/020 and 078. Six isolates from three different countries had a metronidazole MIC of 2 mg/L. Four of the six isolates were characterized as PCR-ribotype 001.

Conclusions. There was no evidence of in vitro resistance of *C. difficile* to any of the four agents tested. However, the results suggest type-specific differences in susceptibility for the treatment agents we investigated. Continuous surveillance of *C. difficile* isolates in Europe is needed to determine the possible clinical implications of ribotype-specific changes in susceptibility to therapeutic agents.

Introduction

Clostridium difficile infection (CDI) is the primary cause of antibiotic-associated diarrhoea and a prevalent disease in healthcare facilities in many European countries. In recent years an increase in CDI has been reported, partly due to the spread of one specific ribotype: PCR-ribotype 027 [1-4]. Another emerging strain of *C. difficile* in Europe and the USA is PCR-ribotype 078, which has been associated with both food animals and humans [5-9]. Clinical manifestations of CDI range from asymptomatic carriage to severe diarrhoea and pseudomembranous colitis with toxic megacolon. The antibiotics used to treat CDI are usually vancomycin or metronidazole. Metronidazole is currently the drug of first choice for mild infections, whereas vancomycin is preferred for the treatment of severe infections [10-16]. Alternative antibiotic agents have been introduced in the USA and Europe for the treatment of CDI [17-19]. Recently fidaxomicin, a new macrocyclic antibiotic, was approved in Europe for the treatment of adults with CDI. Fidaxomicin was shown to have similar efficacy in the initial cure of CDI compared with oral vancomycin [19-21]. However, recurrence of CDI, due to strains other than PCR-ribotype 027, was significantly less frequent in fidaxomicin-treated patients. Data on the use of fidaxomicin compared with guideline-recommended therapies for mild to moderate and life-threatening CDI are not yet available. Although changes in antibiotic resistance and ribotype prevalence have been reported, *in vitro* studies indicate that MICs of metronidazole and vancomycin for endemic *C. difficile* have remained relatively low over the years [22-26]. There have only been occasional reports of resistance to metronidazole [27,28]. Brazier *et al.* [23] concluded that the MICs of metronidazole and vancomycin were not indicative of clinical resistance, but MICs for epidemic ribotypes (027, 106 and 001) were several dilutions higher. Almost a quarter of recent as opposed to historical *C. difficile* ribotype 001 isolates causing CDI were found to have reduced susceptibility to the metronidazole MIC in one UK centre [29]. While decreased clinical effectiveness of metronidazole treatment for specific ribotypes causing CDI has been described [15,30], there are no published reports in which treatment failure has been linked to antimicrobial metronidazole resistance in *C. difficile* [31].

In November 2008 a pan-European period prevalence surveillance study of CDI was performed with support from the European Centre for Disease Prevention and Control (ECDC) [7]. A unique network of 106 laboratories in

34 European countries was established. Given the potential implications of antibiotic resistance for CDI therapy, we have examined the susceptibility of *C. difficile* isolates from this study. Three antibiotic agents used for the treatment of CDI and a novel investigational agent (LFF571, Novartis) were tested against 398 clinical *C. difficile* isolates and appropriate control strains [32]. LFF571 is novel semisynthetic thiopeptide antibiotic, which has been shown to possess potent *in vitro* and *in vivo* activity against *C. difficile* [33,34]. Though LFF571 has no human clinical history, other thiopeptides have been shown to induce single-site mutations of the ribosomal 23S rRNA binding site region, directly affecting thiopeptide affinity with reduced susceptibility [35]. In addition, clinical outcomes of therapy were evaluated in cases from whom isolates were recovered with higher vancomycin and metronidazole MICs. All isolates were further characterized by multilocus sequence typing (MLST), PCR ribotyping and the presence of genes encoding toxin A, toxin B and binary toxin [36-40]. The antibiotic susceptibility profiles were analysed according to ribotype, MLST clade and country of origin.

Materials and methods

C. difficile isolates and characterization of ribotypes and sequence types

In the European *Clostridium difficile* infection study (ECDIS), isolates were collected from 73 hospitals in 26 countries during November 2008 [7]. Of the 404 isolates collected, 398 were available for characterization and antibiotic susceptibility testing in this study. Identification of *C. difficile* was confirmed by an in-house PCR test for the glutamate dehydrogenase gene specific to *C. difficile* [38]. Isolates were further characterized by PCR ribotyping [36]. The presence of toxin A, toxin B and binary toxin genes was investigated by PCR as described elsewhere [36-39].

In addition, *C. difficile* strains were characterized by MLST. Clades were established by MLST using seven housekeeping genes [40,41]. Clade 2 encompasses *C. difficile* PCR-ribotype 027 and closely related PCR-ribotypes, including 016, 036 and 176, all of them belonging to sequence type (ST) 1. Clade 5 contains *C. difficile* PCR-ribotypes 078 and closely related types, such as 033, 045, 066, 126 and 193, all belonging to ST11.

Antibiotics and MIC determinations

Stock solutions of 12.8 mg/mL were prepared for fidaxomicin (Novartis, Switzerland), LFF571 (Novartis, Switzerland), vancomycin (AppliChem, Germany) and metronidazole (Sigma-Aldrich, Germany). Antibiotics were dissolved in DMSO and stored at -20°C. All antibiotic stock solutions were sterilized by filtration through 0.22 μm filters. For preparation of agar plates, the stock solution was diluted in distilled water (fidaxomicin, vancomycin and metronidazole) or in 0.01 M phosphate-buffered saline (PBS), pH 7.5 (LFF571). MICs were determined using the agar dilution method according to CLSI guidelines [42]. Doubling dilutions of antibiotics (0.06-8 mg/L) were made in Brucella Blood Agar (Becton and Dickinson, France) supplemented with haemin and vitamin K1.

Bacterial isolates were cultured on sheep blood agar plates and after 24 h suspended to a concentration equivalent to that of a 0.5 McFarland standard in PBS. The strains were inoculated onto solid medium using multipoint inoculators to a final concentration of 104 cfu per spot. *Bacteroides fragilis* ATCC 25285, *C. difficile* ATCC 700057 and *Clostridium glycolicum* were used as quality controls. Plates were incubated in an anaerobic cabinet (Don Whitley, UK) and after 48 h plates were read. The MIC endpoints were taken as the concentrations at which marked reductions in growth occurred on the test compared with control plates after 48 h. The MIC50 and MIC90 were defined as the antibiotic concentrations at which 50% and 90%, respectively, of the tested strains were susceptible. The MIC50 and MIC90 from the most frequently found PCR-ribotypes and clades in Europe were compared with other ribotypes and clades. Additionally, geographical differences in MIC50 and MIC90 values between European countries were investigated.

Results

In total, 398 *C. difficile* clinical isolates were investigated in the study. Of the original 404 isolates, five were contaminated with other bacteria and were excluded from the study. The MIC results are summarized in Figure 1 and Table 1.

All 398 strains included in this study were previously characterized by PCR ribotyping. The three most frequently found PCR-ribotypes were 001 014/020 and 078 [7]. The MIC50s and MIC90s for ribotypes 001, 014/020 and 078 are shown in Table 2 in comparison with those for ribotype 027 and the remaining ribotypes. Out of 398 isolates, six from three different countries (Germany, Greece and UK) had a metronidazole MIC of 2 mg/L. Four of the six isolates were characterized as PCR-ribotype 001 and the remaining isolates as ribotypes 002 and 078.

Table 1. MIC_{50}s, MIC_{90}s and MIC ranges of the four antibiotics tested against 398 *C. difficile* isolates.

C. difficile infected litter (No. of isolates tested)	Ciprofloxacin	Clindamycin	Erythromycin	Metronidazol	Moxifloxacin	Penicillin	Vancomycin
Farm 1 (1)	> 32	0.5	> 32	0.02	0.38	1.5	0.38
Farm 2 (4)	> 32	2.0	> 32	0.06	0.5	1.5	0.38

a. Breakpoints were as described in *Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria* (Clinical and Laboratory Standards Institute, 2007).

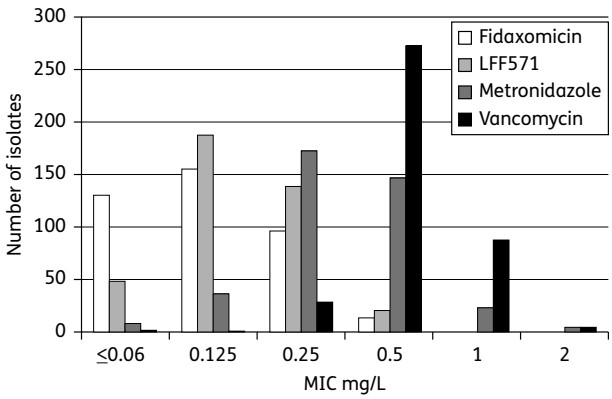

Figure 1. Overall distribution of the MICs (mg/L) of four antibiotics tested against 398 *C. difficile* isolates.

Out of 398 isolates, six from three different countries (Germany, Greece and UK) had a metronidazole MIC of 2 mg/L. Four of the six isolates were characterized as PCR-ribotype 001 and the remaining isolates as ribotypes 002 and 078.

Strains were characterized by MLST: six different clades were identified. The most frequently isolated clades in Europe include 1 (62.3% of isolates), 2 (6.5% of isolates) and 5 (12.3% of isolates). Fifty-seven percent of the isolates in clade 2 were characterized as ribotype 027. Other clades included clades 3, 4 and 6 (6.3%). Fifty of 398 (12.6%) strains were not typeable. In Table 3 the MIC50s and MIC90s according to clades are shown.

Table 2. MIC_{50}s, MIC_{90}s and MIC ranges for the three most frequently found *C. difficile* ribotypes in comparison with those of ribotype 027 and the remaining ribotypes ('other').

PCR ribotype	MIC_{50} (mg/L)	MIC_{90} (mg/L)	MIC range (mg/L)	Number of isolates
014/020				
metronidazole	0.25	0.5	≤0.06–1	63
vancomycin	0.5	1	0.25–1	63
fidaxomicin	0.125	0.25	≤0.06–0.5	63
LFF571	0.125	0.25	≤0.06–0.5	63
001				
metronidazole	0.25	0.5	0.06–2	40
vancomycin	0.5	1	0.25–2	40
fidaxomicin	≤0.06	0.125	≤0.06–0.25	40
LFF571	0.125	0.25	≤0.06–0.5	40
078				
metronidazole	0.25	0.5	0.125–2	32
vancomycin	0.5	1	0.5–1	32
fidaxomicin	0.125	0.25	≤0.06–0.5	32
LFF571	0.125	0.25	≤0.06–0.5	32
027				
metronidazole	0.5	1	0.5–1	18
vancomycin	0.5	0.5	0.125–1	18
fidaxomicin	0.25	0.5	≤0.06–0.5	18
LFF571	0.125	0.25	≤0.06–0.5	18
Other				
metronidazole	0.25	0.5	≤0.06–2	245
vancomycin	0.5	1	≤0.06–2	245
fidaxomicin	0.125	0.25	≤0.06–0.5	245
LFF571	0.125	0.25	≤0.06–0.5	245

When using CLSI breakpoints, no resistance to metronidazole was detected. The clinical outcome of all six patients with a metronidazole MIC of 2 mg/L was evaluated. All six CDIs were healthcare associated. Five patients were treated with oral metronidazole and in one patient the treatment was unknown. CDI complications were defined as CDI that contributed to or caused intensive care unit (ICU) admission or death, or led to colectomy. CDI complications were not reported in any of these six patients. One of the patients (PCR-ribotype 078) had recurrent CDI after initial treatment with metronidazole. The distribution of MIC90s in European countries with >10 evaluable isolates is shown in Figure 2.

Table 3. MIC_{50}s, MIC_{90}s and MIC ranges according to *C. difficile* clades[a]

Clade	MIC_{50} (mg/L)	MIC_{90} (mg/L)	MIC range (mg/L)	Number of isolates
1				
metronidazole	0.5	0.5	≤0.06–2	248
vancomycin	0.5	1	≤0.06–2	248
fidaxomicin	0.125	0.25	≤0.06–0.5	248
LFF571	0.125	0.25	≤0.06–0.5	248
2				
metronidazole	0.5	1	0.25–1	26
vancomycin	0.5	1	0.125–1	26
fidaxomicin	0.25	0.5	≤0.06–0.5	26
LFF571	0.25	0.25	≤0.06–0.5	26
5				
metronidazole	0.25	0.5	≤0.06–2	49
vancomycin	0.5	1	0.5–2	49
fidaxomicin	0.125	0.125	≤0.06–0.5	49
LFF571	0.125	0.25	≤0.06–0.5	49
Other				
metronidazole	0.125	0.5	≤0.06–0.5	25
vancomycin	0.5	1	0.25–1	25
fidaxomicin	0.125	0.25	≤0.06–0.25	25
LFF571	0.125	0.25	≤0.06–0.5	25
Indeterminate				
metronidazole	0.25	0.5	0.125–1	50
vancomycin	0.5	1	0.25–2	50
fidaxomicin	0.125	0.25	≤0.06–0.5	50
LFF571	0.25	0.25	≤0.06–0.5	50

[a]Presence of various *C. difficile* PCR ribotypes in the different clades. Clade 2: 016, 019, 027, 075 and 208. Clade 5: 033, 045, 078 and 126. Clade 1: 001, 002, 003, 005, 009, 010, 011, 012, 014, 015, 018, 025, 026, 029, 031, 037, 050, 051, 053, 056, 057, 064, 070, 081, 084, 087, 106 and 118. Other (clades 3, 4 and 6): 017, 023 and 131. Indeterminate: 013, 024, 039, 046, 063, 090, 093, 097, 101, 107, 110, 137, 139, 150, 154, 159, 161, 176, 202, 205, 207, 228, 229, 230, 231, 232 and 234.

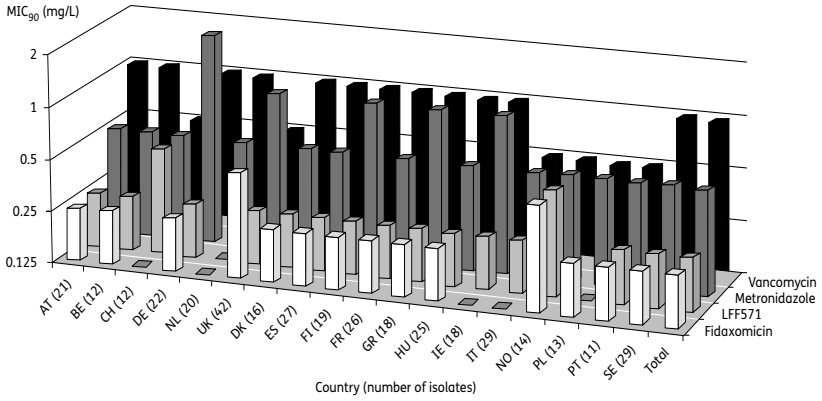

Figure 2. MICs (mg/L) of four antibiotics for *C. difficile* isolates from European countries with >10 isolates. AT, Austria; BE, Belgium; CH, Switzerland; DE, Germany; NL, Netherlands; UK, United Kingdom; DK, Denmark; ES, Spain; FI, Finland; FR, France; GR, Greece; HU, Hungary; IE, Ireland; IT, Italy; NO, Norway; PL, Poland; PT, Portugal; SE, Sweden.

Discussion

In accordance with previous studies, all 398 clinical *C. difficile* isolates collected in 2008 from 28 different European countries showed no *in vitro* resistance to metronidazole according to CLSI breakpoints [43]. Fidaxomicin and LFF571 (MIC range <0.06-0.5 mg/L) were in general 2-4-fold more potent than vancomycin and metronidazole [19,21,33,44]. All isolates were highly susceptible to fidaxomicin and LFF571, including the six isolates with a metronidazole MIC of 2 mg/L. Of 398 isolates 130 (32.7%) had a fidaxomicin MIC <0.06 mg/L, whereas 49 (12.3%) had a LFF571 MIC <0.06 mg/L. However, the MIC50 (0.125 mg/L) and MIC90 (0.25 mg/L) for these newer agents were identical.

Vancomycin and metronidazole MICs ranged from <0.06 to 2 mg/L, although the modal MICs were 0.5 and 0.25 mg/L, respectively. Notably, however, six isolates from three different countries had a metronidazole MIC of 2 mg/L. Four of these six isolates were characterized as PCR-ribotype 001 and were obtained from three different hospitals (and regions) in Germany. In general, PCR-ribotype 001 predominated in Germany (10/22 isolates) in this study. The number of isolates is too small to draw conclusions, although higher

MICs of metronidazole for PCR-ribotype 001 and other common ribotypes have indeed been described before [23,29]. One of the factors that may play a role in the development of antimicrobial resistance to metronidazole is prolonged antibiotic exposure of the most common *C. difficile* clones followed by selection in healthcare facilities. In a study by Zaiß *et al.* [45] in Germany in 2008, PCR-ribotype 001 was the most prevalent and widespread ribotype in German hospitals, but they found no significant differences in the mean MICs of metronidazole for the common ribotypes 001, 078 and 027 compared with other ribotypes. However, metronidazole MICs were determined by E-test, which may be a less reliable method for the detection of reduced susceptibility to metronidazole [46].

In the six patients with a metronidazole MIC of 2 mg/L we evaluated, there was no correlation between the elevated MIC and clinical outcome. Five patients were treated with metronidazole and none of them developed CDI complications. One of these patients developed recurrent CDI within 3 months after the primary infection. In theory, given the gut pharmacokinetic profile of metronidazole in humans, a higher MIC of metronidazole could have implications in clinical cure or recurrences of CDI due to the poor penetration of metronidazole into the colon [15]. Mean antibiotic concentrations reported in faeces of patients receiving oral metronidazole range from <0.25 to 9.5 mg/L, and drug concentrations decrease as diarrhoea resolves [47-49]. In an *in vitro* gut model that simulates CDI, metronidazole was instilled into the system at a dosage that was calculated to achieve concentrations equivalent to the published faecal concentrations. Interestingly, the metronidazole concentrations measured by bioassay were markedly lower than expected, which may be due to inhibition or inactivation, *e.g.* by enterococci in the gut [50-52]. Thus, the modest penetration of metronidazole into the lower gastrointestinal tract may be further compromised by drug inactivation, which increases the chance that CDIs due to strains displaying increased MICs of metronidazole will not be effectively treated using this antibiotic.

There are, however, no published reports in which CDI treatment failure has been linked to metronidazole resistance in *C. difficile*. In a retrospective study, clinical outcome data were compared for 19 CDI cases due to *C. difficile* ribotype 001 strains having reduced susceptibility to metronidazole (MICs >4 mg/L) and 19 control CDI cases (metronidazole MICs <0.5-2mg/L for ribotype 001 strains), of whom 14 and 13, respectively, were treated with metronidazole (median ages 81 and 80, respectively) [53]. Notably, patients

were typically frail and elderly with very poor outcome (21% mortality rate by day 30). Response to metronidazole was generally slow and in all patients it was prone to recurrence (16% of cases and 26% of controls). However, using the endpoints for failure to resolve (need for vancomycin therapy), such as the number of days to resolution of diarrhoea, death by day 30 and recurrence, no difference was seen between the two groups of CDI cases. Much larger study groups would be needed, ideally with less frail patients, to determine the true clinical significance of *C. difficile* strains with reduced susceptibility to metronidazole. There are clear logistical and ethical issues in carrying out such a study prospectively, including the lack of real-time availability of metronidazole susceptibility results and whether it would be acceptable to randomize individuals to metronidazole treatment if isolates are susceptible *in vitro* but have elevated MICs. Furthermore, it should be emphasized that metronidazole should only be used in mild to moderate CDI, and differences in outcome in such cases might be difficult to elucidate.

As shown in Table 3, *C. difficile* isolates from clade 2 had 2-4-fold higher metronidazole and fidaxomicin MIC90s in comparison with the other clades, whereas there were no clade-to-clade variations in MICs of either vancomycin or LFF571. In the present study, 57% of the isolates in clade 2 were characterized as ribo-type 027. Similar fidaxomicin and metronidazole MICs were indeed observed for ribotype 027 compared with other ribotypes. *C. difficile* ribotype 001 isolates had a 2-fold lower fidaxomicin MIC90 compared with the other frequently found ribotypes 014/020 and 078, and a 4-fold lower fidaxomicin MIC90 compared with ribotype 027. Thirty-five out of 40 (87.5%) ribotype 001 isolates had a fidaxomicin MIC of <0.06 mg/L. This was a statistically significantly higher proportion than for other ribotypes (Student's t-test, $P<0.05$). Ribotype 001 isolates (n = 40/398) were obtained from 13 different European countries. These results suggest type-specific differences in susceptibility for the treatment agents we investigated. Notably, clonal spread of *C. difficile* strains displaying reduced susceptibility to metronidazole or vancomycin has been observed [29,54].

Two- to four-fold higher metronidazole MIC90s were found in isolates originating in Germany, UK, Finland, Greece and Ireland compared with 13 other countries. Although the number of isolates is very small, it should be noted that a metronidazole MIC90 >1 mg/L was recorded in only one of the 18 countries with >10 isolates; *C. difficile* ribotype 001 predominated in Germany. Although we emphasize that the geographical distribution

of ribotypes and MIC50/MIC90 values for *C. difficile* isolates in this study does not represent the national epidemiology of *C. difficile* in Europe due to the small number of participating laboratories per country, the ribotype distribution might be suggestive of regional spread. The epidemic and highly pathogenic ribotype 027 was found in only 4.5% of the isolates and in 6/28 countries. However, 11/18 (61%) of these isolates originated from the UK, accounting for 26.1% of all UK isolates. In addition, 12 (28.6%) isolates from the UK were characterized as PCR-ribotype 106. PCR-ribotype 027 as well as ribotype 106 have 2-4-fold higher metronidazole MIC90s compared with other ribotypes [23].

We conclude that there was no evidence of *in vitro* resistance of *C. difficile* to any of the four agents tested in 398 European clinical isolates in this study. Vancomycin and metronidazole MICs for the *C. difficile* strains we investigated were generally low. However, metronidazole MICs were 2-fold higher for clade 2 isolates, which include PCR-ribotype 027, compared with other clades and ribotypes, suggesting ribotype-specific differences in antibiotic susceptibility. All strains were highly susceptible to fidaxomicin and LFF571. Continuous surveillance of *C. difficile* isolates in Europe is needed to determine the possible clinical implications of ribotype-specific changes in susceptibility to therapeutic agents.

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Transparency declarations

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Chapter 7

European Society of Clinical Microbiology and Infectious Diseases: update of the treatment guidance document for *Clostridium difficile* infection (CDI)

Debast SB, Bauer MP, Kuijper EJ, on behalf of the Committee Clinical Microbiology and Infection. 2014. *Accepted for publication*.

Abstract

In 2009 the first European Society of Clinical Microbiology and Infection (ESCMID) treatment guidance document for *Clostridium difficile* infection (CDI) was published. The guideline has been applied widely in clinical practice. In this document an up-date and review on the comparative effectiveness of the currently available treatment modalities of CDI is given, thereby providing evidence-based recommendations on this issue. A computerized literature search was carried out to investigate randomized and non-randomized trials investigating the effect of an intervention on the clinical outcome of CDI. The Grades of Recommendation Assessment, Development and Evaluation (GRADE) system was used to grade the strength of our recommendations and the quality of the evidence. The ESCMID and an international team of experts from eleven European countries supported the process.

To improve clinical guidance in the treatment of CDI, recommendations are specified for various patient groups, *e.g.* initial non-severe disease, severe CDI, first recurrence or risk for recurrent disease, multiple recurrences, and treatment of CDI when oral administration is not possible. Results from individual studies, reviews and meta-analyses on prognostic markers for CDI are evaluated in this document to select prognostic markers that may be useful in clinical practice to distinguish patients with increased risk for severe or recurrent CDI. Treatment options that are considered in this guidance document include: oral and non-oral antibiotics, toxin-binding resins and polymers, immunotherapy, probiotics, faecal or bacterial intestinal transplantation. The choice of antibiotics depends mainly on the stage and severity of disease. Except for very mild CDI, that is clearly induced by antibiotic usage, antibiotic treatment is advised. The main antibiotic treatment agents that are recommended in this guideline are: metronidazole, vancomycin and fidaxomicin. A non-antibiotic treatment modality strongly recommended for multiple recurrent CDI is faecal transplantation. In case of perforation of the colon and/or systemic inflammation and deteriorating clinical condition despite antibiotic therapy, total abdominal colectomy or diverting loop ileostomy combined with colonic lavage is recommended.

Introduction

The previous ESCMID guidance document, which has been applied widely in clinical practice, dates from 2009 [1]. Meanwhile, new treatments for CDI have been developed and limitations of the currently recommended treatment options of CDI are considered. As the current ESCMID treatment guidance document is already implemented in clinical practice, an update of this widely applied guidance document is essential to further improve uniformity of national hospital infection treatment policies for CDI in Europe. In particular, after the recent development of new alternative drugs for the treatment of CDI (*e.g.* fidaxomicin) in US and Europe, there has been an increasing need for an update on the comparative effectiveness of the currently available antibiotic agents in the treatment of CDI, thereby providing evidence-based recommendations on this issue.

