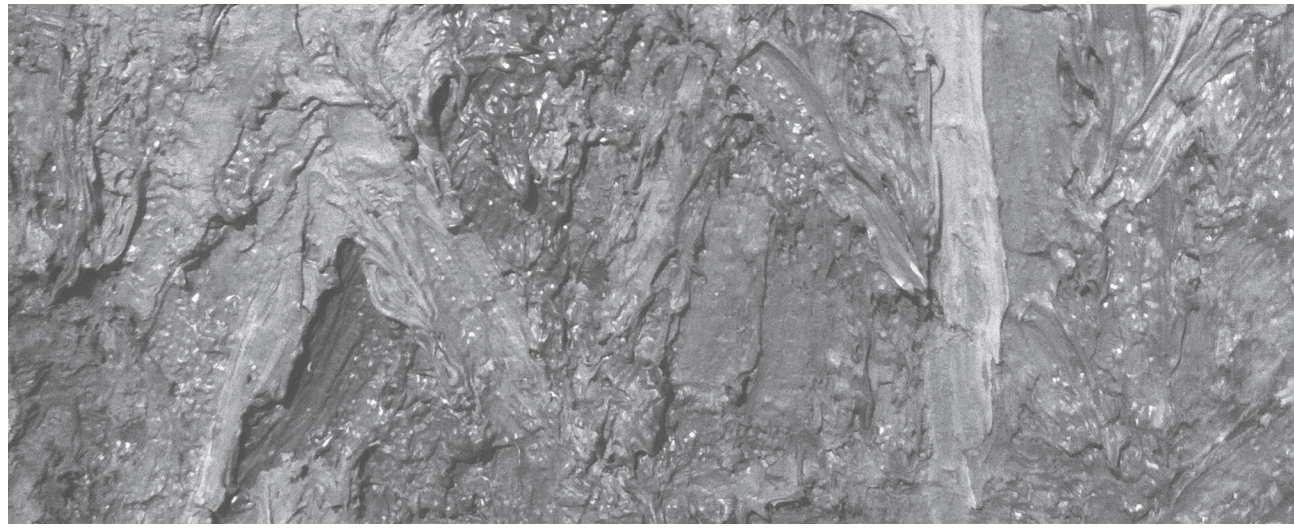
General introduction and outline of the thesis



Background

Clostridium difficile is an anaerobic spore-forming bacillus that can be found in a wide range of habitats, from soil and water to intestines of animals, including humans. The bacterium was identified as the most important infectious cause of antibiotic-associated diarrhea in the 1970s [1]. *C. difficile* infection (CDI) is transmitted via the fecal-oral route. It has been associated mainly with hospitals, where it occurs both endemically and epidemically. However, since the beginning of the new millennium, the epidemiology of CDI appears to be changing. Higher incidence rates of CDI were recorded and large outbreaks with relatively high morbidity and mortality were noticed, first in Canada, followed by the US, the UK and the European mainland [2]. These outbreaks were found to be caused by a specific strain of *C. difficile*, typed as North American pulse field gel electrophoresis type I and PCR ribotype 027 [3]. This change in epidemiology renewed scientific interest in CDI, which led to more advanced understanding of the disease.

Pathogenesis

Much has been learnt about how *C. difficile* causes disease. This has been helped by molecular techniques, such as the construction of *C. difficile* mutants, and the availability of improved animal models. *C. difficile* spores, which are resistant to various physical and chemical attacks, may survive for years. Once they have been ingested and have passed the stomach, they germinate in the intestinal lumen under the influence of the binding of the primary bile acids cholic acid and cheodeoxycholic acid [4] to the receptor CspC [5]. The vegetative forms of the bacterium have to colonize the mucosa, a process that is greatly facilitated by disruption of the resident microbiome, usually as a result of antibiotics. The microbiome of CDI patients has less diversity than that of individuals without CDI. The proportion of lactate-producing bacteria is increased and that of butyrate-producing bacteria is decreased with great proportional losses of firmicutes and bacteriodetes [6]. A healthy microbiome may protect against colonization by *C. difficile* by metabolizing primary bile acids, competing for nutrients and mucosal surface, producing bacteriocins and influencing host defense [7]. The so-called surface layer proteins, especially SlpA, play an important role in adherence to the mucosal surface [8]. Pathogenic *C. difficile* strains produce the toxin TcdB and usually also TcdA. These large clostridial toxins probably leave the bacterial cell through a holin, coded by TcdE [9]. These toxins bind to unknown and probably different surface receptors on epithelial cells, and, after loss of epithelial barrier function, to underlying stromal cells. After binding, the toxins enter the cell through clathrin-mediated endocytosis [10]. Under the influence of decreasing pH in the endosome, TcdB undergoes a conformational change, resulting in its autocatalytic cleavage and release of the N-terminal catalytic domain in the cytoplasm

