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Towards understanding Clostridioides difficile colonization
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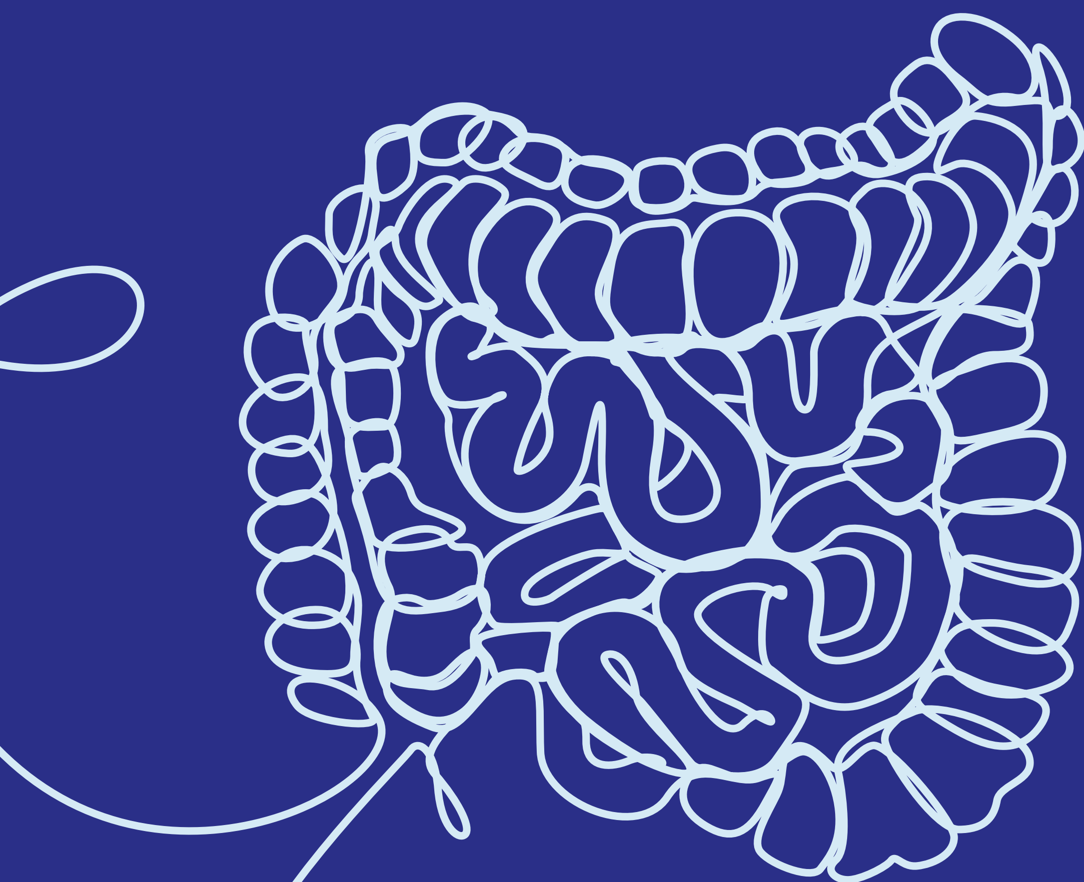
Towards understanding

Clostridioides difficile colonization



Monique J. T. Crobach

Towards understanding *Clostridioides difficile* colonization

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Colophon

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Monique J.T. Crobach
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Towards understanding *Clostridioides difficile* colonization

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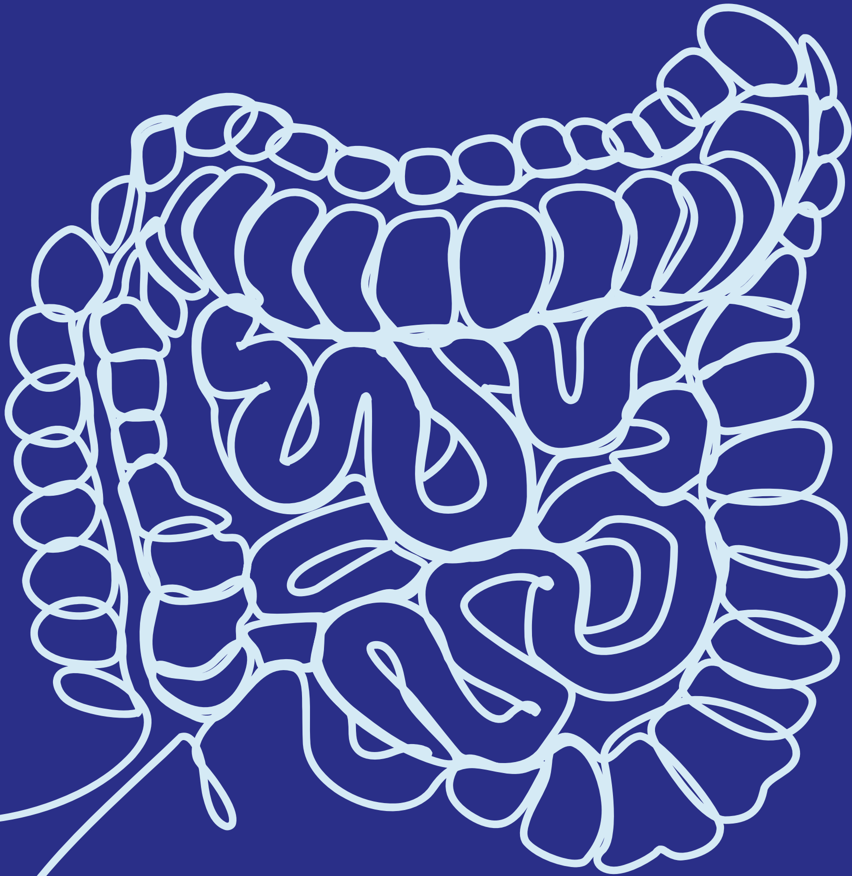
CHAPTER 1
Introduction



Human bodies provide a habitat for microbial communities, such as bacteria, archea, fungi, viruses and parasites. These microbial communities which reside in and on our bodies are together called the microbiota. In a healthy state, there is a symbiotic relationship between the human host and its microbiota. The human gut harbors the densest numbers of microbes, predominantly bacteria. This gut microbiota has a wide range of functions, including degradation of carbohydrates, synthesis of bioactive substances, regulating host immunity and providing colonization resistance against incoming potentially pathogenic microorganisms. An altered microbial composition could potentially disrupt these functions and has been associated with a range of diseases. The most well-known example is the development of *Clostridioides difficile* infection (CDI) in people with a perturbed microbiota after antibiotic use. After the widespread introduction of antibiotics, CDI has become the most reported healthcare-associated infection in Northern-America and Europe. At the beginning of this millennium, the historical view of CDI as a healthcare-associated infection transmitted primarily from other symptomatic CDI patients was challenged. *C. difficile* can also be found as an asymptomatic colonizer of the human gut. These asymptomatically colonized subjects were shown to act as a hitherto underestimated *C. difficile* reservoir, but could also progress to symptomatic CDI upon disturbance of their microbiota. A better understanding of which factors predispose to colonization but at the same time protect from infection is needed. Also, the prevalence of colonization and contribution to *C. difficile* transmission in different settings need to be better defined.

This thesis focusses on *C. difficile* colonization with the aim to better understand this condition and its role in epidemiology of *C. difficile* infections.

This introduction summarizes the essentials about *C. difficile* and the infection it causes.

The bacterium in historical perspective

The bacterium *Clostridioides difficile* was first discovered in faecal samples of healthy infants by Hall and O'Toole in 1935. (1) The bacterium was named *Bacillus difficile* reflecting the initial difficulty to culture the bacterium. Subsequently, it was also discovered as a commensal in the intestines of several animals and also in the environment. (2) It took however until the widespread introduction of antibiotics (and especially clindamycin) that *C. difficile* was recognized as a human pathogen. The increasing application of antibiotics was accompanied by an increasing number of antibiotic-associated colitis. In 1978, experiments by Bartlett and colleagues showed that toxins produced by *C. difficile* were able to induce

colitis. (3) Since then, *C. difficile* has been recognized as the cause of antibiotic-associated pseudomembranous colitis in humans. At that time the name *Clostridium difficile* was introduced, reflecting its rod-shaped appearance (κλωστηρ in Greek). The traditional presentation of *C. difficile* infection was regarded as diarrhea in elderly inpatients or in those recently discharged from healthcare facilities with antibiotic use as the most important precipitating event. However, since the beginning of this millennium, the epidemiology of *C. difficile* infections has changed dramatically. (4-6) Major outbreaks with high mortality rates were reported both in the United States and Canada. These outbreaks were due to the emergence of a new more virulent type, designated as NAP1/PCR ribotype 027, which was associated with more severe disease and a higher transmissibility. Because the NAP1/PCR ribotype 027 strain is resistant to fluoroquinolones, the use of this antibiotic has been considered a significant driver of these outbreaks. (7, 8) In the years that followed, the strain spread to Europe, leading to an increasing CDI incidence and large outbreaks all over the continent. In the Netherlands, the first outbreak due to NAP1/PCR ribotype 027 was reported in July 2005. (9) In reaction to these outbreaks, surveillance systems were set in place to monitor CDI incidence rates and detect outbreaks. Since 2009, a voluntary sentinel surveillance program with a continuous monitoring of CDI cases in hospitalized patients had been established in the Netherlands. (10) Around 20 acute care hospitals located across the Netherlands contributed to this surveillance, supplying data from approximately 1000 CDI cases annually. Clinical data from all CDI cases were collected and isolates were typed and characterized at The National Reference Laboratory for *C. difficile* (a joint initiative from the Department of Medical Microbiology of the Leiden University Medical Center and the National Institute for Public Health and the Environment (RIVM)). In addition, outbreak investigations were offered to all healthcare facilities in the Netherlands with an unanticipated increase in CDI cases. Supplementary to national surveillance programs, an European CDI surveillance program was initiated by ECDC since 2016. (11, 12) Data from the first surveillance year demonstrated that PCR ribotype 027 was the most common ribotype in Europe, accounting for 22.9% of all characterized isolates. (13) CDI became the leading cause of nosocomial infectious diarrhea in adults, with an estimated 10-25% of antibiotic-associated diarrhea cases due to CDI. (14, 15)

Meanwhile, it was noted that CDI was not restricted to the hospital environment. Instead, it has an important share in infectious diarrhea in the community, too. Data from a study performed between 2010-2012 showed that community-associated CDI equalled infections due to *Campylobacter species* and outnumbered infections due to *Salmonella species*. (16, 17) Although part of CDI cases diagnosed in the community can be linked to previous

hospitalization, it was appreciated that another part of community-onset cases are truly acquired in the community. Interestingly, patients with community-acquired CDI often lack the specific risk factors for CDI, like previous antibiotic therapy and older age. (18, 19)

The changing epidemiology of CDI not only led to the initiation of systematic surveillance, but also gave a boost to *C. difficile* research to better identify risk factors for the disease, and optimize diagnostic and treatment options. The molecular typing methods that became available during the last years have initiated multiple studies that explore transmission patterns of *C. difficile* and demonstrated that asymptomatically colonized patients may also contribute to transmission. (20, 21) Innovations in microbiota research techniques have also led to an increased interest in patients with CDI as subjects for microbiota investigations, given that CDI is the classical example of a condition associated with a perturbed and diminished microbiota. (22-24) In 2016, the bacterium was renamed *Clostridioides difficile* based on phenotypic, chemotaxonomic and phylogenetic analyses. (25)

Over the last years, CDI incidence in hospitalized patients in our country has stabilized at a rate of 3 cases per 10,000 patient-days. (10) The proportion of RT027 in the Netherlands is relatively low; it is still found in sporadic cases but outbreaks are rare. (Figure 1) This enables research in transmission pathways and *C. difficile* sources without the disturbing effect of specific strains or specific risk factors associated with outbreaks.

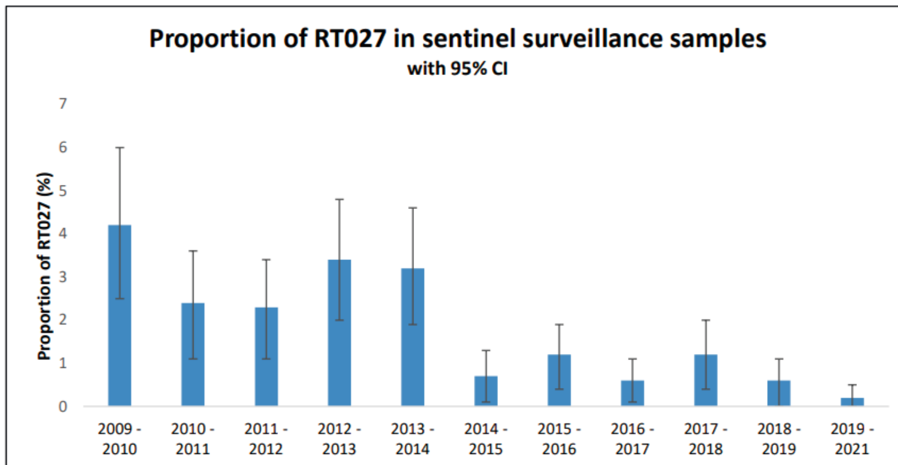


Figure 1. Proportion of RT027 in isolates obtained for Dutch sentinel *C. difficile* infection surveillance 2009-2021.
CI, confidence interval; RT, ribotype. Adopted from Vendrik *et al.* (10)

Pathogenesis

C. difficile is a Gram-positive spore-producing rod. Although the vegetative *C. difficile* forms require strict anaerobic conditions, its spores are resilient to oxygen, heat, detergents and alcohol. Spores enable *C. difficile* to survive outside patients for prolonged periods, as they can remain viable in the (hospital) environment for weeks to years. (26, 27) After ingestion of spores, bile acid induced germination occurs in the small intestine. (28) Thereafter, vegetative cells enter the colon where they can colonize. The extent to which spores germinate and subsequent colonization is established is dependent on the gut microbiota and its metabolome. A healthy gut microbiota offers colonization resistance by competition for nutrients or place and production of metabolites and bacteriocins. However, in case of a disrupted microbiota (e.g after antibiotic use) this defense mechanism is no longer in place and *C. difficile* can start to colonize and outgrow in the gut. For example, a microbiota depleted of bacteria that turn primary bile acids into secondary bile acids may lead to an advantage for *C. difficile*, as primary bile acids stimulate *C. difficile* sporulation while secondary bile acids suppress *C. difficile*. (29, 30)

After colonization has been established, *C. difficile* can remain inactive (asymptomatic colonization) or start to produce toxins. The factors that stimulate or suppress the progression from colonization to toxin production will be explored later in this thesis. The exotoxins Toxin A (TcdA) and Toxin B (TcdB) are most important in causing the *C. difficile* associated symptoms. After secretion, TcdA and TcdB bind to and enter the colonic epithelium. Here they cause production of cytokines, neutrophil influx, disruption of tight junctions, fluid secretion and cell death. (31, 32) This inflammatory response leads to the characteristic formation of pseudo-membranes.

The quantities in which the toxins are produced vary between different *C. difficile* strains. Some strains, including the epidemic RT027, produce a third toxin, called *C. difficile* transferase (CDT, also named binary toxin), which might ease adherence and colonization by forming microtubule-based protrusions, although its definite role in CDI pathogenesis is not clear yet. (33, 34) Strains that are not capable of producing any toxins -the so-called non-toxigenic strains- do not cause symptoms.

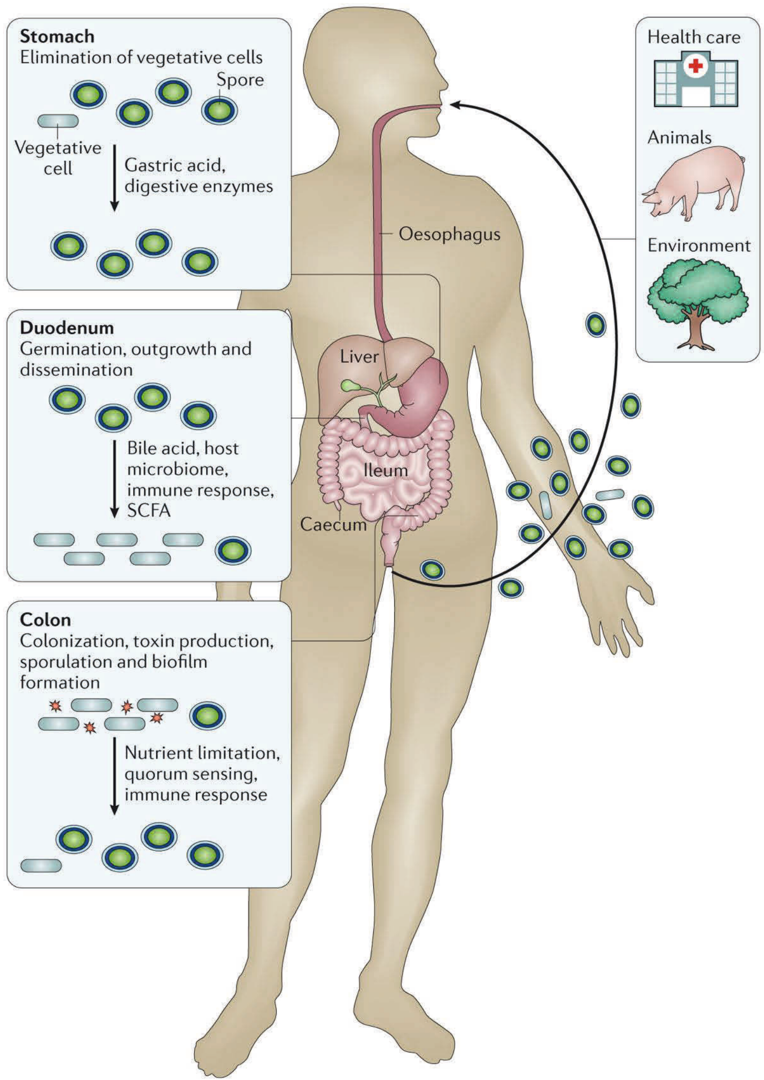


Figure 2. Stages of the *C. difficile* life cycle in the human gastrointestinal tract.
Adopted from Smits *et al.* (32)

Detection and typing methods

Although more than 40 years have passed since the discovery of *C. difficile* as a human pathogen, the optimal approach for detection of CDI is still a matter of debate. The main problem is the absence of an easy and rapid diagnostic test that has optimal sensitivity

and specificity. Moreover, many assays that are used to diagnose CDI do not differentiate between true infection and asymptomatic colonization. (Table 1) Guidelines have been developed to optimize CDI diagnostics (see later in this thesis). (35-38)

Table 1. Methods used for diagnosing *C. difficile* infection (CDI).

Question to be answered	Detection method	Advantages	Disadvantages
Is *C. difficile* present?	Culture	Sensitive, but presence does not equate with infection as many *C. difficile* strains are non-toxigenic. Useful for epidemiological investigation and surveillance.	Slow detection (days). Suboptimal sensitivity in inexperienced hands. Requires anaerobic culturing capability.
	Antigen (glutamate dehydrogenase) detection	Highly negative predictive value. Rapid detection (hours).	Not specific for *C. difficile* and therefore requires supplementary testing.
Is *C. difficile* toxin present?	Cytotoxin assay	Sensitive. High specificity for infection.	Slow (minimum 1-2 days). Requires access to and/or experience of cell culture methods.
	Enzyme immunoassay	Familiar methodoogy than can be used widely. Rapid (hours).	Variable sensitivity and specificity resulting in low positive predictive values, especially in populations with low prevalence of *C. difficile* infection. Requires laboratory facilities.
	Membrane assays	Does not necessarily require laboratory facilities. Rapid (minutes to hours).	Variable sensitivity and specificity resulting in low positive predictive values, especially in populations with low prevalence of *C. difficile* infection.
Does the *C. difficile* have the capacity to produce toxin?	Cytotoxigenic culture	High sensitivity	Uncertain specificity for infection. Slow (days).
	Detection of toxin B gene	High sensitivity. Rapid (hours).	Uncertain specificity for infection. Requires laboratory and molecular expertise. High cost.

Adopted from Rupnik *et al.* (39)

Different *C. difficile* strains exist, all with unique characteristics. The most important characteristic is the capability or inability to produce toxins, dividing strains in toxigenic

versus non-toxigenic strains. Strains may differ in virulence but also in reservoirs: while RT027 is considered mainly a healthcare-associated strain, RT078 is often considered livestock-associated and RT265 is often found among children. (40, 41)

At the moment, different typing methods exist. In Europe, PCR ribotyping has traditionally been used as the standard typing method. It relies on the variability between strains in the size and number of 16S-23S intergenic spacer regions. After PCR amplification, the PCR products can be separated by gel electrophoresis. As the number and size of the PCR products will vary between strains, strain-specific banding patterns will be formed. These banding patterns are referred to as PCR ribotypes. (42, 43) The Dutch Reference Laboratory for *C. difficile* is currently able to recognize 306 different PCR ribotypes. (10) In the US, restriction enzyme analysis (REA) and pulsed-field gel electrophoresis (PFGE) have more frequently been used up till recently. Therefore, strains are often indicated with different names based on the typing method, for example the epidemic strain that caused outbreaks in the beginning of this millennium is designated as PCR ribotye 027/NAP1/B1 based on PCR ribotyping, PFGE typing and REA typing, respectively. Capillary gel-based electrophoresis ribotyping (instead of the conventional agarose gel-based ribotyping) has been standardized and enables comparison of ribotyping results between laboratories and with international reference databases, and should be considered as the gold standard nowadays. (44, 45) Another typing method that allows interlaboratory comparison is the sequence-based genotyping method Multilocus sequence typing (MLST). (46) This method uses polymorphisms in nucleotide sequences of seven housekeeping gene fragments to discriminate between strains. A sequence type (ST) can be allocated to every strain based on the combination of their housekeeping gene variants.

As not all patients with the same PCR ribotype or ST will necessarily have an identical *C. difficile* strain, more discriminant typing methods should be used when studying transmission patterns or outbreaks. Multilocus variable number of tandem repeats analysis (MLVA) is a band-based genotyping method and makes use of the fact that different strains contain a different number of tandem repeats at certain loci. Amplification of these regions results in a sum of tandem repeat number differences between individual strains. The less differences, the more related strains are. (47, 48) MLVA is able to discriminate among strains that have the same PCR ribotype. A minimum spanning tree (MST) can be constructed to graphically present the result of the MLVA.

Even more discriminatory sequence-based genotyping methods that have become available during the last years use whole genome sequencing (WGS). The first typing method based on WGS is single nucleotide polymorphism (SNP) analysis. SNP analysis compares the studied genome with a reference genome to detect SNPs. This method has the highest resolution, but is time-consuming, laborious and lacks standardization. Other WGS-based typing methods are gene-by-gene allelic profiling of the core genome (cgMLST) or whole genome (wgMLST). Both cgMLST and wgMLST are a bit less discriminative but more rapid than SNP analysis and are standardized as the schemes are maintained in publicly or commercially available databases. However, it is important to realize that intra-ribotype allele differences vary per ribotype and ST. Therefore, when studying outbreaks in clades with low intra-ribotype allele differences, even cgMLST may be not discriminative enough.(49)

Table 2. Genotypic methods for typing of *C. difficile*.

Method	Target	Discriminatory power	Reproducibility
Band-based			
PCR ribotyping (conventional)	16S-23S ISR	moderate	moderate
Capillary PCR ribotyping	16S-23S ISR	moderate	good
MLVA	tandem repeats in whole genome	good	moderate
Sequence-based			
MLST	7 housekeeping genes	moderate	good
cgMLST	core genome, gene-by-gene allelic profiling	excellent	good
wgMLST	whole genome, gene-by-gene allelic profiling	excellent	good
SNP typing	whole genome, SNPs	excellent	moderate

Modified from Knetsch *et al.* (50)

Clinical disease and treatment

The symptoms associated with *C. difficile* infection depend on susceptibility of the host and the virulence of the infecting strain. As the bacterium is non-invasive, infection in other organs than the colon is negligible. Typical symptoms include diarrhea, lower abdominal pain, fever, nausea, anorexia and sometimes dehydration. (51) The typical finding by colonoscopy in more advanced cases is a so-called pseudomembranous colitis. Yellowish pseudomembranes are formed through destruction of normal bowel anatomy, ulcerations and hemorrhages. (52) In very severe cases toxic megacolon might develop which is defined

as a dilation of the colon to a diameter more than 6cm. Toxic megacolon may be associated with sepsis and multi-organ failure and mortality associated with toxic megacolon is high. (53) The most well known risk factors for CDI include age > 65 years, antibiotic use and prior hospital admission. (54) In patients with healthcare-associated CDI, carbapenems and third- and fourth-generation cephalosporins are most strongly associated with development of CDI, but fluoroquinolones, clindamycin and β-lactamase inhibitor combination penicillin antibiotics are also known to increase CDI risk. (55) Disruption of the gut microbiota can persist for > 3 months after antibiotic therapy, making patients susceptible for CDI even months after antibiotic treatment. (39) (Figure 3) Proton pump inhibitor use is also frequently cited to predispose to CDI, although the exact association and cause-effect relationship are still a matter of debate. (56)

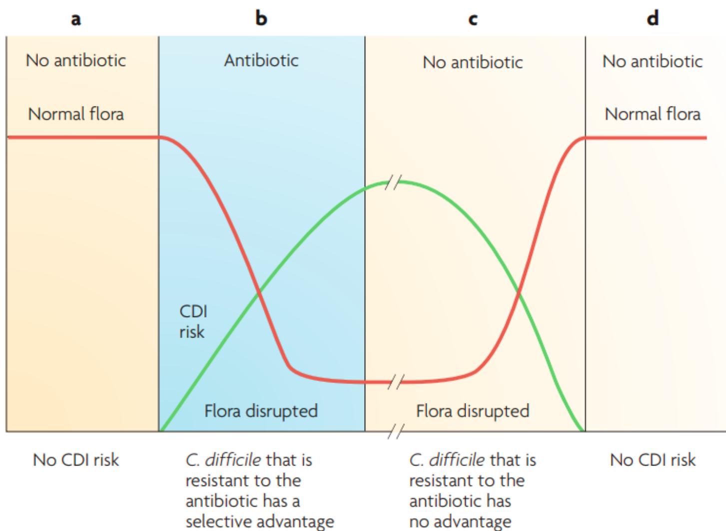


Figure 3. The effect of antibiotics on the normal gut flora and the risk of *C. difficile* infection (CDI). CDI, *Clostridioides difficile* infection. Adopted from Rupnik *et al.* (39)

The first step in treating CDI is discontinuation of the provocative antibiotics, where possible. Further treatment depends on severity of disease and the risk to develop a recurrence. Most recent ESCMID guidelines recommend the antibiotic fidaxomicin as the preferred option for treating a first CDI episode. (57) (Figure 4) Fidaxomicin has a narrow antibiotic spectrum and is therefore less detrimental to the gut microbiota than for example vancomycin, which is suggested as a cheaper alternative for fidaxomicin but associated with more recurrences.

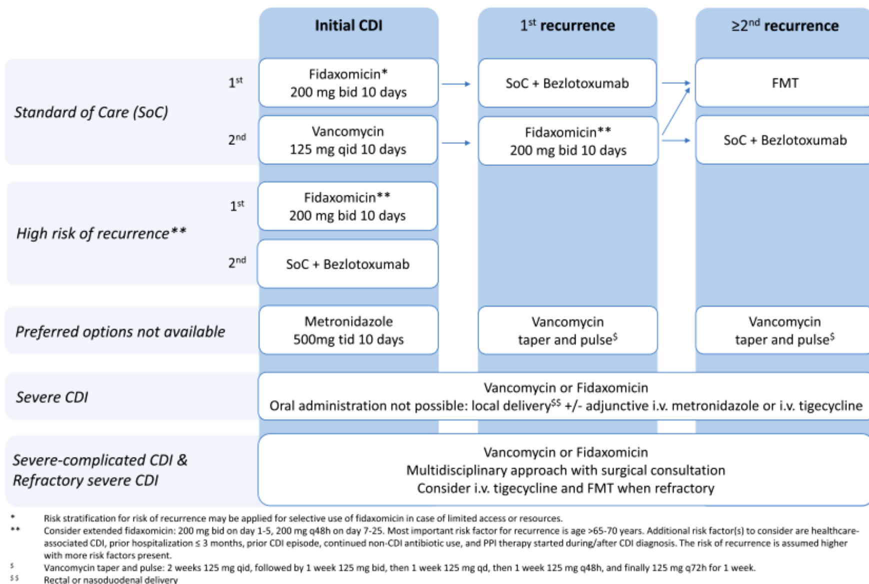


Figure 4. Suggested treatment algorithm according to European Society of Clinical Microbiology and Infectious Diseases: 2021 update on the treatment guidance document for *Clostridioides difficile* infection in adults
CDI, *Clostridioides difficile* infection; FMT, fecal microbiota transplant. Adopted from van Prehn *et al.* (57)

Although antibiotic therapy is able to cure most cases, their use might further disturb the microbiota composition in the gut. This explains the high risk of recurrence in the first weeks after an initial CDI episode. (58, 59) After a first recurrence, there is an even higher risk of a second or third recurrence. (59) Recurrences can either be a relapse of infection with the same strain, or a re-infection with a new strain. Treating these recurrences can be challenging. A potential method to reduce the risk of recurrence is to supplement the antibiotic treatment with Bezlotoxumab, a monoclonal antibody directed against toxin B. (57, 60) A subset of patients however suffers from multiple recurrent CDI every time after cessation of anti-CDI antibiotics. The gut microbiota in these patients is severely and persistently disturbed and is a driving force in maintaining the disease. A treatment option for these patients is restoring the microbiota composition by fecal microbiota transplantation (FMT). (61) In the Netherlands, a National Donor Faeces Bank (NDFB) was set up in 2016 at the Leiden University Medical Center to facilitate transplantation of carefully screened donor faeces. FMT is reported to have sustained cure rates of over 70%. (62) Recent trials have demonstrated that administration of a live biotherapeutic product (like

RBX2660 or SER-109) after standard-of-care antibiotics may also be beneficial for patients at high risk of recurrence. (63, 64) Other future therapeutic or preventive strategies may include concomitant administration of ribaxamase (a poorly absorbed beta-lactamase) when administering broad-spectrum beta-lactam antibiotics or active immunization. (57)

Outline of this thesis

This thesis provides an overview of what is currently known about *C. difficile* colonization (CDC), and will place CDC into the broader context of *C. difficile* infection (CDI) epidemiology. Studies investigating laboratory protocols to diagnose CDI are included, as accurate CDI diagnosis is a prerequisite to discern CDI from CDC. Besides, studies in this thesis focus on risk factors for CDC and transmission of *C. difficile*.

Chapter 2 is an extensive review on the current understanding of *C. difficile* colonization. It includes both epidemiological data and the pathophysiological background of *C. difficile* colonization.

Chapter 3 describes the laboratory methods that can be used to diagnose CDI. It includes a meta-analysis of commercially available assays to determine the sensitivity and specificity of these types of assays. These calculated sensitivity and specificity for the different types of assays were used to propose the most optimal diagnostic algorithms for CDI diagnosis. Findings from this review were formalized in recommendations and endorsed as European Guidelines for diagnosing CDI.

Chapter 4 describes the application of quantitation of the nucleic acid amplification test (NAAT) for diagnosing CDI. Samples from asymptomatically colonized patients as well as symptomatic patients were included in the analysis to assess if this method can differentiate between these two groups.