Therefore the objectives of this document are to:

1) Provide an overview of currently available CDI treatment options
2) Develop an evidence-based update of treatment recommendations

Update methodology

Studies on CDI treatment were found with a computerized literature search of PUBMED and Google Scholar using the terms "*Clostridium difficile* AND (treatment OR trial)". All randomized and non-randomized trials investigating the effect of an intervention on the clinical outcome (resolution or recurrence of diarrhoea; incidence of complications) of CDI published in any language were included. Studies investigating carriage or other purely microbiological parameters were not considered sufficient evidence for treatment strategies. The resulting literature from 1978 was reviewed and analysed. Furthermore, systematic reviews from the most recent Cochrane analysis [2] and the up-dated guidelines of the Infectious Diseases Society of America (IDSA), the Australasian Society for Infectious Diseases, the American College of Gastroenterology, and the HPA/Public Health England guidance document (*http://www.hpa.org.uk*) were evaluated [3-5]. Recommendations were based on a systematic assessment of the quality of evidence. The GRADE system was used to grade the strength of our recommendations and the quality of the evidence [6,7].

Table 1. Definition of the Strength of Recommendation Grade (SoR) ESCMID.

Strength	Definition
A	**Strongly** supports a recommendation for use.
B	**Moderately** supports a recommendation for use.
C	**Marginally** supports a recommendation for use.
D	Supports recommendation **AGAINST** use.*

* *Recommendations* against *use are marked in grey in the Tables*

Draft versions of the guideline were written by the executive committee (consisting of: S. Debast, M. Bauer and E. Kuijper) and criticized by the Executive Committee, advisors and a patient representative. After this, consensus was reached, resulting in the final version. The methods to evaluate the quality of evidence and to reach group consensus recommendations were based on the method described by Ullmann *et al*. [8].

Definition of the strength of recommendation is given in Table 1. The quality of the published evidence is defined in Table 2a. Grouping quality of evidence into three levels only may lead to diverse types of published evidence being assigned specifically a level II. To increase transparency in the evaluation of the evidence an index (Table 2b) to the level II recommendations was added where appropriate.

The guideline followed the Appraisal of Guidelines Research and Evaluation Collaboration (AGREE) self-assessment tool [9].

Table 2a. Definition of the Quality of Evidence (QoE) Level ESCMID.

Quality of Evidence Level	Definition
I	Evidence from at least 1 properly designed randomized, controlled trial.
II	Evidence from at least 1 well-designed clinical trial, without randomization; from cohort or case-controlled analytic studies (preferably from >1centre); from multiple time series; or from dramatic results of uncontrolled experiments.
III	Evidence from opinions of respected authorities, based on clinical experience, descriptive case studies, or reports of expert committees.

Table 2b. Definition of the Quality of Evidence (QoE) Index ESCMID. Adapted from Ref [8].

Quality of Evidence Index	Definition
r	Meta-analysis or systematic review of randomized controlled trials.
t	Transferred evidence *i.e.* results from different patients' cohorts, or similar immune-status situation.
h	Comparator group is a historical control.
u	Uncontrolled trial.
a	Abstract or poster of a study published at an international meeting.

Definitions

Diagnosis

The diagnosis of CDI is based on (1) a combination of signs and symptoms, confirmed by microbiological evidence of *C. difficile* toxin and toxin-producing *C. difficile* in stools, in the absence of another cause, or (2) colonoscopic or histopathologic findings demonstrating pseudomembranous colitis [1,3,10,11,12].

There are many different approaches that can be used in the laboratory diagnosis of *CDI*, however the best standard laboratory test for diagnosis has not been established yet. Diagnostic tests for CDI include: (1) detection of *C. difficile* products: cell culture cytotoxicity assay (CCA), glutamate dehydrogenase (GDH) and Toxins A and/or B, (2) toxigenic culture of *C. difficile*, and (3) nucleic acid amplification tests (NAAT): 16S RNA, toxin genes, GDH genes. Preferably a two- or three-stage algorithm is performed to diagnose CDI, in which a positive first test is confirmed with one or two confirmatory tests or a reference method [3,4,12,13]. Faeces samples could be investigated with an enzyme immunoassay (EIA) detecting GDH, an EIA detecting toxins A and B, or NAAT detecting Toxin B (TcdB). Samples with a negative test result can be reported as negative. Faeces samples with a positive first test result should be re-tested with a method to detect free faeces toxins, or with a method to detect GDH or toxin genes, dependent on the assay applied as first screening test. If free faeces toxins are absent

but *C. difficile* TcdB or GDH is present; CDI cannot be differentiated from asymptomatic colonization. Recently a large study was presented in which several diagnostic algorithms were evaluated to optimise the laboratory diagnosis of CDI [14]. The investigators concluded that two-stage algorithms improve diagnosis of CDI. Two commonly recommended methods in the laboratory diagnosis of CDI are the use of GDH detection in stools as a means of screening for CDI, confirmed by NAAT such as PCR to detect toxigenic strains of *C. difficile* [4]. Furthermore, patients with a positive stool toxin had *C. difficile* disease with an increased risk of mortality as compared to patients with only a positive toxigenic culture, thereby implicating stool toxin testing to be included in a testing algorithm to optimize *C. difficile* diagnostic testing [15].

Diarrhoea is defined as loose stools, *i.e.* taking the shape of the receptacle or corresponding to Bristol stool chart types 5-7, plus a stool frequency of three stools in 24 or fewer consecutive hours or more frequently than is normal for the individual (definition *World Health Organization, http://www.who.int/topics/diarrhoea)* [1, 3, 16-18].

Clinical pictures compatible with CDI are summarized in Table 3.

Table 3. Clinical pictures compatible with CDI [1,3,11,19,20].

Sign/symptom	Definition
Diarrhoea	Loose stools, *i.e.* taking the shape of the receptacle or corresponding to Bristol stool chart types 5-7, plus a stool frequency of three stools in 24 or fewer consecutive hours or more frequently than is normal for the individual.
Ileus	Signs of severely disturbed bowel function such as vomiting and absence of stool with radiological signs of bowel distension.
Toxic megacolon	Radiological signs of distension of the colon (>6 cm in transverse width of colon) and signs of a severe systemic inflammatory response.

Definition of CDI

An episode of CDI is defined as:

- A clinical picture compatible with CDI and microbiological evidence of free toxins and the presence of *C. difficile* in stool without reasonable evidence of another cause of diarrhoea.

or

- pseudomembranous colitis (PMC) as diagnosed during endoscopy, after colectomy or on autopsy [3, 11, 19].

Treatment response

Definition of treatment response

Treatment response is present when either stool frequency decreases or stool consistency improves and parameters of disease severity (clinical, laboratory, radiological) improve and no new signs of severe disease develop. In all other cases, treatment is considered a failure. Treatment response should be daily observed and evaluated after at least three days, assuming that the patient is not worsening on treatment. Treatment with metronidazole, in particular, may result in a clinical response only after three to five days [21-23]. After clinical response, it may take weeks for stool consistency and frequency to become entirely normal [24].

Recurrences

Definition of recurrent CDI

Recurrence is present when, CDI re-occurs within eight weeks after the onset of a previous episode, provided the symptoms from the previous episode resolved after completion of initial treatment [4,11].

It is not feasible to distinguish recurrence due to relapse (renewed symptoms from already present CDI) from recurrence due to reinfection in daily practice [20,25-28].

Table 4. Patient characteristics that could reasonably be assumed to correlate positively with severity of colitis **in the absence of another explanation for these findings.**

Category	Signs/symptoms
Physical examination	- Fever (core body temperature > 38.5ºC). - Rigours (uncontrollable shaking and a feeling of cold followed by a rise in body temperature). - Haemodynamic instability including signs of distributive shock. - Respiratory failure requiring mechanical ventilation. - Signs and symptoms of peritonitis. - Signs and symptoms of colonic ileus. Admixture of blood with stools is rare in CDI and the correlation with severity of disease is uncertain.
Laboratory investigations	- Marked leucocytosis (leukocyte count >15·10⁹/l). - Marked left shift (band neutrophils >20% of leukocytes). - Rise in serum creatinine (>50% above the baseline). - Elevated serum lactate (≥5 mmol/L). - Markedly reduced serum albumin (<30 g/l).
Colonoscopy or sigmoidoscopy	- Pseudomembranous colitis. There is insufficient knowledge on the correlation of endoscopic findings compatible with CDI, such as oedema, erythema, friability and ulceration, and the severity of disease.
Imaging	- Distension of large intestine (> 6 cm in transverse width of colon). - Colonic wall thickening including low-attenuation mural thickening. - Pericolonic fat stranding. - Ascites not explained by other causes. The correlation of haustral or mucosal thickening, including thumbprinting, pseudopolyps and plaques, with severity of disease is unclear.

Severity of disease

Definition of severe CDI

Severe CDI is defined as an episode of CDI with (one or more specific signs and symptoms of) severe colitis or a complicated course of disease, with significant systemic toxin effects and shock, resulting in need for ICU admission, colectomy or death [1,4,29].

CDI without signs of severe colitis in patients with high age (≥65), serious comorbidity, Intensive Care Unit (ICU) admission, or immunodeficiency may also be considered at increased risk of severe CDI [30, 31].

An overview of characteristics in patients with CDI that are assumed to correlate with the severity of colitis is given in Table 4 [32-39]. We must stress that the prognostic value of these markers is uncertain.

Clinical prediction markers

Evidence

Clinical studies indicate superiority of specific treatment strategies depending on the severity of disease. In addition, alternative treatment options have been developed, which may be more effective in preventing recurrences of disease. Unfortunately some of the novel treatment strategies can be very expensive, and may only be cost-effective for a certain group of patients depending on the stage and severity of disease. This emphasizes the importance for better identification of clinical markers, preferably early in the course of disease, which might predict the benefit from specific treatment regimens to decrease CDI related complications, mortality or recurrences. Surprisingly little prospective and validated research has been done on clinical predictors of outcome [40]. Furthermore, for some complications of CDI, such as ICU admission or death, it is difficult to determine to what extent the complication can be attributed to CDI as opposed to the presenting acute illness(es) or comorbidities.

A wide variety of risk factors for severe or recurrent CDI have been suggested in literature, which makes it difficult to set a rigid clinical prediction rule [1, 25, 41-46]. Recently, a systematic review was performed to derive and validate clinical rules to predict recurrences, complications and mortality [46]. A majority of studies was found to have a high risk of bias due to small sample sizes and much heterogeneity in the variables used, except for: leukocytosis, serum albumin and age [46]. Bauer *et al.* used a database of 2 randomized controlled trials, which contained information for a large patient group (1105 patients) with CDI, to investigate the prognostic value of 3 markers for severe CDI. They found both leukocytosis and renal failure are useful predictors of a complicated course of CDI, if measured on the day of diagnosis [45].

A recent meta-analysis of two pivotal randomized controlled trials comparing fidaxomicin and vancomycin revealed: previous vancomycin or metronidazole treatment in the 24 hours before randomization, low eosinophil count ($<0.1 \cdot 10^9$/L), and low albumin level to be independent pre-

dictors of persistent diarrhoea or death in the first 12 days [40]. Recently Miller *et al.* [36] analysed the same two clinical therapeutic trials in order to derive and validate a categorization system to discriminate among CDI patients and correlate the grouping with treatment response. They concluded a combination of five clinical and laboratory variables measured at the time of CDI diagnosis, combined into a scoring system (ATLAS), were able to accurately predict treatment response to CDI therapy with fidaxomicin and vancomycin. These variables include: age, treatment with systemic antibiotics, leukocyte count, albumin and serum creatinine as a measure of renal function.

Strain type has been suggested as an additional cause of excess morbidity, disease severity and higher recurrence rates of CDI. In a Canadian study [47], PCR-ribotype 027 was correlated with more-severe disease and fatal outcome among patients at almost all ages. Some studies on the other hand suggested that PCR-ribotype 027 strains might only be associated with worse outcome in settings where 027 strains are epidemic, and not in an endemic situation [38, 48]. However, these findings are questioned by others [49]. Recently, a large study by Walker and colleagues clearly showed that strain types varied in the overall impact on mortality and biomarkers (predominantly those associated with inflammatory pathways) [50]. Besides *C. difficile* PCR-ribotype 027, other strains are associated with outbreaks and severe *C. difficile* infection as well, *e.g.* PCR-ribotype 078 [51]. Despite increased virulence of specific strain types, the value of the PCR-ribotype as a prediction marker for disease severity may be limited, as the ribotype involved in an infection is commonly not known upon diagnosis. However, in an epidemic situation the PCR-ribotype may be taken into account in deciding on the choice of empiric treatment regimens [21,39].

The level of host immune response to *C. difficile* exposure has been shown to be an important determinant of the severity and duration of clinical manifestations [52-57]. Anti-toxin antibody levels have been demonstrated to be higher in healthy adult controls compared with healthy children, and levels were noticed to fall with increasing age. In addition, anti-toxin antibodies increased after resolution of diarrhoea, which coincided with decreased incidence of CDI recurrence [57]. Inability to mount an adequate humoral immune response (*e.g.* during use of rituximab) may therefore be an important additional prediction marker for severe and/or recurrent CDI [25, 57-62]. Unfortunately in most cases this information is

not available at presentation/diagnosis; also, as the strength of evidence for immunodeficiency as an independent predictor for severe and/or recurrent CDI is still limited, we did not include this risk factor as a separate prediction marker.

Table 5. Prognostic markers that can be used to determine (increased risk of developing) severe CDI.

Characteristics	SoR*	QoE	Ref(s) not exhaustive	Comment(s)
Age (≥ 65 years)	A	IIr	[32, 41, 46]	Large cohort study on CDI mortality at 30 d, and review of studies of factors associated with CDI outcome [41]. Systematic review of studies describing the derivation or validation of Clinical Prediction Rules for unfavourable outcomes of CDI [46]: in general methodological biases and weak validities.
Marked leukocytosis (leukocyte count > $15{\cdot}10^9$/l)	A	IIrht	[32, 37, 39 45, 46, 63, 64]	Systematic review [46]: in general methodological biases and weak validities. Cohort study: severity score on malignancy, white blood cell count, blood albumin, and creatinine [37]. Retrospective cohort study on risk factors for severe CDI: death < 30 d, ICU, colectomy or intestinal perforation [32].
Decreased blood albumin (< 30 g/L)	A	IIr	[32, 37, 40, 46, 65]	Systematic review [46]: in general methodological biases and weak validities.
Rise in serum creatinine level (≥133 μmol/L or ≥1.5 times the premorbid level)	A	IIht	[32, 37, 41, 45]	Depending on the timing of measurement around CDI diagnosis [45].
Comorbidity (severe underlying disease and/or immunodeficiency)	B	IIht	[37, 41, 63, 66]	Comorbidity: wide variety of risk factors described/investigated, including cancer, cognitive impairment, cardiovascular, respiratory and kidney disease [41]. Chronic pulmonary disease, chronic renal disease and diabetes mellitus [66]. History of malignancy [37]. Prior operative therapy, inflammatory disease and intravenous immunoglobulin treatment [63].

** SoR: degree of recommendation to use a (clinical) characteristic as a prognostic marker.*

The results from individual studies, reviews and meta-analyses on prognostic markers for CDI were evaluated to reach a group consensus on a selection of markers that may be useful in clinical practice to distinguish patients with increased risk for severe or life-threatening CDI and recurrences. For detailed recommendations refer to Tables 5 and 6.

Recommendations

CDI is judged as severe when one or more of the clinical markers of severe colitis listed in Table 4 is present, and/or when one or more unfavourable prognostic factors (Table 5) is present:

» Marked leucocytosis (leukocyte count >15 · 10^9/L)
» Decreased blood albumin (<30 g/L)
» Rise in serum creatinine level (≥133 μmol/L or ≥1.5 times the premorbid level)

CDI without signs of severe colitis in patients with high age (≥65), serious comorbidity, Intensive Care Unit (ICU) admission, or immunodeficiency may also be regarded as increased risk of developing severe CDI.

Table 6. Prognostic markers that can be used to determine (increased risk of) recurrent CDI.

Characteristics	SoR*	QoE	Ref (s) not exhaustive	Comment(s)
Age (> 65 years)	A	IIrh	[42, 43, 46, 67]	Meta-analysis: [43]. Systematic review: [46]. Prospective validation study of risk factor: [42].
Continued use of (non-CDI) antibiotics after diagnosis of CDI and/or after CDI treatment	A	IIrh	[42, 43]	Meta-analysis: [43]. Prospective validation study of risk factor: [42].
Comorbidity (severe underlying disease) and/or renal failure	A	IIh	[42, 45, 68]	Prospective validation study of risk factor: comorbidity conditions rated by Horn's index (scoring system for underlying disease severity) [42].
A history of previous CDI (> 1 recurrences)	A	IIt	[26, 40, 69-71]	Data from randomized controlled trials: [26, 70]. Meta-analysis of pivotal randomized controlled trials [40].
Concomitant use of antacid medications (PPI)	B	IIrh	[43, 72]	Meta-analysis on recurrent CDI: [43]. Meta-analysis on CDI:[72].
Initial disease severity	B	IIth	[42, 67]	Prospective validation study of risk factor [42]. Long-term population based cohort study [67].

** SoR: degree of recommendation to use a (clinical) characteristic as a prognostic marker.*

Treatment of CDI

Once CDI is diagnosed in a patient, immediate implementation of appropriate infection control measures is mandatory in order to prevent further spread within the hospital. These include early diagnosis of CDI, surveillance, education of staff, appropriate use of isolation precautions, hand hygiene, protective clothing, environmental cleaning and cleaning of medical equipment, good antibiotic stewardship, and specific measures during outbreaks. Measures for the prevention and control of CDI ("bundle approach") have been described in a ESCMID guideline by Vonberg *et al.* [73].

Additional treatment measures include [1,3,4,72,74]:

- discontinuation of unnecessary antimicrobial therapy
- adequate replacement of fluid and electrolytes
- avoidance of anti-motility medications
- reviewing proton pump inhibitor use

In general it is difficult to compare studies on the treatment of CDI because of the use of variable diagnostic criteria, patient selection and subgroup definitions, stringency of searches for potential enteropathogens, severity of CDI, co-morbidities, exposures to causative and/or concomitant antibiotics, and follow-up. Moreover, studies have employed different definitions of clinical and/or microbiological cure and recurrence [2,75]. The variability in definitions and criteria of randomized controlled trials of antibiotic therapy for CDI is illustrated in Table 7. In 13/17 randomized controlled trials of antibiotic treatment of initial CDI, recurrences and duration of follow-up were defined. Follow-up varied from three to six weeks after treatment for CDI. In 6/17 randomized controlled trials definitions for severity of disease were given. In most of the studies very severe and/or life-threatening CDI was excluded.

Table 7. Randomized controlled trials of antibiotic treatment of initial CDI: definitions and criteria of recurrences, follow-up and severity of infection.

d = days; wk = weeks; m = months; WBC = white blood cell count; Alb = serum albumin.

Trial	Recurrences prior to study	Relapse/recurrences and follow-up	Severity of CDI	Severe CDI excluded/ included
[76]	Previous PMC excluded	Recurrences not defined and follow-up not specified	Not defined	Not specified
[77]	Not described	Reappearance of diarrhoea <21 d	Not defined	Not specified
[78]	Not described	Reappearance of diarrhoea <5 wk	Not defined	Not specified
[79]	Not described	Reappearance of diarrhoea after therapy Follow-up: length not clear	Not defined	Not specified
[80]	Not described	"Recurrence of disease": not further specified Follow-up not defined	No definition but judged by physician	Severe/moderate CDI included, mild CDI excluded
[81]	Not described	Not described No follow-up period	Not defined	Not specified
[82]	Not described	Reappearance of diarrhoea and other symptoms ≥1 m Follow-up not further specified	Not defined	Not specified
[83]	Treatment for CDI <6 wk excluded	Cure followed by return of inclusion criteria CDI <4 wk	Not defined	Not specified
[84]	Not described	Reappearance of diarrhoea and other symptoms <25-30 d	Severity estimated by: number/shape stool, CRP, WBC, ESR	Severe and mild CDI included. Results for PMCspecified
[85]	CDI ≤6 m excluded	Reappearance diarrhoea during 28-33 d	Not defined	Not specified. Severe "medical conditions" excluded
[86]	Not specified Excluded oral vanco/ metro treatment <7 d prior to study (≤2 doses included)	Reappearance of symptoms < 31 d after start of treatment and after at least 1 negative CD toxin test before retreatment	Not defined	Toxic megacolon excluded

Table 7. *[continued]*

Trial	Recurrences prior to study	Relapse/recurrences and follow-up	Severity of CDI	Severe CDI excluded/ included
[87]	Previous CDI excluded	Recurrence of diarrhoea during 30 d	Not defined	Not specified. Ileus and toxic megacolon excluded
[88]	Prior failure of treatment for CDI with study-drugs excluded	Recurrence of CD toxin positive diarrhoea within 21 d	Severe CDI defined as severity assessment score ≥2 (points). Based on: age (1), temperature (1), Alb (1), WBC (1), endoscopic PMC (2), ICU (2)	Severe and mild CDI included: results specified Life-threatening abdominal complications excluded
[89]	>1 recurrence or relapse within 3 m prior to study excluded	Recurrence of CD toxin positive diarrhoea <6 wk	Severity CDI based on: stools/ day, vomiting, ileus, severe abdominal tenderness, WBC, toxic megacolon, life-threatening CDI	Mild to moderately severe CDI included: results not specified Very severe CDI excluded
[90]	>1 recurrence <3 m prior to study excluded Results specified for CDI <90 d before study.	Return of symptoms (toxin positive diarrhoea) <31 d after onset of treatment, or clinical response after empiric re-treatment	Severe CDI defined as severity assessment score ≥2 (points). Based on: age (1), stools/day (1), temperature (1), Alb (1), WBC (1)	Severe and mild CDI included: results specified Unstable vital signs or ICU excluded.
[70]	>1 CDI <3 m prior to study excluded. Results specified for patients with/ without CDI <3 m before study.	Reappearance of CD toxin positive diarrhoea <4 wk and need for retreatment for CDI	Mild, moderate and severe CDI: based on bowel movements/day, WBC	Mild, moderate and severe disease included: results specified. Life-threatening or fulminant CDI and toxic megacolon excluded
[91]	>1 CDI <3 m prior to study excluded Results specified for patients with CDI <3 m before study.	Return of CD toxin positive diarrhoea <30 d and need for retreatment for CDI	Severe and not-severe CDI based on ESCMID criteria [1]: WBC, creatinine, temperature	Severe and not-severe disease included: results specified for severity. Life-threatening or fulminant CDI and toxic megacolon excluded

A Cochrane analysis published in 2011 reviewed 15 studies on the antibiotic treatment for CDI in adults [2]. The risk of bias was rated as high in 12 of the 15 included studies. The authors concluded that a specific recommendation for the antibiotic treatment of CDI could not be made. Nevertheless, and in spite of the observed limitations, it is apparent that a clear and up-to-date guideline on the treatment of CDI is urgently needed for clinical practice. For this purpose the strength of a recommendation and the quality of evidence are assigned in two separate evaluations in this guideline, thus allowing an assessment of the strength of a recommendation independent of the level of supportive evidence (Tables 1 and 2).

To improve clinical guidance in the treatment of CDI, treatment recommendations are specified for various **patient groups**:

A Initial CDI: non-severe disease
B. Severe CDI
C. First recurrence or risk of recurrent disease
D. Multiple recurrent CDI
E. Treatment of CDI when oral administration is not possible

The following **treatment options** are considered:

1. Oral and non-oral antibiotics
2. Toxin-binding resins and polymers
3. Immunotherapy
4. Probiotics
5. Faecal or bacterial intestinal transplantation

A. Initial CDI

Oral antibiotic therapy for non-severe disease

Evidence

The antibiotics commonly used to treat CDI are oral metronidazole or oral vancomycin. Oral metronidazole has been shown to be effective in inducing a clinical response and has the advantage of low cost and is assumed to be associated with reduced vancomycin resistant enterococci (VRE) selection risk. In a pooled intention to treat analysis (treating exclusions, deaths and relapses as treatment failures) of three randomized controlled trials comparing symptomatic cure between metronidazole and vancomycin [77, 84, 88]: no statistically significant differences were found [2, 75]. However, a recently presented pooled analysis of a study on the use of tolevamer showed that overall metronidazole was inferior to vancomycin [92]. In addition the response rate to metronidazole may be slower than with vancomycin [23]. Oral metronidazole is usually recommended for treatment of non-severe disease, whereas oral vancomycin is generally preferred for treatment of severe infections [1, 3-5].

Decreased clinical effectiveness of metronidazole treatment for specific ribotypes causing CDI, *e.g.* PCR-ribotype 027 has been described [93]. Although changes in antibiotic resistance and ribotype prevalence have been reported, *in vitro* studies indicate that MICs of metronidazole and vancomycin for endemic *C. difficile* have remained relatively low over the years. Brazier *et al.* concluded that the MICs of metronidazole and vancomycin were not indicative of clinical failure, but MICs for epidemic ribotypes (027, 106 and 001) were several dilutions higher [94]. Indeed there is increasing evidence of the emergence of reduced susceptibility to metronidazole in some *C. difficile* strains, with evidence for clonal spread [95]. Notably, MIC methodology is crucial to the detection of reduced susceptibility to metronidazole; E-tests in particular under-estimate the MIC [95, 96]. There is also evidence of inferior microbiological efficacy of metronidazole in comparison with vancomycin [21, 22]. Although poor gut concentrations of metronidazole alongside reduced susceptibility to metronidazole could explain reduced treatment efficacy, treatment failures have not been associated with decreased susceptibility [95, 97, 98]. A case-control study found no significant differences in clinical outcome for CDI cases from which strains with reduced susceptibility to metronidazole were recovered versus matched (metronidazole susceptible) controls. Response to metronidazole

was generally poor (slow and prone to recurrence) and the frail elderly patients had a 21% 30-day-mortality. However, much larger study groups are needed to determine the clinical significance of CD isolates with reduced susceptibility to metronidazole [99].