[11]. The autocatalytic activity of the toxins is partly countered by S-nitrosylation in the intoxicated cell [12]. In the cytoplasm, the toxins glycosylate Rho and Ras family GTPases [13, 14]. Glycosylation of RhoA, Rac1 and Cdc42 leads to loss of organization of the cytoskeleton, microscopically visible *in vitro* by characteristic rounding of intoxicated cells [15]. Although they have 48% homology, the toxins probably have different functions. TcdA appears to play a more important role in loss of epithelial cell polarity and epithelial integrity [16] and TcdB is more potent. TcdB-induced activation of Rac1 leads to assembly of the NADPH oxidase complex on endosomes, resulting in the formation of reactive oxygen species and eventually cell necrosis [17]. Both toxins appear to be capable of causing disease on their own [18]. The resulting pathologic effect is cell necrosis, fluid secretion and a massive influx of neutrophils, leading to the formation of cryptabscesses, which coalesce into macroscopically visible pseudomembranes (figure 1) [19]. Pathologic changes occur mainly in the colon, although ileitis has also been described, especially after colectomy [20]. Some strains also produce a third toxin, the binary toxin or *C. difficile* transferase (CDT), coded by the genes CdtA and CdtB. These genes are not part of the so-called Pathogenicity Locus, which contains genes for the large clostridial toxins. The lipolysis-stimulated lipoprotein receptor (LSR) has been identified as the receptor for CDT [21]. CDT modifies actin by binding ADP-ribose to it. Thus, it increases the cell surface available for bacterial adherence by induction of the formation of protrusions on the intoxicated cell [22]. Although this toxin is produced by the epidemic strain PCR ribotype 027, it is unclear how important this toxin is for its virulence. Certainly, *C. difficile* strains can cause severe disease without it.

Epidemiology

Admission to healthcare facilities and the use of antibiotics [23], which increase the risk of CDI for at least three months [24], are considered the most important risk factors for CDI. In addition, risk factors that have repeatedly been associated with the acquisition of CDI include advanced age [25], serious comorbidity [26], use of proton pump inhibitors [25, 27, 28], and failure to mount an antibody response against TcdA and TcdB [29, 30]. It is hard to pinpoint specific comorbidity predisposing to CDI and the severity of the comorbid illness seems more important than the exact nature. Proton pump inhibitor use is extremely difficult to separate from severe comorbidity, even after correction for confounding. Furthermore, a plausible biological mechanism by which proton pump inhibitors might predispose to CDI is lacking, since *C. difficile* spores, by which transmission mainly occurs, are acid-resistant. CDI has been regarded as a hospital-acquired infection, because patients admitted for other diseases develop CDI during their hospital stay and outbreaks have only been described in healthcare facilities. The hypothesis was that, even though *C. difficile* is a ubiquitous bacterium, it found a niche in hospitals and – to a lesser extent – nursing homes, where the most susceptible population of elderly, chronically ill individuals exposed to antibiotics is concentrated. This population might serve as a reservoir, in which *C. difficile* can multiply. The highly resilient spores are easily transmitted between patients, via the hands of hospital personnel, fomites [31] and even the air, which may contain spores in the vicinity of diarrheic patients [32, 33]. However, doubts have risen as to whether this model is entirely true. A large proportion of endemic hospital CDI cases cannot be linked to other cases in the same hospital [34] and community-onset cases with no apparent link to healthcare facilities have been described [35, 36]. Therefore, it seems likely that colonization with *C. difficile* often occurs outside of healthcare facilities and the disease only becomes symptomatic when other factors, such as the use of antibiotics, occur during a subsequent stay in a healthcare facility. If *C. difficile* colonization is acquired outside of healthcare facilities, what could be the reservoir? The meat industry has been implicated, since *C. difficile* has been cultured from meat products, albeit not consistently, and *C. difficile* is known to colonize and cause disease in farm animals, especially pigs [37]. Typing studies that found similarities between strains colonizing humans and animals have lent support to the hypothesis that animals can be a reservoir from which humans are colonized [38]. On the other hand, outbreaks with links to a food source or farm have not been described, and the link between humans and animals may also be explained by transmission from human to animal.

Various typing methods have been used for *C. difficile*, of which PCR ribotyping has gained greatest popularity. This method is based on the amplification of the variable-length spacer region between the two genes coding for the 16S and 23S ribosomal subunits [39]. Notable PCR ribotypes are the above-mentioned type 027, and type 078, which has been associated with farm animals [40]. Both of these PCR ribotypes are characterized by a deletion in TcdC, a putative negative regulator of toxin expression (although this function is debated [41, 42]), and production of CDT.