Chapter 5 describes an analysis of the gut microbiota composition of patients with *C. difficile* colonization, *C. difficile* infection and non-colonized patients. Using 16s rRNA gene amplicon sequencing possible differences in microbiota composition between these 3 groups are explored.

Chapter 6 describes the results of a multi-centre study investigating the prevalence of CDC on admission to the hospital. The risk for colonized patients to develop CDI was

investigated. Whole genome sequencing was used to investigate onwards transmission from colonized patients.

Chapter 7 demonstrates the work-up once an outbreak of CDI is suspected based on data submitted for sentinel CDI surveillance. The outbreak was due to a newly discovered PCR ribotype 826 and characteristics of this newly identified strains are explored.

Chapter 8 describes the characteristics of hospitalized patients with community-onset CDI using data from the sentinel CDI surveillance program. The study describes the share of these patients in total CDI burden and CDI associated complications in the hospital setting.

Chapter 9 provides a general discussion. The main findings of this thesis are underlined and put in perspective.

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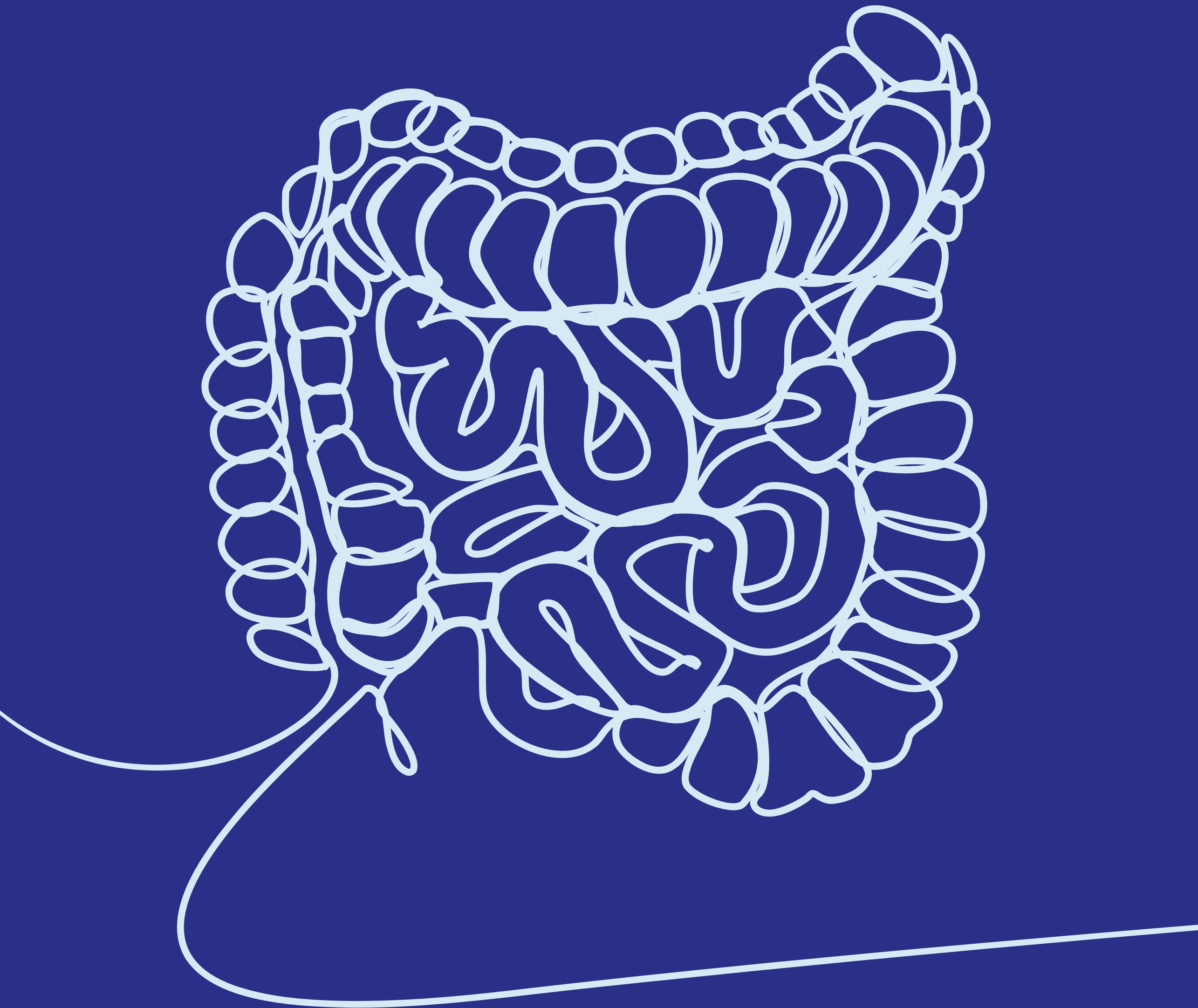
CHAPTER 2

Understanding *Clostridium difficile* colonization



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Summary

Clostridium difficile is the main causative agent of antibiotic-associated and health care associated infective diarrhea. Recently, there has been growing interest in alternative sources of *C. difficile,* other than patients with *Clostridium difficile* infection (CDI) and the hospital environment. Notably, the role of *C. difficile* colonized patients as a possible source of transmission has received attention. In this review, we present a comprehensive overview of the current understanding of *C. difficile* colonization. Findings from gut microbiota studies yield more insights in determinants that are important for acquiring or resisting colonization and progression to CDI. When discussing the prevalence of *C. difficile* colonization among populations and its associated risk factors, colonized patients at admission to the hospital merit more attention as findings from the literature have pointed to their role both in health care associated transmission of *C. difficile* and a higher risk of progression to CDI once admitted. *C. difficile* colonization among patients at admission may have clinical implications, although further research is needed to identify if interventions are beneficial to prevent transmission or overcome progression to CDI.

Introduction

Clostridium difficile is a spore-forming, gram-positive rod causing *Clostridium difficile* infection (CDI), which may range from mild diarrhea to life-threatening pseudomembranous colitis. *Clostridium difficile* infection has been considered as a healthcare associated infection transmitted primarily from other symptomatic CDI patients. Recent studies, notably based on highly discriminatory techniques like whole genome sequencing, have emphasized that assumptions about the sources and transmission of *C. difficile* may not be correct (1-3). The realization that a large proportion of CDI cases are not due to transmission from other CDI cases has underlined the need to re-examine the many diverse potential sources of *C. difficile*, and to determine their contribution to the epidemiology of this disease. Paramount to our understanding is the issue of colonization of *C. difficile*, which is the subject of this review.

Definitions

Definition of *C. difficile* colonization

The authors of this review define "*C. difficile* colonization" as the detection of the organism in the absence of CDI symptoms and "*C. difficile* infection" as the presence of *C. difficile* toxin (ideally), or a toxigenic strain type, and clinical manifestations of CDI (Figure 1). Clinical presentations compatible with CDI include diarrhea (defined as Bristol stool chart type 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 (defined as signs of severely disturbed bowel function such as vomiting and absence of stool with radiological signs of bowel distention) and toxic megacolon (defined as radiological signs of distention of the colon, usually $\geq$10 cm diameter, and signs of a severe systemic inflammatory response) (4).

However, as a previous review highlighted, definitions for CDI used in the Infectious Disease Societies of America (IDSA) and European Society of Clinical Microbiology and Infectious Diseases (ESCMID) guidelines differ (5-7). IDSA guidelines accept a CDI diagnosis if *C. difficile* symptoms are identified in combination with either the presence of a toxigenic strain, free toxin in the stool or histopathological evidence of pseudomembranous colitis, whereas recent ESCMID guidelines require the additional exclusion of alternative etiologies for diarrhea. Differences in definitions for CDI may affect the proportion of patients regarded as asymptomatically or symptomatically colonized instead of having symptomatic CDI.

Moreover, the criteria used to define asymptomatic carriage/colonization vary considerably among studies. Strict definitions of colonization have been described (8, 9), including classifying asymptomatic carriers as those testing positive for *C. difficile* toxins but no signs of CDI for 12 weeks pre- or post-specimen collection, based on a retrospective record review (2). Highly restrictive definitions are difficult to apply in practice, and therefore use of a simplified definition of multiple positive stools from multiple time points to determine colonization has been recommended (10). In contrast, other studies utilized the less strict definition of colonization as a single *C. difficile* positive stool and the absence of diarrhea (11-13). Clearly, this has implications for who is classified as *C. difficile* colonized and how asymptomatic cohorts are perceived as potential transmission sources. Donskey and colleagues demonstrated that a single *C. difficile* positive fecal sample could imply either colonization, transient carriage or even 'pass-through' (10). We thus indicate the importance of further delineation of asymptomatic carriage into transient and persistent colonization, as outlined in a transmission study by Curry *et al.* (2). Differentiating between repeat, persistent detection (carriage) and point detection (colonization) would enable a greater understanding of transmission events and the infection control practices necessary to prevent CDI spread. However, at the moment longitudinal studies on this topic are lacking.

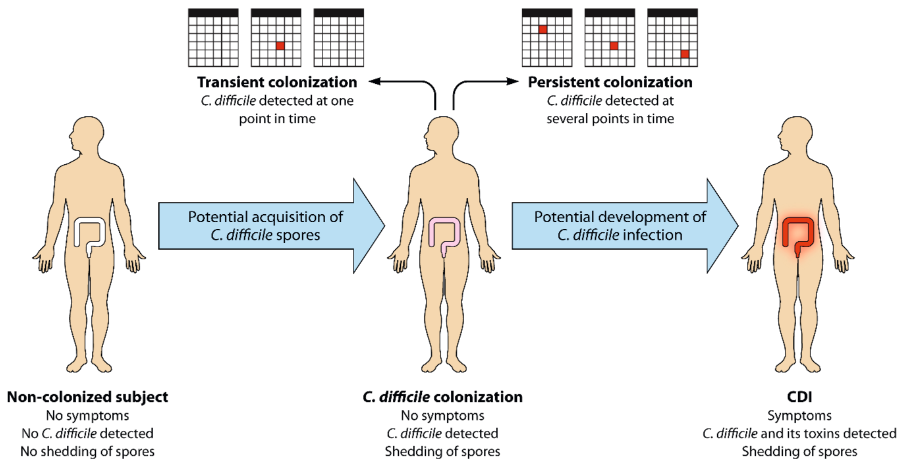

Figure 1. *C. difficile* colonization versus *C. difficile* infection.
CDI, *Clostridium difficile* infection

Assessing asymptomatic colonization

The rates of asymptomatic colonization vary considerably due to the different definitions of diarrhea and laboratory methodological differences.

Standardization of the definition of diarrhea is essential, since McFarland *et al.* defined diarrhea as ≥3 unformed stools for at least two consecutive days (14), whilst others accepted the same number of loose stools, but over a single 24 hour period (12, 15). Therefore, the absence of diarrhea is not synonymous with lack of loose stools, potentially resulting in inconsistent designations of asymptomatic patients.

Besides the disparate definitions for diarrhea, assays or methodologies to test for CDI or *C. difficile* colonization also vary and impact incidence rates of both conditions (13). (See Table 1) Methods used for CDI diagnosis can sometimes also be used for diagnosing *C. difficile* colonization, but on the other hand, some methods used for routinely diagnosing CDI may falsely classify colonized patients with diarrhea (due to a non-*C. difficile* cause) as CDI patients.

Despite its labor intensive and time consuming characteristics and susceptibility to toxin degradation in stool samples with incorrect storage, cell cytotoxicity neutralization assay (CCNA) is frequently considered as the gold standard for CDI due to its high specificity and direct detection of the main virulence factor (toxin) (16, 17). However, as CCNA detects *C. difficile* toxins and not the presence of the organism itself, its utility is limited in detecting *C. difficile* colonization. Nonetheless, in infants, a positive CCNA without clinical symptoms has been used to consider these infants as *C. difficile* colonized (18), indicating the aberrant association between toxin presence and clinical symptoms in this age group.

An alternative gold standard for CDI is toxigenic culture, which includes culture of the organism followed by detection of its in vitro toxin producing capacity by toxin enzyme immunoassay (Tox A/B EIA), CCNA or detection of the toxin genes by nucleic acid amplification test (NAAT). A major study by Planche *et al.* of greater than 12,000 fecal specimens highlighted no increase in mortality in patients harboring a toxigenic *C. difficile* strain without the presence of detectable toxin (19), suggesting that free toxin positivity reflects CDI, while toxigenic culture positivity encompasses some patients with colonization. Therefore, the use of toxigenic culture to diagnose CDI could lead to an over-diagnosis of CDI and hence an underestimation of *C. difficile* colonization. However, if the

goal is detection of toxigenic *C. difficile* colonization in asymptomatic patients, toxigenic culture is a suitable option.

As both gold standard methods for diagnosing CDI are time-consuming and laborious, rapid assays are more appealing for CDI testing in daily practice. When rapid assays are used to test for CDI, it is recommended to use them in an algorithm in order to optimize positive and negative predictive values. Concerning the relationship between free toxins and true disease as described above, the algorithm should include a Tox A/B EIA to test for free toxins in stool. However, in clinical practice, rapid assays and especially NAATs, are often used as stand-alone test instead of as part of an algorithm, and this may again lead to *C. difficile* colonization being erroneously classified as CDI. A study by Polage *et al*. demonstrated that 39.9% of NAAT positive specimens tested negative for toxin by cell cytotoxicity assay (20), showing how reliance on stand-alone NAAT could lead to over-diagnosis of CDI and consequently an underestimation of asymptomatic colonization, similar to the situation described above for TC.

There are some specific limitations that have to be taken into account when assessing *C. difficile* colonizationIn *C. difficile* colonization, bacterial loads can be lower than in CDI. Direct culture of the organism is quite sensitive, although detection rates will differ as the sensitivity of the culture media varies. Nonetheless, culture-independent detection techniques, such as enzyme immunoassays, have lower sensitivity and specificity than culture methods. As stools with lower counts of *C. difficile* could be deemed falsely negative, these assays may lead to underestimation of the asymptomatic colonization rates, making them less suitable for detection of colonization. For example, glutamate dehydrogenase (GDH) screening is regarded as highly specific for detection of *C. difficile* in clinical specimens (7, 21); however, potential issues have been highlighted with the use of this methodology for reporting asymptomatic colonization (22). In a study by Miyajima *et al*., only one out of five positives determined by an enrichment culture method was positive by GDH assay (22), probably due to low levels of GDH antigen in non-diarrheal stools, below the lower limits of detection for this assay.

As the above illustrates, the diagnosis of CDI should not be based on laboratory results alone, but should always be supported by clinical signs and symptoms suggestive of CDI (7, 23). This is especially important when methodologies which cannot discern CDI from colonization (stand-alone NAAT, TC) are applied in routine CDI testing.

Table 1. Diagnostic methodologies detecting *C. difficile* or its toxins.

Diagnostic Test	Target of detection	Able to detect colonization?	Remarks
Direct culture	*C. difficile*	Yes	Does not differentiate between colonization or infection by CD, does not differentiate between tCD and ntCD
Enrichment culture	*C. difficile*	Yes	Does not differentiate between colonization or infection by CD, does not differentiate between tCD and ntCD, thought to be more sensitive than direct culture when low numbers of vegetative cells or spores are present
GDH EIA	GDH	Yes	Does not differentiate between colonization or infection by CD, does not differentiate between tCD and ntCD
Toxigenic culture	Toxigenic *C. difficile*	Yes	Does not differentiate between infection and colonization by tCD
PCR of toxin genes	*tcdA*, *tcdB*, binary toxin genes	Yes	Does not differentiate between infection and colonization by tCD
Toxin A/B EIA	Toxins A and B	No	Detects Toxins A and B and not the presence of the organism, therefore cannot be utilized to identify asymptomatic colonization
CCNA	Toxin B	No	Detects Toxin B and not the presence of the organism, therefore cannot be utilized to identify asymptomatic colonization

tCD – toxigenic *C. difficile*, ntCD – nontoxigenic *C. difficile*, GDH – glutamate dehydrogenase, EIA – enzyme immunoassay, CCNA – cell cytotoxicity neutralization assay, CDI – *Clostridium difficile* Infection, PCR – polymerase chain reaction.

Likewise, we suggest that an optimal diagnostic method for the determination of asymptomatic colonization should include a confirmation of the absence of clinical symptoms (i.e. absence of diarrhea, ileus and toxic megacolon per the criteria described above), or the presence of an alternative explanation for these clinical symptoms. The laboratory methods should include (enrichment) stool culture and either toxigenic culture or PCR confirmation. This combination of sensitive techniques, although expensive, will yield more reliable data and support inter-study comparisons.

Mechanisms of *C. difficile* colonization

After having defined *C. difficile* colonization, a closer look at mechanisms that underlie *C. difficile* colonization is needed. Key factors in acquiring or resisting colonization (and subsequent infection) are the gut microbiota and the host immune response against *C. difficile*.

Disruptions in microbiota

The gut microbiota has a prominent role in the whole life cycle of *C. difficile* from germination and colonization to establishing symptomatic disease. Results from studies on the differences in microbial composition in patients with CDI, asymptomatic carriers and non-infected patients can elucidate which alterations determine either the susceptibility to colonization and/or disease development or colonization resistance (defined as the resistance to colonization by ingested bacteria or inhibition of overgrowth of resident bacteria normally present at low levels within the intestinal tract) (24, 25). The optimal method to study the impact of the microbiota in spore germination, colonization and toxin production by *C. difficile* would be to take luminal samples and biopsies to study both microbiota attached to the intestinal wall and present in the lumen, as *C. difficile* was actually found in biofilm-like structures in the mucus layer of the murine gut and in a human CDI gut model (26, 27). Also, ideally samples should be examined from different locations along the intestine, because it was demonstrated that in mice, *C. difficile* spores did germinate and grow in ileal contents, while this was not possible in cecal contents unless the mice had been treated with specific antibiotics (28). Obtaining these samples in human subjects is not feasible, though ingestible remotely controlled capsules that are capable of taking samples from the small intestinal tract are in development. However, most human studies use easy-to-obtain fecal samples for analyzing the intestinal microbiota, although

these may actually not optimally reflect the microbial composition in the more proximal intestine where bile acid induced germination of the ingested spores occurs (see below).

Alterations in gut microbial composition that have been described for CDI patients include a lower species richness and lower microbial diversity compared with healthy controls (29-31). Between samples from CDI patients, a greater heterogeneity was observed than between individual samples from healthy controls (31). At the phylum level, *Bacteroidetes* were less prevalent in CDI patients than in healthy controls, while there was an increase in *Proteobacteria*. Within the *Firmicutes* phylum, a decrease in the *Clostridia*, especially from the *Ruminococcaceae* and *Lachnospiraceae* families and butyrate-producing anaerobic bacteria from *Clostridium* clusters IV and XIVa was noted in CDI patients (31). In addition to these depletions, increases in the orders of the *Enterobacteriales* and *Pseudomonales (Proteobacteria)* and *Lactobacillales (Firmicutes)* were observed (30, 31). Also, in human fecal samples collected prior to onset of a first CDI episode, a decreased diversity, a decrease in the phylum *Bacteroidetes* and changes within the phylum *Firmicutes* (a decrease in *Clostridiales Incertae Sedis XI* and an increase in *Enterococacceae* from the order *Lactobacillales*) were observed in comparison to samples from hospitalized patients who did not develop CDI (32). A reduction in the family *Clostridiales Incertae Sedis XI* in these samples was demonstrated to be independently associated with CDI development . Moreover, changes in microbial composition comparable to those found in CDI patients have been described for patients with nosocomial diarrhea who tested negative for *C.difficile* or its toxins. These changes included a comparable decrease in species richness and microbial diversity and again a decrease in butyrate producing bacteria from the *Ruminococcaceaea* and *Lachnospiraceae* families in comparison to healthy controls (30, 31, 33). This may indicate that patients with nosocomial diarrhea not due to CDI are also susceptible to development of CDI once they are exposed to *C. difficile* spores. It also suggests that the CDI itself did not much alter the gut microbial composition (31). Among mice that were given clindamycin to render them susceptible to CDI development, luminal samples and biopsies generally confirm the findings in humans and demonstrate a decreased species richness (34). Mice without antibiotic pre-exposure, and therefore undisturbed microbiota, do not develop CDI symptoms after administration of *C. difficile* spores (34). Also, in mice with CDI a microbiota dominated by *Proteobacteria* was demonstrated, instead of a *Firmicutes* and *Bacteroidetes* dominated microbiota as found in healthy mice (34, 35).

Alterations in gut microbial composition in *C. difficile* carriers are less well described, but may give more insight in the mechanisms that allow for colonization whilst protecting against the development of overt disease. One of the few available studies reports a decreased species richness and decreased microbial diversity not only in samples from 8 CDI patients but also in samples from 8 asymptomatic carriers, compared to 9 healthy subjects (29). However, the structure of the microbial community was significantly different among CDI patients and carriers and therefore it is suggested that the absence or presence of certain bacterial taxa is more important in determining the development of CDI or *C. difficile* colonization than the diversity of species richness alone. In carriers, fewer *Proteobacteria* and a higher proportion of *Firmicutes* and *Bacteroidetes* were found than in CDI patients and so this distribution resembled that of healthy individuals more (29). Another study among 98 hospitalized patients (including 4 CDI patients and 4 *C. difficile* colonized patients) showed that, compared with CDI patients, a higher level of *Clostridiales Family XI Incertae Sedis*, *Clostridium* or *Eubacterium* was found just before *C. difficile* colonization was detected, also supporting the notion that the presence of certain bacterial taxa is important to prevent overgrowth or progression from colonization to overt infection (36). Evidence from murine studies also indicates that colonization with certain bacterial taxa may prevent the progression from colonization to CDI; mice precolonized with a murine *Lachnospiracea* isolate showed significantly reduced *C. difficile* colonization (37). Similarly, administration of *Clostridium scindens* in antibiotic-treated mice is associated with resistance to CDI (38). Moreover, in antibiotic-exposed mice who were challenged with *C. difficile* spores, different patterns in microbiota composition were seen in those that developed severe CDI symptoms versus animals who became only *C. difficile* colonized (35). In the first group, a shift towards *Proteoabacteria* was noted, while the latter group had a microbiota that was dominated by *Firmicutes* (including *Lachnospiraceae*) resembling that of mice who had not been exposed to antibiotics. The presence of a *Firmicutes* dominated microbiota seemed to be protective against the development of clinical symptoms in this experiment (35).

Interestingly, a recent longitudinal study in a *C. difficile* colonized infant showed important changes in microbiota composition during weaning. An increase in the relative abundance of *Bacteroides*, *Blautia*, *Parabacteroides*, *Coprococcus* , *Ruminococcus*, and *Oscillospira* was noted suggesting that these bacterial genera likely account for the expulsion of *C. difficile* (39).

In conclusion, there are only a few studies on the intestinal microbiota in patients with asymptomatic *C. difficile* colonization, which are also very limited in sample sizes. However, these studies and findings from mice studies support the idea that a decreased species richness and decreased microbial diversity appear to allow for colonization, although the presence of certain bacterial taxa seems to protect from progression to CDI. Mechanisms by which the microbiome and in particular the presence of certain bacterial taxa may offer colonization resistance and protection against infection will be described below.

Roles of the microbiota

Bile acid metabolism

The first step in establishing *C. difficile* colonization is the germination of spores. Primary bile acids are known to stimulate this germination process (40). The physiological function of primary bile acids is to assist in digesting fat. To be able to do so, after being produced in the liver, primary bile acids are released into and reabsorbed from the small intestine. However, a small amount of the primary bile acids is not reabsorbed and is passed into the colon. In the colon, these primary bile acids are metabolized into secondary bile acids by certain members of the normal gut microbiota. Secondary bile acids inhibit *C. difficile* growth (40). The capacity to metabolize primary bile acids into secondary bile acids by the production of bile acid 7α-dehydroxylating enzymes is shown in members of the *Lachnospiraceae, Ruminococcaceae* and *Blautia* families, all belonging to the phylum *Firmicutes* (28, 41). A disruption in the intestinal microbiota and depletion of *Firmicutes* may therefore cause an increase in primary bile acids and a decrease in secondary bile acids. This was shown in antibiotic-treated mice, where loss of members of the *Lachnospiraceae* and *Ruminococcaceae* families was found to be correlated to a significant loss of secondary bile acids (28). More specifically, this was also shown for one of the members of the *Lachnospiraceae* family, *C. scindens*; the administration of this bacterium was shown to restore physiological levels of secondary bile acid synthesis (38). Loss of secondary bile acids and an increase in primary bile acids creates a favorable environment for *C. difficile.* Support for the role of bile acid metabolism in this susceptibility to *C. difficile* colonization is obtained from both in vitro and in vivo studies. In vitro, spores are able to germinate in the presence of bile acids concentrations found in feces of CDI patients; however, spore germination and vegetative growth was inhibited in the presence of bile acids at concentrations found in patients after fecal microbiota transplant (FMT) or in mice resistant to *C. difficile* (28, 42). In vivo significantly higher levels of primary bile acids and lower levels of secondary bile acids were found in feces from CDI patients compared with controls,

especially in patients with a recurrent CDI episode (43). Notably, the amount of germination in response to bile acids seems to vary between strains, which may be related to mutations in the CspC germinant receptor (called CspC) that recognizes the primary bile acids (42). A *C. difficile* mutant completely deficient for the CspC receptor gene was demonstrated to cause less severe clinical symptoms in a hamster model (40).

Other mechanisms

Apart from the altered bile acid composition, other mechanisms also induced by disruptions of the microbiota are suggested to play a role in conferring susceptibility to *C. difficile*.

First, disruptions in the microbiota that lead to a diminished production of short chain fatty acids (SCFAs) may be of importance. SCFAs are produced from dietary and host-derived carbohydrates mainly by *Lachnospiraceae* and *Ruminococcaceae*, the families that were less abundant in CDI patients and carriers. They may have effect on colonization resistance through reducing the luminal pH (and thereby creating an unfavorable environment for *C. difficile*) (44) and stimulating the defense barrier as one of the SCFAs (butyrate) is the main energy source of the gut epithelium (45, 46). Amino acids may also play a role in the susceptibility to *C. difficile* colonization, as they can enhance germination in the presence of secondary bile acids and may influence the immune system. Moreover, the digestion of carbohydrates in the gut results may impact susceptibility for CDI development. *Bacteroidetes* are mainly responsible for this carbohydrate digestion which results in production of substrates essential for homeostasis of colonocytes (47). A reduction in *Bacteroidetes* may therefore negatively impact colonic health.

Besides the indirect mechanisms described above, the microbiota may also have direct resistant mechanisms against *C. difficile*. These include competition for niches and nutrients and the production of antimicrobials (48, 49).

Roles of the immune system

Innate immunity

The precise protective factors of the innate immunity that prevent colonization and progression to CDI are unknown, but are probably less important than the role of the microbiota and bile acid metabolism. Virulence factors of *C. difficile* induce a rapid innate immune response resulting in an inflammatory response which is necessary to induce adaptive immunity.

CDI is characterized by a severe intestinal inflammatory response in which neutrophils infiltrate the mucosa. TcdA and TcdB play an important role in eliciting this inflammatory response (50). After epithelial barrier disruption, TcdA and TcdB trigger inflammatory signaling cascades through activation of NF-kB, AP-1 and inflammasome, and stimulate production of pro-inflammatory cytokines and chemokines in epithelial cells. This promotes the recruitment of immune cells including neutrophils and induces the production of defensins. Surface proteins also trigger an innate immune response. Challenge of macrophages with *C. difficile* surface proteins (surface layer proteins, SLPs) leads to pro-inflammatory cytokine production such as TNF-α, IL-1β and IL-8 (51).

Additionally, *C. difficile* SLPs interact *in vitro* with TLR4 leading to dendritic cell (DC) maturation, robust Th1 and Th17 responses with production of IFNγ and IL-17, and a weak Th2 response leading to antibody production (52). Ryan *et al*. showed that TLR4 and myeloid differentiation primary-response protein 88 (MyD88) deficient mice were more prone to *C. difficile* infection (53). *C. difficile* flagellin FliC also activates an innate immune response via its interaction with TLR5 inducing predominantly activation of p38 MAPK and, to a lesser extent NF-κB, resulting in up-regulation of the expression of pro-inflammatory cytokine genes and the production of pro-inflammatory factors (54, 55). In vivo, Batah *et al*. showed a synergic effect of *C. difficile* flagellin and toxins in inducing mucosal inflammation (56).

In summary, the innate immune response induces an inflammatory response which promotes an adaptive immune response with memory and long-lasting immunity (see below), but its effects on *C. difficile* colonization are unknown.

Adaptive immunity

The adaptive immunity against colonization or CDI has mainly been studied for its antibody-mediated response whereas the role of the cell-mediated immune response remains unknown.

Serum antibodies against somatic antigens and surface components have been found in asymptomatic carriers and patients recovered from CDI (57, 58), which suggests that surface proteins induce an immune response and modulate disease outcome. Vaccination assays with these proteins have been performed in animal models. Parenteral or mucosal vaccination with the S-layer proteins led to specific antibody production but only partial protection in the hamster model (59, 60). Immunization studies that were performed in

animals with Cwp84 and the flagellar proteins FLIC and FliD by mucosal route resulted in a significant decrease in intestinal *C. difficile* colonization in the mouse model and partial protection in the hamster model (61, 62). Likewise, Ghose *et al.* immunized mice and hamsters intra-peritoneally with FliC adjuvanted with alum, inducing a high circulating anti-FliC IgG response in animal sera, full protection in mice against a clinical 072/NAP1 strain, but only partial protection in hamsters against 630Δerm strain (63). All these results suggest that antibodies against *C. difficile* surface proteins have a protective role against colonization. At the moment, studies with surface protein-based vaccines to prevent colonization in humans are lacking.

Antibodies to TcdA and TcdB do not protect from colonization, but influence disease susceptibility and subsequently the progression from colonization into CDI. Kyne *et al.* studied anti-TcdA IgG antibody levels in patients who became colonized after *C. difficile* exposure. They found that patients who remained asymptomatically colonized had greater increases in anti-TcdA IgG antibodies than patients who progressed from colonization to CDI (64).

Monoclonal antibody (Mab)-based passive immunotherapy directed to toxins was able to protect hamsters from CDI. In humans, two Mabs, one targeting TcdA (actoxumab) and another targeting TcdB (bezlotuxumab) were tested in human clinical trials aimed at the prevention of recurrent disease (65). Bezlotoxumab prevented approximately 40% of the recurrences. A recently published hypothesis suggested that this reduction in recurrences is presumably due to limiting epithelial damage and facilitating rapid microbiome recovery (66), suggesting that reduced (re)colonization may be an important factor, although this should be explored further. Currently, two pharmaceutical firms (Pfizer and Valneva) have vaccine clinical trial development programmes with the two toxins (toxoids or toxin fragments) but no colonization factors as antigens (67); Sanofi Pasteur has recently announced the cessation of its vaccine development programme, which was also based on toxin antigens alone. Therefore these vaccines protect against the toxic effects of *C. difficile* on the intestinal mucosa, and can thereby hinder the progression from colonization to CDI.

In conclusion, a rapid innate immune response induces adaptive immunity to CDI, of which the antibody-mediated response is best understood. Antibodies against *C. difficile* surface proteins are thought to protect against colonization, while antibodies against *C. difficile* toxins protect against disease, directly by its toxin neutralizing effect and possibly also indirectly by limiting epithelial damage and restoring colonization resistance.