Orally administered vancomycin is poorly absorbed from the gastrointestinal tract, and therefore luminal drug levels are very high and orders of magnitude greater than the susceptibility breakpoint concentration for all strains of *C. difficile* tested so far, thereby resulting in a more rapid suppression of *C. difficile* to undetectable levels during therapy and faster resolution of diarrhoea [22, 23]. Metronidazole, on the other hand, is well absorbed from the gastrointestinal tract. Mean antibiotic concentrations reported in faeces of patients receiving oral metronidazole range from <0.25-9.5 mg/L, and drug concentrations in faeces decrease to undetectable levels as mucosal inflammation improves and diarrhoea resolves [100]. Increased MIC for metronidazole could therefore have implications on clinical cure or recurrences in CDI. Although there are no published reports in which treatment failure has been linked to antimicrobial metronidazole resistance in *C. difficile*, the pharmacokinetic properties of vancomycin are considered superior to metronidazole in severe *C. difficile* disease [88].

There is concern that use of vancomycin may be more likely to promote colonization and transmission of VRE by selection pressure. However, both oral metronidazole and oral vancomycin have been associated with the promotion of persistent overgrowth of VRE in stool samples obtained from colonized patients during CDI treatment, thereby increasing the risk of transmission [101]. In a small study of VRE colonized patients with CDI, who experienced frequent faecal incontinence, skin and environmental VRE contamination was common during and after resolution of diarrhoea. It was concluded that the frequency of VRE contamination of skin or the environment was similar between patients treated with metronidazole (n = 17) and those given vancomycin (n = 17), although the study clearly had only limited power to examine this issue [102]. In a large retrospective analysis, increased vancomycin use during an outbreak of CDI was not associated with an increase in VRE colonization during a follow-up period of two years after the outbreak period. The authors concluded that restriction of vancomycin use during CDI outbreaks because of the fear of increasing VRE colonization might not be warranted. However, the interpretation of

the data was complicated by an outbreak of VRE (VanA) cases that was observed after approximately 20 months of increasing preferential use of vancomycin. As the rate of VanA cases subsequently decreased very quickly, the investigators concluded that this temporary increase reflected a localized clonal outbreak unrelated to the CDI therapy at that time [103].

Although vancomycin and metronidazole are effective in the treatment of CDI, they are both broader spectrum agents that cause significant disruption of the commensal colonic microbiota. A disruption in the commensal microbiota may predispose to recurrent CDI and intestinal colonization by healthcare-associated pathogens such as VRE and *Candida* species. Fidaxomicin appears to cause less disruption of the anaerobic colonization microbiota, and has activity against many VRE strains [104]. Therefore it is suggested that the risk of colonization with and transmission of VRE associated with fidaxomicin treatment may be lower as compared with vancomycin therapy. A recent study concluded that fidaxomicin was indeed less likely than vancomycin to promote acquisition of VRE and *Candida* species during CDI treatment. However, selection of pre-existing subpopulations of VRE with elevated fidaxomicin MICs was more common during fidaxomicin therapy [105].

Similar cure rates have been demonstrated for oral vancomycin and oral teicoplanin [82, 84]. For bacteriologic cure oral teicoplanin may even be more effective than vancomycin [2, 82]. Both glycopeptides are very active in vitro against *C. difficile* isolates [106]. Since 2013 Teicoplanin does have a licensed indication for CDI and is available for oral administration. Teicoplanin is not available in the USA. For the purpose of this treatment guideline only oral vancomycin is included in the treatment recommendations.

Tables 8 and 9 report the evidence for oral treatment of initial CDI from randomized trials and observational studies with comments on methodology. Evidence *not* included in the previous ESCMID guideline for the treatment of CDI [1], is highlighted in green.

Although oral metronidazole absorption is very high and potentially can lead to more systemic side effects, adverse effects of oral metronidazole are commonly mild to moderate in severity. The most common adverse reactions reported involve the gastrointestinal tract [107]. Rarely, particularly in

association with long duration therapy, metronidazole has been linked to more severe safety issues, *e.g.* peripheral and optic neuropathy [108] and interactions with warfarines [109].

Oral vancomycin has been shown to be poorly absorbed in most patients, usually producing minimal or sub-therapeutic serum concentrations. However, bowel inflammation may enhance absorption of oral vancomycin, particularly in those with renal failure, thereby increasing the risk for systemic side effects [110]. A recently performed safety analysis of fidaxomicin in comparison with oral vancomycin revealed no differences in serious adverse events between these agents [111].

Note: Fidaxomicin is minimally absorbed. While no specific concerns related to hypersensitivity reactions were identified during the drug development, hypersensitivity reactions associated with fidaxomicin use have been reported to the FDA in the post-marketing phase. The fidaxomicin labeling was revised to include information about the possibility of hypersensitivity reactions.
Ref: Iarikov DE, Alexander J, Nambiar S. Hypersensitivity reactions associated with fidaxomicin use. Clin Infect Dis 2013, doi: 10.1093/cid/cit719.

To evaluate the clinical outcomes of the main antimicrobial agents used in the treatment of CDI, we compared dosages, cure rate, recurrence rate, stated time to response and adverse events of treatment with vancomycin, metronidazole and fidaxomicin. Only randomized controlled trials of antibiotic treatment of initial CDI were included. Results are summarized in Table 10.

Table 8. Randomized controlled trials of oral antibiotic treatment of initial CDI. Initial cure rate, and sustained response rates as a percentage of all patients and relapse rate as a percentage of initially cured patients.

Trial	Treatment	Number of patients	Cure [%]	Recurrence [%]	Sustained response [%]
[76]	Vancomycin, 125 mg qid, 5 days	9	78	0	78
	Placebo	7	14	-	-
	No clear case definition. No description of allocation of treatment. Only data of patients with toxin-positive stool shown. Unclear length of follow-up and incidence or relapse in placebo group. $p < 0.02$ for comparison of cure rates.				
[77]	Vancomycin, 500 mg qid, 10 days	32	100	19	81
	Metronidazole 250 mg qid, 10 days	32	97	6	91
	Only data of patients with toxin-positive stools or pseudomembranous colitis shown. Per-protocol analysis. Follow-up 21 days. Differences not statistically significant.				

Table 8. *(Continued)*

Trial	Treatment	Number of patients	Cure [%]	Recurrence [%]	Sustained response [%]
[78]	Vancomycin, 125 mg qid, 7 days	21	86	33	58
	Bacitracin, 20000 U qid, 7 days	21	76	42	44
	Double-blind. 25% drop-out during follow-up of bacitracin group. Follow-up 5 weeks. Differences not statistically significant.				
[79]	Vancomycin, 500 mg qid, 10 days	15	100	20	80
	Bacitracin, 25000 U qid, 10 days	15	80	42	46
	Double-blind. Patients had leukocytosis, fever or abdominal pain. 29% drop-out in vancomycin group, 12% in bacitracin group. Per-protocol analysis. Unclear definition of failure ('worsening during treatment'). Failing patients crossed over to alternate drug. Interruption of study drug in vancomycin group for a mean of 2.8 days and in bacitracin group for a mean of 1.8 days. Unclear length of follow-up. Differences not statistically significant.				
[80]	Vancomycin, 125 mg qid, mean 10.6 days	24	100	21	79
	Vancomycin, 500 mg qid, mean 10.1 days	22	100	18	82
	Variable duration of therapy. 18% dropout rate. Per-protocol analysis. Unclear length of follow-up. Differences not statistically significant.				
[81]	Vancomycin, 500 mg bid, 10 days	10	100	-	-
	Rifaximin, 200 mg tid, 10 days	10	90	-	-
	Article in Italian. Patients had diarrhoea, abdominal pain and fever. No description of allocation of treatment. Unclear definition of cure. Differences not statistically significant.				
[82]	Vancomycin, 500 mg qid, 10 days	20	10	20	80
	Teicoplanin, 100 mg bid, 10 days	26	96	8	88
	No description of allocation of treatment. Per-protocol analysis. Unclear length of follow-up ('at least 1 month'). Differences not statistically significant.				
[83]	Teicoplanin, 100 mg qid, 3 days, followed by 100 mg bid, 4 days	24	96	35	62
	Teicoplanin, 100 mg bid, 7 days	23	70	50	35
	Double-blind. Outcome of 'improvement, but not cure' (2 loose stools per day or 1 loose stool per day with fever or cramps) was counted as failure. 3 patients with improvement in bid group; 1 in qid group. Follow-up 5 weeks. p = 0.08 for comparison of cure rates.				
[84]	Vancomycin, 500 mg tid, 10 days	31	94	17	78
	Metronidazole, 500 mg tid, 10 days	31	94	17	78
	Teicoplanin, 400 mg bid, 10 days	28	96	7	89
	Fusidic acid, 500 mg tid, 10 days	29	93	30	65
	Follow-up 30 days. Only statistically significant difference was relapse rate of fusidic acid versus teicoplanin (p = 0.042).				
[85]	Metronidazole, 400 mg tid, 7 days	55	93	30	65
	Fusidic acid, 250 mg tid, 7 days	59	83	30	58
	Double-blind. 13% drop-out during treatment; 15% further drop-out during follow-up. Per-protocol analysis. Follow-up 35 days. Differences not statistically significant.				

Table 8. *(Continued)*

Trial	Treatment	Number of patients	Cure [%]	Recurrence [%]	Sustained response [%]
[86]	Metronidazole, 250 mg qid, 10 days	34	82	30	57
	Nitazoxanide, 500 mg bid, 7 days	40	90	6	67
	Nitazoxanide, 500 mg bid, 10 days	36	89	16	75
	No definition of relapse. Double-blind. 23% drop-out during treatment. Per-protocol analysis. Follow-up 31 days. Differences not statistically significant.				
[87]	Metronidazole, 500 mg tid, 10 days	20	65	38	40
	Metronidazole, 500 mg tid + rifampicin 300 mg bid, 10 days	19	63	42	37
	Intention-to-treat analysis. Follow-up 40 days. Differences not statistically significant.				
[88]	Vancomycin, 125 mg qid, 10 days	71	97	7	90
	Metronidazole, 250 mg qid, 10 days	79	84	14	72
	Double-blind. 13% drop-out during treatment. Per-protocol analysis. Follow-up 21 days. p = 0.006 for comparison of cure rates. p = 0.27 for comparison of relapse rates. The original protocol was stratified in a group with mild and a group with severe disease (based on age, fever, albumin level and leukocyte count), which resulted in a larger difference between cure rates in the group with severe disease and a statistically non-significant difference between cure rates in the group with mild disease. Intention-to-treat analysis with dropouts regarded as failures resulted in a statistically significant difference between overall cure rates (initial cure minus relapse; 57 out of 90 versus 64 out of 82; risk ratio 0.91). Other comparisons were not significant anymore in the intention-to-treat analysis.				
[89]	Fidaxomicin, 50 mg bid, 10 days	14	71	8	65
	Fidaxomicin, 100 mg bid, 10 days	15	80	0	80
	Fidaxomicin, 200 mg bid, 10 days	16	94	6	88
	Open-label. Patients with signs of highly severe CDI (>12 bowel movements per day, vomiting, severe abdominal tenderness, ileus, WBC >30, toxic megacolon) were excluded. Cure = complete resolution of diarrhoea. Follow-up 6 weeks after end of treatment.				
[90]	Vancomycin, 125 mg qid, 10 days	27	74	7	69
	Nitazoxanide, 500 mg bid, 10 days	22	77	5	73
	CDI = stool EIA for toxin A or B positive AND (temperature >38.3ºC OR abdominal pain OR leukocytosis). Patients with >1 episode in preceding 6 months were excluded. 12% dropout rate during treatment. Double-blind, placebo-controlled. Modified intention-to-treat analysis. Industry-sponsored. Cure = complete resolution of symptoms during 3 days after completion of therapy. Per-protocol analysis: 87 versus 94% cure. Follow-up 31 days after start of treatment. No differences in severity subgroups. Differences not statistically significant.				
[70]	Vancomycin, 125 mg qid , 10 days	309	86	25	65
	Fidaxomicin, 200 mg bid, 10 days	287	88	15	75
	Placebo-controlled. Industry-sponsored. Very severe CDI and more than one previous episode excluded. Designed as non-inferiority trial. 4 weeks follow-up for recurrences after completion of study drug. Cure = <4 times daily passage of unformed stools AND no necessity for additional treatment. Fidaxomicin was not associated with fewer recurrences in CDI due to PCR ribotype 027 as opposed to non-027. Modified intention-to-treat (patients who received at least one dose of the study drug) and per-protocol analyses were similar.				
[91]	Vancomycin, 125 mg qid , 10 days	257	87	27	64
	Fidaxomicin, 200 mg bid, 10 days	252	88	13	77
	Methods identical to the trial by Louie [70]. Contrary to that trial, this trial did show fewer recurrences in both PCR ribotype 027 and non-027 patients, although the difference was not significant for the former subgroup.				

Table 9. Observational studies of oral antibiotic treatment of initial CDI. Initial cure rate and sustained response as a percentage of all patients and relapse rate as a percentage of initially cured patients.

Trial	Treatment:	Number of patients	Cure [%]	Recurrence [%]	Sustained response [%]
Antibiotics:					
[112]	Vancomycin	79	96	14	83
[113]	Vancomycin	16	100	13	87
[114]	Metronidazole	13	100	15	85
[115]	Vancomycin	189	97	24	74
[106]	Vancomycin 500 mg qid, 10 days	23	100	13	87
	Teicoplanin 200 mg bid, 10 days	22	100	0	100
[116]	Metronidazole	632	98	6	92
	Vancomycin	122	99	10	89
[57]	Metronidazole	44	?	50	-
[117]	Metronidazole	99	62	?	-
[118]	Metronidazole	207	78	28	56
[68]	Metronidazole	1123	84	29	60
	Vancomycin	112	?	28	-
[119]	Difimicin varying dose	45	91	5	86
[120]	Nitazoxanide 500 mg bid, 10 days	35	74	27	54
	Patients first failed metronidazole.				
[101]	Metronidazole	34	>90	12	>79
	Ten patients switched to vancomycin				
	Vancomcyin	18	>90	11	>80
[121]	Tigecycline varying duration	4	100	0	100
	Severe CDI. Follow-up at least 3 months.				
[122]	Rifaximin 400 mg tid	8	100	10	90
	2 weeks follow-up.				

Table 10. Results of randomized controlled trials of oral antibiotic treatment of initial CDI with vancomycin/teicoplanin, metronidazole and fidaxomicin: comparison of dosages, cure rate, recurrence rate, stated time to response or adverse effects due to treatment.

	Trial	Number of patients	Dosages and duration of therapy	Time to initial response (mean)	Cure rate [%]	Recurrence rate [%] and definition	Adverse events [%]
Vancomycin	[76]	9	125 mg qid 5 days	-	78	0 Recurrence not defined, follow-up period not specified	-
	[77]	32	500 mg qid 10 days	3.2 days	100	19 Reappearance of diarrhoea <21 d after therapy	3 Drug intolerance
	[78]	21	125 mg qid 7 days	-	86	33 Reappearance of diarrhoea <5 wk after therapy	-
	[79]	15	500 mg qid 10 days	-	100	20 Reappearance of diarrhoea after therapy Follow-up: length not clear	-
	[80]	24	125 mg qid mean 11 days	4 days	100	21	0
		22	500 mg qid mean 10 days	4 days	100	18 Recurrence of disease not further specified Follow-up not defined	0
	[81]	10	500 mg bid 10 days	3.8 days	100	Not described No follow-up period	0
	[82]	20	500 mg qid 10 days	3.6 days	100	4 Reappearance of diarrhoea and other symptoms ≥1 m after therapy. Follow-up not further specified	0
	[84]	31	500 mg tid 10 days	3.1 days	94	17 Reappearance of diarrhoea and other symptoms <25-30 d after therapy	0
	[88]	71	125 mg qid 10 days	-	97	7 Recurrence of CD toxin positive diarrhoea within 21 d after start of therapy	1 (nausea)
	[90]	27	125 qid 10 days	Median: 96 hrs	74	7 Return of symptoms (toxin positive diarrhoea) < 31 d after onset of treatment, or clinical response after empiric re-treatment for CDI	0
	[70]	30	125 mg qid 10 days	Median: 78 hrs in the MITT	86	25 Reappearance of CD toxin positive diarrhoea < 4 wk after treatment and need for retreatment for CDI	Possibly or definitely related: 9 Serious events related to laboratory test results: 1.2
	[91]	257	125 mg qid 10 days	Median: 58 hrs in the MITT	87	27 Return of CD toxin positive diarrhoea <30 d after treatment and need for retreatment for CDI	Any treatment-emergent adverse event related to study drug: 13.8

d = days; wk = weeks; m = months.

Table 10. *(Continued)*

	Trial	Number of patients	Dosages and duration of therapy	Time to initial response (mean)	Cure rate [%]	Recurrence rate [%] and definition	Adverse events [%]
Teicoplanin	[82]	26	100 mg bid 10 days	3.4 days	96	2 Reappearance of diarrhoea and other symptoms ≥ 1 m after therapy. Follow-up not further specified	0
	[84]	28	400 mg bid 10 days	2.8 days	96	7 Reappearance of diarrhoea and other symptoms < 25-30 d after therapy	0
	[83]	24	100 mg qid, 3 days, followed by 100 mg bid, 4 days	-	96	35	7-8% vomiting, nausea, exanthema, arthralgia, pruritus, hallucinations. No abnormal laboratory results
		23	100 mg bid 7 days		70	50	
Metronidazole	[77]	32	250 mg qid 10 days	3.1 days	97	6 Reappearance of diarrhoea <21 d after therapy	3
	[84]	31	500 mg tid 10 days	3.2 days	94	17 Reappearance of diarrhoea and other symptoms <25-30 d after therapy	10 GI discomfort
	[85]	55	400 mg tid 7 days	Within 5 days	93	30 Reappearance diarrhoea during 28-33 d after treatment	14.5 GI, exanthema, taste
	[86]	34	250 mg bid 10 days	Median: 3 days	82	30 Reappearance of symptoms <31 days after start of treatment and after at least 1 negative CD toxin test before retreatment	related to study drug: 0 serious adverse events not related to study drug: 18.2 intolerance or allergy: 0
	[87]	20	500 mg tid 10 days	6.6 days	65	38 Recurrence of diarrhoea <30 d after treatment	40 (not specified if related to study drug: rash, nausea vomiting)
	[88]	79	250 mg qid 10 days	-	84	14 Recurrence of CD toxin positive diarrhoea <21 d after start of therapy	1.3 (nausea)
Fidaxomicin	[89]	14	50 mg bid 10 days	Median 6.3 days	71	8	20 but not related to study drug.
		15	100 mg bid 10 days	Median 4.8 days	80	0	
		16	200 mg bid 10 days	Median 3.6 days	94	6 Recurrence of CD toxin positive diarrhoea <6 wk after treatment	
	[70]	287	200 mg bid 10 days	Median 58 hours in the MITT	88	15 Reappearance of CD toxin positive diarrhoea <4 wk and need for retreatment for CDI	Possibly or definitely related: 9.7 Serious events related to laboratory test results: 4.7
	[91]	252	200 mg bid 10 days	Median 56 hours	88	13 Return of CD toxin positive diarrhoea <30 d and need for retreatment for CDI	Any treatment-emergent adverse event related to study drug: 11.7

Recommendations

In case of non-severe CDI (no signs of severe colitis) in non-epidemic situations and with CDI clearly induced by the use of antibiotics, it may be acceptable to stop the inducing antibiotic and observe the clinical response for 48 hours, but patients must be followed very closely for any signs of clinical deterioration and placed on therapy immediately if this occurs. Metronidazole is recommended as oral antibiotic treatment of initial CDI in mild/moderate disease. For detailed recommendations on oral antibiotic treatment of initial non-severe CDI refer to Table 11.

Table 11. Recommendations on oral antibiotic treatment of initial CDI: non-severe disease

Treatment	SoR	QoE	Ref(s)	Comment(s)
Metronidazole, 500 mg tid, 10 days	A	I	[77, 84-88]	No statistically significant difference in cure rate between metronidazole and vancomycin or teicoplanin. Statistically significant difference in sustained clinical cure between metronidazole and vancomycin in favour of vancomycin in one study [2, 88] (and pooled results of two randomized controlled trials published only in abstract form [92, 122, 123]).
Vancomycin, 125 mg qid, 10 days	B	I	[70, 76, 78, 80, 82, 84, 88, 90, 91]	Cochrane analysis: teicoplanin significantly better than vancomycin for bacteriologic cure and borderline superior in terms of symptomatic cure [2].
Fidaxomicin, 200 mg bid, 10 days	B	I	[70, 89, 91]	Evidence limited to two Phase III studies. Fewer recurrences as compared to vancomycin, except for *C. difficile* PCR-ribotype 027 [91].
Vancomycin, 500 mg qid, 10 days	C	I	[77, 79-82, 84]	Vancomycin: Equal cure rate 500 mg qid po compared to 125 qid po [80].
Stop inducing antibiotic(s) and observe the clinical response for 48 hours	C	II	[115, 116]	Rate of spontaneous resolution unknown in mild CDI. Studies performed before increased incidence of hypervirulent strains.

A. Initial CDI

Alternative treatment regimens for non-severe disease

Evidence

Tables 12 and 13 report the evidence from randomized trials and observational studies on the non-antibiotic treatment of initial CDI, with comments on methodology. The majority of these alternative treatment strategies are combined with antibiotic treatment. Evidence not included in the previous ESCMID guideline [1], is highlighted in green.

Currently there are no randomized controlled trials on the use of human intravenous gamma-globulins (IVIG). Passive immunizations with IVIG have been reported to be successful in small case series, but the grade of evidence and strength of recommendation of IVIG are too weak to allow recommendations on the use of IVIG in CDI [4,129]. Hypogammaglobulinemia, *e.g.* following solid organ transplants, may predispose to CDI. For this subgroup of patients, IVIG may be beneficial, but more studies are needed before this can be recommended definitively [4].

A recent systematic review on the use of probiotics suggests that probiotics are associated with a reduction in antibiotic associated diarrhoea (AAD) [130]. A recent meta-analysis on probiotic prophylaxis for CDI, concluded moderate-quality evidence suggests a beneficial effect of probiotic prophylaxis in CDI without an increase in clinically important adverse events [131]. However, a Cochrane analysis concluded that there was insufficient evidence to recommend probiotics, in general, as an adjunct to antibiotics in the treatment of *C. difficile* diarrhoea [132]. Although no cases of translocation of microorganisms have been reported in clinical trials with probiotics for AAD or CDI, probiotics should be used with caution. Several studies of invasive disease have been reported, resulting from the use of probiotics such as *Saccharomyces boulardii* in debilitated or immune-compromised patients [133,134]. Moreover, probiotics were associated with increased mortality, partly due to non-occlusive mesenterial ischemia, in a randomized controlled trial in acute pancreatitis [135].

Table 12. Randomized controlled trials of alternative treatment regimens for initial CDI. Initial cure rate and sustained response as percentage of all patients and relapse rate as percentage of initially cured patients.

Trial	Treatment	Number of patients	Cure [%]	Recur-rence [%]	Sustained response [%]
Probiotics:					
[125]	Vancomycin or metronidazole + *Saccharomyces boulardii* 2 · 10^{10} CFU/day, 4 weeks	31	-	19	-
	Vancomycin or metronidazole + placebo	33	-	24	-
	Double-blind. No control for type, duration or dose of antibiotic. Unclear definition of relapse. Follow-up 8 weeks after start of treatment. p = 0.86 for comparison of relapse rates.				
Toxin-binding resins and polymers:					
[24]	Tolevamer 1 g tid, 14 days + placebo	94	60	16	50
	Tolevamer 2 g tid, 14 days + placebo	91	79	7	74
	Vancomycin 125 mg qid, 10 days + placebo	94	91	19	74
	Non-inferiority trial. Patients with stool frequency >12 daily or abdominal pain were excluded. Tolevamer could be prolonged when inciting antibiotic could not be stopped. Double-blind. 23% drop-out. Per-protocol analysis. Cure rate of tolevamer 2 g non-inferior in comparison with vancomycin (Chow-test p = 0.03). Non-inferiority of tolevamer 1 g compared with vancomycin could not be demonstrated. p = 0.05 for comparison of relapse rates of tolevamer 2 g with vancomycin. Relapse rates of tolevamer 1 g and vancomycin not statistically different. Follow-up 6 – 8 weeks.				
[123]*	Tolevamer, 3g tid, 14 days	266	47	3	46
	Vancomycin, 125 mg qid, 10 days	134	81	23	62
	Metronidazole, 375 mg qid, 10 days	143	72	27	53
[124]*	Tolevamer, 3g tid, 14 days	268	42	6	40
	Vancomycin, 125 mg qid, 10 days	125	81	18	66
	Metronidazole, 375 mg qid, 10 days	135	73	19	59
Immunotherapy:					
[71]	Single dose of 10 mg/kg CDA1 and CDB1 (iv. administered human monoclonal antibodies against TcdA and TcdB) with standard antimicrobial therapy	101	93	7	87
	Placebo with standard antimicrobial therapy	99	87	25	65
	Industry-sponsored and -analysed. Patients must have diarrhoea and receive vancomycin or metronidazole at time of enrolment. Diarrhoea = >2 unformed stools on 2 consecutive days or >6 unformed stools on 1 day. Recurrence = new episode of diarrhoea with new positive stool toxin test after resolution of initial diarrhoea. Analysis for recurrence only performed in those who were cured, received >7 days of antimicrobial therapy and did not receive IVIG (93 versus 82). Dropout rate 9 versus 13%, mainly due to deaths not related to CDI. Vancomycin: 30 versus 22%. Follow-up 12 weeks. p < 0.001 for comparison of relapse rates. Intention-to-treat analysis. Primary endpoint was changed during the study before unblinding. Original endpoint: resolution of illness. Subgroup analysis: similar results, although difference much smaller in inpatients than outpatients. Length of hospitalisation did not differ.				