Clinical manifestations

The clinical manifestations of CDI vary. After ingestion of spores and successful colonization of the gut, asymptomatic carriage may follow, but in an estimated 15 to 30% colonized individuals [25, 43], symptomatic disease develops. This disease ranges from mild self-limiting diarrhea to fulminant colitis with a severe systemic inflammatory response, leukemoid reaction and ileus. The latter manifestation, which fortunately is rare, may lead to complications such as septic shock and perforation. This severe complicated form of CDI may be refractory to antimicrobial therapy. Usually though, CDI responds to antimicrobial therapy. In this case, the symptoms gradually improve over days to weeks. However, in some cases diarrhea relapses. The proportion of patients who suffer recurrences varies in studies from 6% to 77% depending on the number of previous CDI episodes [44-46], age [47-50], comorbidity

[50, 51], the need to continue antimicrobials for other infections than CDI [49, 50], humoral immune response [52-56], virulence of the *C. difficile* strain [57], treatment for CDI [58, 59] and, again arguably, the use of proton pump inhibitors [49, 60]. The majority of these recurrences are relapses, although some are reinfections [61, 62]. Without typing methods, which are not part of routine practice in most laboratories, this distinction is obviously difficult to make. An additional problem with this distinction is the fact that in some patients, more than one strain may be found at the same time [63]. The meaning of this is unclear as yet, although it seems most plausible that one of these strains represents the causative agent of the disease, whereas the others represent colonization. In order to distinguish between healthcare-associated and community-associated cases, and relapses and reinfections, arbitrary epidemiological criteria have been developed [64], although the biological ground for these criteria may be debated.

Diagnosis

The diagnosis of CDI is hampered by the fact that the distinction between colonization and disease is not always clear. Diagnostics can be based on the demonstration of free toxin (by cytotoxicity assay, based on demonstration of the above-mentioned characteristic cell rounding after exposure to patient feces *in vitro,* or by ELISA) or the bacterium in feces (by nucleic acid amplification test or culture) [65]. Demonstration of free toxin is thought to correlate better with disease as opposed to carriage [66], although toxin ELISAs are less sensitive than cytotoxicity assays. Diagnostic methods that demonstrate the bacterium instead of toxin may be better at distinguishing colonization from disease if they are quantitative [67].

Treatment

Mild CDI that develops during the use of antibiotics may be cured by stopping the antibiotic without directed treatment [68], but more-severe cases must be treated. As mentioned above, CDI usually responds to antibacterial therapy. Antibiotics that have traditionally been used are oral metronidazole and oral vancomycin (or related teicoplanin). The glycopeptides are generally considered slightly more effective than metronidazole on the basis of clinical studies and pharmacokinetics [69]. These antibiotics have the disadvantage that they cause collateral damage by harming the intestinal microbiome, thus predisposing to recurrences of CDI. Fidaxomicin, an antibiotic that came to market in 2011 for the treatment of CDI, has a narrower spectrum, and appears associated with fewer recurrences [58, 59]. Nevertheless, recurrences still do occur and remain the biggest challenge in treating CDI. Therefore, new antibiotics and other treatment modalities are still being searched for. Antimicrobials that are already available for treatment of other infections have been studied for the treatment of CDI. These include fusidic acid [70, 71], nitazoxanide [72, 73], rifaximin [74] and tigecyclin [75]. Several new compounds have been investigated for the treatment of CDI, of which the non-absorbable oxazolidinone cadazolid [76] and macrocyclic thiopeptide LFF571 [77] have been studied in published phase I clinical trials. The toxin-binding resin tolevamer was shown not to be effective [78]. Other treatment modalities may be divided in immunotherapy and microbial therapy. Immunotherapy concerns the administration of non-specific intravenous immunoglobulins or oral [79] or intravenous polyclonal or monoclonal antibodies directed against *C. difficile* and the large clostridial toxins, in order to supply additional antibodies when the patient fails to mount a sufficient humoral immune response. The intravenous administration of two monoclonal antibodies after antibiotic treatment for CDI resulted in a lower percentage of recurrences as compared to placebo [80]. However, selection bias may have been accountable for this result, because during the study, its endpoint was changed from reduction of symptoms in patients with diarrhea to reduction of recurrences in only those patients who became diarrhea-free. Microbial therapy concerns the administration of probiotics, donor feces or non-toxigenic *C. difficile* strains, in order to restore the microbiome and thus the colonization barrier against *C. difficile.* Of these, 'transplantation' of feces from healthy donors is currently the only therapy supported by a randomized trial [81]. There is no high-grade evidence on how to treat CDI when oral therapy is not possible, e.g., because of ileus. In severe cases of CDI with (imminent) toxic megacolon, surgery is the only remaining effective treatment. This consists of subtotal colectomy with end-ileostomy or, more recently, of the creation of a diverting loop ileostomy, followed by colonic lavage and flushing with vancomycin [82].