Human sources of *C. difficile*

Patients with CDI can shed *C. difficile* not only during the diarrheal episode, but also after completion of therapy. In a study of 52 patients receiving CDI treatment, samples from stool, skin and environmental sites were cultured for *C. difficile* before treatment, every 2-3 days during treatment and weekly after therapy was completed (68). Prior to treatment, 100% of stool samples and approximately 90% of skin and environmental samples were culture positive for *C. difficile*. Stool cultures became *C. difficile* negative in most patients by the time diarrhea resolved at a mean 4.2 days. However, at the same time, skin and environmental contamination with *C. difficile* remained high at 60% and 37% respectively. In addition, stool detection of *C. difficile* was 56% at 1-4 weeks post treatment among asymptomatic patients recovering from CDI. Moreover, 58% had skin contamination with *C. difficile* 1-4 weeks after completion of treatment and 50% had sustained environmental shedding. Persistent skin and environmental contamination was associated with receipt of additional antibiotic therapy. Prior to treatment, the mean density of *C. difficile* in stool samples was significantly higher than at the time that the diarrhea resolved, at end of treatment and at 1-6 weeks post treatment. This study highlights that patients with CDI can be a source of *C. difficile* spores and that they can potentially transmit *C. difficile* to other patients even after diarrhea has resolved. In addition, similar to animal models, continued antibiotic treatment can trigger a "supershedder" state in patients, in which there is *C. difficile* overgrowth and excretion of high concentrations of spores (69).

CDI was historically regarded as a healthcare associated infection transmitted primarily (directly or indirectly) by symptomatic patients, but a growing body of evidence demonstrates that asymptomatic carriers can also transmit the disease.

One study, using MLST (Multi Locus Sequence Typing) could link only 25% of patients with symptomatic CDI to a previously identified CDI patient (1). A follow-up study of the same large patient cohort (>1200 cases) used whole genome sequencing and was able to link at most only 55% (and more likely only 35%) of new cases to previous patients with CDI (3). A much smaller study (~50 cases) using MLVA (Multiple-Locus Variable number tandem repeat Analysis) found that only 30% of new cases could be linked to previously identified cases (2). One could argue that the inability to link new cases to previous ones might be caused by patients with CDI who are clinically undetected. However, strict criteria were used to determine which samples should be tested for CDI in the large UK study (1, 3); although a toxin EIA was used, which is not as sensitive as a reference test, repeat

sampling was carried out according to clinical suspicion of CDI. Depending on the reference test used, the sensitivity of toxin EIA is approximately 60-85%, which means that 15-40% of patients with CDI may go undetected. Nonetheless, this does not account completely for the 45 to 75% of cases that were not closely linked to symptomatic patients (1, 3). This raises the question of what is/are the source(s) accounting for approximately half of new CDI cases? Curry *et al*. examined patients for *C. difficile* carriage who were selected to undergo screening for vancomycin-resistant enterococci. They found that 29% of CDIs could be linked to asymptomatic *C. difficile* carriers (2).

As asymptomatic carriers and the associated shedding of spores usually goes undetected because of lack of routine screening, they can play a role in spread of *C. difficile* to the environment and other patients. Although transmission events from one individual asymptomatic carrier may be rare, as was shown in a relatively small study (15), asymptomatic carriers may still importantly contribute to the transmission of the disease as they likely outnumber symptomatic CDI patients. A recent study showed that 2.6% of patients who were not exposed to *C. difficile* colonized patients developed CDI, while this percentage increased to 4.6% in patients who were exposed (70). Unfortunately, however, the case definition of CDI in this study was based on detection of toxin gene rather than toxin, and so over-diagnosis of true cases likely occurred. Asymptomatic carriers who are colonized at admission appear to contribute to sustaining transmission in the ward. Already in 1992, it was recognized that *C. difficile* strains introduced to the ward by asymptomatic carriers were important sources of onwards health care associated transmission (71), although definitive proof of linkage was hampered by use a non-specific typing technique. More recently, using an epidemiological model of *C. difficile* transmission in healthcare settings, Lanzas *et al*. confirmed that patients colonized on admission likely play a significant role in sustaining ward based transmission (72).

Animal and environmental sources of *C. difficile*

Animals

Similar to humans, CDI or asymptomatic carriage can occur among domestic, farm and wild animals (73-80). Carriage rates in these studies range from 0-100%. These varied observed rates may be related to different culture methodologies and different study settings. Much of this subject has been reviewed in this journal but new information has emerged on possible transmission from domestic and farm animals (81, 82).

C. difficile can cause diarrhea in domestic companion animals such as dogs and cats, but asymptomatic transient carriage of *C. difficile* by household pets is common (11-40%) (73, 78, 83, 84). However, many of these studies did not analyze isolates from humans and pets within the same household. A recent study examined the potential for transmission to pets from 8 patients with recurrent CDI (85), but in this study C. *difficile* was not found in any of the pets. In contrast, Loo *et al.* studied 51 families with 15 domestic pets that included 9 cats, 5 dogs and 1 bird (86). During follow-up visits, toxigenic *C. difficile* was found in cultures of 2 cats and 2 dogs. Probable transmission occurred in 3 of the 15 domestic pet contacts. None of the domestic pets had diarrhea. Typing by pulsed-field gel electrophoresis showed that the profiles of all 4 domestic pet isolates were indistinguishable or closely related to those of their respective index patients. It is conceivable that household pets can serve as a potential source of *C. difficile* for humans.

Transmission from farm animals to humans has been examined using whole genome sequencing using 40 Australian ribotype 014/NAP4 isolates of human and porcine origin (87). A clonal relationship with one or more porcine strains was demonstrated among 42% of human strains underscoring potential interspecies transmission. Similar findings were obtained in a study on 65 *C. difficile* 078/NAP7 isolates collected between 2002 and 2011 that included 12 pairs of human and pig isolates from 12 different pig farms (88). Five (41.7%) of the 12 farmer-pig pairs were colonized with identical and nearly identical *C. difficile* clones (88); the remaining 7 (58.3%) farmer-pig pairs were not clonal suggesting exposure to different sources such as the environment.

Food

With reports that *C. difficile* can be detected among farm animals, studies of *C. difficile* detection in retail food products appeared.

Studies from Canada and the United States report that *C. difficile* can be recovered from retail meat including ground beef, ready to eat beef, ground pork, ground turkey, pork sausage, summer sausage, pork chorizo and pork braunschweiger, with prevalences ranging from 20-63% (89-92). However, the prevalence of *C. difficile* in retail meat products was lower in European countries, ranging from 0-6.3% (93-95). The observed differences in prevalence of *C. difficile* culture positivity in retail meats in North American and Europe is striking. This may be related to seasonal and temporal changes, or may be true observed geographical differences.

Using both quantitative and enrichment culture, Weese *et al*. sought to provide a measure of the degree of contamination from 230 samples of retail ground beef and pork (96). *C. difficile* was isolated from 28 (12%) and notably, approximately 70% of samples were positive by enrichment culture only. Among the samples that were positive on direct culture, the concentration of spores ranged from 20 to 240 spores/gram. Although the infectious dose of *C. difficile* is not known, these findings suggest that although *C. difficile* can readily be recovered from retail meat products, the concentration of *C. difficile* spores is low.

Stabler *et al*. investigated the MLST profiles of 385 *C. difficile* isolates from human, animal and food sources and from geographically diverse regions (97). Animal and food strains were associated with the ST-1 and ST-11 profiles and these strains have been associated with CDI outbreaks in humans. Although the majority of *C. difficile* isolates recovered from retail food products are toxigenic and are of the same ribotypes or MLST to those of human isolates, there have not been any human CDI cases that have been confirmed to be foodborne in origin.

Environment

C. difficile spores can survive in the environment for months or years due to their resistance to heat, drying, and certain disinfectants. Within hospitals, the surface environment is frequently contaminated with *C. difficile*. *C. difficile* has been cultured from many surfaces including floors, commodes, toilets, bed pans and high-touch surfaces such as call bells and overbed tables (14, 98). The frequency of environmental contamination depends on the *C. difficile* status of the patient: fewer than 8% of rooms of culture-negative patients, 8-30% of rooms of patients with asymptomatic colonization and 9-50% of rooms of CDI patients were found to be contaminated with *C. difficile*, respectively (14, 99, 100).

To examine environmental sources outside of the healthcare milieu, Al Saif and Brazier undertook a large study of 2580 samples in Cardiff, South Wales from various sources including water, domestic and farm animals, soil, raw vegetables, surface samples from healthcare facilities, veterinary clinics and private residents (101). One hundred and eighty-four (7.1%) samples were positive. Water samples gave the highest yield of culture positivity at 36%, followed by soil at 21% and healthcare environments at 20%. *C. difficile* was found in 59% of lawn samples collected in public spaces in Perth, Australia and toxigenic ribotypes 014/NAP4 and 020/NAP4 were predominant (102). A Canadian study demonstrated that *C. difficile* was found in 39% of sediments sampled from rivers connected to the discharge

effluent pipe of waste water treatment plants (103). The most common PCR ribotype was 078/NAP7.

In summary, *C. difficile* has been isolated from animals, retail food and the environment. Using ribotyping and whole genome sequencing techniques, there appears to be interspecies and environmental transmission but the directionality of the transmission remains to be elucidated.

Epidemiology of asymptomatic colonization

After having discussed possible sources of *C. difficile* and underlying mechanisms of colonization, a description of the epidemiology of colonization, including the prevalence of colonization rates among different populations, is essential.

Infants (0-24 months)

Asymptomatic colonization rates in neonates and infants (<2 years) are widely reported as high, but range between 4-71% (18, 104-108). Although the clinical relevance of *C. difficile* colonization in infants is considered as less significant, due to low rates of disease in this population (109), its potential as a transmission reservoir for adult populations remains.

An early study researching the prevalence of *C. difficile* in the neonate population found that approximately 30% of all newborns were asymptomatically colonized within their first month of life (18). However, these data included four specimens deemed positive with no identifiable organism, only toxin. Nonetheless, the transient nature of colonization at this early stage was highlighted with only 4 of 10 babies who were culture positive in the first week of life remaining positive at 14 and 28 days. A more recent review corroborated these early figures, pooling data from 5887 subjects to determine a colonization rate of approximately 35% of infants under one year of age (105). This large-scale analysis suggests that colonization peaks between 6-12 months, before substantially decreasing towards adult rates. Although this major review provides a valuable assemblage of data, the variability across methodologies used by the included studies should be taken into consideration.

Geographical differences in infant colonization rates have been identified, with one study indicating a variance of 4-35% across Estonian and Swedish infant populations respectively

(108). The colonization rate was inversely associated with an elevated presence of inhibitory Lactobacilli in Estonian subjects, which may be determined by variation in diet and environmental exposure. A US study of hospitalized infants demonstrated a 20% colonization rate (110) whereas Furuichi *et al*. found no evidence of *C. difficile* colonization amongst Japanese newborns (111). However, the Japanese data were based on culture only, with no attempt to utilize EIA or NAAT to detect low levels of organism. These studies emphasize the variable epidemiology amongst diverse geographical populations.

The source of infant colonization is uncertain, with suggestions that the presence of *C. difficile* in the urogenital tract implicated vaginal delivery as a potential route of transmission to neonates (112). However, later work contradicted this suggestion, failing to detect any *C. difficile* positive vaginal swabs from post-partum mothers (18, 104). Molecular analysis of both infant and environmental isolates demonstrate likely acquisition from environmental sources and patient to patient transmission (113).

Infants are rarely diagnosed with CDI. Bolton and colleagues found that almost 50% of colonized infants carried toxin positive strains, but showed no sign of diarrhea, suggesting that although the relevant toxin genes may be present, they may be minimally (or not) expressed and so fail to cause disease; alternatively, absent or immature toxin receptors may explain the infrequency of CDI despite high colonization rates (18). However, understanding toxigenic strain colonization rates may provide a greater insight into the relevance of this population as a reservoir for transmission to adults. Isolates from infants have shown predominance of ribotypes associated with CDI (106). Adlerberth *et al*. found that 71% of colonized infants had toxigenic strains with more than half identified as ribotypes 001/NAP2 and 014/NAP4 that can cause endemic CDI (114). A comparison of *C. difficile* strains in children (<30 months) with those circulating in the adult (≥18 years) CDI population within the same institution, determined nine shared sequence types among the 20% asymptomatic pediatric subjects (115). This may further implicate infants as a potential reservoir for *C. difficile* dissemination; nonetheless, no direct transmission events were documented in this limited pilot study. Potential community-based transmission from infant carriers to the adult population was alluded to in a longitudinal study demonstrating colonization in all 10 infants at some point in the first year of life, with 3 infants colonized for 4-9 months (116).

Children (2-16 years)

Meta-analysis of studies examining pediatric *C. difficile* epidemiology reported asymptomatic colonization in children older than 1 year at 15%, with prevalence reducing to 5% in those greater than 2 years of age (117). One explanation for the reduction in colonization rates after infancy is that by 12 months the distribution of gut flora begins to closely resemble that of a healthy adult, providing a colonization resistance effect. Nonetheless, contemporaneous studies have reported higher rates of up to 30% asymptomatic colonization amongst non-infant pediatric populations (111, 118, 119). Similarly, Merino and colleagues found that around a quarter of US children aged 1-5 years were colonized by *C. difficile* asymptomatically (120). By using a molecular identification method, classifying groups by the presence of the Toxin A gene (*tcdA),* the Toxin B gene (*tcdB)* and binary toxin genes (*cdtA/B*), they found that although 3/37 asymptomatically colonized children harbored a strain with toxigenic genes *tcdA* & *tcdB*, none carried the binary toxin genes *cdtA/cdtB*. Ferreira *et al.* (121) found low levels of toxigenic *C. difficile* in Brazilian children, arguing that the majority of acute diarrhea in this cohort is likely to be associated with entirely different enteropathogens. These epidemiological variations should be considered in the context of widely differing enteric pathogen populations between developing and developed countries.

Healthy adults

Previous studies indicate that the asymptomatic colonization rates amongst healthy individuals range from 4-15% (Figure 2). However, these studies have often been based on point prevalence detection of *C. difficile,* making a true carriage rate difficult to ascertain. Nevertheless, such a prevalence of even transient colonization by *C. difficile* suggests significant potential for exposure to the bacterium in the community setting among healthy populations.

It is important to note the proportions of toxigenic strains because of their importance for transmission and potential for CDI. Work carried out amongst healthy Japanese adults reported a high colonization rate (15.4%), with around 70% harboring toxigenic strains (122). However, a more recent US study discovered that all strains contributing to a 6.6% asymptomatic colonization rate were toxigenic (13). This rate is higher than seen in large patient transmission studies (2, 12, 71) suggesting that the healthy adult data may be skewed by relatively small study cohorts (n=149 (122); and n=139 (123)).

Ozaki *et al*. identified matching PCR ribotypes amongst a cohort of healthy company employees, as a potential indication of a shared work place as a common source or representing human cross-transmission within this cohort (123). As well, they highlighted the transient nature of colonization, with only 37.5% demonstrating carriage with the same strain within a follow-up period of 1 year. Galdys *et al.* also found that approximately 33% of participants remained positive with the same strain, in samples submitted one month apart (13). Another study used cluster analysis to highlight that although colonization amongst healthy groups acts as a reservoir for community acquired CDI, it may only occur infrequently between families (124). Although a previous study has implicated the family environment as a source of transmission of *C. difficile* (125), Kato *et al*. found only one instance of a shared strain type amongst family members, across 22 families with 1 *C. difficile* colonized index patient.

Patients at admission to a hospital

Patients at admission to a hospital are a considerable reservoir for *C. difficile* and, importantly, a potential source of nosocomial transmission. Asymptomatic colonization rates among patients at admission to a hospital range from 3-21% (11, 12, 98, 126-132). (Figure 2) A large study by Clabots and colleagues reported that 9.6% of admissions to the study ward were colonized; admissions from home had the lowest colonization rate (6%), but nonetheless accounted for the second most prevalent method of *C. difficile* introduction, due to their greater numbers (71). A major Canadian study of over 5000 admissions demonstrated a lower *C. difficile* prevalence rate, with 4.05% asymptomatically colonized (133); this rate was very similar in a more recent large-scale study (4.8%) (134). Kong *et al.* suggested that these low rates may be due to regional distribution, as the majority of *C. difficile* colonized patients in this multi-institution study were based in hospitals with higher proportions of NAP1-associated CDI (133).

A recent meta-analysis of studies reporting toxigenic *C. difficile* colonization rates upon hospital admissions, reported a rate of 8.1% among almost 9000 patients (135). Although this overall rate provides a strong insight into the prevalence of toxigenic *C. difficile* colonization, the meta-analysis excluded certain large studies due to methodology differences, in order to attain maximum compatibility of data sets. Such exclusions may well have impacted on the reported colonization rates.

Two considerably smaller studies have reported higher *C. difficile* colonization rates, highlighting the potential for sampling bias. Hung *et al.* found that 20% of 441 patients admitted to a Taiwanese hospital were *C. difficile* positive, with two thirds carrying toxigenic *C. difficile* (11), whilst Alasmari and colleagues reported a rate of 21.2% (n=259), with almost 75% harboring toxigenic strains (127). Prior healthcare exposure was very common and not statistically different between patients colonized with a toxigenic strain and non-colonized patients (prevalence of prior healthcare exposure 90% and 85%, respectively). However, Leekha and colleagues demonstrated recent health care exposure as a significant risk factor, when reporting a 9.7% toxigenic *C. difficile* colonization rate on admission (129).

Hospitalized patients

Determination of hospital *C. difficile* colonization rates is helpful to understanding the potential for nosocomial transmission. Asymptomatic acquisition during hospital admission has generally been demonstrated to range between 3-21% (11, 12, 14, 71, 98, 131, 136, 137). McFarland *et al.* were able to separate their study cohort into early (<2 weeks) and late (>2 weeks) acquisition relative to hospital admission (14). The majority of patients had early colonization, with a significant increase in disease severity associated with those subjects progressing to CDI after late acquisition. However, this understandably correlates with significant increases in other recognized CDI risk factors, including exposure to antibiotics and multiple comorbidities.

Nevertheless, a study that involved mainly HIV positive (and younger) participants, demonstrated that all 44 *C. difficile* negative patients remained non-colonized throughout the period of hospitalization (138). This study population was largely accommodated in single rooms, which could have diminished the impact of positive carriers on transmission. In addition, Guerrero demonstrated that rectal and skin swabs from hospitalized, colonized patients yielded much lower counts than those from subjects with diarrhea, suggesting a reduced transmission potential associated with colonized individuals (8). Furthermore, Longtin and colleagues were able to show a significant decreasing trend in healthcare-associated CDI cases after the implementation of contact isolation precautions for colonized patients identified upon admission (134).

Length of hospital stay not surprisingly is related to the risk of *C. difficile* colonization; a large study reported a 50% acquisition rate for those patients with a length of stay greater

than 4 weeks. For those patients screened negative on admission, the average duration of hospital stay before a positive *C. difficile* culture, ranges between 12-71 days (11, 14, 137).

Patients in long-term care facilities

Previous reports of *C. difficile* colonization rates amongst residents of long-term healthcare facilities (LTHF) have ranged widely (4-51%) (139-142). A major caveat in the study reporting the highest colonization rate was that it was conducted during a CDI outbreak (143). Furthermore, two studies that found high rates examined relatively small cohorts (n=68 (143) and n=32 (141)). Interestingly, the data from Riggs and colleagues showed 37% of colonized residents harbored the outbreak strain (RT027/NAP1) asymptomatically, whilst Rea and O'Sullivan also isolated a range of outbreak-associated strains from the asymptomatic group, including RT027/NAP1, 078/NAP7, 018, 014/NAP4 and 026 (142). These rates must be considered with caution, as the presence of an epidemic strain in a given community is likely to inflate asymptomatic colonization rates. For example, the asymptomatic colonization rate before and post a CDI outbreak was reported to be 6.5% and 30.1%, respectively (p=0.01) (144).

Arvand *et al.* identified colonization rates that ranged from 0-10% across 11 nursing homes in Germany and concluded that additional factors influenced the asymptomatic colonization prevalence, including antibiotic exposure rates, comorbidities of residents and the individual facility's infection control procedures (140). Ryan *et al.* found similar distributions, likely reflecting differing resident morbidities and regional strain prevalence (139). Arvand and colleagues found that nursing home residents were ten times more likely to be colonized with toxigenic strains than non-toxigenic types (140), similar to other reports (122, 139) demonstrating the presence of the toxin genes, *tcdA* and *tcdB*, in 70% of strains from the asymptomatic cohorts. Conversely, Rogers *et al.* found only toxigenic *C. difficile* in those with asymptomatic colonization (141). In one study where follow up samples from colonized residents (1-3 months after initial screening) were tested, 10/12 displayed persistent carriage by the same *C. difficile* PFGE type, possibly indicating a less transient nature amongst individuals in LTHFs (143). These data demonstrate the variability across studies, which likely reflect multiple confounders including stringency of infection control procedures, strain type, antibiotic use and comorbidities, and issues such as single room versus shared accommodation.

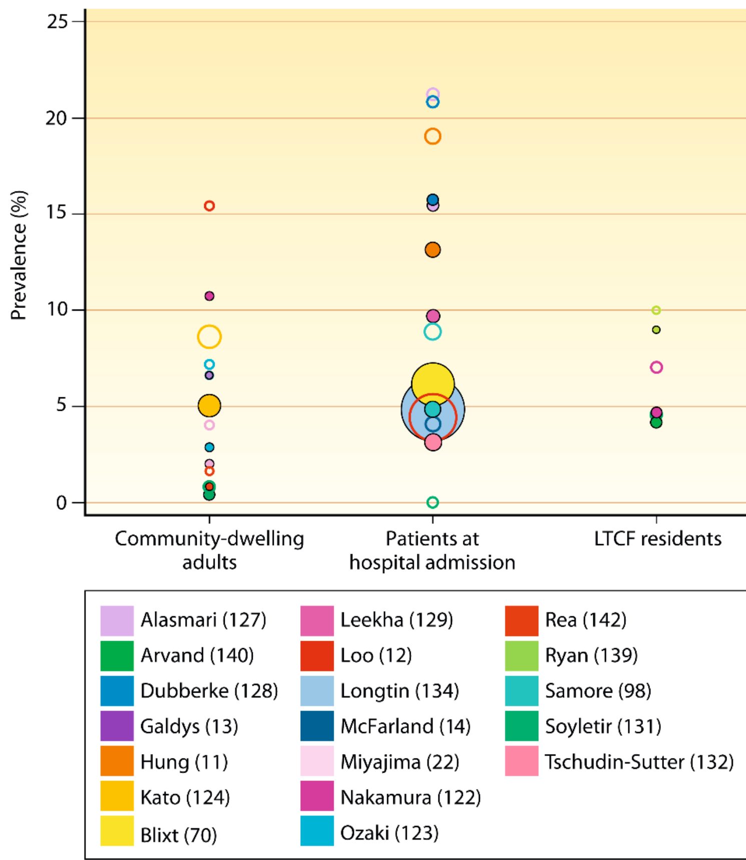

Figure 2. Prevalence of colonization among community-dwelling adults, patients at hospital admission and LTCF residents.
Hollow circles represent CDC prevalences, solid circles represent tCDC prevalences. Size of the circles represents samples size. The different colors represent the different studies (see the legend). See references 11 to 14, 22, 70, 98, 122 to 124, 127 to 131, 133, 138, 139 and 141.
CDC, *Clostridium difficile* colonization (including non-toxigenic and toxigenic strains); tCDC, toxigenic *Clostridium difficile* colonization; LTCF, long term care facility.

Healthcare workers

Asymptomatic gut colonization of healthcare workers (HCW) is a potential, but unproven source for *C. difficile* transmission. HCWs may well have a role in transmission, due to their frequent patient contact, but this could simply be due to transient hand contamination.

Kato *et al*. carried out a large-scale study of Japanese groups including two cohorts of HCWs, and identified 4.2% of hospital employees as colonized by *C. difficile* (124). Van Nood *et al*. attempted to clarify whether intestinal colonization was related to the presence of spores on HCW's hands. Of 50 Dutch hospital workers, 0% and 13% were *C. difficile* culture positive on hand print agar plates and fecal samples, respectively (145). Also, in demonstrating that colonization rates were similar across staff working on wards with and without CDI patients, they highlighted the potential for acquisition and/or transmission by means other than HCW's hands. Unfortunately, no strain typing was carried out in this study and therefore definitive transmission relationships could not be determined.

Several studies demonstrated low to non-existent intestinal colonization levels with 0-1% of healthcare workers being *C. difficile* positive (146-149). Friedman *et al*. did, however, point out the voluntary nature of study recruitment, and thus HCWs with poorer hand hygiene may have opted out, leading to a nonrepresentative cohort (147). Furthermore, these studies only sampled subjects once.

Landelle *et al*. detected *C. difficile* spores on the hands of 24% of HCWs who were directly caring for CDI patients (150). Other studies have also shown that after caring for patients with CDI, the proportion of healthcare workers with hand contamination when gloves are not worn ranged from 8 to 59% (14, 151). This highlights the challenge in determining the relative importance of patients' fecal *C. difficile* burden, versus HCW hand or environmental contamination as potential sources of transmission.

Duration of carriage

There is a paucity of research reporting duration of asymptomatic *C. difficile* carriage. Large-scale, longitudinal studies are required to investigate length of carriage and the associated determinants. Nonetheless, some research does provide follow up data on asymptomatic hosts.

Several studies have assessed duration of short term carriage (98, 152, 153). During weekly follow up of 32 asymptomatic subjects, Samore *et al*. found that 84% remained positive until discharge, although the mean duration of sampling was only 8.5 days (range 7-29 days) (98). Johnson *et al*. continued surveillance on 51 asymptomatic long-term hospital stay patients for up to nine weeks, with no development of CDI during this time (152). Later, when investigating treatment efficacies for asymptomatic carriage, the same investigators found

that 60, 80 and 100% lost *C. difficile* colonization after 40, 70 and >90 days, respectively (in the absence of a targeted intervention) (153). Contemporaneous research demonstrated that only two of six healthy, colonized volunteers retained the same strain one month later (13). Although the data are limited, they indicate the short term, transient nature of symptomless *C. difficile* colonization, at least in the absence of repeated exposure to *C. difficile* risk factors such as antibiotics. Nonetheless, variation among patient cohorts and environments must be considered.

Longitudinal studies of Japanese healthy populations have followed asymptomatic carriers among students, employees and hospital workers. Kato *et al.* performed a longitudinal surveillance on 38 asymptomatic carriers for 5-7 months and determined 12 (31.6%) remained *C. difficile* positive during this time (124). Half of these remained with the same PFGE type, whilst five had acquired a new strain. The remaining participant retained the original strain and acquired a new type. Therefore, only 18.4% of participants retained the same strain after six months, again implying a high rate of transient colonization. Nonetheless, analysis of a single, six-month follow up sample does not permit in-depth analysis of the dynamics of carriage and it remains unclear if carriage was lost after a few days, weeks or months. Testing of 18 asymptomatic subjects in three-month intervals, over one year period found that ten participants (55.6%) only tested positive for *C. difficile* on a single sampling occasion, indicating loss of carriage within three months; only three (16.7%) were persistently colonized throughout (123). This further supports the suggestion that intestinal colonization in healthy adults is largely a transient phenomenon. Of those testing positive on three or four instances, five harbored the same strain on consecutive sampling occasions (3 students, 2 employees), potentially indicating an element of cross-transmission within cohorts sharing common physical areas, and even a possibility of a subject contaminating their own environment and reacquiring the strain later.

A recent study of healthy subjects from Pittsburgh, USA provided analysis of participant demographics and dietary data in relation to the duration of *C. difficile* carriage (13). No correlations were found between previous CDI, prior antibiotics, healthcare exposure, race, ethnicity, consumption of uncooked meat or seafood and duration of carriage.

Ribotype-specific differences

Determining the prevalence of ribotypes among asymptomatically colonized individuals may help to improve the understanding of potential sources of *C. difficile*, and specifically which

toxigenic and common strain types originate from such individuals. Studies of colonizing strains have shown a broad distribution of PCR ribotypes, with reports of 37 ribotypes among 94 isolates (124) and 29 diverse sequence types from 112 carriers (115). Whilst it might be expected that there is a diverse strain distribution among asymptomatically colonized individuals, as with CDI patients, the prevalence of individual strain types is likely to vary depending on the virulence potential of a specific ribotype. Nonetheless, the relationship between ribotype prevalence in CDI patients and strain distribution among asymptomatic carriers remains unclear.

In the context of outbreaks, colonization rates by hyper-virulent strains appear to be markedly increased. Loo *et al.* and Riggs *et al.* found very similar (asymptomatic) colonization rates for PCR ribotype 027/NAP1 strain (36.1% and 37%, respectively) (12, 143). Contemporaneous research highlighted the persistence of PCR ribotype 027/NAP1 in a New York, long-term care facility, where half of the asymptomatic population (19.3% of all residents) carried this strain (154). This is likely to be due to increased prevalence in the patient populations and consequent spore shedding in to the environment (155). Interestingly, three of the five asymptomatically colonized patients that developed subsequent CDI harbored the epidemic 027/NAP1 strain, hinting at its potential superiority in progression from colonization to symptomatic disease.

Other ribotypes have also been implicated as dominant colonizing strains; earlier work reported that 51.7% of asymptomatically colonized, elderly patients were positive for ribotype 001/NAP2 on admission, with the remaining 48.3% consisting of 12 other ribotypes (156). As ribotype 001/NAP2 was deemed to predominate in Welsh hospitals at the time, this may be as expected. Other prevalent European ribotypes (157), including 012/NAP_{cr1}, 014/NAP4 and 020/NAP4 have also been reported as predominant strains among asymptomatic populations (127, 140).

Conversely, in recent studies covering a period of marked reduction in PCR ribotype 027/NAP1-associated CDI (157), asymptomatic colonization rates of this strain were considerably lower (140, 142). These data were supported by a large scale, UK transmission study (15), which also found no evidence of PCR ribotype 027/NAP1 colonization in UK hospitalized patients; no single strain predominated in this study.

Risk factors for *C. difficile* colonization

Clinical and epidemiological risk factors for CDI are well known, but risk factors for colonization with *C. difficile* have only come to attention recently. An important distinction has to be made between risk factors *to be colonized* in the community or *at admission to a hospital,* as opposed to risk factors for acquiring *colonization during hospital admission*.

Colonization in a community setting

Risk factors for being or becoming colonized in the community are not extensively studied. Clusters of colonized patients with identical *C. difficile* types have been identified within community settings (e.g. employees, students) and families, indicating cross-transmission from colonized individuals or acquisition from a common source (124). A study among 106 healthy adults in Pennsylvania found no statistically significant differences in patient's characteristics or exposures between 7 colonized and 99 non-colonized subjects, but this may be due to the small sample size (13). Living in the proximity of livestock farms was not found to be a risk factor in a recent study among 2494 adults in the Netherlands (158). Antibiotic exposure in the 3 preceding months was however associated with a 3.7-fold increased risk of *C. difficile* colonization in the same study (158). A recent study among 338 predominantly healthy infants (<= 2 years of age) showed that *C. difficile* colonization increased with pet dogs (159).