* *poster presentation*

Table 13. Observational studies of alternative treatment regimens for initial CDI. Initial cure rate as a percentage of all patients and relapse rate as a percentage of initially cured patients.

Trial	Treatment	Number of patients	Cure [%]	Recurrence [%]
Toxin-binding resins and polymers:				
[126]	Colestipol 10 g qid, 5 days	12	25	-
	Originally set up as a randomized placebo-controlled trial. Placebo group was merged with historical control, however. Only 6 patients had toxin-positive stool.			
Passive immunotherapy with immune whey:				
[127]	Metronidazole or vancomycin followed by immune whey protein concentrate, 14 days	16	100	0
	56% of patients had recurrent CDI; mean follow-up 333 days.			
[128]	Metronidazole or vancomycin followed by immune whey protein concentrate, 14 days	109	100	10
	109 episodes; 101 patients; 40% of patients had recurrent CDI.			

Recommendations

There is insufficient evidence to support administration of probiotics, toxin binding resins and polymers, or monoclonal antibodies. For detailed recommendations refer to Table 14.

Table 14. Recommendations on alternative treatment regimens for initial CDI.

Type of intervention	Treatment	SoR	QoE	Ref(s)	Comment(s)
Immunotherapy	Human monoclonal antibodies against TcdA and TcdB with standard oral antimicrobial therapy (metronidazole and vancomycin)	C	I	[71]	Evidence limited to Phase II randomized controlled trial. Primary endpoint changed during study. Reduced recurrence of CDI: analysis for recurrence only performed in those who were cured, received >7 day of antimicrobial therapy and did not receive IVIG.
	Passive immunotherapy with immune whey after standard oral antimicrobial therapy	C	II	[128]	Observational study: 101 CDI patients (40% recurrent CDI). Results suggest reduction in recurrence rate.
Probiotics	Oral vancomycin or oral metronidazole + *Saccharomyces boulardii*	D	I	[125, 136]	Comparison of relapse rates: in subgroup analysis efficacy in recurrent CDI, but *not* in initial CDI. Evidence based review: [136].
Toxin binding resins and polymers	Tolevamer, 3 g tid	D	I	[24]	Evidence limited to Phase II randomized controlled trial. Non-inferiority study: tolevamer versus vancomycin.

B. Severe CDI

Oral antibiotic therapy

Evidence

In 6/17 randomized controlled trials severity of disease was defined. Definitions varied among the studies. Only in 4/6 of these trials treatment results were specified for severity of disease (Table 15).

Table 15. Randomized controlled trials of oral antibiotic treatment of initial CDI in which severity of disease is defined and outcome of treatment is specified for severity of disease.

Study	Treatment	CDI severity: Moderate/Mild (M), Severe (S) Nr of patients (%)	Initial cure Nr of patients (%)	Relapse Nr of patients (% of patients with initial cure)	Sustained response rate* Nr of patients (% of all patients)
[88]	Vancomycin, 125 mg qid, 10 days	M 40/71 (56) S 31/71 (44)	39/40 (98) 30/31 (97)	2/39 (5) 3/30 (10)	37/40 (93) 27/31 (87)
	Metronidazole, 250 mg qid, 10 days	M 41/79 (52) S 38/79 (48)	37/41 (90) 29/38 (76)	3/37 (8) 6/29 (21)	34/41 (83) 23/38 (61)
	Intention to treat analysis:				
	Vancomycin, 125 mg qid, 10 days	M 44/82 (49) S 38/82 (46)	39/44 (89) 30/38 (79)	2/39 (5) 3/30 (10)	37/44 (84) 27/38 (71)
	Metronidazole, 250 mg qid, 10 days	M 46/90 (51) S 44/90 (49)	37/46 (80) 29/44 (66)	3/37 (8) 6/29 (21)	34/46 (74) 23/44 (52)
[90]	Vancomycin, 125 mg qid, 10 days	M 17/27 (63) S 10/27 (37)	13/17 (76) 7/10 (70)	1/13 (8) 1/7 (14)	12/17 (71) 6/10 (60)
	Nitazoxanide, 500 mg bid, 10 days	M 12/22 (55) S 10/22 (45)	9/12 (75) 8/10 (80)	0/9 (0) 1/8 (13)	9/12 (75) 7/10 (70)
[70]	Vancomycin, 125 mg qid, 10 days	M 186/309 (60) S 123/309 (40)	156/186 (85) 109/123 (89)	38/156 (24) 29/109 (27)	118/186 (63) 80/123 (65)
	Fidaxomicin, 200 mg bid, 10 days	M 175/287 (61) S 112/287 (39)	161/175 (92) 92/112 (82)	27/161 (17) 12/92 (13)	134/175 (77) 80/112 (71)
[91]	Vancomycin, 125 mg qid, 10 days	M 196/257 (76) S 61/257 (24)	180/196 (92) 43/61 (71)	46/180 (26) 14/43 (33)	134/196 (68) 29/61 (48)
	Fidaxomicin, 200 mg bid, 10 days	M 189/252 (75) S 63/252 (25)	173/189 (92) 48/63 (76)	24/173 (14) 4/48 (8)	149/189 (79) 44/63 (70)

**Sustained response rate: clinical cure and no recurrences during follow-up*

Recommendations

Based on its pharmacokinetic properties vancomycin is considered superior to metronidazole in severe *C. difficile* disease [22, 88]. The use of high doses of vancomycin (500 mg orally qid) was included in the IDSA / SHEA treatment guidelines [3] for management of severe complicated CDI as defined by the treating physician. However, there is insufficient evidence to support the use of doses >125 mg four times daily in the absence of ileus [80].

Fidaxomicin was not inferior to vancomycin for initial cure of CDI, but there are no data available on the efficacy of this drug in severe life-threatening disease [70,91].

For detailed recommendations on oral antibiotic treatment of severe CDI refer to Table 16.

Table 16. Recommendations on oral antibiotic treatment of initial CDI: severe disease.

Treatment	SoR	QoE	Ref(s)	Comment(s)
Metronidazole, 500 mg tid 10 days	D	I	[88]	*Cure rate lower as compared with vancomycin in severe CDI [88]. Intention to treat analysis not reported. Extremely severe CDI excluded. Differences in symptomatic cure of metronidazole versus vancomycin not statistically significant in a pooled analysis [2] ICU admission and hypoalbuminemia (= disease severity) predictors of metronidazole failure [118].
Vancomycin, 125 mg qid 10 days	A	I	[70, 88, 90, 91]	*Cure rate higher as compared with metronidazole in severe CDI [88] See also above
Vancomycin, 500 mg qid 10 days	B	III (I*)	[80]	*Randomized controlled trial on dose effectiveness: no significant differences in measurable responses of high-dose compared to low-dose regimens. However: results not stratified for severity of illness [80].
Fidaxomicin, 200 mg bid 10 days	B	I	[70, 89, 91]	Evidence limited to two Phase III studies. Fewer recurrences as compared to vancomycin 125 mg qid in severe disease (*except for PCR ribotype 027*). **No data on the efficacy in severe life-threatening disease and/or toxic megacolon: excluded from both studies.**

**Two studies reported in abstract form confirm the superiority of vancomycin over metronidazole for treatment of (severe) CDI* [92, 123, 124].

Surgery for complicated CDI

Evidence

Patients with fulminant CDI who fail to respond and progress on to systemic toxicity, peritonitis, or toxic colonic dilatation and bowel perforation require surgical intervention [4]. Mortality rates of emergency surgery in complicated CDI remain high, ranging from 19% to 71% depending on the clinical condition of the patient at the time of surgery [137]. However, recently a systemic review of the existing literature was performed to assess the effect on mortality by colectomy for the treatment of fulminant CDI. The authors concluded that colectomy is associated with a lower mortality than continued medical treatment when this is no longer improving the patient [138]. Several studies suggest that earlier colectomy (time from presentation to surgery) is associated with improved survival [139]. Independent risk factors for mortality in patients who underwent colectomy that have been found among multiple studies include: the development of shock (need for vasopressors), increased serum lactate (≥5 mmol/L), mental status changes, end organ failure, renal failure, and the need for preoperative intubation and ventilation [29,35,137,140,141]. The more negative prognostic signs a patient has, the earlier surgical consultation and operative management should be considered. The established operative management of severe, complicated CDI has been subtotal colectomy with end-ileostomy [139]. However, recently an alternative surgical treatment with creation of a diverting loop ileostomy, followed by colonic lavage, has been shown to reduce morbidity and mortality, while preserving the colon. The surgical approach involves the laparoscopic creation of a diverting loop ileostomy. The colon is then lavaged in an ante-grade fashion through the ileostomy with a high volume of polyethylene glycol 3350 or balanced electrolyte solution and the effluent is collected via a rectal drainage tube. A catheter is placed in the efferent limb of the ileostomy to deliver vancomycin flushes in an ante-grade fashion in the postoperative period. In addition patients receive intravenous metronidazole for 10 days [142]. A multicentre randomized controlled trial is currently being conducted to provide level I evidence for possible implementation of this new treatment into standard practice [http://clinicaltrials.gov/show/ NCT01441271].

Recommendations

Total abdominal colectomy should be performed to treat CDI in case of:

» Perforation of the colon
» Systemic inflammation and deteriorating clinical condition despite maximal antibiotic therapy; this includes the clinical diagnoses of toxic megacolon, acute abdomen, and severe ileus. Colectomy should preferably be performed before colitis is very severe. Serum lactate may, *inter alia*, serve as a marker for severity (operate before lactate exceeds 5.0 mmol/L).

A future alternative to colectomy may be diverting loop ileostomy and colonic lavage, combined with antibiotic treatment (intracolonic ante-grade vancomycin and intravenous metronidazole).

C. First recurrence or (risk of) recurrent CDI

Oral antibiotic therapy

Evidence

In 3/17 randomized controlled trials of antibiotic treatment of initial CDI, results were specified for CDI prior to the study (Table 17).

Table 17. Randomized controlled trials of antibiotic treatment of initial CDI in which relapses are defined, and outcome of treatment is specified for CDI prior to study.

Study	Treatment	CDI prior to study Nr of patients (%)	Initial cure Nr of patients (%)	Relapse Nr of patients (% with initial cure)	Sustained response rate* Nr of patients (%)
[90]	Vancomycin, 125 mg qid, 10 days	5/27 (19)	4/5 (80)	1/4 (25)	3/5 (60)
	Nitazoxanide, 500 mg bid, 10 days	2/22 (9)	2/2 (100)	1/2 (50)	1/2 (50)
[70]	Vancomycin, 125 mg qid, 10 days	54/309 (17)	48/54 (89)	15/48 (31)	33/54 (61)
	Fidaxomicin, 200 mg bid, 10 days	48/287 (17)	42/48 (88)	9/42 (21)	33/42 (78)
[91]	Vancomycin, 125 mg qid, 10 days	36/257 (14)	32/36 (89)	11/32 (34)	21/36 (58)
	Fidaxomicin, 200 mg bid, 10 days analysed in: [142]	40/252 (16)	37/40 (93)	7/37 (19)	30/40 (75)

** Sustained response rate: clinical cure and no recurrences during follow up.*

Recommendations

The incidence of a second recurrence after treatment of a first recurrence with oral metronidazole or vancomycin is similar. Fewer secondary recurrences with oral fidaxomicin as compared to vancomycin after treatment of a first recurrence are reported [70, 91, 143]. However, the evidence on fidaxomicin for this specific subgroup of CDI patients is limited to two phase III studies and based on a retrospective subset analysis of data and a limited number of patients (number of patients in the modified intention to treat analysis: fidaxomicin n = 79 and vancomycin n = 80) [143]. There are no prospective randomized controlled trials performed with metronidazole, vancomycin

or fidaxomicin in this specific patient group. In addition, fidaxomicin was not associated with fewer recurrences in CDI due to PCR-ribotype 027 as opposed to non-027 in one of the randomized controlled trials [70]. Therefore, based on the evidence currently available, the SoR for treating a first recurrence of CDI with oral vancomycin or oral fidaxomicin is considered equal (B-I), unless disease has progressed from non-severe to severe.

For detailed recommendations on oral antibiotic treatment of mild/moderate initial CDI with risk for recurrent CDI or a first recurrence refer to Table 18.

Table 18. Recommendations on oral antibiotic treatment of mild/moderate initial CDI with risk for recurrent CDI or first recurrence.

Treatment	SoR	QoE	Ref(s)	Comment(s)
Vancomycin, 125 mg qid 10 days	B	I	[70, 82, 90, 91]	No statistically significant difference in recurrence rate between vancomycin and teicoplanin [1, 2, 82, 84].
Fidaxomicin, 200 mg bid 10 days	B	I	[70, 89, 91]	Evidence limited to two Phase III studies. Retrospective subset analysis: fewer secondary recurrences with fidaxomicin (n= 16/79 patients) as compared to vancomycin (n = 26/80 patients) after treatment of a first recurrence [143]. **Fidaxomicin was not associated with fewer recurrences in CDI due to PCR ribotype 027 as opposed to non-027** [70].
Metronidazole, 500 mg tid 10 days	C	I	[27, 88]	Recurrence rate: metronidazole not inferior to vancomycin for treatment of mild primary CDI [2, 82, 88] or after a first recurrence [27]. Vancomycin significantly more effective in bacteriological cure than metronidazole in recurrent CDI [69].
Vancomycin, 500 mg qid 10 days	C	III	[80, 84]	One randomized controlled trial on dose effectiveness in primary CDI: no significant differences in responses of high-dose compared to low-dose regimens vancomycin. However results not stratified for recurrent CDI [80].

D. Multiple recurrent CDI

Antibiotic and non-antibiotic treatment strategies

Evidence

Tables 19 and 20 report the evidence from randomized trials and observational studies with comments on methodology. Evidence not included in the previous ESCMID guideline [1] , is highlighted in green.

Table 19. Randomized controlled studies of treatment of recurrent CDI.

Trial	Treatment	Nr. of patients	Failure* [%]
Faecal or bacterial instillation			
[144]	Vancomycin 500 mg qid, 14 days Vancomycin 500 mg qid, 14 days + bowel lavage Vancomycin 500 mg qid, 4 days + bowel lavage + nasoduodenal infusion donor faeces	13 13 16	69 77 19
	3/16 patients with failure after first donor faeces infusion received second infusion from a different donor: 2/3 resolved. Treatment with donor faeces was superior to either of the vancomycin regimens (both P<0.001). Open label. No definition of diarrhoea. Study terminated by use of Haybittle-Peto rule at unplanned interim analysis. Fecotherapy group was older, had more co-morbidities, higher creatinine, and more infections with PCR ribotype 027. Other characteristics were comparable.		
Probiotics			
[125]	Vancomycin or metronidazole + *Saccharomyces boulardii* $2 \cdot 10^{10}$ CFU/day, 4 weeks	26	35
	Vancomycin or metronidazole + placebo	34	65
	Double-blind. No control for type, duration or dose of antibiotic. Unclear definition of relapse. Follow-up 8 weeks after start of treatment. p = 0.04 for comparison of failure rates.		
[145]	Vancomycin 500 mg qid, 10 days, followed by *Saccharomyces boulardii* $2{\cdot}10^{10}$ CFU/d, 4 wks	18	17
	Vancomycin 500 mg qid, 10 days, followed by placebo	14	50
	Vancomycin 125 mg qid, 10 days, followed by *Saccharomyces boulardii* $2{\cdot}10^{10}$ CFU/d, 4 wks	45	51
	Vancomycin 125 mg qid, 10 days, followed by placebo	38	45
	metronidazole 1 g/d, 10 days, followed by *Saccharomyces boulardii* $2{\cdot}10^{10}$ CFU/d, 4 wks	27	48
	Metronidazole 1 g/ day, 10 days, followed by placebo	26	50
	Follow-up 5 months after completion of study. p = 0.05 for the comparison of failure rates in patients who received 500 mg vancomycin qid. 22% drop-out in this group. No further statistically significant differences.		
[146]	Metronidazole 400 mg tid, 10 days + *Lactobacillus plantarum* 299v $5{\cdot}10^{10}$ CFU/d, 38 days	12	42
	Metronidazole 400 mg tid, 10 days + placebo	9	67
	Double-blind. 28% drop-out. Follow-up 70 days. Difference not statistically significant..		
[147]	Vancomycin or metronidazole followed by *Lactobacillus* GG $6{\cdot}10^{11}$ CFU/d, 21 days	8	38
	Vancomycin or metronidazole followed by placebo	7	14
	Patients blinded. No control for type, duration or dose of antibiotic. Follow-up 60 days after completion of antibiotic. Difference not statistically significant.		
Passive immunotherapy with immune whey:			
[148]	Colostral immune whey 200 mL tid + placebo, 14 days	18	44
	Metronidazole 400 mg tid + placebo, 14 days	20	45
	Double-blind. Multi-centre trial. Follow-up 70 days. Difference not statistically significant.		

* *Non-response or relapse*

Table 20. Observational studies for treatment of recurrent CDI.

Trial	Treatment	Nr. of patients	Failure* [%]	Mean follow-up
Antibiotics:				
[149]	Vancomycin taper, 21 days, followed by vancomycin pulse, 21 days	22	0	6 m
[150]	vancomycin 125 mg qid + rifampicin 600 mg bid, 7 days	7	0	12 m
[69]	Vancomycin 1 – 2 g/day	14	71	59 d
	Vancomycin <1 g/day	48	54	59 d
	Vancomycin ≥2 g/day	21	43	59 d
	Vancomycin taper	29	31	80 d
	Vancomycin pulse	7	14	80 d
	Metronidazole <1 g/day	29	45	59 d
	Metronidazole 1.5 g/day	5	40	59 d
	Metronidazole 2 g/day	2	0	59 d
[151]	Vancomycin, 14 days, followed by rifaximin varying dose, 14 days	8	13	233 d
[152]	Rifaximin 400 mg tid, 14 days, followed by rifaximin 200 mg tid, 14 days	5	0	310 d
	Rifaximin 400 mg tid, 36 days	1	100	-
[153]	Rifaximin 400 mg tid, 14 days	25	36	56 d
	Severe CDI excluded. Patients unresponsive to metronidazole 500 mg tid, 5 days. Cure = negative stool PCR for TcdB. All patients had resolution of diarrhoea, but no definition or description of how this was measured is given.			
Probiotics:				
[154]	Metronidazole or bacitracin, 10 days, followed by *Lactobacillus* GG 10^{10} CFU/d, 7–10 days	5	20	-
[155]	*Lactobacillus* GG $6{\cdot}10^{8}$ CFU/day, 14 days	4	0	11 m
Faecal or bacterial instillation §				
[156]	Faecal enema faecal enema n = 15, enteric tube n = 1	16	19	(5d-3y)
[157]	Faecal or bacterial enema 2 faecal and 4 bacterial mixture	6	0	6 m
[158]	Rectal tube	7	0	2 y
[159]	Faecal instillation through colonoscope or gastrostoma	18	17	-
[160]	Lower gastrointestinal tract	6	0	(9-50 m)
[161]	Nasogastric tube, median 3 courses 2 patients died: not CDI related, 15/16 cure after first FT, 1 relapse	16	6	90 d
[162]	Faecal enema	5	0	-
[163]*	Rectal catheter	45	4	(≤1 y)

Trial	Treatment	Nr. of patients	Failure* [%]	Mean follow-up
[164]	Colonoscopy, enema Complete resolution of symptoms in 8/16 and marked reduction in 7/16	16	6	6 wk
[165]	Vancomycin 500 mg qid, followed by faecal instillation by nasoduodenal tube or colonoscopy	7	29 0 after repeated infusion	150 d
[166]	Nasogastric tube	12	17	90 d
[163]#	Faecal enema CDI in refractory IBD	6	0	8 wk
[167]	Nasogastric tube	15	27	median 4 m
[168]	Colonoscopy	37	8	12 m
[169]	Colonoscopy 1/19 non-responders after 1st FT; all cured after 2nd FT	19	5	27 m
[170]	Enema	7	0	9 m
[171]	Colonoscopy	13	15	5 m
[172]	Colonoscopy	12	0	(3 wk-8 yr)
[173]	Gastroscopy or colonoscopy	40	27	80 d
[174]	Colonoscopy	26	8	11 m
[175]	Colonoscopy 7/77 treatment failures within 90 days after treatment (early recurrence). 8/77 recurrence > 90 days after treatment (late recurrence).	77	19	17 m
[176]	Faecal enema	27	7	427 d
[177]	5/27 patients had two FT: 2/5 failures Faecal instillation through coloscope Patients with (14) and without (29) IBD. 6/43 patients had two FT: 2/6 failures	43	14	2 m
[178]	Colonoscopy Initial failures were all PCR-ribotype 027.	70	11	1 y
Immunotherapy:				
[179]	Iv gammaglobulin 400 mg/kg every 3 weeks, 4 – 6 months	5	0	5 m
[180]	Iv gammaglobulin 400 mg/kg day 1 and 21	4	0	7.5 m
	Iv gammaglobulin, varying dose	5	40	2.8 m
[56]	Iv gammaglobulin 300 to 500 mg/kg, 1 to 6 doses	5	40	86 d
[181]	Iv gammaglobulin 150 to 400 mg/kg once	14	71	6.6 m
[182]	Iv gammaglobulin 200 to 300 mg/kg once	18	33 (died or colectomy)	-
[183]	Iv gammaglobulin 75 to 400 mg/kg, 1 to 5 days	21	57 (died)	-

Non-response or relapse; d = days; m = months; wk = weeks; yr, years

*§ Reviewed by Refs. [163, 184-190]; * Louie (2008) abstract only derived from Ref. [163];*

 # Borody (2008) abstract only derived from Ref. [163].

Recommendations

In non-severe second (or later) recurrences of CDI oral vancomycin or fidaxomicin is recommended. Vancomycin and fidaxomicin are equally effective in resolving CDI symptoms, but fidaxomicin has been shown to be associated with a lower likelihood of CDI recurrence after a first recurrence [104, 143]. However, there are no prospective randomized controlled trials investigating the efficacy of fidaxomicin in patients with multiple recurrences of CDI. Vancomycin is preferably administered using tapered and/or pulsed regimen.

Recently the first randomized controlled trial on faecal enteric instillation has been published: faecal transplantation following antibiotic treatment with an oral glycopeptide is reported to be highly effective in treating multiple recurrent CDI [144].

For detailed recommendations on treatment regimens of multiple recurrent CDI refer to Tables 21 and 22.

Table 21. Recommendations on oral antibiotic treatment of multiple recurrent CDI (> 1 relapse).

Treatment	SoR	QoE	Ref(s)	Comment(s)
Vancomycin, 125 mg four times daily for 10 days, followed by pulse regimen (e.g. 125–500 mg/day every 2–3 days) for at least 3 weeks).	B	IIt	[69, 149]	Retrospective case cohort of two placebo/antibiotic trials: [125, 145]. Expert opinion [3].
Vancomycin, 125 mg four times daily for 10 days, followed by taper regimen (e.g. gradually (weekly) decreasing the daily dose by 125 mg per day)	B	IIt	[69, 149]	Retrospective case cohort of two placebo/antibiotic trials: [69, 145]. Expert opinion [3].
Fidaxomicin, 200 mg bid for 10 days	B	IIrt	[75, 143]	Evidence limited to two Phase III studies. [70,91] Retrospective subset analysis: fewer recurrences as compared to vancomycin treatment after **first** recurrence. [143]. Systematic review: [75]. **Efficacy after multiple recurrences was not investigated** [143].
Vancomycin, 500 mg qid 10-14 days	C	IIrt	[69, 75]	Retrospective case cohort of two placebo/antibiotic trials: [125, 145]. Trend for lower recurrence frequency for high-dose vancomycin [69]. Systematic review: [75].
Metronidazole, 500 mg tid 10 days	D	IIrt	[69, 75]	Retrospective case cohort of two placebo/antibiotic trials: [125, 145]. Trend for lower recurrence frequency for high-dose vancomycin and low-dose metronidazole [69]. Systematic review: [75].

Table 22. Recommendations on non-antibiotic treatment (in combination with antibiotic treatment) of recurrent CDI (> 1 relapse).

Type of intervention	Treatment	SoR	QoE	Ref(s)	Comment(s)
Faecal or bacterial instillation	Vancomycin, 500 mg qid, 4 days + bowel lavage + nasoduodenal infusion donor faeces	A	I	[144]	Also many observational studies and meta-analyses. [163,185,188-190].
Probiotics	vancomycin or metronidazole + *Saccharomyces boulardii*	D	I	[125]	Comparison of relapse rates: in subgroup analysis efficacy in recurrent CDI, but *not* in initial CDI. Evidence based review: [136].
	Vancomycin or metronidazole + *Lactobacillus* spp.	D	I	[146, 147]	Evidence based review: [136].
Passive immunotherapy with immune whey	Colostral immune whey	D	I	[148]	Study interrupted early.

E. Treatment of CDI when oral administration is not possible

Evidence

Metronidazole remains the only parental antibiotic therapy supported by case series [191]. Intravenous metronidazole (500 mg IV tid) may be added to oral vancomycin, if the patient has ileus or significant abdominal distension [4, 44]. However, there are no randomized controlled trials available to guide this recommendation.