Prevention and control

Prevention and outbreak control measures are limited to prudent use of antibiotics (if necessary within the context of an antimicrobial stewardship program), adequate hand hygiene (with water and soap) and glove use, and disinfection of medical devices and surfaces of healthcare facilities with chlorine-containing solutions [83]. Isolation and cohorting of CDI patients seems a logical control measure, although there is no high-grade evidence for this. The role of asymptomatic carriers in the spread of *C. difficile* is unclear as yet. There is no convincing evidence that probiotics prevent CDI [84].

It is still unclear what the source of emerging *C. difficile* strains is and how they spread

The emergence of PCR ribotype 027 has been attributed to its acquisition of fluoroquinolone resistance and positive selection pressure from widespread use of these antimicrobials, and to increased virulence [85]. However, where the strain came from is unclear. In general, it is unclear what controls the dynamics of *C. difficile* strains. Where do new strains come from? How do they spread? What drives their spread? In particular, what is the role of the community and what is the role of asymptomatic carriers?

Better predictors of recurrence are needed to guide treatment of CDI

Many episodes of CDI will respond to stopping the inciting antibiotic or a first course of directed antimicrobial therapy. Some patients, however, will suffer one or multiple recurrences of CDI with associated protein-losing enteropathy, malnutrition, hypovolemia and even death. Identifying these patients may influence the choice of treatment. In such patients, an oral glycopeptide may be preferred over metronidazole, in spite of higher cost and (debatable) positive selection pressure for vancomycin-resistant enterococci [86]. In case of a really high risk of recurrence, it may be advantageous to choose costly fidaxomicin up front. A lower risk of recurrences as compared to vancomycin has been shown in patients with a first episode or first recurrence of CDI. Fidaxomicin has not been investigated in randomized trials in patients with multiple recurrences. Although it could be argued that treatment with a small-spectrum agent like fidaxomicin to prevent further loss of diversity of the microbiome in these patients is effective, it could also be argued that the advantage of the microbiome-sparing effect of fidaxomicin is lost in this patient category who have already lost most of the diversity of their microbiome. If the latter were true, it would be even more important to identify patients with a high risk of recurrence during their first episode. Predicting a high risk of recurrence might also lead to the decision to start adjunctive immunotherapy for CDI, or to administer donor feces. Unfortunately, predicting recurrence remains a major challenge, in spite of attempts to construct prediction scores [47].

Outline of this thesis

The research described in this thesis focuses on three issues united by a link to the major clinical challenge of CDI, the risk of a complicated or recurrent course: the distribution of *C. difficile* strains among various populations and recognition of strains associated with complications and/ or recurrences, predicting a complicated or recurrent course of CDI, and choosing therapy in order to minimize the risk of complications and recurrences.

Chapter 1 reviews what is known on community-acquired CDI, illustrated by two case reports of community-onset CDI.

In **chapter 2**, a study is reported that investigates community-onset cases of CDI in The Netherlands, focusing on risk factors and the distribution of *C. difficile* strains.

Chapter 3 reports a study into *C. difficile* carriage among patients with cystic fibrosis, a population in whom CDI is rare, despite the fact that they should be at high risk due to frequent contact with hospitals and high exposure to antibiotics.

Chapter 4 describes a study into the distribution of *C. difficile* strains among CDI cases across European hospitals and their clinical course.

In **chapter 5**, a case-control study is reported that investigated the value of one clinical marker and two biomarkers in predicting primary therapy failure and recurrence after initially successful therapy for CDI.

Chapter 6 describes a study into the association of antibody responses against large clostridial toxins and other *C. difficile* antigens with recurrence of CDI.

Chapter 7 describes a prospective interventional cohort study, in which participants received an experimental product made from whey of cows immunized with killed *C. difficile* and toxoid in addition to antimicrobial therapy for CDI, in order to reduce the risk of a recurrence.

Chapter 8 and **9** are the first version and an update of the guidance document issued by the European Society for Clinical Microbiology and Infectious Diseases for the treatment of CDI.

In the **summary and general discussion**, the conclusions of each chapter are summarized and suggestions for clinical practice and further research are made.

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