Colonization at hospital admission

Recognition of risk factors for being colonized at admission is important, as patients with these risk factors may introduce and spread *C. difficile* into the hospital. Epidemiological and clinical risk factors for (overall or toxigenic) colonization at the time of admission include recent hospitalization (15, 129, 133), chronic dialysis (129), corticosteroid/ immunosuppressant use (15, 129, 133), gastric acid suppressant medication (15), and antibodies against Toxin B (133). (Table 2) The consistent association between previous healthcare contact and colonization by *C. difficile* likely means that hospitals remain important sources of *C. difficile*, related to host factors at time of admission (e.g. altered microbiota composition due to antibiotic use) and increased exposure to strains. However, patients colonized at admission may have acquired *C. difficile* from diverse sources. Notably, the healthcare associated *C. difficile* ribotype 027/NAP1 is less frequently found in carriers at admission, than in those who become colonized during admission (128, 133).

Acquiring *C. difficile* during hospital admission

Previous hospitalization in the last 2 months, use of proton-pump inhibitors H2-blockers or chemotherapy (within the 8 weeks preceding the hospitalization or during hospitalization but before colonization was acquired) and cephalosporin use during admission were significant risk factors for becoming colonized (with toxigenic or non-toxigenic strains) during admission (12, 128). (Table 2) In one study, cefepime use and a toll-like receptor 4 polymorphism were risk factors for acquiring toxigenic *C. difficile* colonization during admission (11). The presence of Toxin B antibodies was associated with asymptomatic colonization during admission (12). Interestingly, antibodies against Toxin B may have protective effect against the development of CDI. Likewise, compared to patients who acquired *C. difficile* and subsequently developed CDI, patients who acquired *C. difficile* colonization but remained asymptomatic had higher levels of IgG antibody against Toxin A at time of colonization (160). These observations may indicate that antibodies and/or acquired immunity (e.g. due to previous hospitalizations) might confer resistance to the development of symptomatic CDI (see before). Patients who acquired *C. difficile* and developed asymptomatic colonization were less frequently colonized with the hypervirulent ribotype 027/NAP1 strain compared to those who developed CDI (12, 128, 160). This suggests that the virulence of the acquired strain can influence the development of colonization or infection.

Colonization by toxigenic versus nontoxigenic strains

A recent study showed that hospitalized patients colonized by toxigenic strains and non-toxigenic strains do not share risk factors. Risk factors for colonization by a toxigenic strain included a higher number of admissions in the previous year, antimicrobial exposure during the current admission and the presence of gastro-esophageal reflux disease. Risk factors for colonization by a non-toxigenic strain were chronic kidney failure and chronic obstructive pulmonary disease. Unfortunately, the design of this study was cross-sectional and therefore the time period of *C. difficile* acquisition (i.e. before at admission or during admission) could not be established in these patients (161). Another study tried to determine if the type of antibiotics used during admission impacts the risk for acquisition of either toxigenic or non-toxigenic *C. difficile*. They found that the use of cephalosporins was a risk factor for both conditions: acquisition of a toxigenic strain was associated with the use of cefepime, while the acquisition of a non-toxigenic strain was associated with the use of cefuroxime. Moreover, the use of glycopeptides was a risk factor for acquiring a non-

toxigenic strain during admission (11). For patients colonized on admission, associations between classes of antibiotics used and the colonization of either toxigenic or non-toxigenic *C. difficile* have also been reported, but multivariate analyses to identify independent risk factors have not yet been performed (127).

Table 2. Studies investigating risk factors for *C.difficile* colonization at hospital admission or acquisition of *C. difficile* during admission. Studies were included if they were published since 1994, investigated either risk factors for colonization at admission or risk factors for colonization acquisition during admission (studies investigating risk factors for being colonized at a certain time point during hospitalization were excluded), had a sample size of > 100 patients, and assessed risk factors by multivariate regression.

Condition	Identified risk factor	Reference
Risk factors for colonization at admission		
CDC	previous hospitalization	133, 15
	previous CDI episode	133
	previous use of corticosteroids or other immunosuppressant medication	133, 15
	presence of antibodies against Toxin B	133
	current loose stools/diarrhea but not meeting CDI criteria	15
tCDC	previous hospitalization	129
	chronic dialysis	129
	use of corticosteroids	129
Risk factors for acquiring colonization during admission		
CDC	previous hospitalization	12
	use of chemotherapy	12
	use of PPI or H2-blockers	12
	presence of antibodies against Toxin B	12
tCDC	TLR4 polymorphism	11
	cefepime use during admission	11

CDC, *Clostridium difficile* colonization; tCDC, toxigenic *Clostridium difficile* colonization.

C. difficile colonization and subsequent CDI

One of the major questions is, do *C. difficile* colonized individuals have an increased risk of developing subsequent CDI, or are they protected against disease? A lower risk for *C. difficile* colonized patients of subsequently developing CDI was found in a frequently cited but older meta-analysis of four studies (162). The major drawback of this review, however, is that patients colonized by toxigenic or non-toxigenic strains were not analyzed separately; this difference may be of importance as 44% of colonized patients in this meta-analysis harbored a non-toxigenic strain. Also, all four studies were performed pre-1994, before the emergence of hypervirulent strains and recognition of community-associated

CDI. Furthermore, colonization was determined at different time points: at admission (71, 98), at start of tube feeding with patients colonized at admission excluded (163) or after a hospital stay of at least 7 days (152). Colonized patients therefore included some patients that acquired colonization during admission. The risk that these latter patients go on to develop CDI during the hospital stay may be different from that for individuals already colonized at admission. A recent meta-analysis aimed to include studies in which patients were colonized *at admission* with *toxigenic* strains only (11, 15, 98, 127, 131, 135, 164-166). However, not all included studies succeeded in obtaining samples within 48hrs or 72hrs of admission (15, 98). Also, a study that included patients at admission to a rehabilitation unit (after an average stay of 30 days in acute care) was included (166). In one study, the distinction between colonization of a toxigenic strain and CDI was difficult to establish, as all patients received a hematopoietic stem cell transplantation and donor lymphocyte infusion; almost all such patients subsequently develop diarrhea. In patients known to carry a toxigenic *C. difficile* strain, diarrhea may have been falsely attributed to CDI (164). Notwithstanding these limitations, all studies pointed to an increased risk for patients colonized with toxigenic *C. difficile* at admission to progress to CDI: overall, the relative risk was 5.86 (95% CI 4.21-8.16). (Table 3) Some recent studies were not included in this meta-analysis. A recent large study, which screened n=3605 of 4508 hospital admissions, found that patients carrying toxigenic strains on admission were at a much increased risk of developing CDI (CDI rates 9.4% vs 2.3% for non-toxigenic *C. difficile* carriers) (70). The risk of CDI in non-colonized patients who were exposed to subjects colonized by a toxigenic strain was also significantly increased (4.6% vs 2.6% for non-exposed patients; odds ratio for CDI if exposed to carrier, 1.79; 95% CI, 1.16–2.76). However, this study appeared to diagnose CDI based on the presence of toxigenic *C. difficile* strains rather than toxin, and so the case incidence is likely to have been overestimated. In turn, the association between colonization by, or exposure to, toxigenic strains and subsequent CDI may have been exaggerated (70). A much smaller study did not report any CDI cases among 37 patients colonized on admission (128) (Table 3).

Two other recent studies describe the risk of colonized ICU patients to develop CDI. The study by Tschudin-Sutter *et al.* in a cohort of 542 ICU patients described a relative risk to develop CDI of 8.6 for patients colonized on admission and a relative risk of 10.9 for patients who became colonized during hospitalization (132). Zhang and colleagues however, identified 6 patients who were colonized on admission to the ICU, but none of them developed CDI. During their study period 4 patients developed CDI, but all were not colonized on admission to the ICU (167). These conflicting results are probably caused by

small samples sizes, a relatively rare outcome event (3 vs 0 colonized patients progressed to CDI) and different predominant strains.

From the above we can conclude that patients asymptomatically colonized by toxigenic strains may progress to CDI during admission. However, for patients asymptomatically colonized by non-toxigenic strains there seems to be no increased risk of progressing to CDI and these patients may even be protected from developing CDI.

Infection control and antimicrobial stewardship implications for asymptomatic carriers

Symptomatic CDI patients are believed to be the main source of nosocomial transmission, and current guidelines recommend their systematic detection and isolation (5). Due to a paucity of data at the time of writing of this review, the isolation of asymptomatic carriers is not recommended. Whether these carriers should be isolated remains an important clinical question stemming from the growing body of literature on the subject. Mathematical modeling of *C. difficile* transmission and simulation of screening and isolation of carriers has shown the intervention to be effective at reducing CDI rates (168, 169). However, a clinically based study to directly answer this question has not been conducted until recently (134).

Longtin *et al.* explored the effect of isolating asymptomatic *C. difficile* carriers on the incidence of hospital acquired CDI in an acute care hospital in Quebec, with high baseline rates of CDI (134). A quasi-experimental design was employed, using change in CDI incidence in other Quebec hospitals as controls. The effect of the intervention (isolation of carriers) was evaluated through a time series analysis. Compared with the pre-intervention period, the incidence of CDI decreased significantly after the intervention. In addition, the effect was confirmed using two methods of analysis, segmented regression analysis and autoregressive integrated moving average (ARIMA) modeling, indicative of the robustness of the results. Incidence rates of CDI in the study hospital remained low a year after the study terminated, demonstrating the sustained effect of this intervention.

This study provides the most convincing evidence to date for the significant effect of isolating carriers. The authors assessed confounding elements; such as intensity of CDI testing, total antimicrobial use and proton pump inhibitor use, which remained stable during the study period. Concurrently, a significant decrease in the use of metronidazole and oral vancomycin suggested true clinical impact from the observed decrease in incidence and

trend. Compliance with hand hygiene increased, but utilized alcohol-based solution not effective against *C. difficile* spores. Some potential confounders that were not assessed include compliance with isolation precautions, environmental cleaning, improvement in appropriate antibiotic use, and knowledge of *C. difficile* carrier status on the management of a patient (170).

Ultimately, these promising findings need to be reproduced in a multicenter, cluster randomized trial, prior to being considered for widespread implementation. If these results are confirmed in various different hospital settings, adoption of screening and isolation of asymptomatic carriers may be an important strategy to decrease CDI rates. However, this will raise several practical questions, such as whether universal versus targeted screening should be adopted and what the optimal screening method is. Given known risk factors for colonization on admission, a reasonable approach may be to selectively target high-risk patients and isolate them on admission to hospital (133). Other issues that would need to be addressed include frequency of screening during hospitalization, the optimal isolation protocol, the impact on patient perception of care and the additional workload burden on frontline healthcare workers and the microbiology laboratory.

Reducing inappropriate antimicrobial use through antimicrobial stewardship programs (ASPs) has been shown to decrease rates of CDI (171-173), but given the lack of widespread screening for asymptomatic carriers, ASPs targeted at this population have not been studied. It does not necessarily follow that targeting colonized patients, as a whole group, would decrease CDI rates, as some of these patients may be long-time colonized patients with immunity and decreased risk of developing symptomatic CDI. These patients are likely different from patients who may still be colonized with *C. difficile* after an episode of symptomatic CDI (10, 68). One study showed a three-fold increase in recurrence of CDI in patients exposed to antimicrobials after resolved CDI, compared with those who were not exposed (174). Therefore, patients with prior CDI, an easily identifiable subset of asymptomatic carriers, probably represent colonized patients at highest risk of developing infection, and may represent suitable targets for focused stewardship efforts.

Table 3. Studies investigating the risk of development of CDI among patients with toxigenic *C. difficile* colonization on hospital admission. Studies were included if they were published since 1994, had a sample size of > 100 patients, compared patients with toxigenic *C. difficile* colonization on admission to controls (patients with nontoxigenic *C. difficile* colonization and noncolonized patients together). Relative risks were calculated as the risk for toxigenic *C. difficile*-colonized patients compared to the risk for noncolonized and nontoxigenic *C. difficile*-colonized patients together and were unadjusted.

Study	Country and period	Setting and patients	Follow up period	Included patients (N)	Prevalence tCDC (%)	CDI among tCDC (%)	CDI among controls (%)	RR for CDI (95% CI)	Remarks
Samore (ref 98)	US 1991	patients with an anticipated LOS of at least 5 days admitted or transferred to general medical and surgical wards and ICUs	until discharge	496	24/496 (4.8)	1/24 (4.2)	8/472 (1.7)	2.46 (0.32-18.87)	90 of 496 samples (18.1%) were not obtained within 72hrs of admission
Soyletir (ref 131)	Turkey published 1996	patients admitted to a general medical ward with a LOS of at least 48hrs	until discharge	202	0/202 (0)	0/0 (0)	0/202 (0)	na	none of the patients was colonized at admission
Gupta (ref 165)	US and Canada 2009-2011	patients >60yrs admitted to general medical and surgical units, on antibiotics	until 30 days after discharge or 60 days in hospital (whichever came first)	1099	91/1099 (8.3)	9/91 (9.9)	11/1008 (1.1)	9.06 (3.86-21.30)	asymptomatic carriage was diagnosed by culture and REA typing but could have included both tCDC and ntDCD
Alasmari (ref 127)	US 2010-2011	adult patients with an anticipated LOS >48hrs admitted to general medical and surgical wards	until 60 days after discharge	259	40/259 (15.4)	1/40 (2.5)	2/219 (0.9)	2.74 (0.25-29.48)	

Table 3. *Continued.*

Study	Country and period	Setting and patients	Follow up period	Included patients (N)	Prevalence tCDC (%)	CDI among tCDC (%)	CDI among controls (%)	RR for CDI (95% CI)	Remarks
Dubberke (ref 128)	US 2010-2012	adult patients admitted to medical or surgical wards with an anticipated LOS >48hrs	until 60 days after discharge	235	37/235 (15.7)	0/37 (0)	2/198 (1.0)	na	partly same patient cohort as Alasmari
Bruminhent (ref 164)	US 2011-2012	patients admitted to a bone marrow transplant unit for an HSCT	until 100 days after HSCT	150	16/150 (10.7)	14/16 (87.5)	23/134 (17.2)	5.10 (3.36-7.72)	distinction between CDI and colonization by toxigenic strains difficult to establish as almost all patients develop diarrhea after HSCT and CDI testing did not include free toxin detection in all cases
Hung (ref 11)	Taiwan 2011-2012	adult patients with an anticipated LOS of at least 5 days admitted to a general medical ward	until discharge from last hospitalization	441	58/441 (13.2)	8/58 (13.8)	6/383 (1.6)	8.80 (3.17-24.46)	
Blixt (ref 70)	Denmark 2012-2013	patients admitted to medical departments at 2 university hospitals	one month (in and outside hospitals)	3464	213/3464 (6.1)	20/213 (9.4)	76/3251 (2.3)	4.02 (2.50-6.44)	
Tschudin-Sutter (ref 132)	US 2013	patients admitted to an ICU within 48hrs of hospital admission	until discharge	542	17/542 (3.1)	2/17 (11.8)	6/525 (1.1)	10.29 (2.24-47.3)	

RR, relative risk; HSCT, hematopoietic stem cell transplantation; tCDC, toxigenic *Clostridium difficile* colonization; ntCDC, nontoxigenic *Clostridium difficile* colonization; CDI, *Clostridium difficile* infection; LOS, length of stay; ICU, intensive care unit; na, not available

Concluding remarks and future directions

The intriguing concept of *C. difficile* colonization has garnered much attention during the last decade. Gut microbiota studies and immunologic studies have provided some insight in the conditions that allow for colonization and protect against disease progression. However, more studies are needed to assess the precise role of changes in microbiota and the precise triggers of spore germination and colonization, as well as changes and initiators that lead to toxin production. It also needs to be explored why some individuals' transition to *C. difficile* carrier status and what interventions could terminate colonization or could block the progression to CDI.

The realization that *C. difficile* colonized patients may be the most important unexplained reservoir for *C. difficile* transmission has led to epidemiological studies investigating colonization rates among different populations and risk factors for this condition. Colonized patients on admission appear to play an important role in introducing and maintaining transmission in the ward and hence, risk factors for colonization on admission are of specific interest. To further study the acquisition and transmission of *C. difficile*, all patients admitted to the hospital should be screened for colonization by (and preferably sustained carriage of) *C. difficile*. *C. difficile* positive individuals should be questioned about risk factors for acquisition and should be followed during admission for the development of symptomatic CDI. Epidemiological investigations and molecular typing methods should be applied to examine possible linkage of *C. difficile* colonized individuals to CDI cases. In this way, risk factors for *C. difficile* colonization can be identified and the role of *C. difficile* positive individuals in transmission of the disease can be elucidated. It would be interesting to determine if there are host and pathogen factors that affect transmissibility of *C. difficile*. More evidence from different settings is needed to determine whether specific control measures targeting colonized patients may be justified to prevent spread. In addition, the protective effects of *C. difficile* vaccines are being examined, but information on the consequences of colonization and spread to non-vaccinated individuals would be relevant.

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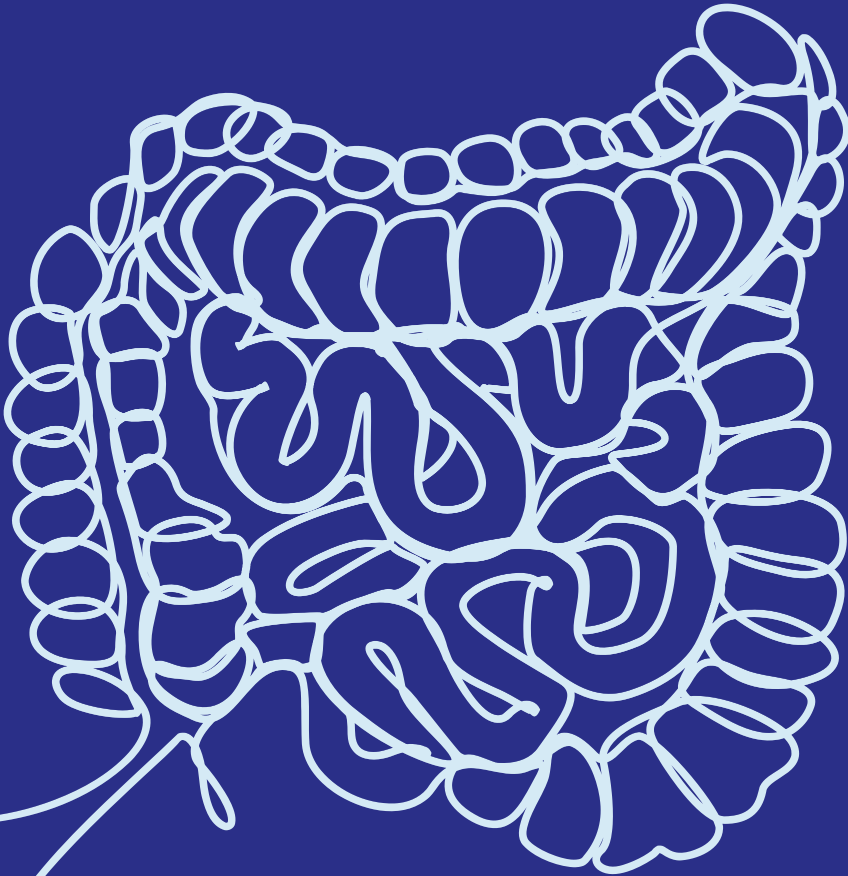
CHAPTER 3

European Society of Clinical Microbiology and Infectious Diseases: update of the diagnostic guidance document for *Clostridium difficile* infection



Clinical Microbiology and Infection, 2016

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Abstract

In 2009 the first European Society of Clinical Microbiology and Infectious Diseases (ESCMID) guideline for diagnosing *Clostridium difficile* infection (CDI) was launched. Since then newer tests for diagnosing CDI have become available, especially nucleic acid amplification tests. The main objectives of this update of the guidance document are to summarize the currently available evidence concerning laboratory diagnosis of CDI and to formulate and revise recommendations to optimize CDI testing. This update is essential to improve the diagnosis of CDI and to improve uniformity in CDI diagnosis for surveillance purposes among Europe. An electronic search for literature concerning the laboratory diagnosis of CDI was performed. Studies evaluating a commercial laboratory test compared to a reference test were also included in a meta-analysis. The commercial tests that were evaluated included enzyme immunoassays (EIAs) detecting glutamate dehydrogenase, EIAs detecting toxins A and B and nucleic acid amplification tests. Recommendations were formulated by an executive committee, and the strength of recommendations and quality of evidence were graded using the Grades of Recommendation Assessment, Development and Evaluation (GRADE) system. No single commercial test can be used as a stand-alone test for diagnosing CDI as a result of inadequate positive predictive values at low CDI prevalence. Therefore, the use of a two-step algorithm is recommended. Samples without free toxin detected by toxins A and B EIA but with positive glutamate dehydrogenase EIA, nucleic acid amplification test or toxigenic culture results need clinical evaluation to discern CDI from asymptomatic carriage.

Introduction

The previous European Society of Clinical Microbiology and Infectious Diseases (ESCMID) guidance document for *Clostridium difficile* infection (CDI) was published in 2009. (1) Since then many laboratories in Europe have implemented a diagnostic algorithm for diagnosing CDI. However, many new diagnostic tests have become available in the meantime, especially nucleic acid amplification tests (NAATs). Although several of these tests have been marketed, their role in the diagnosis of CDI needs to be clarified. Also, the importance of free toxin detection in stool needs to be addressed. This update of the previous guidance document is essential to improve the diagnosis of CDI; to optimize its management, prevention and control; and to improve uniformity in CDI diagnosis for surveillance purposes across Europe.

The main objectives of this guidance document are to summarize the currently available evidence concerning laboratory diagnosis of CDI and to formulate recommendations to optimize CDI testing. This guideline is intended for use among medical microbiologists, gastroenterologists, infectious disease specialists and infection control practitioners. The target population is diarrhoeal patients suspected of having CDI.

Material and Methods

To be able to revise our previous recommendations, an update of the 2009 meta-analysis was performed. In addition, other guidelines and recent literature concerning the diagnosis of CDI were reviewed.

Update of meta-analysis

Search strategy

Studies evaluating laboratory assays for diagnosing CDI were searched in PubMed, Embase, Web of Science, Central and the Cochrane Library. Searches were performed in June 2014 with the support of a trained librarian. The search was restricted to articles published since 2009 in the English language. Meeting abstracts were excluded. The search strategy is displayed in Supplementary Material 1.

Reference tests

A reference test is the best available test and is the standard against which other assays are compared. Cell cytotoxicity neutralization assay (CCNA) and toxigenic culture (TC) are regarded as reference tests for diagnosing CDI. (2)

CCNA demonstrates the presence of free toxin B. For this test, stool filtrates are inoculated onto a monolayer of a cell culture which is then observed for a toxin B-induced cytopathic effect (rounding of the cells). The cytopathic effect is evaluated at 24 and 48 hours. Cell lines commonly used for CCNA include Vero cells, HeLa cells, human foreskin fibroblast cells and Hep-2 cells. Neutralization of the cytopathic effect is necessary to determine the specificity of this effect and can be done by using *Clostridium sordelli* antitoxin or *C. difficile* antitoxin. (3) This reference test takes 1 to 2 days to perform and requires cell culture and laboratory expertise, so it is not routinely used in most diagnostic laboratories.

TC demonstrates the presence of *C. difficile*, which is able to produce toxins in vitro. Stools are incubated anaerobically for at least 48 hours on selective media. Many different culture media exist for this purpose, all aiming to enhance the recovery of *C. difficile* while inhibiting the overgrowth of other faecal flora. (4) Pretreatment with alcohol shock (5) or heat shock can also be used to decrease overgrowth of normal faecal flora. (4) Also, broth enrichment before plating onto a solid medium is sometimes used (also called enriched culture). (4) Furthermore, a chromogenic medium (ChromID agar; bioMérieux) for the recovery of *C. difficile* has been developed which is designed to isolate and identify *C. difficile* within 24 hours. However, no consensus exists on which culture medium and/or culture method is the most appropriate to use. Colonies suspicious for *C. difficile* can be recognized by Gram staining, colony morphology, 'horse manure' odour, biochemical testing, gas-liquid chromatography, ultraviolet light fluorescence, latex agglutination and matrix-assisted desorption ionization-time of flight mass spectrometry. (6) Isolates from positive cultures are either tested for in vitro toxin production by the use of CCNA or toxin A/B enzyme immunoassay (EIA) or tested for the presence of toxin A/B genes by NAAT.

Index tests

Index tests are the tests whose performance is being evaluated compared to the reference tests. The index tests we reviewed comprise all commonly applied and commercially available laboratory tests for diagnosing CDI other than the reference tests. These include EIAs that detect glutamate dehydrogenase (GDH), EIAs that detect toxins A and B and NAAT.

GDH EIAs detect glutamate dehydrogenase, an enzyme that is produced by both toxigenic and nontoxigenic strains of *C. difficile*. GDH EIAs are available in well-type format (results are displayed as a colour change which can be detected visually or photospectrometically) or membrane-type format (results can be visually read from a membrane).

Toxin A/B EIAs detect toxins A and B and are also available in well-type or membrane-type format. Most EIAs detecting only toxin A have been replaced by EIAs detecting both toxins A and B, as strains that only produce toxin B and not toxin A are reported.

Several membrane-type tests that include both an EIA detecting GDH and an EIA detecting toxins A and B are also available (*C. diff* Quik Chek Complete, Techlab, Combo *C. difficile*; Theradiag).

NAATs include assays that use PCR, helicase-dependent amplification and loop-mediated isothermal amplification. Most assays detect conserved regions within the gene for toxin B (*tcdB*), but assays that detect a highly conserved sequence of the toxin A gene (*tcdA*) have also been developed (Illumigene, Meridian, Bioscience and Amplivue, Quidel). (7, 8) NAATs that not only detect *tcdB* but also the binary toxin genes (*cdt*) and the deletion at nucleotide 117 on *tcdC* are also available (*Verigene C. difficile* test, Nanosphere and Xpert, Cepheid) and offer the potential advantage of detecting PCR ribotype 027, although highly related PCR ribotypes may also be detected by these tests (without distinguishing them from PCR ribotype 027). (9) NAATs that detect multiple targets at the same time, including *C. difficile* toxin genes, are also available (Seeplex Diarrhea ACE detection, Seegene, xTAG Gastrointestinal Pathogen Panel, Luminex, FilmArray Gastrointestinal Panel, BioFire Diagnostics).

Test performance

The numbers of truly positive, falsely positive, falsely negative and truly negative index test results are generally displayed in a 2 x 2 table (Table 1). Test performance can be derived from this 2 x 2 table. The sensitivity of a test is defined as the probability that the index test result will be positive in a person with disease (a/a+c). The specificity of a test is defined as the probability that the index test result will be negative in a person without disease (d/b+d). The positive predictive value (PPV) of a test is the probability that a person has the disease, given the positive test result (a/a+b). The negative predictive value (NPV) of a test is the probability that a person is free of disease, given the negative test result (d/c+d). PPV and NPV are dependent on disease prevalence in the tested population (http://training-old.

cochrane.org/sites/training-old.cochrane.org/files/uploads /DTA/1.3_Introduction_to_test_accuracy/story. html).

Table 1. 2x2 table used to calculate test characteristics

	Diseased or reference test positive	Non-diseased or reference test negative
Index test positive	(a) True positive	(b) False positive
Index test negative	(c) False negative	(d) True negative

Eligibility criteria

Studies eligible for inclusion had to: (1) describe original research, (2) compare an index test (one commercially available in Europe) with a reference test (CCNA or TC), (3) perform the tests on *C. difficile*-negative and -positive clinical human stool samples and (4) provide sufficient information to recalculate sensitivity and specificity and their confidence intervals. Culture without determining the toxigenic status was accepted as a reference test if only assays detecting GDH were evaluated.

Studies were excluded if: (1) the reference test was not performed on all samples but only on positive, negative or discordant samples (to exclude partial verification bias), (2) not all samples were tested by the same reference test, (3) the reference method was a composite of more than one test, (4) the reference method included clinical data for its interpretation, (5) the index test was partly used as reference method, (6) the index test did not follow manufacturers' instructions for testing or sample collection, (7) for CCNA, samples were not stored correctly before testing (refrigerated or frozen at -20°C and thawed only once) or neutralization to determine the specificity of the cytopathic effect was not executed and (8) only selected samples were included.

Selection process

Study eligibility was assessed in a two-step selection process by two independent investigators (MC, ET). Inconsistencies were resolved by consensus and by consultation of a third and fourth investigator (EK, TP).

Outcome measures, data extraction and quality assessment

The principal measures of outcome were the sensitivity and specificity of different index tests compared to one of the 2 reference tests. Toxin A/B EIAs, GDH EIAs and NAATs were compared to CCNA and TC. GDH EIAs were additionally compared to culture. From

each study we extracted the number of true-positive, false- positive, false-negative and true-negative findings to be able to calculate the sensitivity and specificity of the index test evaluated in that study. Data were extracted by two independent investigators (MC, ET) using a data extraction form (Supplementary Material 2). Additional data that were extracted included year of publication, storage conditions of the samples, information about the study population and information about the execution of the index test and reference test. The quality of the studies was assessed by the same two independent investigators using a quality assessment tool. This quality assessment tool (Supplementary Material 3) consisted of items from the Quality Assessment for Studies of Diagnostic Accuracy (QUADAS) tool (10), supplemented with items concerning the appropriate handling of specimens and appropriate execution of reference tests.

Statistical analysis

For all index tests in all studies, the sensitivity and specificity and their respective confidence intervals were calculated from the number of true-positive, false-positive, false-negative and true- negative findings supplied in these studies. Wherever possible, the results after initial testing (instead of results after retesting of indeterminate results) were used to calculate the sensitivity and specificity. Random effects logistic regression was used to pool the mean sensitivities and specificities for the different index tests and the different types of index tests. In case of fewer than four studies, a fixed effect model was used. NPVs and PPVs were calculated using a hypothetical prevalence of CDI of 5, 10, 20 and 50% in the tested population. We used Stata 12.0 software (StataCorp) for all statistical analyses.

Guidelines and additional studies

An electronic search was performed on topics concerning laboratory diagnosis of CDI not included in our meta-analysis (e.g. repeated testing, sample selection). Published guidelines on CDI testing were also studied. These included guidelines from the Society for Healthcare Epidemiology of America/Infectious Diseases Society of America (published in 2010) (11), guidelines from the Australasian Society for Infectious Diseases (published in 2011) (12), guidelines from the American College of Gastroenterology (published in 2013) (13), guidelines from the American Academy of Pediatrics (published in 2013) (14) and guidelines from the UK National Health Service (update published in 2012). (15)

Formulation of recommendations

The guideline was developed according to the Appraisal of Guidelines for Research and Evaluation (AGREE II) instrument. (16) Findings of the literature review and meta-analysis results were discussed with the members of the executive committee, and recommendations were formulated. We slightly modified the GRADE system to grade the strength of the recommendations and the quality of evidence (Table 2). (17) A good practice statement could be made instead of a formal graded recommendation for domains where this was deemed appropriate. (18) The drafting group (consisting of experts in the field) and a patients' representative were invited to comment on the recommendations, and results from these discussions were incorporated in the final recommendations.

Table 2. Scoring system for grading quality of evidence and strength of recommendations.