It is still unknown how to best treat patients with ileus due to CDI. There are some anecdotal reports on delivery of vancomycin to the gut by other means than orally, mainly through intracolonic delivery. Questions regarding the efficacy, optimal dosing and duration of treatment with intracolonic vancomycin remain unanswered [192, 193]. Prospective clinical trials with other antibiotics, like tigecycline, have not yet been performed to support general use [121, 194].

Recommendations

When oral treatment is not possible, parenteral metronidazole is recommended, preferably combined with intracolonic or nasogastric administration of vancomycin. Parenteral tigecycline as salvage therapy is only recommended with marginal strength. For detailed recommendations refer to Table 23.

Table 23. Recommendations on non-oral antibiotic treatment of initial CDI: mild and severe disease.

Patient subgroup	Treatment	SoR	QoE	Ref(s)	Comment(s)
Non-severe disease	Metronidazole iv 500 mg tid iv for 10 days	A	IIu	[191]	Retrospective uncontrolled study [191].
Severe disease complicated or refractory CDI	Metronidazole 500 mg tid iv for 10 days + vancomycin retention enema 500 mg in 100 mL normal saline qid intracolonic	A B	IIru III	[191-193]	Retrospective uncontrolled study [191]. Systematic review [192, 193]. Expert opinion [3].
	Metronidazole 500 mg tid iv for 10 days + vancomycin 500 mg qid by oral/ nasogastric tube for 10 days	A B	IIru III	[191-193]	Retrospective uncontrolled study [191]. Systematic review [192, 193]. Expert opinion [3].
	Tigecycline iv 50 mg bid for 14 days	C	III	[121]	Observational study/case report [121].

Summary of definitions

Episode of Clostridium difficile infection (CDI)

A clinical picture compatible with CDI and microbiological evidence of free toxins and the presence of *C. difficile* in stool, without reasonable evidence of another cause of diarrhoea

or

pseudomembranous colitis (PMC) diagnosed during endoscopy, after colectomy or on autopsy.

Clinical pictures compatible with CDI

Diarrhoea: loose stools, *i.e.* taking the shape of the receptacle or corresponding to Bristol stool chart types 5-7, plus a stool frequency of three stools in 24 or fewer consecutive hours, or more frequently than is normal for the individual.

Ileus: signs of severely disturbed bowel function such as vomiting and absence of stool with radiological signs of bowel distension.

Toxic megacolon: radiological signs of distension of the colon (>6 cm in transversal width of colon) and signs of a severe systemic inflammatory response.

Severe CDI

Severe or life-threatening CDI is defined as an episode of CDI with (one or more specific signs and symptoms of) severe colitis or a complicated course of disease, with significant systemic toxin effects and shock, resulting in need for ICU admission, colectomy or death.

One or more of the following unfavourable prognostic factors can be present without evidence of another cause:

- Marked leucocytosis (leukocyte count >15 · 10^9/L)
- Decreased blood albumin (<30 g/L)
- Rise in serum creatinine level (≥133 μmol/L or ≥1.5 times the premorbid level)

Recurrent CDI

Recurrence is present when CDI re-occurs *<8 weeks* after the onset of a previous episode, provided the symptoms from the previous episode resolved after completion of initial treatment.

Treatment response

Treatment response is present when after therapy either stool frequency decreases or stool consistency improves and parameters of disease severity (clinical, laboratory, radiological) improve and no new signs of severe disease develop.

Treatment response should be daily observed and evaluated after at least 3 days, assuming that the patient is not worsening on treatment. Treatment with metronidazole, in particular, may result in a clinical response only after 3–5 days. After clinical response, it may take weeks for stool consistency and frequency to become entirely normal.

Summary of treatment recommendations

Strength of Evidence (SoE: I to III) and Strength of Recommendation (SoR: A to D) are shown between brackets. For grading definitions we refer to Tables 1 and 2.

Asses severity and identify recurrent disease (or risk of recurrent disease) before initiation of treatment.

A. Initial CDI: non-severe disease

Non-antibiotic treatment

In non-epidemic situations and with (non-severe) CDI clearly induced by the use of antibiotics, it may be acceptable to stop the inducing antibiotic and observe the clinical response for 48 hours, but patients must be followed very closely for any signs of clinical deterioration and placed on therapy immediately if this occurs (C-II).

Oral antibiotic treatment

Metronidazole po 500 mg tid for 10 days (A-I)
Vancomycin po 125 mg qid for 10 days (B-I)
Fidaxomicin po 200 mg bid for 10 days (B-I)

B. Severe CDI

Oral antibiotic treatment

Vancomycin po 125 mg qid for 10 days (A-I)

Fidaxomicin po 200 mg bid for 10 days (B-I)

Notes:

» *It can be considered to increase the vancomycin dosage to 500 mg qid for 10 days (B-III)*

» *There is no evidence that supports the use of fidaxomicin in life-threatening CDI (D-III)*

The use of oral metronidazole in severe CDI or life-threatening disease is strongly discouraged (D-I).

Surgical treatment

Total abdominal colectomy with ileostomy should be performed in case of:

» Perforation of the colon

» Systemic inflammation and deteriorating clinical condition not responding to antibiotic therapy; including toxic megacolon, an acute abdomen and severe ileus.

Surgical treatment should preferably be performed before colitis is very severe. Serum lactate may, inter alia, serve as a marker for severity (operate before lactate exceeds 5.0 mmol/L).

A future alternative to colectomy may be diverting loop ileostomy and colonic lavage, combined with *antibiotic treatment* (intracolonic ante-grade vancomycin and intravenous metronidazole).

C. First recurrence or risk of recurrent disease

Oral antibiotic treatment

Fidaxomicin po 200 mg bid for 10 days (B-I)

Vancomycin po 125 mg qid for 10 days (B-I)

Metronidazole po 500 mg tid for 10 days (C-I)

Note: Fidaxomicin was not associated with fewer recurrences in CDI due to PCR-ribotype 027 as opposed to non-027 ribotypes.

D. Multiple recurrent CDI

Oral antibiotic treatment

Fidaxomicin po 200 mg bid for 10 days (B-II)

Vancomycin po 125 mg qid for 10 days followed by pulse strategy (B-II)

or

Vancomycin po 125 mg qid for 10 days followed by taper strategy (B-II)

Non-antibiotic treatment in combination with oral antibiotic treatment

For multiple recurrent CDI unresponsive to repeated antibiotic treatment, faecal transplantation in combination with oral antibiotic treatment is strongly recommended (A-I).

E. Treatment of CDI when oral administration is not possible

Antibiotic treatment

Non-severe CDI: metronidazole iv 500 mg tid for 10 days (A-II)

Severe CDI: metronidazole iv 500 mg tid for 10 days (A-II) + vancomycin retention enema 500 mg in 100 mL normal saline qid intracolonic or vancomycin 500 mg qid by oral/nasogastric tube for 10 days (B-III)

A schematic overview of currently available therapeutic regimens for CDI, including the quality of evidence (QoE: I to III) and strength of recommendations (SoR: A to D) are shown in Figure 1.

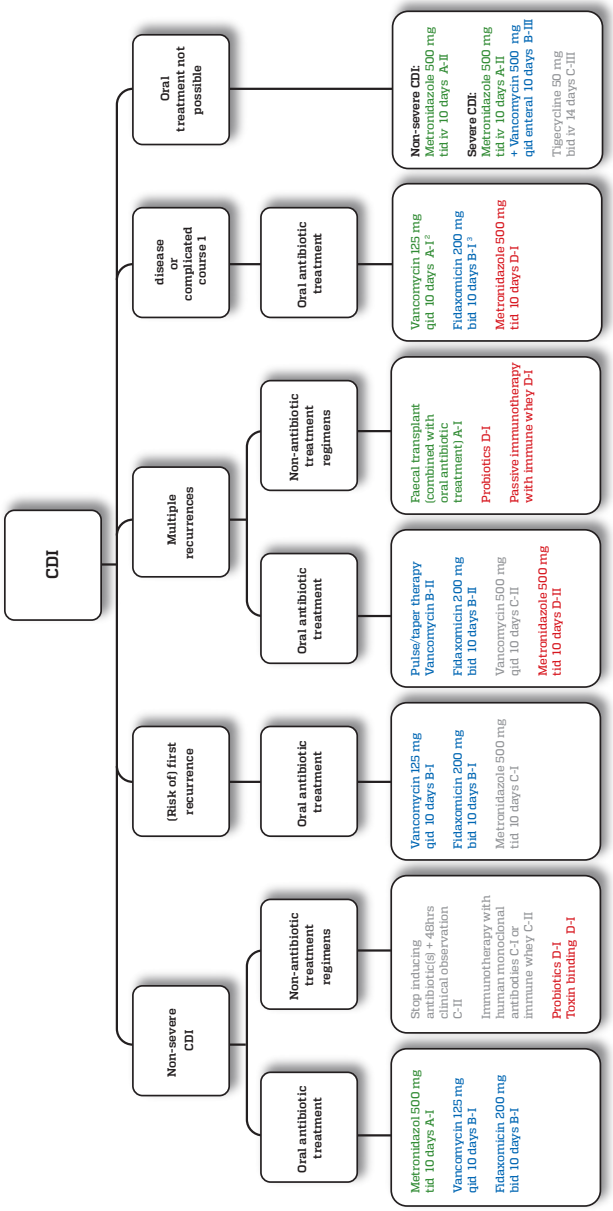

Figure 1. Schematic overview of therapeutic regimens for CDI. [1]Severe CDI or complicated course: surgical therapy not included in this overview; [2]It can be considered to increase the oral dosage of vancomycin to 500 mg qid 10 days (B-III); [3]There is no evidence that supports the use of fidaxomicin in **life threatening** CDI (D-III); *SoR A=green* (**Strongly** *supports a recommendation for use); SoR B=blue* (**Moderately** *supports a recommendation for use); SoR C=grey* (**Marginally** *supports a recommendation for use); SoR D=red* (Recommendation **against** use).

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Authorship

Four draft versions of this guideline document were written by three authors (MB, EK, SD) and critiqued by the Committee and Advisors. A consensus was reached, resulting in the final version.

Transparency Declaration

Authors

The authors declare that they have no conflicts of interest.

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Acta est fabula

Chapter 8

General Discussion

Introduction

In the last decade, CDI has become evidently the leading cause of healthcare-associated diarrhoea in Europe [1-3]. Compared to other important healthcare-associated infections (HCAI), CDI is even so underestimated given the rates of morbidity and mortality (15-25 % within 30 days of infection in outbreaks [4] and up to 10% in endemic situations [2,5]). The seriousness of CDI as an HCAI was illustrated for example in a study, which included 28 community hospitals in southern United States [6]. This study revealed that *C. difficile* has replaced methicillin-resistant *Staphylococcus aureus* (MRSA) as the most common cause of HCAI [6]. In German hospitals, nosocomial CDI incidence was twice as high as that of nosocomial MRSA [7]. Obviously, much can still be improved in infection prevention control measures, as this study also showed that nosocomial MRSA and CDI were associated statistically significant [7].

The first PCR-ribotype 027 *C. difficile* outbreaks signalled the beginning of a continuous rise of the incidence of CDI worldwide [1,3]. In the US, rates of hospital discharges with CDI as any of the listed diagnoses rose from an averaged 3.82 per 1,000 discharges in 2000 to an average of 8.75 per 1,000 discharges in 2008 (Figure 1). In particular in elderly patients (Figure 1), a strong association between a hypervirulent PCR-ribotype 027 infection, severe CDI and mortality attributable to CDI was demonstrated [8].

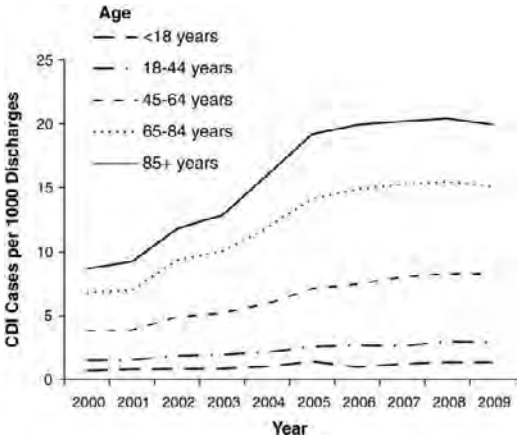

Figure 1. Age-categorized discharge rates for CDI from US short-stay hospitals [9].

Outbreaks with this hypervirulent *C difficile* strain were just reported in Canada [10], United States [11] and United Kingdom [12], when in 2005 the first Dutch hospital outbreak of severe CDI with PCR-ribotype 027 in a hospital in Harderwijk was discovered (Chapter 2). It was the onset for the investigations presented in this thesis.

Shortly after this first acknowledged outbreak, a second epidemic was revealed in a hospital distanced 35 km from the Harderwijk hospital (Chapter 2). The reporting of these cases provoked three other Dutch hospitals to report their observed increased incidences of severe CDI, which could also be ascribed to PCR-ribotype 027 *C. difficile*. In fact, retrospectively, CDI in two of these hospitals had increased evidently in 2002 and 2004 (results not shown). Subsequently, an increase in CDI incidence was noticed in two new hospitals when they sent their samples to the Dutch *C. difficile* reference laboratory for PCR-ribotyping [13]. Analysis of these samples also disclosed the involvement of the hypervirulent ribotype 027.

These events emphasized the need for a better awareness of CDI, continuous surveillance and PCR-ribotyping in the early detection of CDI outbreaks in healthcare facilities, at least in the Netherlands. As soon as CDI is detected, control measures have to be enforced to prevent and/or limit further spread of the infection [13]. This thesis describes and substantiates in Chapter 2 the importance of swiftly launched adequate and dedicated control measures in terms of hygiene and antibiotic stewardship, known as the "bundle approach", as soon as the cause of the HCAI by *C. difficile* and, preferably, the identity of the ribotype, is known.

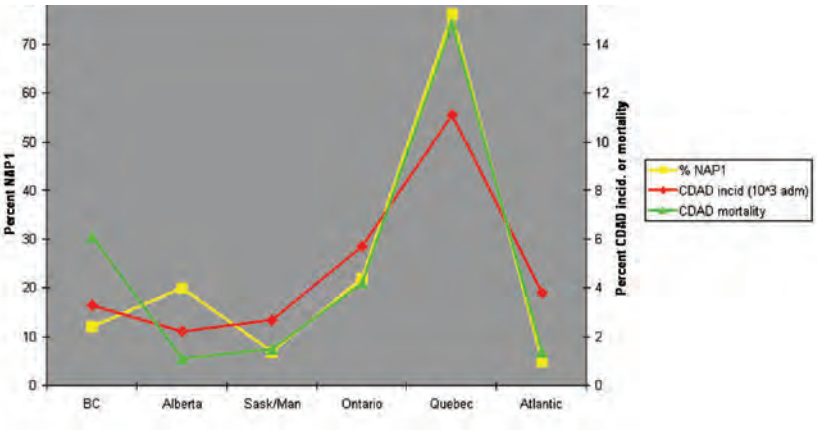

Figure 2. *C. difficile*-associated disease (CDAD) incidence, CDAD-attributable mortality and fraction of *C. difficile* PCR-ribotype 027 isolates (%027 (NAP1) as a proportion of provincial total), in Canada in 2005 by province [8].

Outbreak control

Antibiotic stewardship

Despite intensive hygiene measures, cohorting patients in a separate ward, education and instruction of staff and intensified environmental cleaning, the CDI outbreak in the Harderwijk hospital continued (Chapter 2).

Only after very restricted use of cephalosporins and a complete ban on the application of fluoroquinolones, in which all involved physicians completely cooperated, the outbreak could be halted and the causative microorganism eradicated. The decay of CDI incidence after restrictive use of specific antibiotics and its increase again upon the reintroduction of fluoroquinolones, revealed an important role of these pharmaceuticals in the spread of PCR-ribotype 027-related CDI (Chapter 2).

The importance of antibiotic stewardship, and of a very restricted use of fluoroquinolones in particular in CDI outbreak control, was confirmed by Kallen *et al.* [14]. In compliance with our results, these authors demonstrated also a significant decline in CDI after a restriction, but not complete ban, on the use of fluoroquinolones (Figure 3). With our current knowledge of the

pathology and epidemiology of the infection, the relative slow decay of incidence (Figure 3) could have been much steeper if the fluoroquinolones were banned instead of restricted in their use. In the case of a CDI outbreak on a surgical ward, a non-027 *C. difficile* PCR-ribotype (ribotype 106) highly resistant to clindamycin was involved [15]. The outbreak was most likely associated with the administration of clindamycin and ciprofloxacin, as the outbreak was ended by complete removal of these two antibiotics from the involved unit and use within the surgical directorate was restricted.

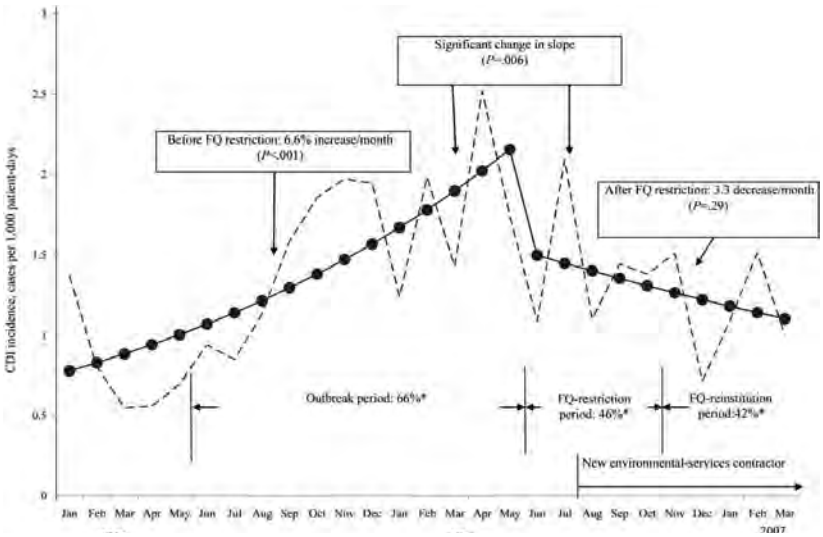

Figure 3. Rate of hospital-onset CDI (dashed line), this rate when predicted from an interrupted time-series model (solid line) and percentage of epidemic *C. difficile* strain isolates (asterisks) [14]. FQ, fluoroquinolone.

Labbé *et al.* found evidence that continued selective antibiotic pressure is associated with the development of antibiotic-resistant *C. difficile* clones and of CDI caused by, in particular, fluoroquinolone-resistant ribotype 027 and clindamycin-resistant ribotype 001 [4]. Although its mechanism is not clear, the application of fluoroquinolones may contribute to the spread and severity of CDI by inducing spore and cytotoxin production [16,17]. Saxton *et al.* demonstrated that ciprofloxacin, moxifloxacin and levofloxacin stimulate germination and cytotoxin production by *C. difficile* PCR-ribotypes 027 and 001, despite differences in their extent of inhibiting gut flora [17]. In their study, early toxin production was observed only for the PCR-ribotype 027 variants suggesting strain-specific responses towards fluoroquinolone exposure.

Recently, the effects of sub-inhibitory concentrations of ciprofloxacin on Toxin A and B gene expressions and protein production in two PCR-ribotype 027 clinical isolates were investigated [18]. One strain had a high and the other strain had a low-level ciprofloxacin resistance. *In vitro,* the strains exhibited distinct differences in exotoxin production following ciprofloxacin exposure. These results demonstrate that identical *C. difficile* PCR-ribotypes can respond differently towards antibiotic pressure with increased toxin production being highest in highly resistant strains [18]. With this outcome it can be anticipated that fluoroquinolones will increase the incidence and severity of CDI when patients are colonized with highly fluoroquinolone-resistant *C. difficile* PCR-ribotype 027. The conclusion underpins the importance of determining the antimicrobial susceptibility of clinical isolates.

C. difficile PCR-ribotype 027 isolates have shown widespread resistance to ciprofloxacin, but also to newer fluoroquinolones such as moxifloxacin and levofloxacin [19]. Acquisition of fluoroquinolone resistance in PCR-ribotype 027 *C. difficile* isolates has been associated with a single transition mutation in DNA gyrase A (GyrA) [17,20-23]. The mutation (C to T) results in the substitution of Thr-82 by the amino acid Ile (Thr-82-Ile) in the active site of GyrA [17,21,23]. Spigaglia *et al.* described this mutation as the most common cause of resistance in 73 multidrug-resistant isolates affiliated with 10 ribotypes collected in 14 countries in Europe [21].

The antimicrobial resistance-driven selection of specific PCR-ribotypes is considered an important factor that has led to an increase in the incidence of hypervirulent *C. difficile* strains and the global change in the epidemiology of CDI [24,25]. The apparent spreading of epidemic *C. difficile* strains may be the result of selective pressure by widespread fluoroquinolone use and thus development of resistance in *C. difficile*. Mena *et al.* showed that application of levofloxacin could select Thr-82-Ile GyrA mutants *in vivo*, conferring resistance also to newer fluoroquinolones [20]. Because identical mutations, like the Thr-82-Ile GyrA, are found in epidemic (*e.g.* PCR-ribotypes 027 and 001) as well as non-epidemic strains (*e.g.* PCR-ribotype 014 and 046), it is suggested that fluoroquinolone resistance alone cannot explain the sudden increase in prevalence of PCR-ribotype 027 [19].

The work presented in Chapters 2 and 4 confirmed that fluoroquinolones represent a critical and independent risk factor for CDI. The risk of developing CDI was extremely high in patients receiving a combination

of cephalosporins and fluoroquinolones (Chapter 2). This was a surprising finding crucial for deducing effective control measures (Chapter 2). The fact that the OR in these patients was much higher than simply summing the ORs for the separate antibiotics, suggested a synergistic effect of cephalosporins and fluoroquinolones in the aetiology of CDI. We were not able to elucidate this synergy, and more studies are needed to unravel the biological effects of (combinations of) antibiotics and/or other agents on the growth, spore-forming capability and toxin-production of the pathogenic bacterium.

Several studies confirmed that stringent antibiotic stewardship measures combined with aggressive infection control are required to combat outbreaks of *C. difficile* infections. This so-called "bundle approach" in outbreak control of CDI is described in this thesis in Chapters 2 and 4 and has been recommended by the ESCMID [26].

The bundle approach includes *i*) early diagnosis of CDI, *ii*) surveillance of CDI cases, *iii*) education of staff, *iv*) appropriate use of isolation precautions, *v*) hand hygiene, *vi*) protective clothing, *vii*) environmental cleaning and cleaning of medical equipment, *viii*) good antibiotic stewardship and *ix*) other very specific measures during outbreaks.

The general outbreak measures are [26]:

1. Infection control staff should always be informed when there is an increased number or augmented severity of CDI cases.

2. All hygiene rules should be enforced in case of a CDI outbreak.

3. Review the standard of environmental cleaning to ensure high-quality and high frequency of decontamination. If possible, implement a designated, well-trained and well-instructed cleaning team especially for the rooms where CDI patients reside.

4. Perform good antibiotic stewardship. Antimicrobial prescribing (frequency, duration and types of agents) should be reviewed as soon as possible, with emphasis on avoiding the use of high-risk agents (*i.e.* cephalosporins, fluoroquinolones and clindamycin) in at-risk patients. Use these agents only when medically needed and alternatives are exhausted.

5. Faecal samples from all CDI cases should be collected and stored for the purpose of culturing and typing, retrospectively if needed.

6. In order to elucidate the epidemiology of *C. difficile*, isolates from infected patients should ideally be compared using molecular methods.

7. Implement interim policies for patient admissions, placement and staffing as needed to prevent *C. difficile* transmission.

8. Implement isolation procedures and dedicate nursing staff.

9. When transmission continues despite the assignment of dedicated staff, close the unit or facility for new admissions.

10. When transmission continues despite all of the above measures, vacate the unit for intensive environmental cleaning to eliminate all potential environmental reservoirs of *C. difficile*.

Existing local protocols and practices for the control of *C. difficile* should be carefully reviewed and modified according to these advised measures.

Chapter 4 shows that risk factors for the development of CDI may depend on the PCR-ribotype involved. General and ribotype-specific risk factors as well as outcome parameters for CDI due to ribotype 027 or 017 were investigated during a hospital outbreak in which both PCR-ribotypes occurred simultaneously. We found that nasogastric intubation, recent hospitalization and use of cephalosporins and clindamycin were general risk factors for the development of CDI. A ribotype-specific risk factor is 'older age' for 017 and 027 in comparison with other PCR-ribotypes. The use of clindamycin and immunosuppressive agents were specific risk factors for PCR-ribotype 017, and the use of fluoroquinolones for ribotype 027.

Resistance to clindamycin (MIC >256 mg/L) was found in nearly all ribotype 017 isolates (95%), whereas all ribotype 027 isolates were susceptible for clindamycin showing MICs ≤4 mg/L for this drug (Chapter 4). However, although both ribotypes 027 and 017 were resistant to ciprofloxacin, high exposure to fluoroquinolones was a specific risk factor exclusively for PCR-ribotype 027. This is an intriguing finding. To explain this difference it was suggested that fluoroquinolones affect the host defence against ribotype

027 by specific changes of the microbiota [17]. Alternatively, fluoroquinolones may increase the spread of ribotype 027 by stimulated sporulation and toxin production of this germ specifically [17]. This implies that besides general outbreak control measures, ribotype-specific measures may have to be taken to prevent and/or combat outbreaks. Good antibiotic stewardship in CDI outbreaks is thus not only steered by the information available on its antimicrobial susceptibility, but also on the involved PCR-ribotype of the outbreak strain.