Quality of evidence	
High quality	Evidence from at least 1 properly designed cross sectional or cohort study in patients with diagnostic uncertainty and direct comparison of all test results with an appropriate reference standard.
Moderate quality	Evidence from: (1) at least 1 cross sectional or cohort study in selected patients and/or no or partial comparison of test results with an appropriate reference standard, (2) case control studies
Low quality	Evidence from opinions of respected authorities, based on clinical experience, descriptive case studies or reports of expert committees
Strength of a recommendation	
Strong recommendation for use	Desirable effects clearly outweigh undesirable effects
Weak recommendation for use	Desirable and undesirable effects are closely balanced or recommendation is based on low quality evidence
Weak recommendation against use	Desirable and undesirable effects are closely balanced or recommendation is based on low quality evidence
Strong recommendation against use	Undesirable effects clearly outweigh desirable effects
Good practice statement	Desirable effects clearly outweigh undesirable effects, but no or only indirect evidence is/will become available

Results

Literature search and selection process

A total of 795 unique citations were identified by our current search. On the basis of title and abstract, 693 articles were excluded, leaving 102 full-text articles for detailed assessment. In total, 61 studies were excluded after detailed assessment. Reasons for exclusion were (some studies had more than one reason for exclusion): not all samples were tested by the (same) reference method (23 studies), no or an inadequate reference test was used (16 studies), samples were selected inadequately (13 studies), not enough information was provided (seven studies), the study did not describe original research (five studies), no clinical human stool samples were included (three studies), no commercial diagnostic test was investigated (two studies) and stool samples were incorrectly collected in transport medium (one study).

From all 43 studies included in the previous meta-analysis (1), 28 were excluded. Twenty-four of these studies evaluated tests that were no longer available (mainly EIAs detecting toxin A only). Two other studies were excluded because they did not evaluate a commercial test (both studies evaluated an in-house PCR), one study was excluded because not all samples were tested by the same reference test and one study was excluded because samples were stored incorrectly for CCNA testing. A total of 56 studies (15 from the previous meta-analysis and 41 published since 2009) were included in the meta-analysis. (7, 8, 19-72) A summary of the selection process is shown in Fig. 1.

Study characteristics

Twenty-four different laboratory assays were evaluated: one well-type EIA for GDH, three membrane-type EIAs for GDH, five well-type EIAs for toxins A and B, four membrane-type EIAs for toxin A and B and 11 NAATs (Table 3). In total, 133 comparisons between index tests and reference tests were available, including 53 comparisons to CCNA, 69 comparisons to TC and 11 comparisons to culture. Studies were published between 1996 and 2014. The number of evaluated index tests per study ranged from one to ten, and the number of included samples ranged from 60 to 12 369. The CDI prevalence in the tested population ranged from 6 to 48%. Table 4 lists the characteristics of included studies.

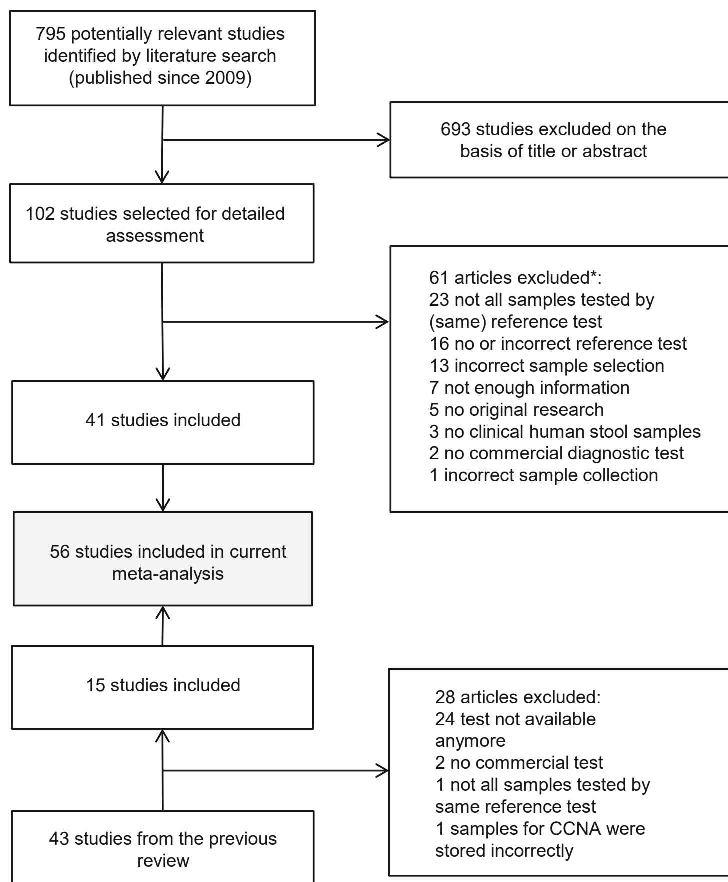

Figure 1. Summary of selection process.
*Some studies had more than one reason for exclusion.

Table 3. Index tests included in meta-analysis

Assay type	Test	Manufacturer	Target(s)	Method
(A) Well-type EIA GDH	C. diff Chek-60	Techlab	GDH	Well-type EIA
(B) Membrane-type EIA GDH	C. diff Quik Chek	Techlab	GDH	Membrane-type EIA
	Immunocard *C. difficile*	Meridian	GDH	Membrane-type EIA
	Quik Chek Complete-GDH[a]	Techlab	GDH	Membrane-type EIA

Table 3. *Continued.*

Assay type	Test	Manufacturer	Target(s)	Method
(C) Well-type EIA toxins A&B	Premier tox A/B	Meridian	Toxins A and B	Well-type EIA
	Remel ProSpecT	Oxoid	Toxins A and B	Well-type EIA
	Ridascreen tox A/B	Biopharm	Toxins A and B	Well-type EIA
	Clostridium diffiicle Tox A/B II	Techlab	Toxins A and B	Well-type EIA
	Vidas CDAB	Biomérieux	Toxins A and B	Automated EIA
(D) Membrane-type EIA toxins A&B	Immunocard tox A/B	Meridian	Toxins A and B	Membrane-type EIA
	Quik Chek Complete-tox A/Ba	Techlab	Toxins A and B	Membrane-type EIA
	Tox A/B Quik Chek	Techlab	Toxins A and B	Membrane-type EIA
	Xpect	Oxoid	Toxins A and B	Membrane-type EIA
(E) NAAT	Advansure CD	LG Life Sciences	*tcdA. tcdB*	RT-PCR
	Amplivue	Quidel	*tcdA*	Isothermal helicase-dependent amplification
	BD GeneOhm	Becton, Dickinson	*tcdB*	RT-PCR
	BD Max Cdiff	Becton, Dickinson	*tcdB*	RT-PCR
	GenomEra	Abacus Diagnostica	*tcdB*	RT-PCR
	Illumigene	Meridian	*tcdA*	LAMP
	Portrait	Great Basin	*tcdB*	Isothermal helicase-dependent amplification
	Prodesse ProGastro Cd Assay	Hologic Gen-Probe	*tcdB*	RT-PCR
	Seeplex Diarrhea ACE Detection[c]	Seegene	*tcdB*	RT-PCR
	Verigene	Nanosphere	*tcdA, tcdB, cdt*[b], *tcdC* deletion nt 117[b]	PCR/nanoparticle-based microarray
	Xpert C. *difficile*	Cepheid	*tcdB, cdt, tcdC* deletion nt 117	RT-PCR

EIA, enzyme immunoassay; GDH, glutamate dehydrogenase; LAMP; loop-mediated isothermal DNA amplification; RT-PCR, real-time PCR.

[a] Part of an EIA that detects both toxins A/B and GDH

[b] Only for epidemiologic purposes

[c] Multiplex PCR system

Table 4. Characteristics of included studies

Author	Year	Country	Reference test	Index test(s)	Total no. samples	Study population	Consistency stool samples	Prev. CDI (CCNA)	Prev. CDI (TC)
Barkin	2012	US	TC	Premier tox A/B, Immunocard *C. difficile*, Illumigene	272	Adult inpatients of a large community teaching hospital with diarrhea, risk factors for CDI and for whom a CDI test was requested by their physician	unformed		13.1
Berg, van den	2005	The Netherlands	CCNA	Immunocard tox A/B	367	unformed stools of adults with a specific request for CDI testing or hospitalized >72hrs that were submitted to the laboratories of 3 university hospitals	unformed	6.3	
Berg, van den	2007	The Netherlands	CCNA	Premier tox A/B	540	unformed stools of patients suspected of having CDI or hospitalized >72hrs in 4 university medical centres	unformed	5.7	
Berry	2014	UK	CCNA	Xpert	1034	inpatients in two acute care hospitals > 15yrs old with suspected CDI for whom CDI testing was requested by the treating physician	unformed	6.0	
Boer, de	2010	The Netherlands	CCNA	Xpect	161	clinical stool specimens from patients for whom a request for CDI testing was issued, prospectively collected at a laboratory for infectious diseases	unclear	9.9	

Table 4. *Continued.*

Author	Year	Country	Reference test	Index test(s)	Total no. samples	Study population	Consistency stool samples	Prev. CDI (CCNA)	Prev. CDI (TC)
Bruins	2012	The Netherlands	TC	Immunocard tox A/B, Quik Chek Complete, Premier tox A/B, Illumigene	986	hospitalized and non-hospitalized patients with diarrhea who had a stool sample sent to the laboratory of a major hospital, preferably from those patients known to have CDI-associated symptoms or risk factors	unformed		7.4
Buchan	2012	US	TC	Portrait, GeneXpert, GeneOhm, Illumigene	540/275/ 169/96	stool specimens from patients> 2yrs old suspected of having CDI collected at 4 institutions	unformed		22.5
Calderaro	2013	Italy	TC	Illumigene, Quik Chek Complete	306	patients attending a university hospital with a suspicion of CDI	unclear		19.6
Carroll	2013	US	TC	Verigene	1875	leftover stool samples submitted specifically for CDI testing according to the institution's routine practice to 5 geographically diverse clinical microbiology laboratories	formed and unformed		8.4 (direct), 14.7 (enriched)

Table 4. *Continued.*

Author	Year	Country	Reference test	Index test(s)	Total no. samples	Study population	Consistency stool samples	Prev. CDI (CCNA)	Prev. CDI (TC)
Eastwood	2009	UK	CCNA	Premier tox A/B, Xpect, Tox A/B Quik Chek, Ridascreen tox A/B, tox A/B II, ProSpecT, VIDAS CDAB, Immunocard tox A/B, C. diff Chek-60, BD GeneOhm	488	stool specimens submitted for CCNA testing at the laboratory of a teaching hospital, 10 samples were randomly chosen each day	unformed	18.1	
Eckert	2014	France	CCNA, TC	Amplivue, C. diff Quik Chek	308	inpatients in 4 university-affiliated hospitals >2yrs old with suspected CDI for whom CDI testing was requested by the treating physician or if diarrhoea occurred after day 3 of hospitalization	unformed	7.5	11.7
Fenner	2008	Switzerland	culture	C. diff chek-60	1468	stools of adults patients suspected of having CDI at a university hospital	unclear		12.7 culture positive
Hart	2014	Australia	culture, TC	Illumigene, BD GeneOhm, Quik Chek Complete	150	stools of children collected at the laboratory of a pediatric hospital fulfilling the criteria for CDI testing in this hospital[a]	formed (4%) and unformed (96%)		30.0

Table 4. *Continued.*

Author	Year	Country	Reference test	Index test(s)	Total no. samples	Study population	Consistency stool samples	Prev. CDI (CCNA)	Prev. CDI (TC)
Hirvonen	2013	Finland	TC	GenomEra	310	stool specimens from inpatients (7-95yrs old), collected prospectively according to routine hospital practice for antibiotic-associated diarrhea at a large teaching hospital	unformed		24.9
Huang	2009	Sweden	CCNA	Xpert	220	consecutve stool specimens from patients >2yrs old and who were symptomatic and had a request for CDI testing at a university hospital	unformed	10.5	
Jacobs	1996	Israel	culture, TC	Immunocard C. *difficile*	258	stool samples from patients who developed diarrhea during hospitalization in a community teaching hospital and control samples from 24 patients without diarrhra	formed and unformed		7.0
Jong	2012	The Netherlands	TC	Immunocard tox A/B, VIDAS CDAB	150	hospitalized adult patients in a tertiary teaching hospital who had a stool specimens submitted for CDI testing	unclear		9.7
Kawada	2011	Japan	culture, TC	Quik Chek Complete, Immunocard C. *difficile*, Tox A/B Quik Chek	60	patients hospitalized at a geriatric hospital and diagnosed as having antibiotic-associated diarrhea	unformed		46.7

Table 4. *Continued.*

Author	Year	Country	Reference test	Index test(s)	Total no. samples	Study population	Consistency stool samples	Prev. CDI (CCNA)	Prev. CDI (TC)
Kim	2014	Korea	TC	Quik Chek Complete, VIDAS CDAB	608	suspected CDI patients in a tertiary care teaching hospital	unformed		9.0
Kim	2012	Korea	TC	AdvanSure, VIDAS CDAB	127	diarrhoeal stool specimens submited to a hospital laboratory for *C. difficile* culture	unformed		8.8
Lalande	2011	France	TC	Illumigene	472	consecutive stools from patients suspected of having CDI	unformed		10.4
Larson	2010	US	CCNA	C. diff Quik Chek	699	stool samples submitted for CDI testing from adult patients at a university hospital	unformed	6.7	
Le Guern	2012	France	TC	BD Max Cdiff, BD GeneOhm, Tox A/B Quik Chek	360	diarrheal stool specimens collected from inpatients at a university hospital	unformed		12.2
Leitner	2013	Austria	TC	BD Max Cdiff, Premier tox A/B	180	stool specimens from adults and children with a specified request for CDI testing at a medical university	unformed		16.7
Massey	2003	Candada	CCNA	Tox A/B II	557	stools samples of adult hospitalized patients suspected of having CDI at a large teaching hospital	unformed	25.7	
Mattner	2012	Germany	TC	Ridascreen	254	all liquid stool samples sent to a university microbiology laboratory	unformed		16.4

Table 4. *Continued.*

Author	Year	Country	Reference test	Index test(s)	Total no. samples	Study population	Consistency stool samples	Prev. CDI (CCNA)	Prev. CDI (TC)
Musher	2007	US	CCNA	Premier tox A/B, Immunocard tox A/B, Tox A/B II, ProSpecT	446/131	consecutive stool samples submitted to the laboratory of a medical center for CDI testing	unclear	17.0/41.2	
Noren	2011	Sweden	CCNA	Illumigene	272	consecutive stool specimens from adults and children submitted for CDI testing from hospitals and communities	unclear	13.2	
Novak-Weekley	2010	US	TC	Xpert, Premier A/B	432	leftover stool samples from patients >2yrs old with suspected CDI for whom toxin EIAs were ordered according to the institution's standard practices at regional reference laboratories serving hospitals and associated medical clinics	unformed		16.8
O'Connor	2001	Ireland	CCNA	Tox A/B II, Premier tox A/B	200	consecutive stools of adult patients suspected of having CDI submitted to the laboratories of university hospitals	formed and unformed	30.5	
Ota	2012	USA	CCNA	C. diff Quik Chek Complete, Premier tox A/B, Illumigene	141	consecutive stool specimens prospectively collected at a children's hospital from patients 1-18 years of age and submitted for CDI testing	unformed	18.4	

Table 4. *Continued.*

Author	Year	Country	Reference test	Index test(s)	Total no. samples	Study population	Consistency stool samples	Prev. CDI (CCNA)	Prev. CDI (TC)
Pancholi	2012	USA	CCNA	Illumigene, Xpert	200	consecutive and prospectively collected stools from adult patients submitted to a university medical center laboratory for routine CDI testing.	unformed	11.6	
Planche	2013	UK	CCNA, TC	Xpert, C. diff Chek-60, Premier tox A/B, Tox A/B II	8827/12365/ 9192/12369	faecal samples from hospital and community patients submitted for routine CDI testing according to a routine protocol[b] submitted to 4 hospital diagnostic laboratories serving major teaching hospitals and their communities	unformed	5.9	8.4
Qutub	2011	Saudi Arabia	CCNA	C. diff Chek-60, Tox A/B II	150	stool samples from consecutive inpatients with suspected CDI	unformed	34.7	
Reller	2007	US	culture	C. diff chek-60	439	stool samples from hospitalized adults and children suspected of having CDI	unclear		36.7 culture positive
Reller	2010	US	CCNA	C. diff Chek-60, Quik Chek, Tox A/B Quik Chek	600	sequential weekday stool samples submitted to a university hospital microbiology laboratory for CDI testing	unformed	7.7	
Shin	2009	Korea	TC	Vidas CDAB	1596	stool samples from patients admitted to a tertiary teaching hospital with clinical signs compatible with CDI			19.6

Table 4. *Continued.*

Author	Year	Country	Reference test	Index test(s)	Total no. samples	Study population	Consistency stool samples	Prev. CDI (CCNA)	Prev. CDI (TC)
Shin	2009	Korea	TC	Vidas CDAB	555	patients >2yrs old with suspected CDI from two hospitals	formed (51%) and unformed		20.3
Shin	2012	Korea	TC	Seegene, BD GeneOhm	243	fresh stool specimens from patients with clinical signs compatible with CDI who were hospitalized in 3 teaching hospitals	unclear		28.8
Shin	2012	Korea	TC	Xpert/epi, Vidas CDAB	253	consecutive stool specimens from suspected CDI patients in a tertiary hospital	unformed		18.4
Sloan	2008	US	TC	Premier tox A/B, Xpect, Immunocard A/B	200	stools of patients suspected of having CDI submitted to a clinical micorobiology laboratory of a large tertiary care teaching hospital	unformed		22.0
Snell	2004	Canada	culture, TC	C. diff chek-60, Tox A/B II	497	stools of inpatients suspected of having CDI at a large teaching hospital	unformed		10.5
Soh	2014	Korea	TC	AdvanSure CD, Illumigene	203	stool samples collected at a tertiary university teaching hospital	unformed		12.8
Stamper	2009	US	CCNA	BD GeneOhm	401	symptomatic adults patients who had a stool sample submitted for routine CDI testing in a tertiary care university medical center	unformed	11.0	

Table 4. *Continued.*

Author	Year	Country	Reference test	Index test(s)	Total no. samples	Study population	Consistency stool samples	Prev. CDI (CCNA)	Prev. CDI (TC)
Stamper	2009	US	CCNA, TC	ProGastro CD	280	stool samples submitted for routine CDI testing from symptomatic patients >2yrs old at a tertiary-care university medical institution	unformed	11.0	15.7
Staneck	1996	US	CCNA	Immunocard *C. difficile*	906	stool samples submitted to 3 hospital microbiology laboratories	unclear	14.1	
Swindells	2010	UK	culture, CCNA, TC	C. diff Quik Chek Complete, Vidas CDAB, Xpert, GeneOhm	150	consecutive stool specimens from inpatients >65 yrs old who developed diarrhea at least 48hrs after admission	unformed	10.0	12.0
Tenover	2010	US/Canada	TC	Xpert	2296	leftover stool specimens from patients >2yrs old from 7 health care organizations (6 US, 1 Canada) for whom CDI testing was ordered according to the institution's practices	unformed		10.8 (direct), 14.7 (enriched)
Terhes	2009	Hungary	CCNA	BD GeneOhm	600	inpatients and outpatients at a local university hospital who had a diarrheal stool sample sent to the laboratory for CDI testing	unformed	6.4	

Table 4. *Continued.*

Author	Year	Country	Reference test	Index test(s)	Total no. samples	Study population	Consistency stool samples	Prev. CDI (CCNA)	Prev. CDI (TC)
Ticehurst	2006	US	CCNA	C. diff chek-60	266	stools of patients suspected of having CDI submitted to the laboratories of two acute-care hospitals	unclear	9.0	
Turgeon	2003	US	CCNA	Immunocard C. *difficile*	1003	consecutive stools of adults and childern suspected of having CDI at 5 major hospitals	unformed and formed	10.1	
Vanpoucke	2001	Belgium	CCNA	Ridascreen	156	stool specimens submitted to the laboratory of a university hospital with a request for CDI testing	unformed	31.8	
Viala	2012	France	TC	BD GeneOhm, Xpert, Illumigene	94	fresh stool specimens from symptomatic patients collected at a university hospital, 45 TC+ and 49 TC- were selected	unformed		47.8
Walkty	2013	Canada	TC	Illumigene, C. diff Quik Chek	428	all diarrheal stool specimens from patients >1yrs old submitted for CDI testing to 3 microbiologcial laboratories serving major hospitals and surrounding communities	unformed		14.7
Wren	2009	UK	culture, TC	C. diff Quik Chek, Tox A/B Quik Chek	1007	stool samples submitted for CDI testing from patients who developed diarrhoea after being admitted to major university hospitals	unformed		8.6

Table 4. *Continued.*

Author	Year	Country	Reference test	Index test(s)	Total no. samples	Study population	Consistency stool samples	Prev. CDI (CCNA)	Prev. CDI (TC)
Zheng	2004	US	culture	C. diff chek-60	992	stool samples submitted for routine CDI testing because of antibiotic-associated diarrhea collected from hospital laboratoires and supplied to TechLab, a large medical center and the reference laboratory	unclear	13.8	

CCNA, cell cytotoxicity neutralization assay; CDI, *Clostridium difficile* infection; TC, toxigenic culture

[a] Criteria were: oncology/haematology patient, specific request for CDI testing by treating physician, history of diarrhoea developed while receiving antibiotics, or pseudomembranous colitis

[b] Criteria were: all unformed faecal samples not clearly attributable to an underlying disease, or treatment from all hospital patients >2years and from individuals in the community >65 years irrespective of *C. difficile* or other testing requests

Quality assessment

None of the studies fulfilled all our quality assessment criteria, mainly because required information was frequently missing (Fig. 2, Supplementary Material 4). The process used to select samples was adequately reported in 23 (41%) of 56 studies. A minority of studies (6/56, 11%) reported that they did not exclude formed samples from CDI testing. In around half of the studies, conditions of storage for the samples before testing with the index test were not (or were insufficiently) reported. Samples tested by GDH EIA, toxin A/B EIA and NAAT were reported to be stored according to manufacturer's instructions in 10 (46%) of 22, 14 (45%) of 31 and 15 (50%) of 30 studies, respectively. In the remaining 12, 16 and 15 studies, respectively, storage conditions did not or not completely comply with manufacturer's instructions. In 18 (72%) of 25 studies using CCNA as the reference test, samples were stored according to our predefined storage requirements: samples were either refrigerated and tested within 5 days (15 studies) (8, 25, 27, 36, 45-48, 58-61, 63, 65, 68) or were frozen at -20°C and thawed no more than once (three studies) (44, 66, 67). In the remaining seven studies (28%), storage conditions for CCNA were not or incompletely described. Storage conditions for samples tested by TC were reported in 23 (68%) of 34 studies, but no specific requirements for storage of samples tested by TC were set. The execution of the reference test was described in sufficient detail in 44 (79%) of 56 studies. In 2 (8%) of 26 studies using CCNA as reference test, the incubation period was only 24 hours. (61, 63) In studies using TC as reference test, ethanol shock was reported to be performed in 18 of 35 studies (19, 21, 23, 32, 35, 37, 38, 47, 51-55, 57, 61, 69-71), and heat shock was performed in three of 35 studies. (22, 49, 58) Eight studies (23%) used an enrichment broth before plating onto a solid agar. (19, 22-24, 32, 43, 58, 62) Toxigenicity was confirmed by PCR (15/32, 47%) (21, 23, 29, 33-35, 37, 51-57, 70), CCNA (9/32, 28%) (7, 8, 22, 24, 43, 47, 58, 61, 62), toxin EIA (7/32, 22%) (19, 30, 32, 38, 40, 69, 71) or both PCR and CCNA (1/32, 3%). (26) Blinding (index test interpreted without knowledge of reference test or vice versa) was reported in 8 (14%) of 56 studies. Thirty- one studies (55%) reported if any indeterminate results (i.e. invalid, 'no call' or difficult-to-interpret results) were found. Indeterminate results actually occurred in 28 studies and were reported for one membrane-type GDH EIA (ImmunoCard C. difficile), three membrane-type toxin A/B EIAs (Tox A/B Quik Chek, ImmunoCard Tox A/B, Xpect), one automated EIA (Vidas) and nine NAATs. The amount of indeterminate results ranged from 0.3 to 6.8% of tested samples. Repeat testing of samples after an initial indeterminate result was done in 24 (86%) of these 28 studies. Of these, 22 presented results only after repeat testing (7, 8, 20, 21, 24, 29, 30, 34, 35, 37, 38, 43, 46, 47, 54, 58, 59, 62, 65, 69, 70), and two presented results of both initial and repeat testing. (27, 63)

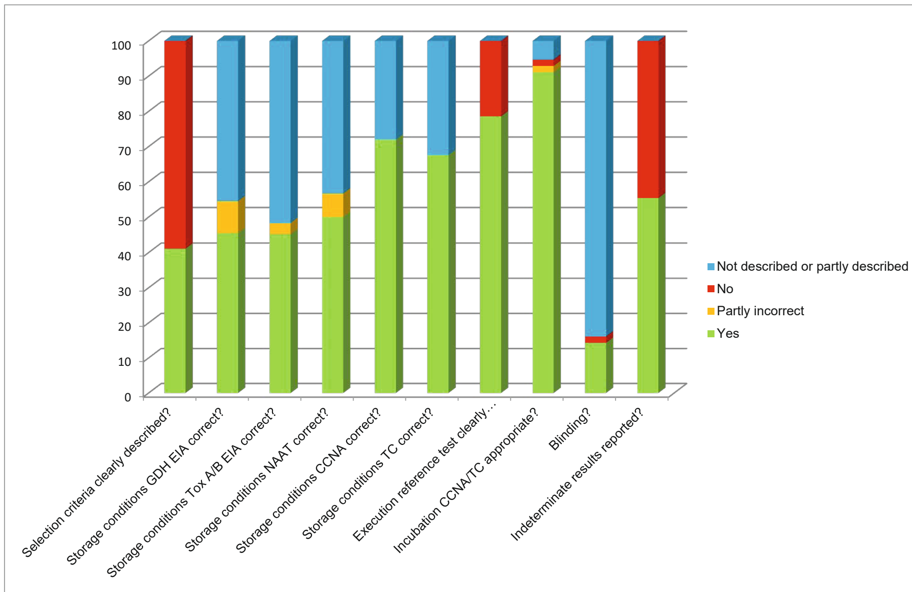

Figure 2. Quality assessment of included studies.

Test performances

Sensitivity and specificity of the index tests were calculated on the basis of the numbers provided in the articles. Discrepancies between calculated sensitivity or specificity and published data were found in two articles; the correct data were provided by both authors upon request. (38, 39) In Table 5, sensitivity and specificity of index tests are compared to CCNA. Reported estimates of sensitivity ranged from 0.80 to 1.00 for GDH EIAs, from 0.44 to 0.99 for toxin A/B EIAs and from 0.83 to 1.00 for NAATs. Reported estimates of specificity ranged from 0.82 to 0.95 for GDH EIAs, from 0.87 to 1.00 for toxin A/B EIAs and from 0.87 to 0.98 for NAATs. Table 6 lists sensitivity and specificity compared to TC. Sensitivities ranged from 0.83 to 1.00, 0.29 to 0.86 and 0.77 to 1.0 for GDH EIAs, toxin A/B EIAs and NAATs, respectively. Specificities ranged from 0.88 to 1.00, 0.91 to 1.00 and 0.83 to 1.00, respectively. In Table 7, sensitivity and specificity of GDH EIAs are compared to culture. Sensitivities ranged from 0.71 to 1.00, and specificities ranged from 0.67 to 1.00. In Table 8, estimates of pooled sensitivity and pooled specificity for the different categories of index tests are shown. The estimated pooled sensitivities and specificities compared to CCNA were used to compute PPVs and NPVs of the categories of index tests at different hypothetical CDI prevalences (Table 9, Supplementary Material 5). At a CDI prevalence of

5%, PPVs ranged from 34 to 81%, and NPVs ranged from 99 to 100%. At a CDI prevalence of 50%, PPVs ranged from 91 to 99%, while NPVs ranged from 83 to 98%.