The results from continuous surveillance of the incidence and of antimicrobial susceptibility of circulating PCR-ribotypes within healthcare facilities will assist the choice of specific measures. Such choices are for example a restriction in or ban on the use of fluoroquinolones in the case of an outbreak with ribotype 027 versus restriction of clindamycin in the case of an outbreak with PCR-ribotype 017.

In addition, risk factors for endemic CDI may differ from epidemic CDI [27,28]. In a study by Hensgens *et al.*, risk factors that have been ascribed to epidemic CDI, such as use of fluoroquinolones and proton pump inhibitors, did not influence the risk of endemic CDI [27]. Independent risk factors for endemic CDI were the use of second-generation cephalosporins, previous hospital admission and previous stay at the intensive care unit (ICU). The use of third-generation cephalosporins was a risk factor for diarrhoea in general.

To enable targeted preventive and/or infection control measures in endemic or epidemic CDI, it is clear that much more research on the role of ribotype- and/or strain-specific risk factors is needed. The specific research questions are formulated in the "Future Perspectives and Recommendations" at the end of this Chapter.

Laboratory diagnosis

An important observation in the study presented in Chapter 3 is that repeated testing of stools for *C. difficile* toxin is of value in controlling outbreaks of *C. difficile* infection. CDI was diagnosed in 5% from follow-up samples obtained within one week after a first negative test. The significance of this finding is that the availability of a highly sensitive and specific screening test in order to identify CDI patients as quickly as possible in the course of an outbreak is essential.

Guidelines for the diagnosis of CDI recommended the analysis of sequential stool samples for *C. difficile* toxins when the first laboratory sample was negative but clinical suspicion of CDI persisted [29]. Our increased diagnostic yield by repeated testing with EIA was reproduced by others [30,31]. This relatively small sensitivity improvement by repeated testing in case of a first negative result was, however, disputed [30,32,33]. On the other hand, repeated testing has been shown to increase the diagnostic yield of the toxin-EIA even more than 5-10% in specific patients groups, such as in irritable bowel diseases (IBD) patients. Approximately one in five IBD patients with CDI required repeated testing to yield a toxin-positive result [34].

It should be noted here, however, that repeated toxin-testing of stool samples had only been evaluated in non-outbreak situations and/or by evaluating data from the laboratory without any correlation to patients' symptoms [30-34]. In an epidemic setting with high prevalence of CDI, the negative predictive value of the toxin-assay will be lower. We concluded that in such setting, repeated testing of stools will be of value to detect additional cases.

Besides sensitivity, Litvin *et al.* demonstrated the importance of the specificity of a diagnostic test in repeated testing of CDI [35]. The authors showed that repeated testing entails a greater chance of a false positive test, which might lead to the call for false CDI outbreaks. False outbreaks result in unnecessary CDI prevention measures, which increase healthcare costs, and which may have various adverse effects on patients. In general, the toxin-detecting EIA has been shown to be less accurate than cell cytotoxicity assays and toxigenic culture, and its use as a stand-alone test results in missed CDI cases (false negatives) and cases being incorrectly assigned to CDI (false positives) [36-39].

Choice of laboratory tests

Two tests are currently of interest for medical microbiological laboratories to implement in routine diagnostics of CDI; the glutamate dehydrogenase (GDH) test and PCR. The enzyme GDH, is produced in large amounts by all strains of *C. difficile* and can be exploited as marker for the presence of *C. difficile* [40]. Assays detecting this enzyme have been introduced as an alternative for the detection of *C. difficile* in stool samples [36,40-44]. In a meta-analysis on its usability and fitness to confirm the presence of *C. difficile* in faeces, it was concluded that GDH detection has a high relative diagnostic accuracy,

sensitivity (>90%) and specificity (>90%), when compared to selective culture as reference method. However, GDH is expressed by toxigenic as well as non-toxigenic strains of *C. difficile*. The GDH test is therefore only a powerful tool for the identification of pathogenic *C. difficile* in a two-step testing algorithm, in which a positive GDH result is followed by a second confirmatory test detecting toxins and/or toxin genes.

Most of the diagnostic PCRs to diagnose CDI are directed to TcdA and/or TcdB. Because a positive PCR-analysis alone cannot differentiate infection from asymptomatic carriage, a two-step testing algorithm that includes a toxin test, is recommended by the ESCMID as well [35]. A combination of real-time PCR assays and GDH detection is considered to be superior to Toxin A and B-detecting EIA's as a standard rapid diagnostic test in epidemic situations [45].

In general, rapid and accurate diagnosis of CDI is essential for patient management, implementation of infection control measures and thus intervention of the spreading of the infection. Recently, Barbut *et al.* compared the impact of three different diagnostic strategies on patient care: *i*) stool cytotoxicity assay/toxigenic culture, *ii*) PCR, and *iii*) a two-step algorithm based on GDH detection followed by PCR. When applying a PCR test (*ii*) or a two-step algorithm (*iii*), the time-to-result is significantly shorter compared to a culture (*i*), so that CDI patients were treated earlier and empirical therapy of patients without CDI decreased [46].

Despite the recommendation of a two-step testing algorithm by the ESCMID, the EIA for toxin detection is still often used as a stand-alone test in Europe [47], which may have hindered adequate intervention in past CDI cases. There is thus a clear need for a consensus on optimal and conscientious application of agreed testing protocols for *C. difficile* infections in order to further optimize diagnostics and improve CDI surveillance in Europe.

Epidemiology

Surveillance

Continuous surveillance is an important and useful tool to assess the epidemiology of CDI. Surveillance results are also used to assist and evaluate prevention and control measures. After the first outbreaks of *C. difficile* PCR-ribotype 027 in the Netherlands in 2005, a national CDI surveillance was started by the Leiden University Medical Centre (LUMC) and the Centre for Infectious Disease Control (CIb) of the National Institute for Public Health and the Environment (RIVM). This surveillance transformed into a continuous Sentinel surveillance in 20 hospitals to evaluate the changes in epidemiology and distribution of circulating *C. difficile* PCR-ribotypes nation-wide [27].

Since 2005, national guidelines have been developed to rapidly recognize ribotype 027 infections and prevent further spreading. By 2009, a significant decrease in PCR-ribotype 027-associated CDI in the Netherlands was reported [53]. This decrease was only possible and is the result of the high-degree of participation of healthcare facilities, in particular of hospitals, and the consciousness, understanding and good collaboration of all stakeholders, including laboratories, practitioners of multiple disciplines, infection control practitioners and many other experts. The vast stream of information developed valuable awareness for CDI not only of medical personnel, but also of the patients themselves. The doctors in charge supported the measures for the prevention of infections and ordered a higher level of hygiene in the facilities (personal professional experience).

The distribution of the five most common PCR-ribotypes in the Netherlands between April 2005 and June 2009 is depicted in Figure 4 [53]. A decrease was seen in the number and incidence of ribotype 027 after the second half of 2006. In the first half of 2009, the percentage of ribotype 027 isolates among all CDI cases decreased to 3.0%, whereas ribotype 001 increased to 27.5%. PCR-ribotype 014 was present in 9.3% of the isolates and *C. difficile* ribotype 078 slightly increased to 9.1%.

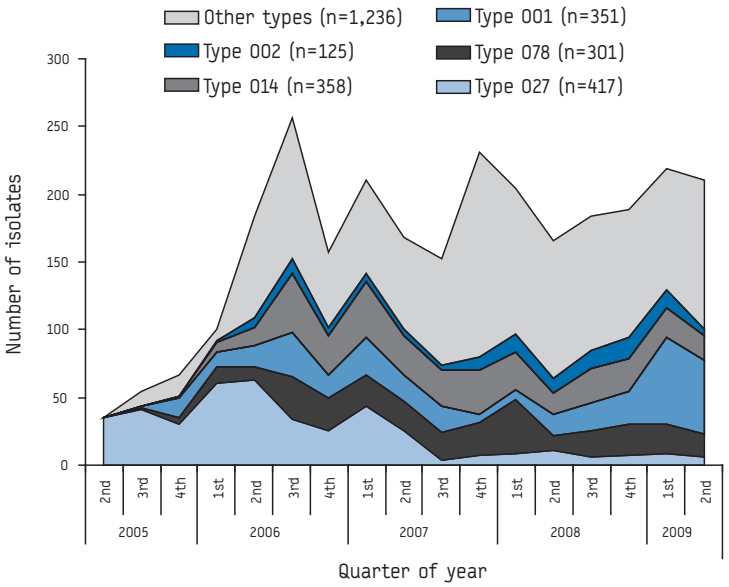

Figure 4. Prevalence of *C. difficile* PCR-ribotypes in the Netherlands (April 2005 to June 2009) [53].

Recently the seventh annual report of the sentinel surveillance described that despite the decrease by 2009, PCR-ribotype 027 was, unexpectedly, found more frequently (20%) between May 2012 and May 2013, compared to the year 2011-2012 (15%) [48]. The re-emergence of PCR-ribotype 027 appeared to be attributable to a large outbreak in one hospital and its surrounding nursing homes.

The unexpected rise of ribotype 027 and its explanation shows the importance of continuous surveillance of CDI cases in all types of healthcare facilities. *C. difficile* has namely been identified as the most common cause of non-epidemic acute diarrheal illness in nursing homes [49-51]. It should be noted that microbiological diagnostics of diarrhoea is not routinely performed in nursing homes [52], so that the real incidence of CDI may be more problematic than at first glance. The prevalence of *C. difficile* colonization in nursing home residents in the absence of a recognized outbreak, ranges from 4% to 20% [49,52]. It is still subject of research to value the contribution of CDI-associated disease in nursing and elderly home residents in the total CDI load. Undoubtedly, nursing home residents, who are transferred for medical care to a hospital, can be a source for (propagation of) CDI infections in

the clinic. This is not to exclude the *vice versa* route; discharged patients returning to their elderly or nursing home may be a potential source for CDI in these homes through carriership or infection.

Clostridium difficile ribotyping networks (CDRN) were established in The Netherlands in 2004 and in the UK in 2007. Both networks became part of enhanced CDI surveillance to facilitate the recognition and control of epidemic strains. Wilcox *et al.* reported changes in CDI epidemiology during the first three years [54]. By providing timely data on ribotypes to infection prevention teams across England, the CDRN enabled interventions in high-incidence CDI settings and particularly those with a high prevalence of ribotype 027. In a similar way as in the Dutch situation (see last few paragraphs above), the proportion of CDIs caused by ribotype 027 declined markedly as a result of the timely interventions facilitated by the network. On the other hand, the English CDRN also reported a significant increase in prevalence of other *C. difficile* ribotypes, such as 014/020, 015, 002, 005, 023, 016 and 078, [54]. By 2011, 15 European countries followed this success and had installed a national or regional network for CDI surveillance.

As a consequence of intensified surveillance, which included PCR-ribotyping of CDI isolates, we became more familiar with the distribution and incidence of toxigenic *C. difficile*. The increase of ribotype 078 that was noticed in the Netherlands since 2006 [55] is a direct outcome of this well-organised surveillance on a national level. In the first trimester of 2008, 19% of all samples collected from 14 Dutch hospitals were ribotype 078-positive [27]. By 2009 ribotype 078 had become the third most common *C. difficile* strain in the Netherlands. In several other European countries, the emergence of ribotype 078 was observed as well [2].

It is obvious that the current (professional) interactions, patient-patient, nursing personnel-patient and environmental contacts in healthcare and nursing facilities are very complex. It warns us that a dense and high-quality level of surveillance should include all relevant (health)care facilities at risk of CDI. Standards for analysis of samples must comprise ribotyping to identify the spreading of *C. difficile* strains. Acquired and interpreted information should then be disseminated correctly and without delay to notify and update all actors in CDI prevention. In this way, prevention of CDI is secured to the best of our know-how and in an advanced fashion.

C. difficile in animals and humans

The national surveillance data and several reports on veterinary cases of CDI in animals, prompted us to inspect outbreaks of disease involving watery and pasty diarrhoea in piglets more closely. It was already shown that *C. difficile* PCR-ribotype 078 is a predominant strain in several farm animals, in particular, in pigs and dairy calves [56,57]. Worrisome was the finding of this ribotype in retail meat products [58]. In addition, this was the ribotype of which its presence is increasing not only in the Netherlands [55], but in several other countries as well [2].

This thesis showed that strains isolated from CDI-affected piglets displaying comparable clinical signs as humans suffering from CDI, were pheno- and genotypically indistinguishable from *C. difficile* PCR-ribotype 078 strains extracted from human CDI patients (Chapter 5). This was repeated in another study focussing on isolates obtained from piglets shortly after their birth [59]. Piglets up to seven days old can be affected and present diarrhoea varying from yellow to orange and from pasty, slimy to watery [60]. Some piglets with CDI are non-diarrheic, but may be constipated or obstipated, although colitis was seen at necropsy of such animals [61]. Although mortality attributed to CDI in piglets is usually very low [60], morbidity of these animals in a farrowing facility may be as high as 97-100% [61]. In piglets, PCR-ribotype 078, besides some 045 carriage [62], is the prevalently identified ribotype that causes disease in the animal. Possibly, other ribotype strains do not have all necessary biochemical tools to survive and cause disease in pigs.

Of concern was that *C. difficile* may shed easily over sows, other piglets and the environment [59]. Because the emergence of *C. difficile* ribotype 078 in humans is linked epidemiologically to its presence in piglets, calves, and their environment, zoonotic transmission is suggested [60,63]. A high *C. difficile* carriage rate of 21% (15/70) was found recently among persons with daily to weekly contact with pigs and concerned all ribotype 078 except on one farm it was ribotype 045 [62]. This rate is higher than the carriership rate of less than 5% in non-hospitalized adults [64].

An important finding in the study described in Chapter 5 was that the antimicrobial susceptibility of the strains isolated from pigs is consistent with that of strains isolated from humans. In accordance with human-

derived strains, the *porcine C. difficile* PCR-ribotype 078 strains were resistant for ciprofloxacin (MIC >256 mg/L), but sensitive for the last generation quinolones.

Keessen *et al.* who also compared the antimicrobial profiles of human- and piglet-derived *C. difficile* strains reproduced this result [65]. Human and *porcine* isolates were susceptible to clindamycin (96%) and resistant to ciprofloxacin (96%). Moxifloxacin resistance was found in 16% of the human and of the *porcine* isolates. This was in fact a surprising result, while the susceptibility patterns for the fluoroquinolones tested in human and porcine ribotype 078 isolates are similar, the antimicrobial pressure in humans and pigs is not comparable at all [65]. Here, it must be noted that the piglets studied in Chapter 5 were not treated with fluoroquinolones, which are generally hardly used in pigs [66].

Fluoroquinolones are not frequently used in the Dutch animal production chain [67] being about three metric tonnes in 2012 (1.3% of the total antibiotics sales in metric tonnes; 0.41% being the 'newer' fluoroquinolones [66]). This was also reflected by the antibacterial sensitivity of *porcine*-isolated indicator *E. coli*. In the reporting group of member states, the resistance levels for tetracyclines, streptomycin, sulphonamides and ampicillin were 48%, 44%, 37% and 21%, respectively, whereas 1.1% of the *E. coli* isolates from Dutch pigs showed reduced susceptibility for ciprofloxacin [66]. The level of resistance to both ciprofloxacin and nalidixic acid was only 2%, whereas cefotaxime resistance was 1% (varying between 0% and 5%). Compared to other EU member states, the Dutch data on swine isolates showed moderate to high resistance [67].

Arruda *et al.* concluded that the administration of antibiotics was no major risk factor for CDI in piglets [68]. This appears to be in contrast with the observations by Belloc *et al.,* who found that quinolone treatment in pigs caused a strong selective pressure in the *E. coli* population of treated sows and their piglets [69]. This was in accordance with the study of Taylor *et al.*, who reported that the use of fluoroquinolones was the most important factor associated with finding resistant *E. coli* and/or *Campylobacter* strains [70]. In addition, quinolone-resistant bacteria may spread between pig farms [70]. Recently, the role of pigs as a potential source for epidemic multidrug resistant *C. difficile* strains in Spain was suggested [71]. So far, there is no strong scientific evidence for shared or overlapping routes of

infection of animals and humans. Although pigs can have infected humans (the zoonotic route), humans can have infected pigs *vice versa* (the reversed zoonotic route). In addition, a common infection source for both animals and humans strains is possible as well.

One of the possible explanations for similar infections in humans and pigs, despite the very different antibiotic exposures and limited use of (fluoro) quinolones in pigs, is the protective role of gut flora. The disruption of microbiota due to antibiotic administration is one of the main risk factors for the development of CDI [72]. Britton *et al* reviewed potential mechanisms for the mediation of *C. difficile* colonization by the normal microbiota [72]. These mechanisms include: (1) modulation of the intestinal bile composition, which may impact the antimicrobial properties of bile, (2) exclusion of toxigenic *C. difficile* by colonization with nontoxigenic *C. difficile*, (3) direct antagonism by the intestinal bacteriocins produced by specific microbiota.

Specific organisms of the gut microbiota have been shown to inhibit *C. difficile in vitro*. Skraban described changes in faecal microbiota associated with *C. difficile* colonization in poultry [73]. Microbes associated with *C. difficile* colonisation in poultry were different than those reported for humans and included bacteria (*e.g. Acidaminococcus intestine*) as well as fungi. Interestingly, another recent study by Skraban in humans indicates that not only the presence of a single species/group (*Bifidobacterium longum*) of microbiotia is important in preventing colonization with *C. difficile*, but that certain combinations of gut microbes are associated with *C. difficile* carriage and that some ribotypes (*e.g.* PCR-ribotype 027) might be associated with more disturbed microbiota than other ribotypes [74]. This implies that specific antibiotic regimens that spare organisms important for colonization resistance could be preferentially used in humans and animals to decrease the risk of *C. difficile* colonization.

A recent study by Harlow *et al.* [75] clearly illustrates that disruption of specific gastrointestinal microbiota (*e.g.* cellulolytic bacteria) in horses can lead to high level colonisation by enteric pathogens such as *C. difficile* or *Salmonella*. Within 24 hr after administration of trimethoprim-sulfadiazine a group of healthy horses became highly colonized with *C. difficile*, without showing signs of disease. The bacterium remained detectable at least one week after withdrawal of the antibiotic. The sows described in Chapter 5 were also treated peripartum for 1 week with trimethoprim-sulfadiazine.

Unfortunately, there are no comparable studies investigating the role of trimethoprim-sulfadiazine in the disruption of microbiota and subsequent colonization with *C. difficile* in pigs. However, it can be reasoned that the antibiotic, may increase the risk of symptomless-colonization of pigs with *C. difficile*, thereby increasing the risk of transmission.

These observations in combination with the results shown in this thesis on the role of cephalosporins, fluoroquinolones and clindamycin in the aetiology of CDI, the medical community, veterinarians and physicians, have to deliberate about appropriate use of antibiotics. Despite the very limited application of quinolones in pig production, unrestrained, unaccounted and irresponsible use of antibiotics is not acceptable. Antibiotics may not only stimulate the emergence of antibiotic-resistant bacteria, but may give advantage to the spreading of toxigenic *C. difficile* strains in particular too.

A factor in the spreading of toxigenic *C. difficile* is the role of the environment. The consumption of quinolones is increasing in the community (through primary health care and nursing homes), which lead to increasing resistance rates [67]. For comparison, high-level resistance to ciprofloxacin in broiler chickens was 4.5% of the *E. coli* isolates in 2012 [67], whereas quinolone consumption accounted for 22% of the total antibiotic use on broiler farms [66]. It is shown that the degree of quinolone resistance is correlated to the extent of toxin expression [18]. In other words, the resistant variants of the circulating *C. difficile* strains may have a selective advantage over non-resistant variants and are possibly able to manifest themselves in more virulent fashion.

It should be noted that antibiotics are not the only environmental risk for the augmented spreading of toxigenic strains. For example, certain disinfectants intensify sporulation and are associated positively with the spreading of *C. difficile* as well [76].

In the Netherlands, PCR-ribotype 027 CDI restricts to the healthcare facilities and has not been found in animals or in the community. In contrast, PCR-ribotype 078 is more frequently associated with community-acquired CDI and to a lesser extent bound by spatial barriers. Goorhuis *et al.* also showed that, compared to patients with ribotype 027-associated CDI, patients with CDI due to PCR-ribotype 078 were generally younger [77]. So it seems that the interaction of the microorganism with its environment and host determine

i) the risk of CDI, and *ii*) the PCR-ribotype, which is most likely to infect human or animal, and by that the severeness of the disease.

The reason for the emergence of ribotype 078 in humans is still under discussion. One explanation could be the increased use of fluoroquinolones in patients with ribotype 078-associated CDI. However, it was shown that the majority of patients with CDI due to ribotype 078 were not treated with fluoroquinolones [77]. Therefore, increased fluoroquinolone use alone cannot explain the recent emergence of this ribotype in humans. An additional selection mechanism that may favour of this hypervirulent genotype has not yet been uncovered. One other possible explanation is that ribotype 078 emerged from animals close to humans, including cattle, pigs, dogs, elephants, horses and ostriches [57,62,78-84], but to our opinion a common source is also possible. The fact that CDI due to ribotype 078 is predominantly a community-associated disease is also in line with a role for animals and/or common source for humans and animals in the environment.

It is obvious that veterinary and human medical scientists have to put the relationship between specific (drug-resistant) *C. difficile* strain carriership and development of CDI in animals and in humans on their mutual agenda. The One Health concept that is currently attracting increasingly attention [85], is a very suited platform for this. It stimulates and invites interdisciplinary collaboration and communication, which is apparent and highly needed in this matter. Involved researchers will have an important and essential task to unravel the epidemiology and infection control of quinolone-resistant *C. difficile* in humans and livestock.

Treatment

Antimicrobial susceptibility

The antibiotics used to treat human CDI are usually vancomycin or metronidazole. Metronidazole is currently the drug of first choice for mild infections, whereas vancomycin is preferred for the treatment of severe infections [41,86]. Because the emergence of vancomycin and/or metronidazole resistance may have very serious consequences for the treatment of CDI, it is important to monitor the antimicrobial susceptibility of *C. difficile* [87]. Chapter 6 of this thesis describes the resistance profiles of

nearly 400 clinical *C. difficile* isolates obtained from 26 European countries. This investigation showed no evidence of *in vitro* resistance of *C. difficile* to any of the four (potential) treatment agents tested, including vancomycin and metronidazole. However, the results suggested ribotype-specific differences in MICs for the investigated agents. In several studies the MICs of metronidazole and vancomycin, especially for epidemic ribotypes (027, 106 and 001), were several dilutions higher [88-90]. Therefore, the options for optimal antibiotic treatment of CDI may depend on the PCR-ribotype involved. In this context it must be noted that sub-optimal efficacy of metronidazole treatment has been associated with *C. difficile* PCR-ribotype 027 outbreaks [10,91]. Hitherto, metronidazole resistance is not linked to CDI treatment failure.

Metronidazole resistance of other anaerobic microorganisms, such as *Bacteroides* spp., has been shown to be associated with the presence of specific nitroimidazole (nim) resistance genes [92]. However, Pelaez *et al.* were not able to demonstrate this mechanism through these genes in metronidazole resistant *C. difficile* isolates which were collected in Spain [93]. They investigated whether metronidazole resistance in *C. difficile* may manifest through hetero-resistance, which is selected via *in vitro* and, possibly, *in vivo* exposure to the drug. However, this ribotype of resistance to metronidazole in *C. difficile* appeared to be very unstable, and the measured MIC depended on the method used to determine antimicrobial susceptibility (E-test versus agar-dilution and disk-diffusion methods). An important conclusion for common laboratory practice was that metronidazole hetero-resistance of *C. difficile* isolates may go undetected if metronidazole MICs are determined by the CLSI standard agar dilution method after the isolates are thawed. In Chapter 6 we applied an agar dilution method, which may have hampered the detection of metronidazole hetero-resistant strains.

Recently, Lynch *et al.* characterized the first stable metronidazole resistance in a *C. difficile* PCR-ribotype 027 isolate found in Canada [94]. Following the isolation of the strain from the stool sample, the MIC value was 256 μg/mL by agar diffusion and 32 μg/mL by E-test. The metronidazole-resistant strain followed an aberrant growth in broth and showed elongated cell morphology relative to a metronidazole-susceptible wild ribotype strain. Additionally, comparative genomic analysis revealed single nucleotide polymorphism (SNP) level variation within genes affecting core metabolic

pathways such as electron transport, iron utilization and energy production. It is clear that more research is needed to elucidate completely the exact mechanisms of resistance in and related fitness of the germ.

Resistance to the glycopeptide vancomycin was first described in *enterococci*, and has spread to other Gram-positive bacteria. Therefore, there is much concern about the potential risk of the development of vancomycin resistance in *C. difficile*. Vancomycin resistance in *enterococci* is generally due to VanA and VanB determinants [95]. The VanG-type determinant in *enterococci* is characterized by a low-level resistance to vancomycin (MIC 16 μg/mL) and by susceptibility to teicoplanin [96]. In 2006, the complete genome sequence of *C. difficile* revealed the presence of a VanG cluster designated "VanG-like" [97]. The VanG-like *C. difficile* cluster displayed a high degree of identity with VanG in *E. faecalis* [98]. Amman *et al.* found a high prevalence of the VanG-like cluster among clinical isolates of *C. difficile* [99]. Fortunately, despite the presence of these genes homologous to the VanG operon, *C. difficile* continues to be susceptible to vancomycin [98].

The results and conclusions from the monitoring in Chapter 6 underline the necessity to establish the monitoring of *C. difficile* susceptibility to critical drugs in clinical isolates on regular basis. However, research is needed to optimize the methods to detect and monitor susceptibility of the critical therapeutic drugs in clinical practice, as illustrated by the metronidazole resistance detection case here above. Additionally, research is also needed to elucidate the mechanisms of metronidazole and vancomycin resistance in *C. difficile*. It is namely expected that (ribotype-specific) development of reduced susceptibility and/or antimicrobial resistance to vancomycin and metronidazole will become a more important element in future therapeutic guidance for CDI.

Updated treatment guidelines for CDI

In 2009, the first ESCMID treatment guidance document for CDI was published [100]. The guideline has been applied widely in clinical practice. Since then, new treatments for CDI were developed, and the limitations of the recommended treatment of CDI have surfaced. In Chapter 7 an updated comparative effectiveness of currently available antibiotics in modern treatment of CDI is outlined. The comparison provides an evidence-based

recommendation on CDI treatment. The main antibiotic treatment agents that are recommended in the new ESCMID guideline are fidaxomicin, metronidazole and vancomycin. The choice for one of these antibiotics depends mainly on the stage and severity of disease, which is explained in detail in Chapter 7.

It must be noted that *C. difficile* resistance against these three antibiotics, including the commonly used therapeutics metronidazole and vancomycin, has not (yet) been shown in Europe (Chapter 6) and they should continue to be effective in the proposed treatments of CDI in the elaborated guideline. Metronidazole, however, is less successful in the treatment of epidemic PCR-ribotype 027-associated CDI [101]. As data on *in vivo* efficacy of this therapeutic in specific PCR-ribotypes is largely missing, the guideline does not advise ribotype-specific administration of certain antibiotics. It is expected that this will change as soon as we have learned more on the effectiveness of the drugs on each ribotype strain causing CDI *in vivo*. When severe ribotype 027-associated CDI manifests, however, the antibacterial of choice is vancomycin or fidaxomicin.

The study in Chapter 7 demonstrated the need for identification of improved clinical markers early in the course of the disease. These markers can predict the merits from specific treatment regimens to decrease CDI-related complications, mortality or recurrences. Unfortunately, almost no prospective and validated research has been carried out on the clinical predictors of CDI treatment outcomes.

The new ESCMID guideline is to a large extent in line with recently published other guidance documents [41,102,103]. In contrast to these guidelines, we included an evidence-based recommendation on the use of fidaxomicin in addition to metronidazole and vancomycin.

Fidaxomicin is one of the latest developed alternative drugs for the treatment of CDI. It is a macrocyclic antibiotic with activity against Gram-positive aerobes and anaerobes, including *C. difficile* [104]. The pharmaceutical lacks activity against Gram-negative microorganisms and will consequently preserve normal gastrointestinal flora [105]. This is of importance, as preservation of intact gastrointestinal flora is associated with a reduced risk of recurrence of CDI. In addition, fidaxomicin achieves very high faecal concentrations with minimal systemic absorption [104,106].

When the data from two large phase-III studies with fidaxomicin were assessed retrospectively [107,108], reduction of persistent diarrhoea, recurrence and death by 40% compared with vancomycin through day 40 was revealed [109]. A reduction in recurrences is considered to be one of the most important advantages of fidaxomicin administration [110]. In a subgroup analysis, however, the significantly fewer recurrences in CDI due to PCR-ribotype 027 as opposed to non-027 PCR-ribotypes by fidaxomicin treatment could not be repeated [108,109]. In addition, patients with multiple CDI recurrences were not included in prospective, multicentre, double-blind and randomized trails. The cited studies had also not included patients with fulminant CDI. Therefore, as stated in Chapter 7, the role of this antibiotic in multiple recurrent CDI, in fulminant CDI and in PCR-ribotype 027-affected patients remains unclear and needs more studies, preferable independent from the pharmaceutical industry.

A limitation in the treatment of CDI with fidaxomicin is that the antibiotic is quite expensive and treatment with this drug may therefore be more costly than using its alternatives. Indeed, Stranges *et al*. state that the treatment with fidaxomicin may only be cost-effective for a certain group of patients [111]. National cost analyses have compared fidaxomicin and vancomycin [107-109,111]. The analyses were based on the clinical data from the two pivotal phase-III studies. In the cost analysis performed by the Scottish Medicines Consortium [112] two subgroups of patients were evaluated. The cost effectiveness of fidaxomicin was demonstrated in patients with a first CDI recurrence, but not for the population of patients with severe CDI. The Irish National Centre performed another pharmaco-economical examination and concluded that fidaxomicin was dominant (less costly and more effective) for patients with non-severe and severe CDI, and patients with a first recurrence [113]. The All Wales Therapeutic and Toxicology Centre published an advice on the use of fidaxomicin, in which several limitations in the cost analysis provided by the manufacturer are discussed [114].

In conclusion, the current cost analyses are based on a limited number of patients of two trial populations with severe CDI and recurrences. The favourable outcome for fidaxomicin as compared to vancomycin depends mainly on the assumed reduction in the re-infection rate in specific patient groups. The hypothetical cost-effectiveness of fidaxomicin remains therefore uncertain and is likely to vary across countries and settings with different local and specific cost structures.

To give a substantiated cost-effectiveness analysis of any appropriate antibiotic therapy, more prospective randomized trials comparing specific patient subgroup populations (*e.g.* multiple recurrent CDI and severe CDI) are necessary. In addition, further prospective randomized trials are needed to investigate and compare the (long-term) effectiveness of a treatment with respect to the specific PCR-ribotype involved in the infection.

Successful treatment of multiple recurrent CDI is achieved with antibiotics in combination with a therapy not based on the use of antibiotics, such as inoculation of patients with a faecal preparation from healthy donors (Chapter 7). In fact, faecal transplant is one of the main advances in combined non-antibiotic and antibiotic therapies. It is included as an important treatment instrument in the guideline presented in Chapter 7. Based on the recently published first prospective randomized controlled trial, faecal transplant was strongly recommended. However, the practical implementation and implications of faecal transplantation in a hospital setting have to be further elaborated. Moreover, consensus has to be reached on the screening of faecal donors, *i.e.* whether provided faecal flora contains key microorganisms, but also whether harmful (non-bacterial) micro-organisms, residues of pharmaceuticals, allergens and other potentially health-threatening substances are present.

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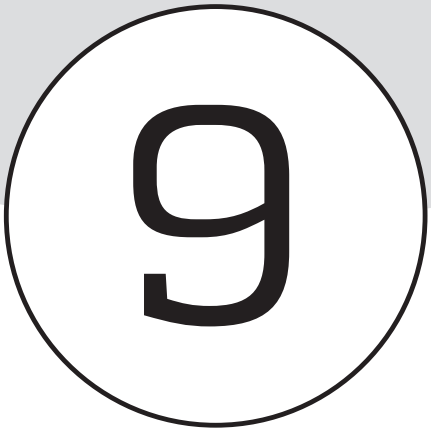

Chapter 9

Future Perspectives and Recommendations

Future Perspectives and Recommendations

This thesis summarizes our findings with outbreak control, diagnosis of *C. difficile*, identification of PCR-ribotype-specific risk factors and treatment of CDI after the discovery of the emergence of *C. difficile* PCR-ribotype 027 in the Netherlands. The studies illustrate the role of antibiotics in relation to persistence, severeness and spreading of CDI. Antibiotics are shown to be a primary risk factor for the development of (ribotype-specific) CDI and an essential part of the outbreak control measures, namely antibiotic stewardship. The use of antibacterials is a risk for selection of novel endemic *C. difficile* strains in *e.g.* animals, which introduce an increasing risk of alternative zoonotic transmission routes.

Forthcoming research should give more insight into the mechanisms of induction, selection and virulence of specific *C. difficile* strains by antibiotics or by combinations of drugs. It is important to realize that the intestinal microbiota probably determines whether *C. difficile* can colonize and/or produce toxins with subsequent development of disease. Future research should be directed toward the precise role of the microbiota in de defence against CDI, enabling us to develop new interventions. Regarding the general increase of antibiotic-resistant bacteria causing nosocomial infections, and the consequent limitations in antibiotic treatment over the past years, more knowledge on PCR-ribotype specific antibiotic stewardship will be needed to prevent and control outbreaks with CDI. In addition a local, national and European network for the surveillance of antibiotic susceptibility in *C. difficile* strains is essential for up-to-date treatment recommendations. More research is necessary to elucidate the (ribotype-specific) mechanisms by which colonic microbiota may mediate colonization resistance against *C. difficile in vivo*. It may explain the success of faecal transplantation. More knowledge on this mechanism will be input for the development of novel treatment procedures for CDI and development of strategies preventing infections.

Looking to the future, many scientific questions remain to be answered. For example, how can we further optimize, facilitate, and more importantly, standardize CDI diagnosis and subsequent ribotyping of *C. difficile* strains? What is the value of PCR-based rapid diagnostics in outbreak control? Should we screen hospitalized patients and/or nursing home residents for *C. difficile* carriership? How can we improve the recognition of persons at

risk for developing (severe) CDI? Should pre-emptive barrier precautions and antibiotic treatment of carriers of known epidemic and pathogenic strains be part of preventive and/or outbreak-control measures? How may specific antibiotics disrupt the microbiota and alter the colonization resistance? Can we identify specific bacteria in the gut microbiota that interfere with *C. difficile*? What is the role of the host immune system in mediating colonization resistance against *C. difficile*? How can specific antibiotics induce *C. difficile* spore germination and subsequent toxin production?

In particular, high-quality knowledge on *C. difficile* spore germination and toxin production is of paramount importance for the development of sophisticated strategies for the prevention of CDI. If, unfortunately, CDI occurs, such understanding of the responses of the bacterium will contribute to the availability of novel effective treatments.

The cost effectiveness of infection interventions and novel treatment options for CDI need to be investigated in more detail and with more underpinning data in order to estimate the benefits on clinical outcome and medical cost savings. Given the increasing elderly population, we expect that unless we are *able to increase the awareness of patients, healthcare workers and of policy makers,* the economic burden associated with nosocomial and community-acquired CDI will increase for primary and recurrent infection. Such an increase of medical costs should be avoided, not only from an economical point of view, but the expectation of high costs will discourage decision-makers to make right and firm decisions as fast as possible after the discovery of a commencing outbreak. CDI is too contagious and too serious in many of its aspects to delay effective intervention.

Samenvatting [Summary in Dutch]

Achtergrond

In 1977 werd voor het eerst ontdekt dat de anaerobe bacterie, *Clostridium difficile* (*C. difficile*), "antibiotica geassocieerde diarree" veroorzaakt. Tot die tijd werd diarree tijdens een (langdurig) verblijf in het ziekenhuis of na gebruik van antibiotica, beschouwd als een vervelende doch onvermijdbare complicatie en bijwerking van een opname of behandeling. De ontdekking van *C. difficile* als een belangrijke verwekker van nosocomiale (= in het ziekenhuis ontstane) diarree markeert dan ook de start van veel wetenschappelijk onderzoek naar de diagnostiek, behandeling en preventie van deze infectie. *C. difficile* geassocieerde infectie (CDI) heeft zich inmiddels ontwikkeld tot de meest voorkomende ziekenhuis-gerelateerde diarree.

Een van de bijzondere eigenschappen van *C. difficile* is de vorming van sporen. Eenmaal uitgescheiden in de omgeving kan de bacterie in de vorm van sporen zeer lang (jaren) overleven. Sporen zijn zeer resistent tegen hitte, uitdroging, lucht, reinigingsmiddelen en veel gebruikte desinfectantia zoals alcohol. De vorming van sporen is dan ook een belangrijke reden waarom de bacterie zich snel kan verspreiden in een ziekenhuisomgeving. Infectie ontstaat door inademing of inname via de mond van sporen, die vervolgens kunnen ontkiemen in de darm. Niet alle patiënten met *C. difficile* worden ziek (dragerschap). Het ziekmakend vermogen van *C. difficile* wordt door bacterie en gastheer factoren bepaalt. Alleen *C. difficile* stammen die toxinen kunnen produceren veroorzaken ziekte: uiteenlopend van milde diarree tot ernstige en/of gecompliceerde darmontsteking en dood. Het aantonen van *C. difficile* toxine is dan ook belangrijk voor het stellen van de diagnose (**Hoofdstuk 3**). Een van de belangrijkste gastheerfactoren voor het ontwikkelen van ziekte is de verstoring van de normale darmflora (microbiota) door het gebruik van antibiotica.

Het laatste decennium is CDI, mede door uitbraken met snel verspreidende en ernstige infecties veroorzakende *C. difficile* stammen, wereldwijd sterk toegenomen. In 2003 werden een aantal grote uitbraken van CDI beschreven in ziekenhuizen in Canada, gevolgd door vergelijkbare uitbraken in de VS en VK. Deze uitbraken kenmerkten zich niet alleen door een significante

toename in het aantal ziektegevallen veroorzaakt door *C. difficile*, maar met name door een ongekend ernstig beloop, een hoge mortaliteit en meer complicaties. Al gauw bleek de toename in de ernst van de ziekte toe te schrijven te zijn aan de opkomst van een hoog virulent (= zeer ziekmakend) ribotype van deze bacterie: later geïdentificeerd als het PCR-ribotype 027.

Het onderzoek beschreven in dit proefschrift begint in die periode, met de ontdekking van de eerste uitbraak met dit bijzonder virulente *C. difficile* ribotype in een Nederlands ziekenhuis in 2005. Op dat moment was er nog weinig bekend over aspecten zoals: epidemiologie, pathogenese, risicofactoren, behandelstrategieën, diagnostiek en preventieve en uitbraak beheersmaatregelen. Omdat antibioticagebruik een bekende risicofactor voor het ontstaan van CDI was en ribotype 027 *in vitro* ongevoelig bleek te zijn voor verschillende antibiotica die frequent gebruikt worden voor de behandeling van diverse infecties (zoals fluorochinolonen), was er grote behoefte aan meer onderzoek naar de rol van antibiotica in de epidemiologie, uitbraakbeheersing en behandeling van CDI.

Sinds de uitbraken met het PCR-ribotype 027, werden er in toenemende mate CDI uitbraken beschreven met andere (virulente) PCR-ribotypen (zoals PCR-ribotype 001 en 017). Naast het identificeren van CDI specifieke risicofactoren, was het dus belangrijk te onderzoeken of er ook PCR-ribotype specifieke risicofactoren zijn. Meer kennis van (eventuele PCR-ribotype-specifieke) risicofactoren van patiënt, omgeving en bacterie voor het ontwikkelen van een (ernstige) CDI, is belangrijk voor de bestrijding van uitbraken (**Hoofdstukken 2 en 4**).

Continue surveillance toonde de laatste jaren een stijging in incidentie in *community acquired* (= niet gerelateerd aan een gezondheidsinstelling) CDI, waarbij ook andere opkomende PCR-ribotypen gesignaleerd worden, zoals het PCR-ribotype 078. Met de toename van CDI buiten het ziekenhuis, rijst de vraag of en welke specifieke risicofactoren zoals antibioticagebruik hiermee geassocieerd zijn en of en met welke potentiele bronnen er buiten het ziekenhuis rekening gehouden moet worden. Omdat CDI ook beschreven wordt in dieren, en het antibioticumgebruik in de veehouderij en humane geneeskunde de laatste jaren een punt van zorg en aandacht zijn, is het verkrijgen van meer inzicht in de een mogelijke epidemiologische relatie tussen CDI in mens en dier belangrijk (**Hoofdstuk 5**)

In de meeste gevallen wordt CDI antibiotisch behandeld. De belangrijkste antibiotica voor de behandeling van CDI zijn metronidazol en vancomycine. Gezien de zorgwekkende algemene toename in resistentieontwikkeling van micro-organismen wereldwijd, is het van belang dat ook eventuele resistentieontwikkeling van *C. difficile* onderzocht en gevolgd wordt (**Hoofdstuk 6**). Met de komst van nieuwe antibiotica en niet-antibiotische behandelstrategieën, is het belangrijk dat behandelrichtlijnen voor CDI regelmatig herzien worden (**Hoofdstuk 7**).

In dit proefschrift worden diverse aspecten van CDI onderzocht en beschreven: het onderzoek richt zich daarbij met name op de rol van **antibiotica**. Het proefschrift bestaat uit drie secties: Sectie I behandelt **de beheersing van uitbraken** in het ziekenhuis **(Hoofdstukken 2, 3 en 4)**.

Sectie II richt zich op de **epidemiologie** van nieuw opkomende PCR-ribotypen bij mens en dier **(Hoofdstuk 5)**.

Sectie III focust op de (antibiotische en niet-antibiotische) **behandeling** van *C. difficile* **(Hoofdstukken 6 en 7)**.

De studies die beschreven worden in secties I en II van dit proefschrift spitsen zich toe op drie PCR-ribotypen van *C. difficile*: 027, 017 en 078.

De belangrijkste onderzoeksdoelstellingen van dit proefschrift zijn:

i) Het vaststellen van het belang van *antibiotic stewardship* als een van de CDI uitbraak beheersmaatregelen in een ziekenhuis.
ii) Het identificeren van (PCR-ribotype specifieke) risico factoren voor de ontwikkeling van CDI, zodat preventieve en uitbraak beheersmaatregelen verder verbeterd kunnen worden.
iii) Het onderzoeken of CDI bij dieren een potentiële bron zou kunnen zijn voor de opkomst van specifieke PCR-ribotypen bij dier en mens.
iv) Het bestuderen van de antimicrobiële gevoeligheid van *C. difficile* in Europa, om mede daarmee de Europese richtlijnen voor de behandeling van CDI te herzien, te optimaliseren en te moderniseren.

Onderzoeken in dit proefschrift

Sectie I: Uitbraakbeheersing

In **Hoofdstuk 2** wordt de eerste uitbraak met de zeer virulente *C. difficile* PCR-ribotype 027 in een Nederlands ziekenhuis beschreven: specifieke risicofactoren voor CDI en uitbraak beheersmaatregelen werden onderzocht.

Doelstelling en opzet

In een retrospectieve case-control studie werden 3 groepen patiënten vergeleken voor het identificeren van CDI specifieke risicofactoren: (1) patiënten met CDI, (2) patiënten zonder diarree en (3) patiënten met non-infectieuze diarree. Daarnaast werden uitbraak beheersmaatregelen geëvalueerd; met name de rol van *antibiotic stewardship*.

Resultaten

Onafhankelijke risicofactoren voor CDI met PCR-ribotype 027 waren: leeftijd ouder dan 65 jaar), opnameduur in het ziekenhuis, en antibioticagebruik. Met name cefalosporinen en fluorochinolonen werden geïdentificeerd als belangrijkste riscofactoren voor het ontwikkelen van CDI. Dit risico was significant groter in geval van gebruik van een combinatie van een cefalosporine met een fluorochinolon. De uitbraakmaatregelen die genomen werden bestonden uit: intensivering van hygiëne (w.o. handen wassen met water en zeep, en intensieve reiniging en desinfectie van gebruikte patiëntmaterialen en van omgeving), cohorteren van patiënten op een speciaal voor CDI-patiënten bestemde afdeling, de invoering van versnelde diagnostiek naar CDI en educatie van de ziekenhuispersoneel. Desondanks zette de uitbraak zich voort; pas na de invoering van een zeer stringent antibioticumbeleid naast alle overige maatregelen ("*bundle approach*"), waarin het gebruik van cefalosporinen werd verminderd en het gebruik van fluorochinolonen volledig stop gezet werd, kon de uitbraak beëindigd worden.

Conclusie

Antibiotica (cefalosporinen en fluorochinolonen) zijn een belangrijke risicofactor voor het ontstaan van CDI door PCR-ribotype 027. *Antibiotic stewardship* (in dit geval: restrictie van het gebruik van cefalosporines en het stoppen van fluorochinolonen) is een van de essentiële uitbraak beheersmaatregelen bij CDI met ribotype 027 in een ziekenhuis.

Hoofdstuk 4 beschrijft een uitbraak met simultaan twee verschillende PCR-ribotypen (027 en 017) in een enkel ziekenhuis.

Doelstelling en opzet

In een retrospectieve case-control studie werden karakteristieken van vijf patiëntengroepen vergeleken om ribotype specifieke riscifactoren te onderzoeken: 1) patiënten met PCR-ribotype 027, 2) patiënten met PCR-ribotype 017, 3) patiënten met overige PCR-ribotypen, 4) patiënten met niet-infectieuze diarree, en 5) patiënten zonder diarree. Daarnaast werd de klonale verspreiding van de verschillende PCR-ribotypen onderzocht met behulp van *multilocus variable number tandem repeat analysis* (MLVA).

Resultaten

PCR-ribotype specifieke risicofactoren waren: leeftijd en (recente) ziekenhuisopname (PCR-ribotypen 027 en 017 in vergelijking met overige endemisch voorkomende ribotypen), gebruik van clindamycine en immunosuppressiva (PCR-ribotype 017), gebruik van fluorochinolonen (PCR-ribotype 027). De MLVA analyse liet een verspreiding van beide klonale, persistente ribotypen zien.

Conclusie

Patiënten met CDI hebben ribotype-specifieke risicofactoren. De studie onderschrijft het belang van continue surveillance (=bewakingsonderzoek) inclusief PCR-ribotypering in ziekenhuizen voor de preventie en beheersing van uitbraken.

Hoofdstukken 2 en 4 laten zien dat antibiotica belangrijke risicofactoren voor CDI zijn. Daarnaast tonen beide studies aan dat de ribotypen verschillend reageren op gebruikte antibiotica. Het gevolg hiervan is dat het instellen van ribotype-specifieke restricties in het antibioticagebruik een belangrijke uitbraak beheersmaatregel is. Het wil ook zeggen dat de druk van een specifiek antibioticum van invloed is op de selectie van specifieke PCR-ribotypen in een ziekenhuis.

In **Hoofdstuk 3** wordt een uitstapje gemaakt naar de rol van snelle diagnostiek als onderdeel van uitbraakbeheersing. Gezien de snelle verspreiding van CDI wordt het snel en adequaat stellen van een diagnose essentieel geacht.

Doelstelling en opzet

De meerwaarde van het effect van het sequentieel testen van feces gedurende een ziekenhuisuitbraak met CDI PCR-ribotype 027 werd onderzocht gebruikmakend van sneldiagnostiek met een immunochemische test (immunoassay) voor de detectie van *C. difficile* toxinen A en B in feces.

Resultaten

In het merendeel van de CDI patiënten werden toxinen in een eerste feces monster aangetoond (86%). Additioneel werd bij 5% van de patiënten de diagnose gesteld in een vervolgmonster die binnen één week na het eerste feces monster werd afgenomen. De overige 9% van de patiënten hadden alsnog een positieve test na meer dan een week na de eerste negatieve test. De diagnose werd in alle herhaalde monsters bevestigd door het kweken van toxinogene *C. difficile*.

Conclusie

Uit de resultaten van deze studie werd geconcludeerd dat het herhaald testen van feces tijdens een uitbraak een toegevoegde waarde heeft voor het snel identificeren van patiënten met CDI.

Omdat de in **Hoofdstuk 3** gebruikte test een lage gevoeligheid heeft en daarnaast nieuwe snelle gevoeligere technieken ontwikkeld zijn, wordt de laatste jaren de voorkeur gegeven aan een twee- of drie-stappen procedure om de microbiologische diagnose CDI te stellen. Hierbij worden feces monsters met een snelle gevoelige methode getest op de aanwezigheid van *C. difficile* (bv. door middel van het aantonen van glutamaat dehydrogenase (GDH) met behulp van een enzym immunoassay (EIA) en/of het aantonen van bacteriële genen die coderen voor toxinen A en/of B). Indien deze eerste test positief is bevonden, wordt met behulp van een tweede techniek de aanwezigheid van toxine producerende *C. difficile* bevestigd.

Sectie II: Epidemiologie

In **Hoofdstuk 5** wordt de relatie tussen CDI in mens en CDI in dier onderzocht.

Doelstelling en opzet

In een prospectieve studie werden fecale monsters van CDI-verdachte biggen bacteriologisch onderzocht op feno- en genotype van de veroorzaker

van de infectie. Daarnaast werd de genotypische relatie tussen porciene en humane *C. difficile* stammen bestudeerd. Voor dit onderzoek werd de feces van biggen van twee Nederlandse varkenshouderijen met verschijnselen van diarree onderzocht op de aanwezigheid van toxine producerende *C. difficile*.

Resultaten

Kweek en PCR analyse toonden aan dat toxinogene *C. difficile* PCR-ribotype 078 de verwekker van de diarree in de onderzochte biggen was. Een MLVA analyse liet zien dat de bij de varkens geïsoleerde stammen genetisch nauw verwant zijn aan humane PCR-ribotype 078 stammen. Aanvullend werd een klonaal complex geïdentificeerd dat zowel varkens als humane isolaten omvatte. De antimicrobiële gevoeligheid van de porciene stammen was gelijk aan die van de Nederlandse humane stammen.

Conclusie

Op basis van de feno- en genotypische analyses, kan geconcludeerd worden dat de *C. difficile* stammen van varkens met CDI niet verschillen van de in toenemende mate voorkomende *C. difficile* PCR-ribotype 078 stammen in humane infecties in de Nederlandse populatie.

Sectie III: Behandeling

In **Hoofdstuk 6** wordt het voorkomen van antimicrobiële resistentie van *C. difficile* tegen antibiotica die gebruikt (zouden kunnen) worden voor de behandeling van CDI, onder de loep genomen.

Doelstelling en opzet

De antimicrobiële gevoeligheidspatronen van de in Europese ziekenhuizen voorkomende *C. difficile* PCR-ribotypen werd onderzocht. Van 398 *C. difficile* stammen afkomstig van 73 ziekenhuizen in 26 Europese landen werd met behulp van de agar verdunningsmethode de MIC bepaald. De volgende middelen werden getest: vancomycine, metronidazol, fidaxomicine en LFF571 (een nieuw experimenteel middel).