Table 5. Sensitivity and specificity of index tests compared to CCNA

Index test	Study	Sensitivity (95% CI)	Specificity (95% CI)
(A) Well-type EIA GDH			
C. diff Chek-60	Eastwood [27]	0.90 (0.82–0.95)	0.93 (0.90–0.95)
C. diff Chek-60	Planche [47]	0.96 (0.95–0.98)	0.92 (0.92–0.93)
C. diff Chek-60	Qutub [48]	0.94 (0.84–0.99)	0.88 (0.80–0.94)
C. diff Chek-60	Reller [50]	0.91 (0.79–0.98)	0.90 (0.87–0.92)
C. diff Chek-60	Ticehurst [64]	0.96 (0.79–1.00)	0.90 (0.86–0.94)
(B) Membrane-type EIA GDH			
C. diff Quik Chek	Eckert [8]	1.00 (0.85–1.00)	0.92 (0.88–0.94)
C. diff Quik Chek	Larson [36]	1.00 (0.92–1.00)	0.90 (0.87–0.92)
C. diff Quik Chek	Reller [50]	1.00 (0.92–1.00)	0.83 (0.79–0.86)
ImmunoCard *C. difficile*	Staneck [60]	0.84 (0.77–0.90)	0.92 (0.90–0.94)
ImmunoCard *C. difficile*	Turgeon [65]	0.80 (0.71–0.87)	0.92 (0.91–0.94)
Quik Chek Complete-GDH	Ota [45]	0.81 (0.61–0.93)	0.82 (0.73–0.88)
Quik Chek Complete-GDH	Swindells [61]	1.00 (0.78–1.00)	0.95 (0.90–0.98)
(C) Well-type EIA toxins A/B			
Clostridium difficile Tox A/B II	Eastwood [27]	0.91 (0.84–0.95)	0.96 (0.93–0.97)
Clostridium difficile Tox A/B II	Massey [39]	0.75 (0.67–0.82)	0.98 (0.96–0.99)
Clostridium difficile Tox A/B II	Musher [41]	0.96 (0.87–1.00)	0.87 (0.77–0.94)
Clostridium difficile Tox A/B II	O'Connor [44]	0.80 (0.68–0.89)	0.99 (0.96–1.00)
Clostridium difficile Tox A/B II	Planche [47]	0.83 (0.80–0.86)	0.99 (0.99–0.99)
Clostridium difficile Tox A/B II	Qutub [48]	0.73 (0.59–0.84)	1.00 (0.96–1.00)
Premier toxins A/B	Berg, van den 2007 [67]	0.97 (0.83–1.00)	0.94 (0.92–0.96)
Premier toxins A/B	Eastwood [27]	0.92 (0.85–0.96)	0.97 (0.95–0.98)
Premier toxins A/B	Musher [41]	0.99 (0.93–1.00)	0.97 (0.95–0.99)
Premier toxins A/B	O'Connor [44]	0.82 (0.70–0.91)	0.99 (0.96–1.00)
Premier toxins A/B	Ota [45]	0.58 (0.37–0.77)	1.00 (0.97–1.00)
Premier toxins A/B	Planche [47]	0.67 (0.63–0.71)	0.99 (0.99–0.99)
Remel ProSpecT	Eastwood [27]	0.90 (0.83–0.95)	0.93 (0.90–0.95)
Remel ProSpecT	Musher [41]	0.91 (0.80–0.97)	0.97 (0.91–1.00)
Ridascreen toxins A/B	Eastwood [27]	0.67 (0.57–0.75)	0.95 (0.93–0.97)
Ridascreen toxins A/B	Vanpoucke [68]	0.57 (0.43–0.70)	0.97 (0.92–0.99)
(D) Membrane-type EIA toxins A/B			
ImmunoCard toxins A/B	Berg, van den (2005) [66]	0.91 (0.72–0.99)	0.97 (0.95–0.99)
ImmunoCard toxins A/B	Eastwood [27]	0.85 (0.76–0.91)	0.99 (0.98–1.00)
ImmunoCard toxins A/B	Musher [41]	0.96 (0.89–0.99)	0.99 (0.97–1.00)
Quik Chek Complete-Tox A/B	Ota [45]	0.50 (0.30–0.70)	1.00 (0.97–1.00)
Quik Chek Complete-Tox A/B	Swindells [61]	0.73 (0.45–0.92)	1.00 (0.97–1.00)

Table 5. *Continued.*

Index test	Study	Sensitivity (95% CI)	Specificity (95% CI)
Tox A/B Quik Chek	Eastwood [27]	0.84 (0.76–0.91)	0.99 (0.98–1.00)
Tox A/B Quik Chek	Reller [50]	0.61 (0.45–0.75)	0.99 (0.98–1.00)
Xpect	Boer, de [25]	0.44 (0.20–0.70)	1.00 (0.97–1.00)
Xpect	Eastwood [27]	0.83 (0.74–0.90)	0.99 (0.98–1.00)
(E) Automated EIA toxins A/B			
VIDAS CDAB	Eastwood [27]	0.98 (0.93–1.00)	0.99 (0.98–1.00)
VIDAS CDAB	Swindells [61]	0.53 (0.27–0.79)	1.00 (0.97–1.00)
(F) NAAT			
Amplivue	Eckert [8]	0.96 (0.78–1.00)	0.95 (0.91–0.97)
BD GeneOhm	Eastwood [27]	0.92 (0.85–0.97)	0.95 (0.93–0.97)
BD GeneOhm	Stamper (2009–1) [59]	0.91 (0.78–0.97)	0.95 (0.92–0.97)
BD GeneOhm	Swindells [61]	1.00 (0.78–1.00)	0.98 (0.94–1.00)
BD GeneOhm	Terhes [63]	0.95 (0.82–0.99)	0.96 (0.94–0.98)
Illumigene	Noren [42]	1.00 (0.90–1.00)	0.93 (0.89–0.96)
Illumigene	Ota [45]	0.88 (0.70–0.98)	0.97 (0.93–0.99)
Illumigene	Pancholi [46]	0.87 (0.66–0.97)	0.91 (0.86–0.95)
Prodesse ProGastro Cd assay	Stamper (2009–2) [58]	0.83 (0.65–0.94)	0.96 (0.92–0.98)
Xpert *C. difficile*	Berry [20]	1.00 (0.94–1.00)	0.94 (0.92–0.95)
Xpert *C. difficile*	Huang [31]	0.96 (0.78–1.00)	0.87 (0.82–0.92)
Xpert *C. difficile*	Pancholi [46]	1.00 (0.85–1.00)	0.89 (0.83–0.93)
Xpert *C. difficile*	Planche [47]	0.98 (0.96–0.99)	0.93 (0.92–0.94)
Xpert *C. difficile*	Swindells [61]	1.00 (0.78–1.00)	0.97 (0.93–0.99)

CI, confidence interval; CCNA, cell cytotoxicity neutralization assay; EIA, enzyme immunoassay; GDH, glutamate dehydrogenase; NAAT, nucleic acid amplification test

Table 6. Sensitivity and specificity of index tests compared to TC

Index test	Study	Sensitivity (95% CI)	Specificity (95% CI)
(A) Well-type EIA GDH			
C. diff Chek-60	Planche [47]	0.94 (0.93–0.96)	0.94 (0.94–0.95)
(B) Membrane-type EIA GDH			
C. diff Quik Chek	Eckert [8]	0.97 (0.85–1.00)	0.95 (0.92–0.97)
C. diff Quik Chek	Walkty [70]	0.83 (0.71–0.91)	0.97 (0.95–0.98)
ImmunoCard *C. difficile*	Barkin [19]	1.00 (0.90–1.00)	1.00 (0.98–1.00)
ImmunoCard *C. difficile*	Jacobs [32]	0.60 (0.32–0.84)	0.76 (0.68–0.83)
Quik Chek Complete—GDH	Bruins [21]	0.97 (0.90–1.00)	0.98 (0.96–0.98)
Quik Chek Complete—GDH	Kawada [33]	1.00 (0.88–1.00)	0.88 (0.71–0.96)
Quik Chek Complete—GDH	Swindells [61]	1.00 (0.81–1.00)	0.97 (0.92–0.99)
(C) Well-type EIA toxins A/B			
Clostridium difficile Tox A/B II	Planche [47]	0.58 (0.55–0.61)	0.99 (0.98–0.99)
Clostridium difficile Tox A/B II	Snell [56]	0.85 (0.72–0.93)	0.98 (0.96–0.99)
Premier toxins A/B	Barkin [19]	0.86 (0.71–0.95)	0.91 (0.86–0.94)
Premier toxins A/B	Bruins [21]	0.41 (0.30–0.53)	0.99 (0.98–0.99)
Premier toxins A/B	Leitner [38]	0.40 (0.21–0.61)	1.00 (0.98–1.00)
Premier toxins A/B	Novak-Weekley [43]	0.58 (0.46–0.70)	0.95 (0.92–0.97)
Premier toxins A/B	Planche [47]	0.46 (0.42–0.49)	0.99 (0.99–0.99)
Premier toxins A/B	Sloan [55]	0.48 (0.32–0.63)	0.98 (0.94–1.00)
Ridascreen toxins A/B	Mattner [40]	0.52 (0.36–0.68)	0.98 (0.95–0.99)
(D) Membrane-type EIA toxins A/B			
ImmunoCard toxins A/B	Bruins [21]	0.41 (0.30–0.53)	0.99 (0.98–1.00)
ImmunoCard toxins A/B	de Jong [26]	0.47 (0.23–0.72)	0.99 (0.96–1.00)
ImmunoCard toxins A/B	Sloan [55]	0.48 (0.32–0.63)	0.99 (0.95–1.00)
Quik Chek Complete—Tox A/B	Bruins [21]	0.55 (0.43–0.66)	1.00 (1.00–1.00)
Quik Chek Complete—Tox A/B	Calderaro [23]	0.68 (0.55–0.80)	0.89 (0.84–0.92)
Quik Chek Complete—Tox A/B	Hart [29]	0.29 (0.16–0.44)	1.00 (0.97–1.00)
Quik Chek Complete—Tox A/B	Kawada [33]	0.79 (0.59–0.92)	0.97 (0.84–1.00)
Quik Chek Complete—Tox A/B	Kim (2014) [35]	0.64 (0.50–0.76)	0.98 (0.96–0.99)

Table 6. *Continued.*

Index test	Study	Sensitivity (95% CI)	Specificity (95% CI)
Quik Chek Complete—Tox A/B	Swindells [61]	0.61 (0.36–0.83)	1.00 (0.97–1.00)
Tox A/B Quik Chek	Kawada [33]	0.71 (0.51–0.87)	0.94 (0.79–0.99)
Tox A/B Quik Chek	Le Guern [37]	0.43 (0.28–0.59)	1.00 (0.98–1.00)
Tox A/B Quik Chek	Wren [71]	0.40 (0.30–0.51)	1.00 (1.00–1.00)
Xpect	Sloan [55]	0.48 (0.32–0.63)	0.84 (0.77–0.89)
(E) Automated EIA toxins A/B			
VIDAS CDAB	Jong, de [26]	0.71 (0.42–0.92)	0.95 (0.90–0.98)
VIDAS CDAB	Kim (2012) [34]	0.64 (0.31–0.89)	1.00 (0.97–1.00)
VIDAS CDAB	Kim (2014) [35]	0.76 (0.61–0.87)	0.97 (0.96–0.99)
VIDAS CDAB	Shin (2009–1) [52]	0.68 (0.62–0.73)	0.96 (0.95–0.97)
VIDAS CDAB	Shin (2009–2) [51]	0.69 (0.59–0.78)	0.97 (0.94–0.98)
VIDAS CDAB	Shin (2012–2) [54]	0.44 (0.30–0.60)	1.00 (0.98–1.00)
VIDAS CDAB	Swindells [61]	0.44 (0.22–0.69)	1.00 (0.97–1.00)
(F) NAAT			
Advansure CD	Kim (2012) [34]	1.00 (0.72–1.00)	0.98 (0.94–1.00)
Advansure CD	Soh [57]	0.85 (0.65–0.96)	0.98 (0.95–1.00)
Amplivue	Eckert [8]	0.86 (0.71–0.95)	0.98 (0.95–0.99)
BD GeneOhm	Buchan [22]	0.97 (0.86–1.00)	0.98 (0.95–1.00)
BD GeneOhm	Hart [29]	0.89 (0.76–0.96)	0.99 (0.95–1.00)
BD GeneOhm	Le Guern [37]	0.95 (0.85–0.99)	1.00 (0.98–1.00)
BD GeneOhm	Shin (2012–1) [53]	0.96 (0.88–0.99)	0.97 (0.93–0.99)
BD GeneOhm	Swindells [61]	0.94 (0.73–1.00)	0.99 (0.96–1.00)
BD GeneOhm	Viala [69]	0.96 (0.85–0.99)	0.98 (0.89–1.00)
BD Max Cdiff	Le Guern [37]	0.98 (0.88–1.00)	1.00 (0.98–1.00)
BD Max Cdiff	Leitner [38]	0.96 (0.80–1.00)	0.99 (0.96–1.00)
GenomEra	Hirvonen [30]	1.00 (0.95–1.00)	0.99 (0.96–1.00)
Illumigene	Barkin [19]	1.00 (0.90–1.00)	1.00 (0.98–1.00)
Illumigene	Bruins [21]	0.93 (0.85–0.98)	1.00 (0.99–1.00)
Illumigene	Buchan [22]	0.93 (0.68–1.00)	0.95 (0.88–0.99)
Illumigene	Calderaro [23]	1.00 (0.94–1.00)	0.83 (0.78–0.87)
Illumigene	Hart [29]	0.89 (0.76–0.96)	1.00 (0.97–1.00)
Illumigene	Lalande [7]	0.92 (0.80–0.98)	0.99 (0.98–1.00)
Illumigene	Soh [57]	0.92 (0.75–0.99)	0.99 (0.97–1.00)
Illumigene	Viala [69]	0.87 (0.73–0.95)	1.00 (0.93–1.00)
Illumigene	Walkty [70]	0.73 (0.60–0.83)	1.00 (0.98–1.00)
Portrait	Buchan [22]	0.98 (0.94–1.00)	0.93 (0.90–0.95)

Table 6. *Continued.*

Index test	Study	Sensitivity (95% CI)	Specificity (95% CI)
Prodesse ProGastro Cd assay	Stamper (2009–2) [58]	0.77 (0.62–0.89)	0.99 (0.97–1.00)
Seeplex ACE	Shin (2012–1) [53]	0.90 (0.80–0.96)	0.97 (0.93–0.99)
Verigene	Caroll [24]	0.91 (0.87–0.94)	0.93 (0.91–0.94)
Xpert *C. difficile*	Buchan [22]	1.00 (0.94–1.00)	0.92 (0.87–0.95)
Xpert *C. difficile*	Novak-Weekley [43]	0.94 (0.86–0.98)	0.96 (0.94–0.98)
Xpert *C. difficile*	Planche [47]	0.95 (0.93–0.96)	0.96 (0.96–0.97)
Xpert *C. difficile*	Shin (2012–2) [54]	1.00 (0.93–1.00)	0.95 (0.91–0.98)
Xpert *C. difficile*	Swindells [61]	1.00 (0.81–1.00)	0.99 (0.96–1.00)
Xpert *C. difficile*	Tenover [62]	0.93 (0.90–0.96)	0.94 (0.93–0.95)
Xpert *C. difficile*	Viala [69]	0.98 (0.88–1.00)	0.98 (0.89–1.00)

CI, confidence interval; EIA, enzyme immunoassay; GDH, glutamate dehydrogenase; NAAT, nucleic acid amplification test; TC, toxigenic culture.

Table 7. Sensitivity and specificity of index tests compared to culture

Index test	Study	Sensitivity (95% CI)	Specificity (95% CI)
(A) Well-type EIA GDH			
C. diff Chek-60	Fenner [28]	0.93 (0.88–0.97)	0.97 (0.95–0.97)
C. diff Chek-60	Reller (2007) [49]	1.00 (0.98–1.00)	0.67 (0.61–0.72)
C. diff Chek-60	Snell [56]	0.94 (0.86–0.98)	0.98 (0.96–0.99)
C. diff Chek-60	Zheng [72]	0.71 (0.63–0.78)	0.88 (0.85–0.90)
(B) Membrane-type EIA GDH			
C. diff Quik Chek	Wren [71]	0.95 (0.90–0.98)	0.99 (0.98–1.00)
Quik Chek Complete—GDH	Bruins [21]	0.95 (0.89–0.99)	0.99 (0.98–0.99)
Quik Chek Complete—GDH	Hart [29]	0.87 (0.75–0.95)	0.97 (0.91–0.99)
Quik Chek Complete—GDH	Kawada [33]	1.00 (0.88–1.00)	0.93 (0.78–0.99)
Quik Chek Complete—GDH	Swindells [61]	1.00 (0.82–1.00)	0.98 (0.93–1.00)
ImmunoCard *C. difficile*	Jacobs [32]	0.75 (0.59–0.87)	0.90 (0.83–0.95)
ImmunoCard *C. difficile*	Kawada [33]	0.80 (0.61–0.92)	1.00 (0.88–1.00)

CI, confidence interval; EIA, enzyme immunoassay; GDH, glutamate dehydrogenase; NAAT, nucleic acid amplification test.

Discussion

In the present meta-analysis, we evaluated the diagnostic accuracy of various commercial laboratory assays for diagnosing CDI. Toxin A/B EIAs tended to be the most specific assays, while GDH EIAs and NAATs were more sensitive tests. Although many toxin A/B EIAs belong to the least sensitive tests, the sensitivity of this category of assays is not as low as reported earlier. (1) This is because only currently available tests were included in the present analysis, and the newer generation of toxin A/B EIAs turns out to be more sensitive than the earlier toxin A EIAs.

We compared all categories of the index tests (GDH EIAs, toxin A/B EIAs and NAATs) to both of the reference tests, CCNA and TC. However, not only are the targets of these three categories of index tests somewhat different, but also the targets of the two reference tests differ: CCNA detects in vivo toxin production, while TC detects the presence of a toxigenic *C. difficile* strain.

This explains why sensitivities and specificities were different for each reference test that was used as a comparator. For example, toxin A/B EIAs were less sensitive compared to TC instead of CCNA: toxin EIAs will not (like the TC) detect all samples containing toxigenic *C. difficile* strains but only (some of) those with free toxin present. It also explains why NAATs were less specific compared to CCNA instead of TC: NAATs are not able (like CCNA) to discern samples with in vivo toxin production from samples with in vitro toxin production.

We included both CCNA and TC as reference tests, as there has always been debate which of these tests best defines CDI cases. Recently a large study reported that CCNA positivity (i.e. demon- stration of free toxin) but not TC positivity (i.e. demonstration of toxin-producing capacity) correlated with clinical outcome. Therefore, at least all samples with a positive CCNA can be considered to represent true CDI cases. (47) However, samples with a positive TC but negative CCNA are difficult to interpret. These samples could either belong to *C. difficile* carriers (harbouring a toxigenic *C. difficile* strain not producing detectable toxins at that moment) or to patients with CDI with toxin levels below the threshold of detection.

To guarantee a certain level of uniformity and quality, only studies that met our eligibility criteria were included in the meta- analysis. Still, studies differed from one another in many aspects. For CCNA, diverse dilutions of faecal filtrate and diverse cell lines were used. For TC, diverse culture media and diverse methods to demonstrate toxigenicity were applied.

Table 8. Pooled sensitivities and specificities of categories of tests

Type	Test	Compared to CCNA			Compared to TC			Compared to culture		
		No. of studies	Sensitivity (95% CI)	Specificity (95% CI)	No. of studies	Sensitivity (95% CI)	Specificity (95% CI)	No. of studies	Sensitivity (95% CI)	Specificity (95% CI)
EIA GDH	Total	12	0.94 (0.89–0.97)	0.90 (0.88–0.92)	8	0.96 (0.86–0.99)	0.96 (0.91–0.98)	11	0.94 (0.86–0.97)	0.96 (0.92–0.98)
	Well type	5	0.94 (0.91–0.97)	0.92 (0.92–0.93)	1	0.94 (0.93–0.96)	0.94 (0.94–0.95)	4	0.89 (0.86–0.91)	0.91 (0.90–0.92)
	Membrane type	7	0.98 (0.78–1.00)	0.90 (0.87–0.93)	7	0.97 (0.84–1.00)	0.96 (0.90–0.99)	7	0.93 (0.84–0.97)	0.98 (0.95–0.99)
EIA toxins A/B	Total	27	0.83 (0.76–0.88)	0.99 (0.98–0.99)	29	0.57 (0.51–0.63)	0.99 (0.98–0.99)			
	Well type	18	0.85 (0.77–0.91)	0.98 (0.96–0.99)	16	0.60 (0.52–0.68)	0.98 (0.97–0.99)			
	Membrane type	9	0.79 (0.66–0.88)	0.99 (0.98–0.99)	13	0.53 (0.45–0.61)	0.99 (0.97–1.00)			
NAAT		14	0.96 (0.93–0.98)	0.94 (0.93–0.95)	32	0.95 (0.92–0.97)	0.98 (0.97–0.99)			

CI, confidence interval; CCNA, cell cytotoxicity neutralization assay; EIA, enzyme immunoassay; GDH, glutamate dehydrogenase; NAAT, nucleic acid amplification test; TC, toxigenic culture.

Also, none of the studies satisfied all our quality assessment criteria. Notwithstanding these differences, all included studies met the minimal -quite strict- requirements we set. We therefore think that it is justifiable that we calculated summary estimates of sensitivity and specificity, especially because we intended to provide a general overview of test performances of different categories of laboratory assays instead of pointing out one 'best' assay. It is, however, important to realize that test performances of individual assays may have been influenced by the design of included studies analysing these tests. Besides, test characteristics presented here should not be considered unchanging over time and should not be considered fixed characteristics. This is because procedures of commercial assays are sometimes revised to enhance test performance, and also because assays may perform differently among different populations (e.g. high- vs. low-risk patients). Also, in all categories, new assays were marketed. The introduction of newer toxin A/B EIAs leading to a better sensitivity of this category of assays is a good example of the latter.

Table 9. PPV and NPV for different categories of index tests at hypothetical CDI prevalences of 5, 10, 20 and 50%

	CDI prevalence 5%		CDI prevalence 10%		CDI prevalence 20%		CDI prevalence 50%	
Test type	PPV	NPV	PPV	NPV	PPV	NPV	PPV	NPV
Well-type EIA GDH	38	100	54	99	72	98	91	94
Membrane-type EIA GDH	34	100	52	100	71	99	91	98
Well-type EIA toxins A/B	69	99	83	98	91	96	98	87
Membrane-type EIA toxins A/B	81	99	90	98	95	95	99	83
NAAT	46	100	64	100	80	99	94	96

Pooled estimates of sensitivity and specificity compared to cell cytotoxicity neutralization assay were used to calculate the predictive values.
CDI, *Clostridium difficile* infection; EIA, enzyme immunoassay; GDH, glutamate dehydrogenase; NAAT, nucleic acid amplification test; NPV, negative predictive value; PPV, positive predictive value.

On the basis of the review results, PPVs and NPVs were calculated at different hypothetical prevalences of CDI in the tested population. The prevalence of CDI can be seen as the pretest probability of having CDI and would typically be around 5-10% in an endemic setting. (73) At a CDI prevalence of 5%, even the most specific tests (toxin A/B EIAs) would have PPVs of only 69-81%. On the contrary, NPVs would be very high for all index tests. If the prevalence of CDI would rise to 50% among the tested patients, the PPV would consequently raise to 98.8% for the most specific test, but the NPV would drop to 82.5% for the least sensitive tests. Both suboptimal PPV and NPV have implications. A low PPV will result in many patients with false-positive results. These noninfected patients may receive

unnecessary treatment for CDI, and unnecessary isolation precautions may be taken. A low NPV will result in many undetected cases, which may not only have implications for individual patients but also for further transmission of C. *difficile*. It is therefore important to be aware not only of the sensitivity and specificity of an assay but also of the CDI prevalence in the tested population, as the predictive values and hence the clinical utility of the assays depend on them.

The easiest way to diagnose CDI would be to use a single rapid laboratory test that is able to reliably predict disease status. A rapid CDI diagnosis is associated with more prompt CDI treatment and less unnecessarily treated patients. (74) However, two problems arise if the rapid assays are used as stand-alone test for diagnosing CDI. First, as described above, the PPVs of even the most specific tests are inadequate at low disease prevalence. If toxin EIAs were to be used in an endemic situation (CDI prevalence of 5% in the tested population, PPV 81%), an unacceptably high percentage (19%) of patients with a positive test result would not actually have CDI. Second, as the targets identified by the index tests are (just like the targets of the reference test) different from each other, a positive index test does not necessarily indicate a real CDI case. Two of the three categories of index test are not able to differentiate carriers from CDI patients: both GDH EIAs and NAATs do not detect free toxins. Using NAAT as a stand-alone test and relying on clinical symptoms to discern patients with CDI from asymptomatic carriers is not an optimal approach: patients colonized by a toxigenic *C. difficile* strain may very well develop diarrhoea due to other causes, and no specific clinical symptoms exist to differentiate CDI from other causes of diarrhoea. From the above, we conclude that neither GDH EIA nor toxin A/B EIA or NAAT can reliably be used as a stand-alone test to diagnose CDI.

Because no single test is suitable to be used as a stand-alone test, it is best to combine two tests in an algorithm in order to optimize the diagnosis of CDI. The advantage of an algorithm is that tests can be combined in such a way that the percentage of false-positive results can be decreased. This can be done by testing all samples with a first test, then performing reflex testing on samples with a positive first test result only. The first test should be a test that reliably classifies samples with a negative test result as non-CDI; these samples will not be tested further. This first test should therefore be a test with a high NPV (i.e. a highly sensitive test). Thus, in our case, this first test can either be a GDH EIA or NAAT. The choice between these two categories of assays can be made by each individual laboratory. The second test should be a test with a high PPV (i.e. a highly specific test), so that all samples with a positive second test result can reliably be classified as CDI. Toxin

A/B EIAs can very well be used for this purpose, because besides being the most specific tests, these tests also have the advantage of detecting free toxin. Thus, after application of a first sensitive test (GDH EIA or NAAT), the toxin A/B EIA can then be performed as a second step on all samples that tested positive by NAAT or GDH EIA (Fig. 3(a)). Samples with a positive second test result can be classified as CDI likely to be present. However, samples with a first positive test result but a negative toxin A/B EIA need to be clinically evaluated. Among these samples, CDI (with toxin levels below the threshold of detection or a false-negative toxin A/B EIA result) or *C. difficile* carriage is possible. A recent large study tried to establish the optimum diagnostic algorithm for CDI. (47) In this study, 12 420 faecal samples were tested by diverse commercial assays, TC and CCNA. The overall performance of combined tests was superior to individual tests. The combination of a NAAT (Xpert) and toxin A/B EIA (Techlab Tox A/B II) was the optimal algorithm compared to the CCNA test, but the GDH EIA (C. diff Chek-60)-toxin A/B EIA algorithm performed almost identically. (47) These findings can be seen as a validation of our more theoretical approach to establish the best testing strategy, and they endorse the conclusion that NAAT-toxin A/B EIA, or alternatively GDH EIA-toxin A/B EIA, are two of the best algorithms to diagnose CDI (Fig. 3(a)).

An alternative algorithm is to test simultaneously with both a GDH and toxin A/B EIA. An assay is available that includes both these targets in one system (*C. diff* Quik Chek Complete; Techlab), but the sensitivity of the toxin component is unclear and may not be as a high as some individual toxin EIAs (Tables 5-7). Samples that test negative for both GDH and toxin A/B can reliably be classified as non-CDI, while samples that test positive for both GDH and toxin A/B can be classified as CDI likely to be present. Samples with a GDH-positive result but that are negative for toxin could undergo reflex testing by NAAT to determine if a toxigenic *C. difficile* strain is present (Fig. 3(b)). Samples with a negative GDH result but that are positive for toxin need to be retested, as this is an invalid result. Only one study evaluating this kind of algorithm and comparing it to a reference test was identified in the literature. (45) In this specific study, samples were screened by *C. diff* Quik Chek Complete, and inconclusive results underwent reflex testing by Illumigene. The overall sensitivity for this algorithm compared to CCNA was 81%, while specificity was reported to be 100%. The overall sensitivity and specificity of this and the aforementioned algorithm depend, however, on the individual assays that are included.

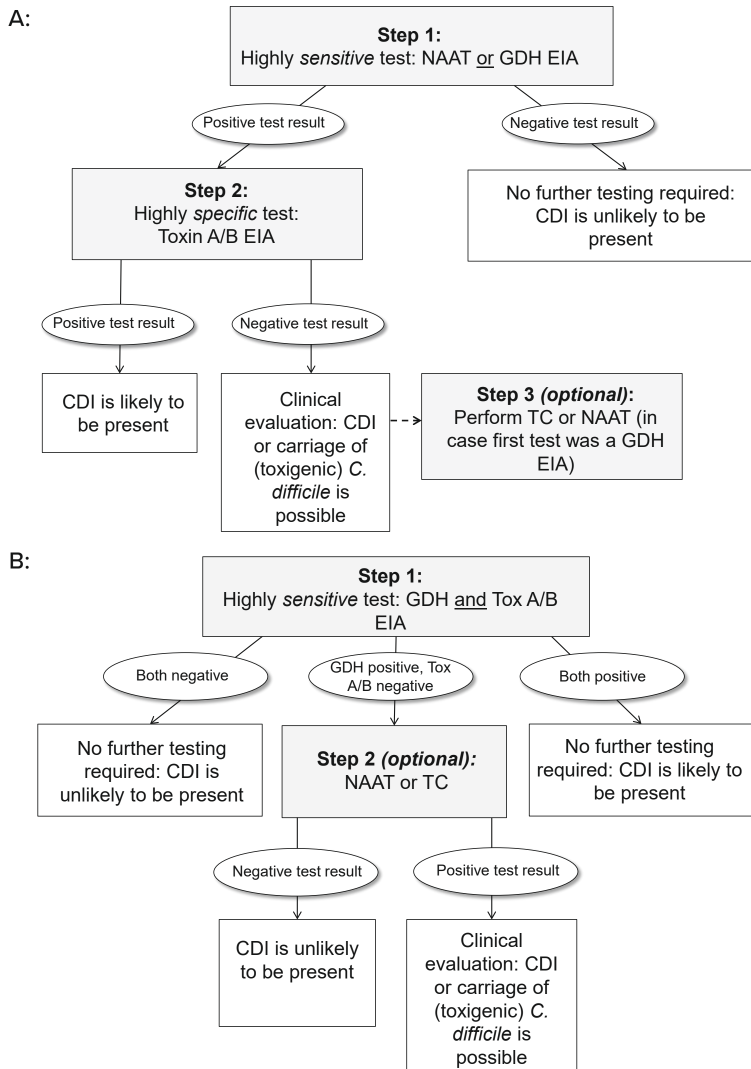

Figure. 3. Recommended algorithms for CDI testing. (a) GDH or NAAT–Tox A/B algorithm. (b) GDH and Tox A/B–NAAT/TC algorithm.
CDI, *Clostridium difficile* infection; GDH, glutamate dehydrogenase; NAAT, nucleic acid amplification test; TC, toxigenic culture; Tox A/B, toxin A/B; EIA, enzyme immunoassay.

Although we recommend the use of an algorithm for CDI testing based on two rapid assays, every laboratory should also be able to isolate *C. difficile*, ideally via TC from selected samples, for two reasons. First, TC offers the ability to perform molecular typing and susceptibility testing on recovered isolates from positive samples and can be used for outbreak investigations. (75) Second, samples with a positive GDH EIA and/or NAAT but a

negative toxin A/B EIA may either be samples that tested falsely positive on GDH EIA/NAAT or samples containing *C. difficile*, but without detectable free toxin. To be able to discern between these two conditions, a third-stage reflex test to either a TC or NAAT or GDH (if not yet performed) can be performed on samples with discordant results. For patients with evidence of *C. difficile* but negative toxin A/B EIA, clinical evaluation is needed, and clinical considerations come into play to determine a case as either positive or negative; these patients can either be CDI patients with undetectable toxin levels, or false- negative toxin A/B EIA results or potential carriers of toxigenic *C. difficile*. Although *C. difficile* carriers may play an important role in the spread of the disease (76, 77), the indication for treating these patients for CDI remains controversial. In addition, the need for isolation precautions for these patients remains to be clarified. Therefore, performing TCs on these samples can be of importance for epidemiologic purposes, but it is not yet a prerequisite for patient management.

The decision to treat CDI is ultimately a clinical decision, guided by laboratory results. No tests are infallible, so it may be clinically justified to treat a patient for CDI despite negative test results; treatment should not be withheld on the basis of laboratory tests alone. However, patients with toxin-negative specimens should have alternative diagnoses considered and excluded; provided an adequate testing strategy is followed, most patients with negative results for CDI will truly not have this infection, and thus treatment will be unnecessary.

Besides the question which assay or algorithm should be used for CDI detection, another issue is the number of specimens per patient that should be submitted for testing. Before the introduction of algorithms to diagnose CDI, lack of confidence in the tests for CDI detection (mainly toxin EIAs) led to the practice of multiple sample submission. However, the diagnostic gain of repeat testing within a 7-day period with both toxin A/B EIA and PCR was demonstrated to be very low. (78) If one of the above proposed algorithms is used, then the adequate NPV at low disease prevalence is based on original studies which did not test samples repeatedly by index test and only once by reference test. This adequate NPV indicates that routine submission of multiple samples after a first negative test round has to be discouraged; these samples can reliably be classified as non-CDI.

However, in cases of ongoing clinical suspicion during an endemic situation, the submission of a repeat sample may be justified, as these specific algorithms will have adequate PPVs even in a low-prevalence situation.

In outbreak situations with a higher CDI prevalence in the tested population, the NPV of the algorithm will fall. In such an outbreak situation, submitting a repeat sample in case of ongoing clinical suspicion will be of value, as has been shown for toxin A/B EIA . (79) Testing for cure is not recommended, as patients can shed spores and even toxins of *C. difficile* for a prolonged time after resolution of diarrhoea . (80, 81) The infection can be considered resolved when symptoms of diarrhoea have resolved.

Selection of which of submitted stool samples should be tested for CDI is also important. Recognition of potential CDI cases may be burdensome, as it is increasingly being recognized that CDI is not only acquired in healthcare facilities by patients with well-known risk factors for the disease. In the Netherlands, *C. difficile* was relatively frequent among patients with diarrhoeal complaints in general practice. (82) Community-onset CDI can affect all age groups, and many patients do not have known risk factors. (83, 84) A recent study showed that on a single day in Spain, two of every three CDI episodes were underdiagnosed or misdiagnosed owing to nonsensitive tests (19.%) but more importantly to lack of suspicion and request (47.6%). (85) Especially for nonhospitalized patients and younger patients, CDI tests were not requested. (85)This trend was also seen in a study involving almost 500 hospitals in 20 countries across Europe: on two sampling days, 23% of samples with a positive CDI test result were initially missed due to lack of suspicion. (73) Hence, restricting testing to samples with a physician's request for CDI testing will lead to underdiagnosis.

Empirical testing of all unformed stool samples submitted to the laboratory was shown to increase the diagnostic yield. (73, 86) We recommend testing all unformed faecal samples submitted to the laboratory (except samples from children under age 3). In infants, high rates of asymptomatic colonization with both toxigenic and nontoxigenic strains have been described. (87) Even in the case of toxin production, infants rarely develop clinical disease. However, CDI can occur in infants and young children. (88) A recently released policy statement from the American Academy of Pediatrics recommends to test for CDI only if age-specific clinical criteria are met. (14) According to their statement, searching for alternative aetiologies should be performed even in the case of a positive CDI test for children under 3 years of age. Concerning the problematic interpretation of positive test results in this population, we indeed recommend to limit testing of samples from children under age 3 to samples with a physician's request only. Unformed stool samples of children 3 years and older can be managed in the same way as described above.

Clinical signs and symptoms are essential to CDI diagnosis. Therefore, formed stool samples should not be tested for CDI, as these do not meet the clinical criteria of CDI. However, sometimes only solid parts of diarrhoeal faeces may be collected and submitted for *C. difficile* testing. Local protocols therefore need to enable *C. difficile* testing on specific samples to take place. Also, an exception has to be made for patients suspected of CDI who have ileus. In these patients, a rectal swab can be used with adequate sensitivity and specificity for (toxigenic) culture, NAAT or GDH EIA. (89, 90) The use of perirectal swabs for NAAT or GDH EIA testing might also be an alternative in selected patient populations but may depend on the presence of faecal staining of the swab. (89-91) However, the use of (peri)rectal swabs has not been evaluated for toxin EIA, and therefore clinical judgement remains essential in these cases to discern colonized patients from patients with CDI.

Recommendations

Sample selection

- We recommend that CDI testing should not be limited to samples with a specific physician's request. (Strong recommendation, high-quality evidence)
- We suggest that at least all submitted unformed stool samples from patients 3 years or older should be tested for CDI. (Weak recommendation, low-quality evidence)
- We suggest to limit testing of samples from children under age 3 to samples with a physician's request only. (Weak recommendation, low-quality evidence)
- Formed stool samples should not be tested for CDI (except in case of paralytic ileus). (Good practice statement)
- In patients suspected of ileus, a rectal swab can be used for (toxigenic) culture, NAAT or GDH EIA. (Strong recommendation, moderate-quality evidence)

Testing protocol

- The diagnosis of CDI should be based on clinical signs and symptoms in combination with laboratory tests. Decision for treatment for CDI is a clinical decision and may be justified even if all laboratory tests are negative. (Good practice statement)
- We recommend against the use of a single rapid test as a stand- alone test due to inadequate PPV in an endemic situation. (Strong recommendation, moderate-quality evidence)

- We recommend the use of a 2-step algorithm (Fig. 3(A)). (Strong recommendation, moderate-quality evidence)
- This algorithm should start with either NAAT or GDH EIA. Samples with a negative first test result can be reported as negative. (Strong recommendation, moderate-quality evidence)
- Samples with a positive first test result should be tested further with a toxin A/B EIA. Samples with a positive second test results can be reported as CDI-positive. (Strong recommendation, moderate-quality evidence)
- An alternative algorithm is to screen samples with both a GDH and toxin A/B EIA (Fig. 3(B)). Samples with concordant positive or negative results can be reported as such. Samples with a negative GDH result but positive for toxin need to be retested as this is an invalid result. (Strong recommendation, moderate- quality evidence)
- Samples with a positive first test result and negative second test result (Fig. 3(A)) and samples with a GDH-positive test result but negative toxin A/B test result (Fig. 3(B)) may represent samples with CDI or C. difficile carriage and may optionally be tested with TC or NAAT (if not performed yet). (Weak recommendation, moderate-quality evidence)
- We recommend to perform TC and molecular typing of recovered isolates in case of outbreak situations. (Good practice statement)

Repeated testing

- Repeated testing after a first positive sample during the same diarrhoeal episode is not recommended in an endemic situation. (Strong recommendation, moderate-quality evidence)
- Repeated testing after a first negative sample during the same diarrhoeal episode may be useful in selected cases with ongoing clinical suspicion during an epidemic situation or in cases with high clinical suspicion during endemic situations. (Strong recommendation, moderate-quality evidence)
- A test of cure is not recommended. (Good practice statement)

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Transparency Declaration

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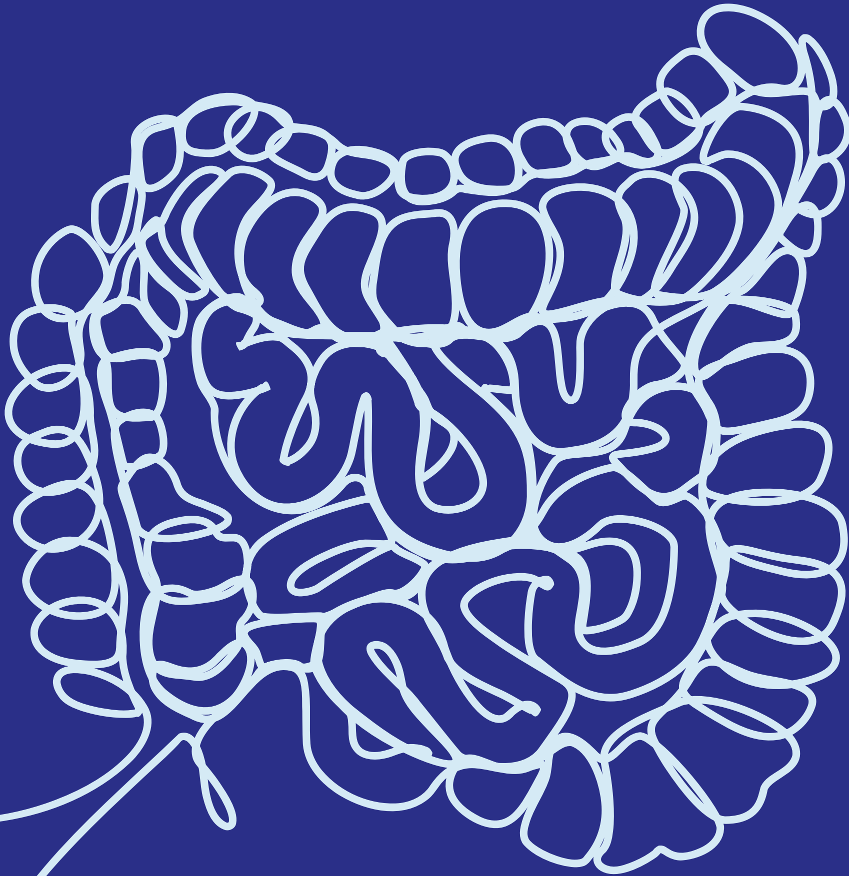
CHAPTER 4

Nucleic acid amplification test quantitation as predictor of toxin presence in *Clostridium difficile* infection



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Abstract

Multi-step algorithmic testing in which a sensitive nucleic acid amplification test (NAAT) is followed by a specific toxin A and B Enzyme Immunoassay (Tox A/B EIA) is among the most accurate methods for *Clostridium difficile* infection (CDI) diagnosis. The obvious shortcoming of this approach is that multiple tests must be performed to establish a CDI diagnosis, which may delay treatment. As such, we sought to determine whether a preliminary diagnosis could be made on the basis of the quantitative result of the first test in algorithmic testing, which provides a measure of organism burden. To do so, we retrospectively analyzed two large collections of samples (n=2669 and n=1718, respectively) that were submitted to the laboratories of two Dutch hospitals for CDI testing. Both hospitals apply a two-step testing algorithm in which a NAAT is followed by a Tox A/B EIA. From all samples, 208 and 113 samples tested positive by NAAT, respectively. Within these NAAT-positive samples, significantly lower mean *Cq* values were found in patients whose stool eventually tested positive for toxin than in patients who tested negative for toxin (24.4 vs 30.4 and 26.8 vs 32.2, $p<0.001$ for both cohorts). Receiver operating characteristics (ROC) curve analysis was performed to investigate the ability of *Cq* values to predict toxin status and yielded areas under the curve (AUCs) of 0.826 and 0.854. Using the optimal *Cq* cut-off values, prediction of the eventual Tox A/B EIA results was accurate in 78.9% and 80.5% of samples, respectively. In conclusion, *Cq* values can serve as predictors of toxin status, but due to the suboptimal correlation between the two tests, additional toxin testing is still needed.

Introduction

Clostridium difficile (recently reclassified as *Clostridioides difficile* based on phenotypic, chemotaxonomic and phylogenetic analyses (1) – for simplicity and consistency with previous literature *C. difficile* will be used in this paper) is an anaerobic, spore-producing bacterium that is responsible for *C. difficile* infection (CDI), the leading cause of nosocomial infectious diarrhea (2). Symptoms range from mild self-limiting diarrhea to potentially life-threatening fulminant colitis (3, 4). CDI occurs when alterations in the gut microbiome, particularly antibiotic-induced disruptions, create conditions favorable for *C. difficile* proliferation (5). Proliferation is followed by production of one or two enterotoxins, known as toxins A and B (Tox A/B), and in some strains a binary toxin, *C. difficile* transferase (CDT), whose inflammatory and necrotic effects on colonic tissue mediate the clinical symptoms of CDI (2).

CDI diagnostic methods continue to present problematic shortcomings. Establishing a CDI diagnosis is dependent on demonstrating the presence of toxin or toxigenic organism in stool samples (6). The two reference methods for doing so, cell cytotoxicity neutralization assay (CCNA) and toxigenic culture (TC), are lengthy, laborious techniques whose clinical implementation is unrealistic. Therefore, rapid tests with the same aims in mind have been developed. Enzyme immunoassays (EIA) can be used to either detect toxin (Tox A/B EIA) or glutamate dehydrogenase (GDH EIA), an abundant enzyme whose presence is indicative of *C. difficile* (both toxigenic and non-toxigenic strains). Similarly, nucleic acid amplification tests (NAAT) can detect the presence of toxin-producing genes. While these rapid tests are easily carried out in a clinical setting, they too suffer from drawbacks. Tox A/B EIA use was once widespread, given the etiologic relationship between toxin and clinical symptoms, but recognition of its low sensitivity (6) has changed this paradigm. Increasingly, NAATs have gained popularity, given their ease of use and high sensitivity. However, there is considerable debate about whether the presence of toxigenic organism alone warrants a diagnosis of CDI or should instead be considered *C. difficile* colonization (7-10). This has prompted the creation of multi-step algorithms, where a first sensitive test, a NAAT or GDH EIA, is used to screen for the organism, which in the event of a positive result reflexes to a highly specific second test for toxin detection, the Tox A/B EIA (6).

The algorithmic approach is currently recommended by common guidelines, such as those published by the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) (6). And while algorithms do well to minimize false positive and negative results,

their obvious shortcoming is that in the event of a positive first test, a second test must be performed to establish a diagnosis, potentially delaying treatment and isolation of true CDI patients, or leading to premature treatment of non-CDI patients. In light of this shortcoming, we sought to determine whether the quantitative result of the first test, a Tox A/B or Tox B NAAT, in a two-step algorithm could predict the eventual outcome of the second test, a Tox A/B EIA.

Methods

Study design and population

This study was performed using CDI testing data from two Dutch hospitals, the Leiden University Medical Center (LUMC) (a tertiary care university-affiliated hospital) and Amphia Hospital (a large general hospital). In both hospitals CDI diagnoses are established using a two-step algorithm recommended by ESCMID, in which a NAAT for the toxin A producing gene (*tcdA*) and/or toxin B producing gene (*tcdB*) is, in the event of a positive result, followed by a Tox A/B EIA. All consecutive stool samples (both from inpatients and outpatients) that underwent CDI testing by this algorithm were considered. Samples from infants were only included if a specific request for CDI testing was made. For the LUMC samples were included from January 2016 – March 2017, and for Amphia Hospital samples were included from January 2016 – January 2017. Additionally, LUMC data from adult asymptomatic patients who, upon admission for non-CDI-related reasons agreed to have their stool tested for *C. difficile* and were found positive by culture, were included as a control. In the LUMC only, culture and ribotyping was performed on NAAT positive samples.

Diagnostic tests

Both hospitals use an in-house NAAT targeting *tcdB* only (LUMC) or both *tcdB* and *tcdA* (Amphia). For both sites, we used the *tcdB Cq* value for all calculations. LUMC's NAAT was performed as previously described (11). For the in-house NAAT in the Amphia hospital, DNA extraction was performed using the Nuclisens EasyMag system (bioMerieux, Marcy-l'Etoile, France). This in-house assay has been been validated internally and complies with the quality criteria described in the requirements of the International Organization for Standardization (ISO 15189:2012). In short, feces of approximately the size of a pinhead was suspended in 1ml of stool transport and recovery (STAR) buffer (Roche Diagnostics, Almere, The Netherlands) and frozen before further processing. After thawing, samples

were first homogenized in the Magnalyser (Roche Diagnostics, Almere, The Netherlands) (30 seconds, speed 6000xg) and thereafter centrifuged (1 minute, speed 14.000xg). A total of 100 microliters supernatant was used for automated nucleic acid extraction using the EasyMag system. Amplification of the *tcdA* and *tcdB* genes was performed on an ABI Taqman 7500 real time PCR system (Applied Biosystems, Nieuwerkerk a/d Ijssel, The Netherlands). Primers and probes that were used for the Amphia NAAT have been described before (12). Taqman Universal PCR Master Mix (Applied biosystems, Nieuwekerk a/d IJssel, the Netherlands) and PCR plates were prepared using the PIRO pipetting robot (Dornier, Lindau, Germany) and contained 20microliters of mastermix and 5 microliters of extracted DNA. The amplification protocol included 5 min at 50C, 10 min at 95C followed by 45 cycles of amplification; and 95C for 10s, 60C for 32 seconds. Both laboratories used phocine herpes virus as internal control to test for PCR inhibition. For both hospitals, NAAT results were quantitated by measuring quantification cycle (*Cq*); the cycle value at which fluorescence from amplification exceeds the background fluorescence, serving as an indirect measure of how many copies of DNA were present in the sample tested. At the LUMC a VIDAS® *C. difficile* Toxin A & B (bioMerieux, Marcy-l'Etoile, France) was performed; values greater than 0.37 were considered positive according to the manufacturer's instructions. Amphia used an Immuno*Card* Toxins A&B (Meridian Bioscience, Cincinnati, OH, USA); results were not quantitative, instead presented as positive/negative. Both assays were performed according to manufacturer's instructions.

On working days, NAATs were performed on day of receipt. During weekend days and holidays, NAAT was performed on the following working day. In case of a positive NAAT, Tox A/B EIA was performed on the same or following day. Samples were stored at 4°C until tested. Culture and ribotyping of NAAT positive samples from the LUMC cohort was performed as previously described (13).

Statistical analysis

Average *Cq* values were compared by t-test and ANOVA. The ability of *Cq* values to predict toxin presence was assessed by receiver operating characteristic (ROC) curves. Positive predictive values and negative predictive values were calculated for different *Cq* cut-off points. Results were considered significant below the 0.05 level. Analyses of data were performed using SPSS version 23.0 statistical software (IBM, Armonk, NY, USA) and STATA SE version 12.1 statistical software (StataCorp, Texas, USA).

Results

LUMC

In total, 2669 unformed stool samples from patients suspected of CDI were tested by an in-house NAAT. Of these, 2424 had a negative result and 17 showed inhibition on NAAT and were excluded from further analysis. Of the remaining 228 NAAT positive samples 20 were excluded from further analysis as the Tox A/B EIA was not performed (either because there was insufficient feces to perform the assay or because the assay was stopped for any other reason). The remaining samples underwent testing by Tox A/B EIA, yielding a final sample size of 208 (Figure 1a). Quantification cycle (*Cq*) values in patients with positive (n=78) and negative (n=130) Tox A/B EIA results and in asymptomatic individuals who were found to be asymptomatically colonized by *C. difficile* upon hospital admission via culture are shown in Figure 2a. Comparable mean *Cq* values were observed in symptomatic patients negative for toxin (30.4, 95% CI 29.5-31.3) and asymptomatic carriers (29.2, 95% CI 27.3-31.2), while symptomatic patients with a positive toxin result had significantly lower mean *Cq* values per ANOVA (24.4, 95% CI 23.5-25.3, $p<0.001$). Seventeen outliers that were positive by Tox A/B EIA with high *Cq* values were retested by *tcdB* NAAT. The mean *Cq* value in these samples did not decrease after retesting. Samples were evaluated for PCR inhibition or irregular amplification curves, but neither was found to be a cause for these anomalies. Clinical data showed that only one of these samples was submitted during metronidazole treatment for CDI, 14 samples were submitted while no CDI antibiotics were used and for 2 samples antibiotic use was not clear. All but one of these 17 samples were positive in culture, yielding 11 different ribotypes. The only culture negative sample was from a patient with a clinical suspicion of a CDI recurrence 4 months after a previous episode. After the positive CDI test result, the patient was treated with oral metronidazole.

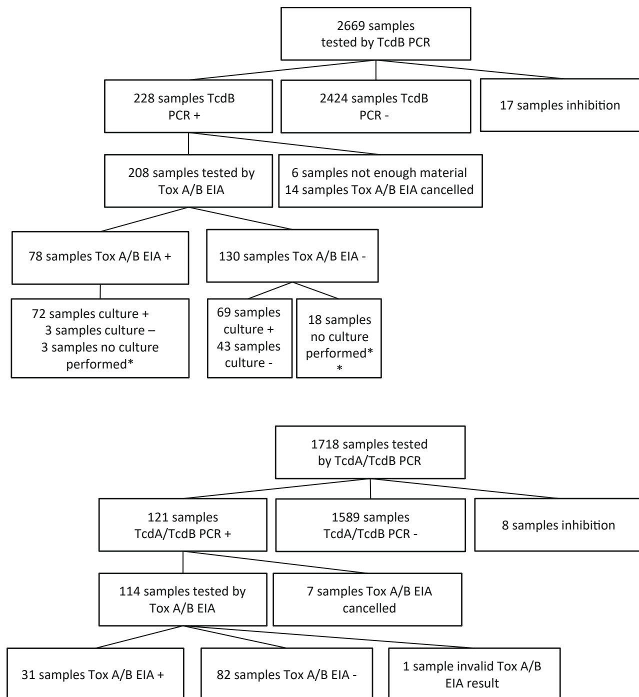

Figure 1. Flowchart of included samples. 1a. LUMC cohort. 1b. Amphia cohort.
* no culture performed because a culture with positive result was performed within the previous week.
** no culture performed because a culture with positive result was performed within the previous week (n=1) or because the positive *tcdB* result was obtained retrospectively during implementation phase of the *tcdB* NAAT, when samples were routinely tested by Tox A/B EIA only.

Based on the significantly lower *Cq* values observed in toxin positive samples, a receiver operating characteristic (ROC) curve was generated to calculate *Cq* values' ability to predict Tox A/B EIA outcome (Fig 3a). The area under the curve (AUC) was found to be 0.826 ($p<0.001$), with an ideal cut-off value of 25.3 cycles (the value best able to discriminate between outcomes – 78.9% of samples would be correctly classified as Tox A/B EIA positive or negative using this *Cq* cut-off value). Measures of accuracy of the ideal cut-off value and others are shown in Table 1.

As LUMC data included PCR ribotypes, we investigated whether ribotype had an effect on our findings. Ribotype distribution was comparable between Tox A/B EIA positive and negative patients by chi-squared test (p=0.26) and we did not find any differences in the mean *Cq* values between different ribotype categories (p=0.55 for toxin negative samples and p=0.11 for toxin positive samples, respectively) (Table 2).

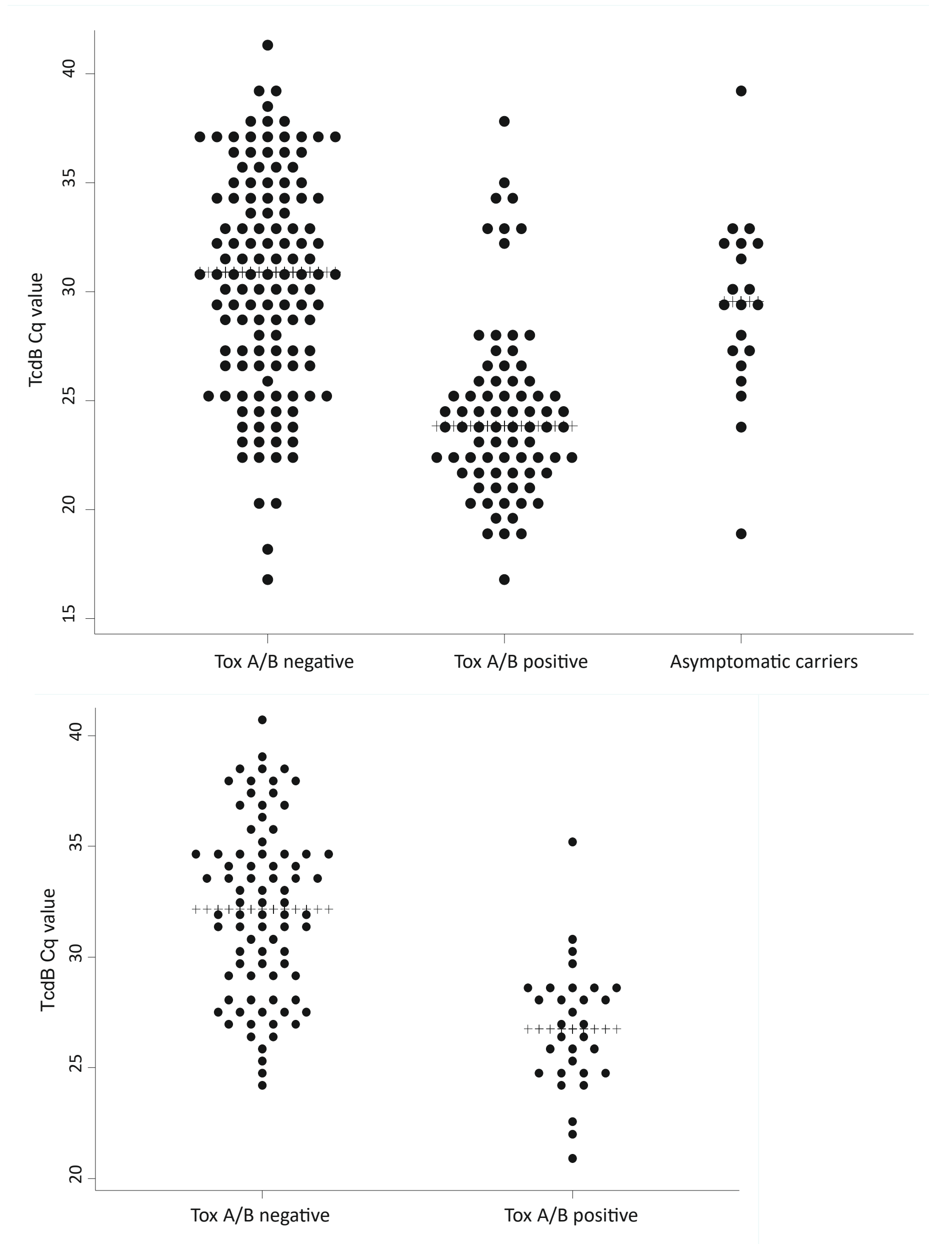

Figure 2. Dotplots of observed *Cq* values. 2a. LUMC cohort and asymptomatic carriers. 2b. Amphia cohort.

Table 1. Accuracy of the ability of NAAT *Cq* cutoff values to predict Tox A/B EIA outcomes.

Cq cut-off value	sensitivity (%) (95% CI)	specificity (%) (95% CI)	accuracy (%) (95% CI)	PPV (%) (95% CI)	NPV (%) (95% CI)
LUMC cohort					
22.0	24.36 (15.35-35.40)	96.92 (92.31-99.16)	69.71 (62.98-75.87)	82.61 (62.65-93.08)	68.11 (65.23-70.86)
23.0	41.03 (30.01-52.75)	93.85 (88.23-97.31)	74.04 (67.52-79.86)	80.0 (66.02-89.17)	72.62 (68.68-76.24)
24.0	51.28 (39.69-62.77)	88.46 (81.68-93.4)	74.52 (68.03-80.29)	72.73 (61.25-81.81)	75.16 (70.50-79.30)
25.3	71.79 (60.47-81.41)	83.08 (75.51-89.08)	78.85 (72.66-84.19)	71.79 (62.92-79.25)	83.08 (77.36-87.58)
26.0	75.64 (64.6-84.65)	77.69 (69.56-84.52)	76.92 (70.59-82.47)	67.05 (59.04-74.17)	84.17 (78.06-88.82)
27.0	82.05 (71.72-89.83)	73.08 (64.60-80.48)	76.44 (70.08-82.03)	64.65 (57.49-71.20)	87.16 (80.67-91.69)
28.0	87.18 (77.68-93.68)	68.46 (59.73-76.33)	75.48 (69.05-81.17)	62.39 (55.94-68.42)	89.90 (83.14-94.14)
29.0	89.74 (80.79-95.47)	64.62 (55.75-72.80)	74.04 (67.52-79.86)	60.34 (54.38-66.02)	91.30 (84.33-95.35)
Amphia Hospital cohort					
24.0	9.68 (2.04-25.75)	100 (95.60-100)	75.22 (66.22-82.86)	100	74.55 (72.3-76.67)
25.0	29.03 (14.22-48.04)	97.56 (91.47-99.7)	78.76 (70.07-85.89	81.82 (50.72-95.16)	78.43 (74.33-82.04)
26.0	38.71 (21.85-57.81)	95.12 (87.98-98.66)	79.65 (71.04-86.64)	75.0 (51.13-89.59)	80.41 (75.55-84.51)
27.0	51.61 (33.06-69.85)	91.46 (83.2-96.5)	80.53 (72.02-87.38)	69.57 (51.01-83.38)	83.33 (77.55-87.86)
28.0	64.52 (45.37-80.77)	78.05 (67.54-86.44)	74.34 (65.26-82.09)	52.63 (40.63-64.33)	85.33 (78.12-90.46)
29.0	87.1 (70.17-96.37)	74.39 (63.56-83.4)	77.88 (69.10-85.14)	56.25 (46.46-65.57)	93.85 (85.83-97.46)
30.0	90.32 (74.25-97.96)	68.29 (57.08-78.13)	74.34 (65.26-82.09)	51.85 (43.44-60.16)	94.92 (86.31-98.22)
31.0	96.77 (83.30-99.92)	64.63 (53.30-74.88)	73.45 (64.32-81.32)	50.85 (43.40-58.26)	98.15 (88.45-99.73)
32.0	96.77 (83.30-99.92)	56.10 (44.7-67.04)	67.26 (57.79-75.79)	45.45 (39.29-51.77)	97.87 (86.89-99.69)

Values were calculated as follows: sensitivity= samples with a *Cq* value beneath the cut-off/all toxin positive samples; specificity= samples with a *Cq* value above the cut-off/all toxin negative samples; accuracy= all correctly classified specimens; positive predictive value = chance of positive Tox A/B EIA result among samples with a *Cq* value beneath the cut-off; negative predictive value= chance of negative Tox A/B EIA result among samples with a *Cq* value above the cut-off

Amphia Hospital

A total of 1,718 unformed stool samples suspected of CDI were tested by an in-house NAAT (different from the LUMC NAAT). Of these, 1,589 had a negative result and 8 showed inhibition and were excluded from further analysis. Seven of the 121 NAAT positive samples were not tested by Tox A/B EIA (2 were repeat samples from the same patient on the same day, 1 sample was a gut biopsy and 4 other samples were not tested otherwise). The remaining 114 samples underwent Tox A/B EIA testing. One sample had an invalid result on the second test (no detectable color in the reaction port) and was also excluded from further analysis, yielding a final sample size of n=113 (Figure 1b). *Cq* values in toxin positive (n=31) and negative (n=82) samples are shown in Figure 2b. Significantl lower mean *Cq* values were found in toxin positive patients compared to toxin negative patients (26.8, 95% CI 25.8-27.9 vs 32.2, 95% CI 31.3-33.0, p<0.001). Evaluation of the one outlier positive by Tox A/B EIA with a high *Cq* value revealed a normal shape of the amplification curve but no diarrhea anymore at time of results (without treatment).

Like the other cohort, an ROC curve was generated for determining *Cq* values' ability to predict the outcome of Tox A/B EIA testing (Fig 3b). AUC was 0.854 (p<0.001), with an ideal cut-off value of 27.0 (80.5% of samples correctly classified). Measures of accuracy of the ideal cut-off value and others are shown in Table 1

Discussion

This study sought to determine whether quantitation of NAAT results could predict the presence or absence of toxin in subsequent testing. Significantly lower *Cq* values were found in stool samples that tested positive for toxin in two large cohorts from different hospitals. Concomitant ROC curves in both cohorts showed that, using the optimal *Cq* cut-off value, the toxin result could be predicted in at least 78% of the samples. With the recent emergence of NAATs as stand-alone tests or as first step in an algorithm, there has been increasing interest in the use or non-use of quantitation of NAAT results. There is a growing body of work showing an association between *Cq* values and toxin presence: toxin-positive samples are associated with lower *Cq* values or higher bacterial load (14-21). Toxin presence is generally thought to be associated with CDI severity and outcome (7, 8). Some studies indeed found *Cq* values to be predictors of clinical severity or outcome, probably mediated through the presence or absence of toxins (21-23), although this was not confirmed in all studies (24, 25). A very recent paper was the first to describe the

Table 2. Ribotype distribution and mean *Cq* values for toxin positive and toxin negative samples (LUMC cohort).

Ribotype	Mean Ct value for Tox A/B EIA – samples(n)	for Tox A/B EIA + samples (n)
001	35.55 (2)	26.38 (4)
002	32.23 (4)	21.15 (2)
003		22.35 (2)
005	30.93 (3)	22.83 (3)
012	26.6 (2)	22.35 (2)
013		22.75 (2)
014/020	28.46 (14)	22.56 (15)
015		22.96 (5)
017	24.7 (1)	
019		32.0 (1)
023		30.55 (2)
026		22.2 (1)
031	23.9 (1)	
034	30.3 (1)	
037	32.65 (2)	
050	34.4 (1)	21.8 (1)
053	26.3 (1)	
057	33.4 (1)	27.3 (1)
070	30.9 (1)	21.9 (2)
076		27.7 (1)
078/126	28.3 (15)	25.79 (15)
081	24.2 (2)	
104		32.7 (1)
123	30.9 (1)	
127		24.1 (1)
154		23.9 (1)
156		18.6 (1)
168	30.2 (1)	
198		23.6 (1)
258		22.8 (1)
262	25.3 (1)	
265	31.0 (3)	29.55 (2)
293	23.95 (2)	
328	27.78 (4)	
356	23.4 (1)	
unknown	26.92 (5)	25.06 (5)

performance characteristics of NAAT *Cq* cut-offs for discriminating toxin-positive and toxin-negative stool samples (26). Our study adds to the literature by confirming that *Cq* values can indeed be used to predict toxin status. In our cohorts, the optimal *Cq* cutoff detected toxin-positive samples with a positive and negative predictive value of 71.8% and 83.1% and 69.6% and 83.3%, respectively. Our study also indicates that local assessment of NAAT performance is warranted to determine a cut-off value that can be used for clinical use, as *Cq* values are semi-quantitative, and depend on many factors concerning sample material, used materials and assay.