Resultaten

De MICs van fidaxomicine en LFF571 waren lager dan die van vancomycine en metronidazol. Isolaten behorende clade 2 (een groep van genetisch verwante PCR-ribotypen), waaronder PCR-ribotype 027, vertoonden een- tot

twee verdunningen hogere MIC50 en MIC90 waarden voor fidaxomicine en metronidazol. *C. difficile* PCR-ribotype 001 was *in vitro* gevoeliger voor fidaxomicine in vergelijking tot andere vaak voorkomende PCR-ribotypen 014/020 en 078. Zes isolaten afkomstig uit drie verschillende landen hadden een verhoogde metronidazol MIC van 2 mg/L. Vier van deze isolaten betroffen PCR-ribotype 001.

Conclusie

Alle *C. difficile* stammen waren *in vitro* gevoelig voor de vier geteste middelen. Er werden wel ribotype specifieke verschillen in MICs aangetoond. Om de klinisch implicaties van ribotype specifieke MIC veranderingen voor de behandeling van CDI te bepalen, is een continue surveillance van de gevoeligheid van *C. difficile* isolaten in Europa nodig.

In **Hoofdstuk 7** wordt een overzicht gegeven van en aanbeveling gegeven voor de huidige behandelstrategieën van CDI.

Doelstelling en opzet

In dit onderzoek wordt een overzicht gegeven van gerandomiseerde en niet-gerandomiseerde studies die vorsen naar het klinisch effect van een behandeling van CDI, en die tussen 1978 tot 2013 gepubliceerd zijn. Op basis van de studies werd een *evidence-based*, herziene richtlijn ontwikkeld voor de behandeling van CDI.

Resultaten

De behandelopties die in dit onderzoek werden bestudeerd zijn: orale en niet-orale antibiotica, toxine-bindende middelen, immunotherapie, probiotica, intestinale feces/bacterie transplantatie. De aanbevolen behandelstrategieën werden onderverdeeld naar ernst van de ziekte, het voorkomen van recidieven en/of niet-orale antibiotische behandeling.

Conclusie

Behalve voor CDI met milde diarree, die duidelijk gerelateerd is aan antibiotica gebruik, wordt een behandeling met antibiotica aanbevolen. De keuze van de gebruikte antibiotica hangt met name af van de ernst van de ziekte. De belangrijkste antibiotica die in de richtlijn aanbevolen worden, zijn: metronidazol, vancomycine en fidaxomicine. Een behandelstrategie die sterk geadviseerd wordt in geval van multipele recidieven van CDI, is antibiotische behandeling gevolgd door feces transplantatie. In geval van

perforatie van het colon en/of systemische ontstekingen (inflammatie) met ernstige verslechtering van de klinische toestand ondanks antibiotische behandeling, is chirurgisch ingrijpen geïndiceerd. In dat geval wordt totale abdominale colectomie, of zogenaamde "*diverting loop ileostomie*", gecombineerd met een colon-lavage geadviseerd.

Maatschappelijke Relevantie
[Social Relevance]

De bescherming van onzichtbare micro-organismen tegen ziekten enerzijds en de bedreiging erdoor van onze gezondheid anderzijds, is een delicate balans. De eerste uitbraak met een zich snel verspreidende zeer ziekmakende en voorheen onbekende variant van de bacterie Clostridium difficile in een Nederlands ziekenhuis in 2005 bewees dit weer eens. Onderzoek naar en ervaringen met deze variant waren nog beperkt en gebaande paden voor uitbraakcontrole bleken er voor deze sporenvormer niet te zijn. Gesteund door ziekenhuisbestuur, medische staf, mijn promotor en vele collega's, was dit voor mij de kans om bij te dragen aan het vinden van oplossingen voor een aandoening die inmiddels een grote impact op de gezondheidszorg heeft.

De natuurlijke darmflora beschermt ieder mens tegen invloeden van buiten. Antibioticagebruik is geassocieerd met een verstoring van deze natuurlijke weerstand, waardoor *Clostridium difficile* de kans krijgt zich in de darm te nestelen. Behalve dit, stimuleren bepaalde antibiotica Clostridium difficile ook om zijn gifstoffen af te geven, waaraan deze bacterie zijn ziekmakend vermogen ontleent. Het vroegtijdig identificeren van hoog-risico patiënten leidt tot een effectievere preventie en behandeling van deze infectie.

In de media wordt een "ziekenhuisbacterie" nog vaak geassocieerd met MRSA, maar schattingen tonen dat *Clostridium difficile* infecties (CDI) significanter kunnen zijn dan iedere andere in een zorginstelling ontstane infectie. Een uitbraak heeft directe gevolgen voor welzijn, genezing, opnameduur en overlevingskansen van de patiënt, en de kosten van het bestrijden ervan zijn hoog. Het beïnvloedt de bedrijfsvoering van een zorginstelling op dramatische wijze en ook de naasten van een patiënt ondervinden gevolgen. De oplossing voor uitbraakcontrole vraagt multidisciplinair management, strikte opvolging van hygiënemaatregelen en het beperken van het gebruik van bepaalde antibiotica. Het is evident dat de maatschappelijke relevantie van dit CDI onderzoek groot is.

List of Abbreviations

AAD	Antibiotic-associated diarrhoea
Bp	Base pair
CIb	Centrum Infectieziektebestrijding
CC	Clonal complexes
CDAD	Clostridium difficile associated disease
CDI	*Clostridium difficile* infection
CLSI	Clinical and Laboratory Standards Institute
CodY	Transcriptional repressor protein
CcPA	Carbon catabolite control protein
EIA	Enzyme immunoassay
ESCMID	European Society of Clinical Microbiology and Infection
ermB	Erythromycin ribosomal methylase B
GDH	Glutamase dehydrogenase
gluD	Glutamate dehydrogenase gene
GyrA	DNA gyrase A gene
HCAI	Healthcare-associated Infections
ICTAB	ImmunoCard Toxins A and B
ICU	Intensive care unit
Ile	Isoleucine
LAMP	loop-mediated isothermal amplification
MIC	Minimum inhibitory concentration
MLVA	Multiple-Locus Variable number tandem repeat Analysis
MST	Minimum spanning tree
Nim	Nitroimidazole
OR	Odds ratio
PCR	Polymerase chain reaction
RIVM	Rijksinstituut voor Volksgezondheid en Milieu
SNP	Single nucleotide polymorphism
STRD	Summed tandem repeat difference
tcdA	Toxin A encoding gene
tcdB	Toxin B encoding gene
tcdC	Gene encoding TcdC: anti-sigma factor; (negative) regulator toxin genes
tcdR	Gene encoding TcdR: alternate sigma factor; (positive) regulator toxin transcription
Thr	Threonine
VanA	Vancomycin-resistance gene A
VanB	Vancomycin-resistance gene B
VanG	Vancomycin-resistance gene G
WBC	White blood cell count

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Curriculum Vitae

Sylvia Debast kwam op 30 juli 1966 te Xanten (Duitsland) ter wereld. Na het Cals College (Nieuwegein), startte zij aanvankelijk met de studie Scheikunde aan de Universiteit Utrecht (UU), maar na haar propedeuse begon zij in 1985 Geneeskunde aan dezelfde Universiteit te studeren. Het keuze-co-assistentschap Medische Microbiologie werd aangevuld met een 9-maands onderzoeksstage bij de afdeling Medische Microbiologie van het Universitair Medisch Centrum (UMC) Utrecht (prof dr. J. Verhoef) en het bedrijf U-Gene Research (dr. R. Torensma en dr. A.D.C. Fluit). Na het arts-examen in 1992, volgde haar specialisatie medische microbiologie aan het Radboud UMC (opleider: prof. dr. J.A.A. Hoogkamp-Kostanje). In het jaar dat ze er als arts-microbioloog in hetzelfde ziekenhuis werkte, verdiepte ze zich in de serologie en verrichtte in de afdeling Virologie (prof. dr. J.M.D. Galama) en het Laboratorium Kindergeneeskunde & Neurologie (Dr. K.J.B. Lamers) onderzoek naar de serologische diagnostiek van infecties van het centraal zenuwstelsel.

In 1998 trad Sylvia toe tot de maatschap Medische Microbiologie Amersfoort-Harderwijk. Tot 2006 werkte zij als all-round arts-microbioloog en staflid van het Ziekenhuis St Jansdal te Harderwijk en het Meander Medisch Centrum (MMC) te Amersfoort. In 1999 werd zij hoofd van het laboratorium Medische Microbiologie in het Ziekenhuis St Jansdal. Daarnaast participeerde zij in de Ghana-commissie van dit ziekenhuis om samen met het St Mary's Hospital in Drobo humanitaire projecten te realiseren. Na het beëindigen van haar werkzaamheden in het Ziekenhuis St Jansdal in 2006, zette zij haar werk voort in het MMC, waar zij eenheidsmanager werd van de afdeling Medische Microbiologie-Medische Immunologie.

In 2005 werd zij geconfronteerd met de eerste Nederlandse ziekenhuisuitbraak van een zeer ziekmakende variant van *Clostridium difficile*. Gesteund door de raad van bestuur van het Ziekenhuis St Jansdal (dr. C.M.M.L. Bontemps-Hommen) en prof. dr. E.J. Kuijper (Leids Universitair Medisch Centrum en Nederlands Referentielaboratorium *C. difficile*) werd in 2006 gestart met een eerste studie. De uitbraak markeerde het startpunt van het onderzoek dat tot het onderhavige proefschrift leidde.

In 2009 verrichtte zij samen met dierenarts dr. L.A.M.G. van Leengoed en prof. dr. A.A. Bergwerff een pilotstudie naar het voorkomen van *C. difficile* in varkens. Deze studie was aanleiding voor een onderzoeksproject van de faculteit Diergeneeskunde van de UU (prof. dr. A.A. Bergwerff, prof. dr. F. van Knapen en dr. L.J.A. Lipman) en het laboratorium Medische Microbiologie van het LUMC (prof. dr. E.J. Kuijper).

Naast haar werk als arts-microbioloog is Sylvia sinds 2010 bestuurslid van de Werkgroep Openbare Gezondheidszorg van de Nederlandse Vereniging van Medische Microbiologie.

In 2012 beëindigde zij haar werkzaamheden in het MMC te Amersfoort en is thans werkzaam in het Radboud UMC.

Sylvia is gehuwd met Aldert Bergwerff en zij vormen samen met hun vier kinderen en twee honden een meesterlijk team.

Dankwoord [Acknowledgements]

Ik wil alle mensen danken die aan de totstandkoming van dit proefschrift hebben bijgedragen. Daar het risico om namen te vergeten groot is, zal ik met een kleine uitzondering, niet specifiek op namen ingaan. De weg naar dit proefschrift is niet altijd eenvoudig geweest. Daarom ben ik een ieder die mij ondanks de hindernissen geholpen en gesteund heeft bijzonder dankbaar. Dankzij velen heb ik dit onderzoek kunnen afronden om uiteindelijk deze mijlpaal in mijn carrière te bereiken.

Uiteraard wil ik mijn promotor Ed Kuijper bedanken. Door een onverwachte ziekenhuis uitbraak met een voorheen onbekende variant van *Clostridium difficile* en de confrontatie met de ernstig ziekmakende werking op patiënten, werd mijn interesse in en de wens onderzoek te doen naar ziekteverwekker en infectie gewekt. Ed, het was het moment dat ik jou, deskundig op dit gebied, om advies vroeg en van waaruit een langdurige samenwerking en vriendschap is ontstaan. Dit onderzoek heeft uiteindelijk geleid tot dit proefschrift. Dank voor je vertrouwen in mij. Ik hoop dat wij onze samenwerking ook in de toekomst voort zullen zetten.

Aldert, we zijn in zoveel opzichten een waar team. Je hebt mij gesteund in mijn besluit een nieuwe weg in mijn carrière in te slaan om mij tijdelijk op de afronding van mijn promotie te kunnen richten. Daar ben ik je ongelooflijk dankbaar voor. Persoonlijk, maar ook op wetenschappelijk gebied ben je een grote steun en stimulans voor mij. Wij hebben elkaar leren kennen tijdens de scheikunde studie. Daarna hebben wij ieder onze eigen wetenschappelijke weg gekozen. Overtuigd dat het over de grenzen van onze vakgebieden heen kijken de belangrijkste voorwaarde is om nieuwe dingen te ontdekken, hebben onze gezamenlijke discussies aan de keukentafel er uiteindelijk toe geleid dat door jouw inzet een onderzoeksproject samen met de faculteit diergeneeskunde opgezet kon worden en wij onze eerste gezamenlijke publicaties hebben geschreven.

Ursula, Anna-Marij, Thijmen en Fabian, eindelijk is het proefschrift klaar! Zonder jullie geduld, liefde en gezelligheid was dit werk niet tot stand gekomen. Ik ben ongelooflijk trots dat ik dit moment samen met jullie mag beleven.

Lieverds, dat we nog lang samen nieuwe dingen mogen ontdekken. Amor omnia vincit.

Tot slot wil ik mijn familie bedanken. In het bijzonder mijn ouders en zus Stephanie; zonder wie ik niet zou staan waar ik nu sta. Jullie hebben mij een solide, liefdevolle basis gegeven en geleerd nooit op te geven. Lieve papa, wat had ik dit graag met je gedeeld.

paar jaar geleden is [illegible]
Medisch Centrum in Amsterdam. Hoeveel levens de bacterie kost, kan Kuijper niet zeggen.
Besmettelijkheid: De clostridium difficile is een zogeheten sporenvormende bacterie, wat betekent dat de bacterie zich stevig inkapselt met een beschermende laag. Daardoor is hij goed bestand tegen hitte en uitdroging. De bacterie kan maandenlang overleven.

het hebben van diarree [illegible]
den om een patiënt niet over te plaatsen. Dat is nu anders. Van patiënten met diarree wordt eerst een kweek gemaakt.

Het Harderwijker ziekenhuis heeft nog geen speciale maatregelen getroffen voor het overplaatsen van patiënten naar andere ziekenhuizen.

Bacterie clostridium difficile is onschadelijk voor gezond mens

van een onzer verslaggevers
HARDERWIJK

Bezoekers van ziekenhuis St. Jansdal hoeven niet bang te zijn voor de gevolgen van een eventuele besmetting met de bacterie clostridium difficile, die in het ziekenhuis bij enkele tientallen mensen voor problemen zorgde en twee levens eiste.

De bacterie veroorzaakt pas infecties na een verstoring van de flora in de dikke darm. Dat is bijvoorbeeld het geval na een operatie aan de dikke darm of bij het gebruik van bepaalde antibiotica.
Ook gezonde mensen kunnen drager zijn van de bacterie, maar worden er niet ziek van. Voor een ziekenhuis is een besmetting met clostridium difficile echter bijzonder vervelend. De sporen van de bacterie kunnen weken tot maanden in leven blijven en zijn bestand tegen uitdroging, verhitting en chemische stoffen. De omgeving van een besmette patiënt in het ziekenhuis is dan ook vaak zwaar besmet, vooral vloeren en badkamers, maar ook bijvoorbeeld stoelen, nachtkastjes en thermometers.
Desinfecteren alleen heeft daarom weinig effect. In feite moet de omgeving waar de zieke [illegible] hebben verbleven grondig worden gemaakt. Met het sporenlijke worden ook de sporen derd.
Hoe de bacterie het ziekenhuis nengekomen is niet bekend. het zou heel goed kunnen door zogeheten asymptomatische dragers is geweest: mensen bacterie bij zich dragen, zelf

ziekteverschijnselen vertonen. Tot de familie van clostridium difficile behoren ook de bacteriën die tetanus en botulisme veroorzaken.

men urinewegen als gevolg van tie.
Wondinfecties operatie vo

Onrust onder patiënten en

UITGELICHT

vervolg van voorpagina
door LO VAN DER WAL
HARDERWIJK

Het St. Jansdalziekenhuis heeft gistermiddag veel verontruste reacties te verwerken gekregen. Patiënten en hun familieleden wilden weten wat er precies aan de hand is met de darmbacterie en wat de risico's zijn nu twee ernstig zieke patiënten zijn overleden nadat ze besmet waren.

Er belden ook mensen die hun onderzoek wilden uitstellen. In verband met de onrust overweegt het ziekenhuis vandaag patiënten die komen voor een polikliniesche behandeling bij de polikliniek te informeren.
Ook mevrouw Van de Hoorn uit Putten, die in de ziekenhuiscafetaria net lekker achter een kopje koffie en een broodje zit, schrikt als haar wordt verteld wat er aan de hand is. Ze gaat straks op bezoek bij haar man die in verband met een ademhalingsonderzoek een nachtje in het ziekenhuis moet blijven. 'Dit maakt me onrustig. Ik had het in ieder geval graag eerder willen weten'. Een andere bezoeker die z'n dochter opzoekt die net bevallen is, heeft het nieuws wél gehoord. 'Geen reclame voor het ziekenhuis', meent hij, 'als je naam op zo'n manier elk uur op de radio voorbij komt'. Maar ongerust? Hij niet, niet echt tenminste.
Andere reacties zijn er ook. Patiënte Heleen Koolmoes uit Lelystad vindt het maar 'flauwekul', zegt ze. Ook dat er twee al ernstig zieke mensen zijn overleden nadat ze besmet waren vat ze rustig op. 'Een toevalstreffer'.
Ze wil geen kwaad woord horen over het St. Jansdal, net als trouwens mede-patiënte J.E. Mouw uit Ermelo met wie ze voor de hoofdingang een sigaretje rookt. Bij de Ermelose was de bacterie helemaal geen onderwerp op zaal, op de zaal van Heleen Koolmoes werd er maar even over gesproken..

Woordvoerster Constantine van der Veen vindt het 'heel jammer en ook onnodig' dat onrust is ontstaan. Ze wijst erop dat de kans dat gezonde mensen worden besmet bijna uit te sluiten is. Om die reden gelden dan ook geen beperkingen voor bezoekers met uitzondering van de familie van de vijf besmette patiënten die nu nog in het ziekenhuis liggen. Hen is gevraagd niet ook andere zieken te bezoeken. Buiten het ziekenhuis mogen ze niet iedereen contact hebben.
Het Harderwijker ziekenhuis heeft overigens nog geen speciale maatregelen genomen ten aanzien van het overplaatsen van patiënten naar andere ziekenhuizen. Uiteraard worden de vijf besmette patiënten, die elk in een aparte kamer liggen, niet overgeplaatst. Normaal is het hebben van diarree geen reden om een patiënt niet over te plaatsen. Dat is nu anders. Van patiënten met diarree wordt

Een à twee keer ziek per 10.000 opnames

Voorkomen: Clostridium difficile is een bacterie waarvan patiënten 1 à 2 keer per 10.000 ziekenhuisopnames ziek worden. Dat is een voorzichtige eerste schatting van medisch microbioloog E. Kuijper van het Leids Universitair Medisch Centrum (LUMC) op basis van onderzoek dat hij nog verder moet uitwerken. Hij deed zijn onderzoek in vijf ten Nederlandse ziekenhuizen.
Epidemie: In het St. Jansdal Ziekenhuis in Harderwijk is sprake van een epidemie, met acht tot negen ziekegevallen per duizend opnames. Dit is in Nederland vaker voorgekomen zegt Kuijper. Een paar jaar geleden is nog een uitbraak beschreven in het Academisch Medisch Centrum in Amsterdam. Hoeveel levens de bacterie kost, kan Kuijper niet zeggen.
Besmettelijkheid: De clostridium difficile is een zogeheten sporenvormende bacterie, wat betekent dat de bacterie zich stevig inkapselt met een beschermende laag. Daardoor is hij goed bestand te

Het Harderwijker ziekenhuis heeft nog geen spec

Bacterie patiënten fataal

Twee doden door besmetting in ziekenhuis Harderwijk

Van een onzer verslaggevers

HARDERWIJK **Het Sint Jansdal Ziekenhuis in Harderwijk is getroffen door een besmettelijke darmbacterie, waardoor al twee patiënten op leeftijd zijn overleden. In drie maanden tijd zijn 29 mensen besmet geraakt met de bacterie. Vijf van hen liggen nog in het ziekenhuis.**

Het is voor het eerst dat dit type van de darmbacterie clostridium difficile rondwaart in een Nederlands ziekenhuis. Zij kan hevige diarree en ernstige darmklachten veroorzaken. Patiënten die ernstig zijn verzwakt en bepaalde antibiotica krijgen toegediend, lopen het grootste risico de bacterie te krijgen en daaraan te overlijden. De bacterie is moeilijk uit te roeien omdat ze sporen achterlaat die nauwelijks met desinfecteermiddelen te doden zijn. „Het is ons een raadsel waar de bacterie vandaan komt", aldus een woordvoerster van het ziekenhuis. „De antibiotica die de kans op besmetting vergroten, worden in ons ziekenhuis inmiddels niet meer gebruikt."

In april begon de ellende nadat patiënten op verschillende afdelingen dezelfde ziekteverschijnselen kregen. Omdat het ziekenhuis het niet vertrouwde, is de gezondheidsinspectie ingeschakeld en is uiteindelijk een crisisteam in het leven geroepen. De kans dat de bacterie overwaait naar andere ziekenhuizen, wordt vooralsnog klein geacht. In enkele buitenlandse

difficile. Het type dat in het Sint Jansdal Ziekenhuis rondwaart, is volgens hem zeer waarschijnlijk een nieuw, agressief type dat tot nu toe alleen in de VS, Canada en Engeland is waargenomen. Of het inderdaad om deze variant gaat, wordt nog onderzocht.

Om nieuwe bacteriebesmettingen te voorkomen, zijn in het ziekenhuis maatregelen genomen. Verplegers en medici moeten extra vaak hun handen wassen. Ook wordt extra schoongemaakt. Bezoekers worden over de bacteriebesmetting geïnformeerd. Het bezoek van de vijf besmette patiënten is verzocht niet bij andere zieken op bezoek te gaan. Geen van de vijf is in kritieke toestand.

FOCUS ZIEKENHUISBACTERIËN

Bacteriën waren steeds vaker rond in ziekenhuizen en infecteren daar ernstig zieke patiënten, vooral op de intensive care-afdelingen. Een groeiend aantal bacteriën is nauwelijks nog met desinfecteermiddelen bestrijden. Ze zijn ongevoelig geworden voor antibiotica. Uit voorzichtige schattingen blijkt dat 5 tot 10 procent van alle patiënten een ziekenhuisinfectie krijgt.
Een veel voorkomende ziekenhuisbacterie is mrsa, die voor ernstig verzwakte mensen levensgevaarlijk kan zijn. Patiënten moeten

Het verhaal van de darmbacterie, waarbij een kleine dertig patiënten zijn betrokken, begint in april.

Bacterie is vervelende primeur voor St Jansdal

or LO VAN DER WAL
ARDERWIJK

d het St Jansdal onrust kunn voorkomen door eerder en kaart te spelen over de rmbacterie? Voorzitter Maa Bontemps van de Raad van stuur vindt van niet. 'Ik zou t opnieuw zo gedaan heb'.

t verhaal van de darmbacte waarbij een kleine dertig paten zijn betrokken, begint pril. In een paar weken tijd rdt de darmbacterie enkele len ontdekt in de diarree van patiënten. Het St Jansdal is er vast van overtuigd dat dit ligt aan de nieuwe kweekmethode die nauwkeuriger werkt.
Er is ook geen reden voor paniek. Het is vrij normaal dat af en toe een darmbacterie opduikt in ziekenhuizen. Meestal blijft een behandeling achterwege en gaat de diarree vanzelf weer over. Alleen patiënten die veel last hebben krijgen antibiotica.
Begin mei beginnen de eerste bellen te rinkelen, wanneer weer vier patiënten diarree hebben. Bontemps: 'We hebben toen twee dingen gedaan. Actief zoeken, dat wil zeggen dat we de kweken hebben opgestuurd naar het Universitair Medisch Centrum Leiden met de vraag of de bacteriën van dezelfde stam komen en we hebben hygiënische maatregelen genomen, zoals schorten voor, handschoenen aan en vaak handen wassen'.
Medio juni wordt duidelijk dat alle bacteriën inderdaad van dezelfde stam zijn en vorige week donderdag bericht 'Leiden' dat het om de Canadese variant gaat. Harderwijk heeft daarmee een twijfelachtige primeur, want deze tak die ziekmakender is dan de 'gewone' darmbacterie, is nog niet eerder in ons land opgedoken.
Bontemps: 'We hebben ervoor gekozen dat niet vrijdag, vlak voor het weekend, naar buiten te brengen, maar op maandag. Wel hebben we meteen de hygiënische maatregelen aangescherpt. Ik hoop niet dat mensen denken dat we informatie hebben achtergehouden. We hebben naar ons beste weten gehandeld. Patiënten die de bacterie bij zich hadden en hun familie hebben we ingelicht, want iedere patiënt heeft het recht alles over zijn ziekte te weten, zeker als er risico bij is. Sinds bekend is dat het om de ziekmakender tak gaat proberen we zo maximaal mogelijk tegemoet te komen aan de informatiebehoefte.
Tegelijkertijd loopt het onderzoek naar de oorzaak en proberen we de bacterie verder in te dammen door de maatregelen die we hebben genomen. Het belangrijkste is dat mensen hun angst kwijt raken. Ik zou het heel vervelend vinden als mensen onnodig angstig zijn dat hen of hun vader of moeder iets overkomt in het ziekenhuis'.
Verhalen over de darmbacterie staan ook op internet: www.destentor.nl/jansdal