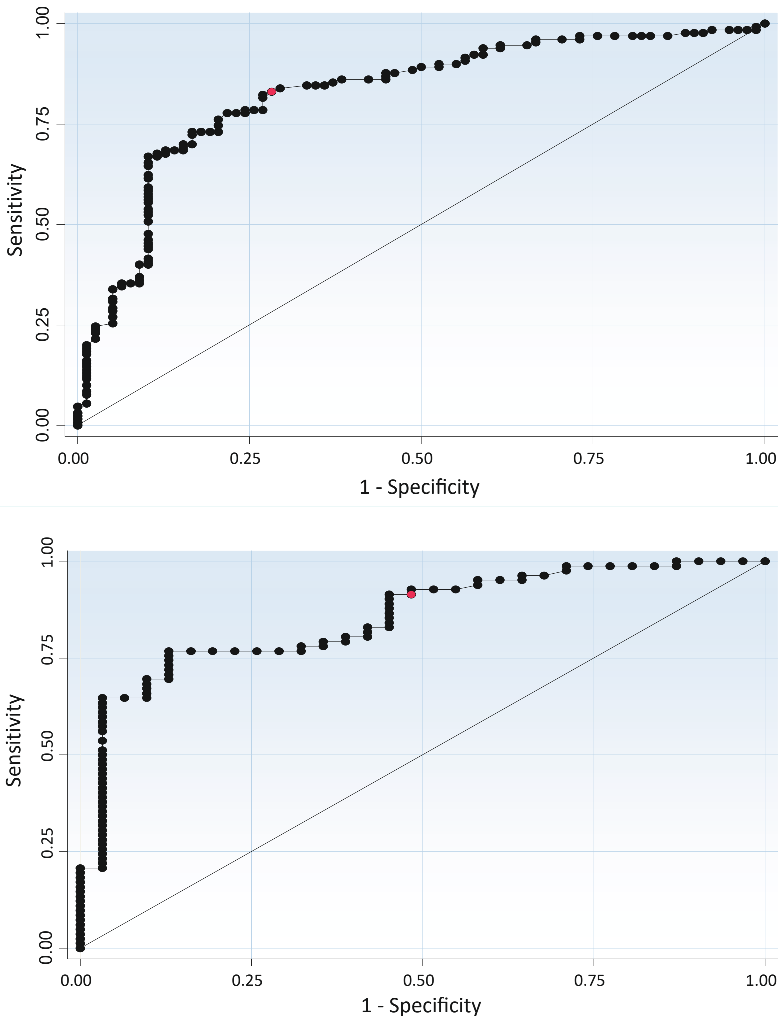

Figure 3. ROC curves assessing the ability of *Cq* values to predict presence of toxin. Optimal cut-off point shown in red. (Top) LUMC cohort. (Bottom) Amphia Hospital cohort.

Clinical implementation of these findings may be beneficial. Algorithmic testing requires more time to establish a CDI diagnosis than standalone tests, which has been shown to negatively impact patient care (27). One way of addressing this problem would be to use *Cq* values to establish a preliminary diagnosis. This can be either done by using the optimal *Cq* cut-off to consider samples likely toxin positive or negative. Using this approach, as many samples as possible will be classified correctly. One might however also argue, that toxin positive samples should not be missed, as delayed treatment or delayed isolation measures may negatively impact patient care and *C. difficile* transmission. In that case, a *Cq* cut-off with a high negative predictive value should be chosen to classify samples with *Cq* values above this cut-off as probable toxin negative. As an example, *Cq* cut off values of 29.0 and 32.0 for the LUMC and Amphia cohort, would correctly classify 91.3% and 97.9% of samples with a *Cq* value above this cut-off as negative, respectively. A preliminary diagnosis based on one of these two approaches might for instance be used when a clinician considers CDI treatment of a patient before results of toxin testing are available. However, we do recognize that the correlation between *Cq* values and toxin positivity and the positive and negative predictive values of the diverse cut-off values are far from perfect. We therefore think that *Cq* values may be helpful in doubtful cases, but NAAT quantitation should not be seen as a surrogate for free toxin testing or clinical judgment. It would be interesting to investigate if the incorporation of *Cq* values in an algorithm improves patient outcomes, compared to testing where a diagnosis, and consequent treatment, is exclusively dependent on demonstrating presence of toxin.

Our study had some limitations. First, we used an EIA to detect toxin, tests that are known to suffer from low sensitivities. Automated Tox A/B EIA such as the VIDAS® *C. difficile* Toxin A & B used by LUMC have reported sensitivities ranging from 53 to 98% – 0.98 compared to CCNA; membrane-type Tox A/B EIA such as the Immuno*Card* Toxins A&B have sensitivities ranging from 85 to 96% compared to CCNA (6). It is possible that some of the outliers we observed, with low *Cq* values but no toxin present, were actually false negatives in the Tox A/B EIA. Ideally CCNA, the gold standard of toxin detection, should have been used instead, but as these analyses were conducted retrospectively using clinical data where toxin testing is done by Tox A/B EIA, this was not possible. In the study by Senchyna and colleagues a membrane-type EIA detecting both GDH and Tox A/B, CCNA and a well-type Tox A/B EIA were combined to detect toxin positive samples (26). Using these combined tests as the reference standard, the optimal CT cutoff detected toxin-positive samples with a bit higher positive predictive value of 81.7% than in our cohorts, which may thus be explained by the more sensitive reference standard they used. A second limitation of our

study is that we analysed all samples that were tested for CDI and did not exclude samples from the same patient, samples from children, samples during the same diarrheal episode or samples submitted during or after treatment. The heterogeneity of the cohorts may therefore have obscured some associations, like higher *Cq* values for certain ribotypes, as was previously reported for ribotype 014 (20), or an aberrant association between *Cq* values and toxin positivity in children. However, the inclusion of all submitted samples led to a cohort that is representative for the actual situation. Information on repeat samples and CDI treatment is often lacking and the eventual ribotype (if CDI is confirmed) is not available yet at the moment the samples arrive at the laboratory. We therefore think that this study demonstrated the usefulness of NAAT quantitation in two unbiased cohorts which were highly representative for samples that are submitted for CDI testing, both in a universal and a general hospital.

Besides the representative cohorts that were used, there were some other strengths in our study. First of all, samples from the LUMC cohort underwent culture and PCR ribotyping and we were therefore able to evaluate any differences in *Cq* levels between different ribotype categories. Culture and ribotyping results were also used to evaluate the outliers. As 16/17 outlier samples had positive cultures and the one remaining sample had a clear clinical suspicion of CDI, false positive Tox A/B EIA results were considered less likely. A laboratory and clinical evaluation including retesting by *tcdB* NAAT was performed, but no clear explanation for the outliers with high *Cq* values but positive Tox A/B EIA was found. Another strength of our study is the unique comparison to a third group of asymptomatic carriers, which clearly demonstrated that *Cq* levels in asymptomatic carriers and symptomatic patients testing negative for toxins are comparable, suggesting that the latter group indeed represents CDI carriers with diarrhea not due to CDI.

In conclusion, we found *Cq* values to be predictors of toxin status in two large representative cohorts, although the suboptimal accuracy underscores the need of additional Tox A/B EIA testing. Additional studies are needed to determine if the inclusion of *Cq* values in algorithmic testing may aid clinicians in a faster but still accurate preliminary CDI diagnosis while awaiting the results of free toxin testing.

Acknowledgments

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Potential conflicts of interest

None to declare

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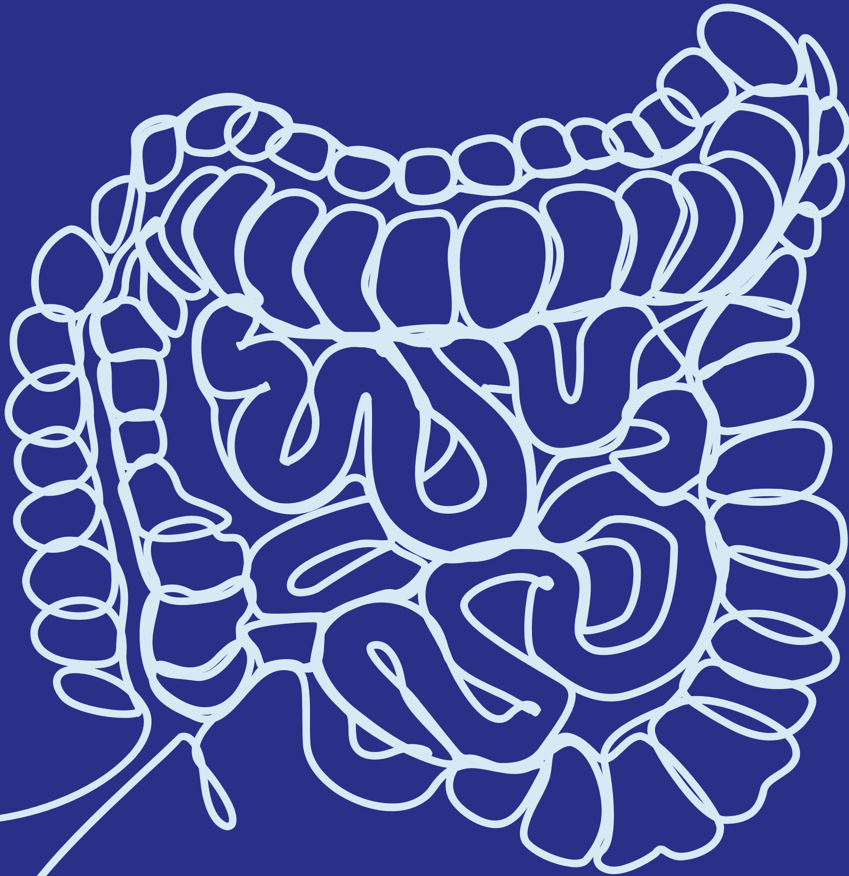
CHAPTER 5

The bacterial gut microbiota of adult patients infected, colonized or noncolonized by *Clostridioides difficile*



Microorganisms, 2020

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Abstract

Gut microbiota composition in patients with *Clostridioides difficile* colonization is not well investigated. We aimed to identify bacterial signatures associated with resistance and susceptibility to *C. difficile* colonization (CDC) and infection (CDI). Therefore, gut microbiota composition from patients with CDC (n = 41), with CDI (n = 41), and without CDC (controls, n = 43) was determined through 16S rRNA gene amplicon sequencing. Bacterial diversity was decreased in CDC and CDI patients ($p < 0.01$). Overall microbiota composition was significantly different between control, CDC, and CDI patients ($p = 0.001$). Relative abundance of *Clostridioides* (most likely *C. difficile*) increased stepwise from controls to CDC and CDI patients. In addition, differential abundance analysis revealed that CDI patients' gut microbiota was characterized by significantly higher relative abundance of *Bacteroides* and *Veillonella* than CDC patients and controls. Control patients had significantly higher *Eubacterium hallii* and *Fusicatenibacter* abundance than colonized patients. Network analysis indicated that *Fusicatenibacter* was negatively associated with *Clostridioides* in CDI patients, while *Veillonella* was positively associated with *Clostridioides* in CDC patients. Bacterial microbiota diversity decreased in both CDC and CDI patients, but harbored a distinct microbiota. *Eubacterium hallii* and *Fusicatenibacter* may indicate resistance against *C. difficile* colonization and subsequent infection, while *Veillonella* may indicate susceptibility to colonization and infection by *C. difficile*.

Introduction

Clostridioides difficile, formerly named *Clostridium difficile*, is an anaerobic, Gram-positive, spore-forming bacillus. It is the main causative agent of nosocomial diarrhea, with antibiotic use as its most important risk factor. Nowadays, community-associated diarrhea due to *C. difficile* is also increasingly reported. Clinical symptoms arise when *C. difficile* spores germinate within the intestine and the viable bacteria start to produce toxins. The secretion of Toxin A (TcdA) and Toxin B (TcdB) leads to inflammation of the large intestine. (1) The clinical presentation may range from mild diarrhea to a life-threatening toxic megacolon. (1) However, the ingestion of *C. difficile* spores does not always lead to the development of symptomatic disease. *C. difficile* can also be silently present in the gut, without causing any symptoms. This condition is called asymptomatic *C. difficile* colonization. (2) Patients colonized with *C. difficile* play an important role in disease epidemiology, as they act as a reservoir for onward transmissions (3, 4); they may also progress to infection themselves, especially in the presence of an underlying illness. (5-7)

It is believed that the bacterial gut microbiota plays an important role in determining the susceptibility to colonization and subsequent infection with *C. difficile*. In patients with *C. difficile* infection (CDI), a lower richness and diversity, and decreased relative abundances of Bacteroidetes, *Ruminococcaceae* and *Lachnospiraceae* members have been described. (8, 9) The gut microbiota in *C. difficile* colonized patients is less well characterized (8, 10), but may give more insight into mechanisms that allow for colonization whilst protecting against infection. A previous study identified specific gut metabolites associated with colonization and infection by *C. difficile*, but did not determine microbiota composition. (11) In order to identify which bacterial signatures are associated with resistance and susceptibility to *C. difficile* colonization and CDI, we compared the gut microbiota of CDI patients, patients with *C. difficile* colonization (CDC) at hospital admission, and patients without CDI or CDC at admission.

Materials and Methods

Subjects and sample collection

This study was designed to compare the gut microbiota between three groups: patients with *C. difficile* colonization (CDC) on hospital admission, patients without *C. difficile* colonization on hospital admission (controls), and hospitalized patients with CDI.

For the first two groups, fecal samples were obtained from CDC and control patients admitted to Leiden University Medical Center (LUMC) or Amphia hospital as part of the CDD ("*Clostridium difficile* dragerschap" (carriership)) study, a study designed to determine the prevalence of CDC at hospital admission, conducted between January 2015 and March 2016. Adult patients admitted to predefined medical and surgical wards were eligible for enrolment. Stool samples were requested within 72h of hospital admission. If patients were discharged home within 72h, a stool sample was collected at home and returned to the hospital by mail or in person. Colonized patients were defined as patients who tested positive for *C. difficile* by stool culture and were not clinically suspected of CDI within the first 72 h of admission. For each colonized patient, the first consecutive patient with a negative stool culture for *C. difficile* was included to form the control group. For the third group, fecal samples were obtained from adult patients hospitalized in the LUMC and diagnosed with CDI between July 2015 and May 2017. All CDI cases had to comply with the definitions valid in the Dutch surveillance protocol (12), and CDI diagnosis was based on CDI symptoms in combination with laboratory CDI testing in agreement with the recommendations of the European Centre for Disease Control and Prevention.(13) *C. difficile* culturing and molecular diagnostics were performed as described below in Section 2.2. Patients initially participating in the CDD study but diagnosed with CDI within 72h of admission were included in the CDI group.

The LUMC institutional review board served as the central institutional review board and had no objection to the performance of the study. At the Amphia hospital, the directing board had no objection to the performance of the study. Stool samples from CDC and control patients were collected under verbal consent, and written informed consent from these patients was obtained for collection of additional data (see below). A waiver for informed consent from CDI patients was obtained.

Microbiological analyses

Microbiological analyses were performed at the National Reference Laboratory for *Clostridium difficile* (LUMC, The Netherlands). Fecal samples were initially stored at 2–6 °C and tested on the day of receipt, or the following working day in case of weekends or holidays.

Fecal samples from CDC and control patients were cultured on CLO plates (containing cefoxitin, amphotericin B and cycloserin, BioMérieux, The Netherlands) and after ethanol

shock on CLO plates and CNA plates (containing colistin and nalidixinic acid, BioMérieux, The Netherlands). Suspicious colonies were tested by GDH PCR to confirm the presence of *C. difficile*. (14) In addition, a multiplex PCR for *tcdA*, *tcdB*, and binary toxin genes was performed on the isolates to determine if CDC patients were colonized by a toxigenic or nontoxigenic strain. (15)

Fecal samples from patients with suspected CDI were tested according to standard operating procedures, which included an assay to detect free *C. difficile* toxins. (16) In addition, positive tested samples were cultured for presence of *C. difficile* as described above.

C. difficile isolates from CDC patients and CDI patients were PCR ribotyped as previously described. (17)

Patient data collection

Demographical data and data about medication use during the last three months (until admission for CDC patients and controls or until sample submission for CDI patients), previous hospitalization in the last year, and previous CDI episodes (ever and within the last eight weeks) were collected by questionnaires and electronic chart review (CDC and control patients) or chart review only (CDI patients). Recurrent CDI was defined as a new diarrheal episode within two to eight weeks after a previous diarrheal episode due to *C. difficile* and *C. difficile* reinfection as a new diarrheal episode more than 8 weeks after the previous diarrheal episode due to *C. difficile*.

Epidemiological analyses were performed to compare characteristics between control, CDC, and CDI patients by one-way ANOVA or chi-squared test using STATA SE version 15.1 (StataCorp, College Station, TX, USA).

Microbiota analysis

Samples

A total of 125 fecal samples were included: 43 samples from control patients, 41 samples from CDC patients, and 41 samples from CDI patients. Samples from control and CDC patients were in 74/84 patients (88%) obtained within 72 h after admission. Fecal samples

were submitted from home by 15/84 patients (17.9%), while from the other 69/84 patients (82.1%) fecal samples were collected in the hospital.

DNA extraction, library preparation and sequencing

DNA was extracted from 0.1 g feces using the Quick-DNA™ Fecal/Soil Microbe Miniprep Kit (ZymoResearch, CA, USA). Quality control, library preparation, and sequencing were performed by GenomeScan B.V. (Leiden, The Netherlands) using the NEXTflex™ 16S V4 Amplicon-Seq Kit (BiooScientific, TX, USA) and the Illumina HiSeq4000 platform (paired-end, 150bp). An average of 2,117,322 (707,362–5,742,717) reads per sample was obtained. Raw sequencing data are available in the European Nucleotide Archive (http://www.ebi.ac.uk/ena) under study accession PRJEB30586.

Sequencing data analysis

Read filtering, operational taxonomic unit (OTU)-picking, and taxonomic assignment were performed using the NGTax 0.4 pipeline with following settings: forward read length of 150, reverse read length of 120, ratio OTU abundance of 2.0, classify ratio of 0.9, minimum threshold of 1×10^{-7}, identity level of 97%, and error correction of 98.5, using the Silva_132_SSU Ref database. (18-20) The obtained OTU-table was filtered for OTUs with a number of sequences less than 0.005% of the total number of sequences. (21) A couple of technical duplicates were included for DNA extraction (n = 3 samples) and sequencing (n = 6 samples) procedures, indicating high replicability of results (Figure S1). Three negative controls were included from DNA extraction onwards and contained less than 1% of the number of reads obtained from fecal samples.

All microbiota analyses and data visualization were performed in R (v3.5.1), using the packages phyloseq (v1.24.2), vegan (v2.5-2), ggplot2 (v3.0.0), DESeq2 (v1.20.0), microbiome (v1.2.1), and SpiecEasi (v0.1.4). (22-27) Visualization of network analysis was performed in Cytoscape (v3.7.0). (28) Results were considered significant if $p \leq 0.05$, or Benjamini-Hochberg corrected $p \leq 0.05$ for differential abundance analysis. Prior to differential abundance testing (DESeq2) and network analysis (SpiecEasi), the OTU-table was filtered for OTUs present in less than 25% of samples. Nucleotide sequences belonging to the *Clostridioides* genus were blasted using the NCBI standard nucleotide blast, with 16S ribosomal sequences (Bacteria and Archaea) selected as the reference database, to determine if the sequence had a better hit to *C. difficile* or *C. mangenotii*. Kruskal–Wallis followed by post-hoc Dunn's testing was performed to compare Shannon diversity indices between the patient groups. Permutational multivariate analysis of variance (PERMANOVA)

was performed using the “adonis” function with 999 permutations and Bray–Curtis distances to separately investigate associations between microbiota composition and various clinical variables. Each clinical variable was tested separately using PERMANOVA. SpiecEasi, using the Meinshausen–Buhlman method for graph estimation of the network, was performed for network analysis with lambda.min.ratio = 0.01, nlambda = 20 and rep. num = 99. This method is robust to many characteristics of 16S amplicon data, such as compositionality and dimensionality. (22) OTUs without a direct edge connection to another OTU were removed for visualization purposes.

Results

Epidemiology

Thirty CDI episodes were primary episodes, seven were recurrent episodes, and four were *C. difficile* reinfections. From four patients, two different episodes were included in this study. This mixture of primary episodes, recurrences, and reinfections reflects the true CDI population, as recurrence and reinfection are common. Previous CDI, both within and beyond the last eight weeks, was common among CDI patients (17.1% for both), whereas it was uncommon in CDC patients (2.4% and 7.3%), and no previous CDI was recorded in controls. Antibiotics were used in the last three months in 97.6% of CDI patients, 73.2% of CDC patients, and 59.5% of control patients ($p < 0.001$). The *C. difficile* PCR ribotype distribution in CDC and CDI patients is shown in Figure S2. Patient characteristics are shown in Table 1.

Bacterial community structure

To elucidate characteristics of the bacterial community structure, several tests were performed. We determined bacterial diversity using the Shannon index, performed PERMANOVA to relate microbiota composition to clinical factors, and clustered samples based on both weighted and unweighted UniFrac distance metrices for between-sample comparisons. Bacterial diversity was significantly higher in controls than in CDC and CDI patients ($p < 0.01$), but did not differ between CDC and CDI patients (Figure 1).

Table 1. Subject characteristics.

	CDI patients (n=41)	CDC patients (n=41)	Control patients (n=43)	p-value
Age in years, mean (SD)	57.5 (17.6)	55.3 (18.7)	57.8 (13.5)	0.76
Sex				0.47
Male	22/41 (53.7%)	22/41 (53.7%)	28/43 (65.1%)	
Female	19/41 (46.3%)	19/41 (46.3%)	15/43 (34.9%)	
Previous CDI				
Last 8 weeks	7/41 (17.1%)	1/41 (2.4%)	0/42 (0%)	0.003
>8 weeks earlier	7/41 (17.1%)	3/41 (7.3%)	0/42 (0%)	0.02
Current CDI episode				
primary episode	30/41 (73.2%)			
persistent primary episode	2/41 (4.9%)			
1st recurrence of primary episode	3/41 (7.3%)			
2nd recurrence of primary episode	1/41 (2.4%)			
5th recurrence of primary episode	1/41 (2.4%)			
1st reinfection	1/41 (2.4%)			
2nd reinfection	2/41 (4.9%)			
2nd recurrence of first reinfection	1/41 (2.4%)			
Previous hospitalisation (last year)	29/41 (70.7%)	30/41 (73.2%)	19/42 (45.2%)	0.01
Comorbidities				
IBD	2/41 (4.9%)	7/41 (17.1%)	2/42 (4.8%)	0.08
Solid organ transplant	17/41 (41.5%)	9/41 (22.0%)	2/42 (4.8%)	<0.001
Solid malignancy	5/41 (12.2%)	6/41 (14.6%)	11/42 (26.2%)	0.2
Haematological malignancy	9/41 (22.0%)	0/41 (0%)	2/42 (4.8%)	0.001
Previous medication use (last 3 months)				
Antibiotics	40/41 (97.6%)	30/41 (73.2%)	25/42 (59.5%)	<0.001
Immunosuppressants	30/41 (73.2%)	17/41 (41.5%)	13/42 (31.0%)	<0.001
Chemotherapy	10/41 (24.4%)	2/41 (4.9%)	5/42 (11.9%)	0.03
PPI or antacids	31/41 (75.6%)	30/41 (73.2%)	19/42 (45.2%)	0.006

CDI, *Clostridioides difficile* infection; IBD, inflammatory bowel disease; PPI, proton pump inhibitor; SD, standard deviation

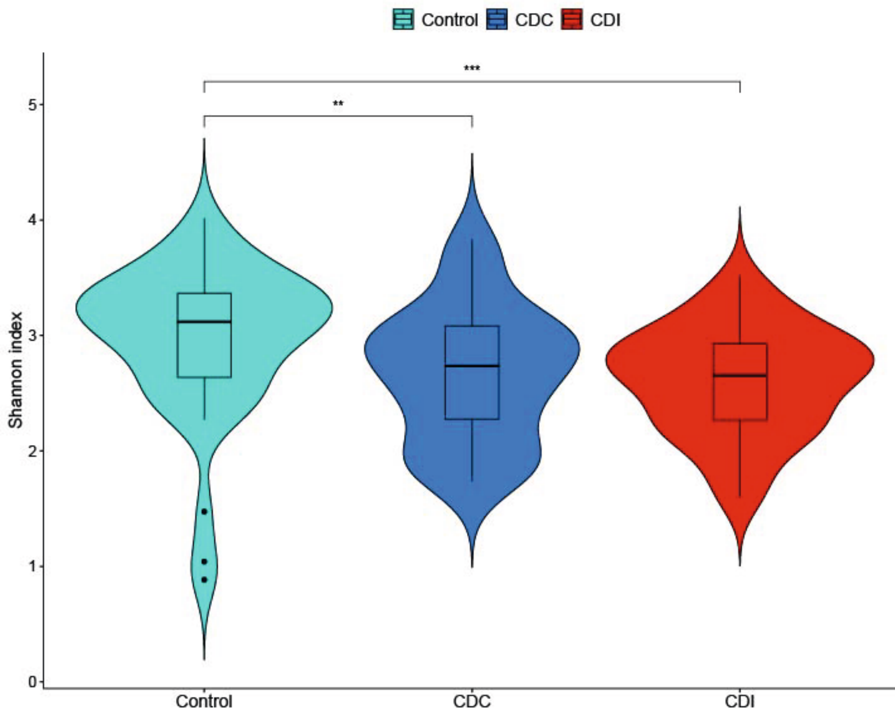

Figure 1. Violin plot of alpha diversity, as measured by the Shannon index, in control, *C. difficile* colonization (CDC) and *C. difficile* infection (CDI) patients.
The box plot shows the median, 25th and 75th percentile and whiskers indicate 1.5* interquartile range. *p<0.05, **p<0.01, ***p<0.001. CDC, *Clostridioides difficile* colonization; CDI, *Clostridioides difficile* infection

The most important clinical factor associated with microbiota composition was the patient group (PERMANOVA, p = 0.001, R^2 = 0.075). Additional pairwise comparisons revealed that microbiota composition of control, CDC, and CDI patients all differed from each other (PERMANOVA, $p < 0.01$). The difference in microbiota composition between groups could also be observed via sample clustering based on unweighted UniFrac distance, but was less apparent, although still visible, using weighted UniFrac distance (Figure 2), reflecting differences in presence/absence of bacterial taxa rather than in their relative abundance. Here, microbiota composition of CDC patients are scattered, with some samples being more similar to CDI patients and others to controls. Clustering analysis solely on the CDC group showed no differentiation in microbiota composition by toxinogenic or non-toxinogenic *C. difficile* carriership, or by any other variable (data not shown).

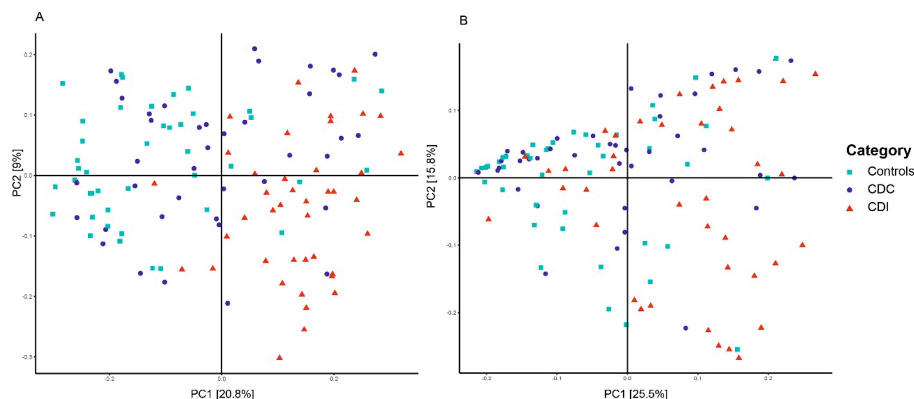

Figure 2. Principal Coordinates Analysis (PCoA) based on unweighted (A) and weighted (B) UniFrac distances.
Each sample is represented by a shape and colour according to its category. The percentage of variation explained by the two first PCoA dimensions is indicated on the respective axes. CDC, *Clostridioides difficile* colonization; CDI, *Clostridioides difficile* infection.

In addition to patient group, overall microbiota composition was significantly affected by solid organ transplantation, previous CDI, PPIs/antacids, immunosuppressants, and specific antibiotics, including vancomycin and metronidazole, which are commonly prescribed antibiotics for CDI treatment (Table S1). However, effect sizes were smaller for these clinical variables than for segregation by patient group. Since antibiotics are known to alter gut microbiota composition, we explored whether antibiotic use in the previous three months affected microbiota composition within the control and CDC group. This was indeed the case for control patients (PERMANOVA, $p = 0.035$, $R^2 = 0.044$), but not for CDC patients (PERMANOVA, $p = 0.409$, $R^2 = 0.031$). Within the control group, antibiotic use also impacted bacterial diversity, with a trend for increased diversity in the nonantibiotic group ($p = 0.0518$). For these reasons, the control group was separated in controls with (C+AB) and without (C-AB) previous antibiotic use for differential abundance analysis.

Relative abundance of individual bacterial taxa

In order to study the differential abundance of bacterial taxa between the patient groups, DESeq2 analysis was performed. Relative abundance of *Clostridioides* showed a significant stepwise increase from C-AB (<0.01 ± <0.01%), C+AB (0.05 ± 0.2%) to CDC (0.7 ± 2.2%), and CDI patients (2.5 ± 2.9%) (Table S2A,B). It is, however, important to take prevalence into consideration, as *Clostridioides* reads were detected in only 26/41 CDC patients (63.4%) and in 38/41 CDI patients (92.7%). The nucleotide sequence belonging to this *Clostridioides* OTU

resulted in a 100% sequence identity with two *C. difficile* strains, but only a 94% sequence identity with *C. mangenotii*.

Compared to CDC patients, C+AB and C-AB had an increased relative abundance of *Eubacterium hallii* (Figure 3, Table S2A,B). As expected, more and larger differences were observed between C-AB and CDC patients than between C+AB and CDC patients. In addition to an increase in *E. hallii,* the relative abundance of *Fusicatenibacter* was significantly higher in C-AB than in CDC patients, while the relative abundance of several *Enterococci*, *Ruminococcus gnavus,* and *Lachnoclostridium* were significantly lower (Figure 3, Table S2A,B).

Compared to C+AB and CDC patients, microbiota of CDI patients was characterized by a higher relative abundance of *Clostridioides*, *Bacteroides,* and *Veillonella*, and by a lower abundance of genera belonging to the *Ruminococcaceae* family and Actinobacteria phylum (Figure 3, Table S2A,B). Many of these lower abundant genera are known short-chain fatty acids (SCFA)-producers and carbohydrate degraders. Additionally, CDI patients had increased relative abundance of *R. gnavus* and *Lachnoclostridium* compared to C+AB patients. To avoid antibiotic use bias, CDI patients were not compared to C-AB patients.

Bacterial networks

To investigate connectivity of the differentially abundant *Clostridioides* genus with other bacterial genera, network analysis was performed on microbiota composition profiles of CDC and CDI patients. In CDI patients, *Fusicatenibacter* was negatively associated with *Clostridioides* (Figure 4A). In CDC patients, a positive association between *Clostridioides* and *Veillonella* was observed (Figure 4B).

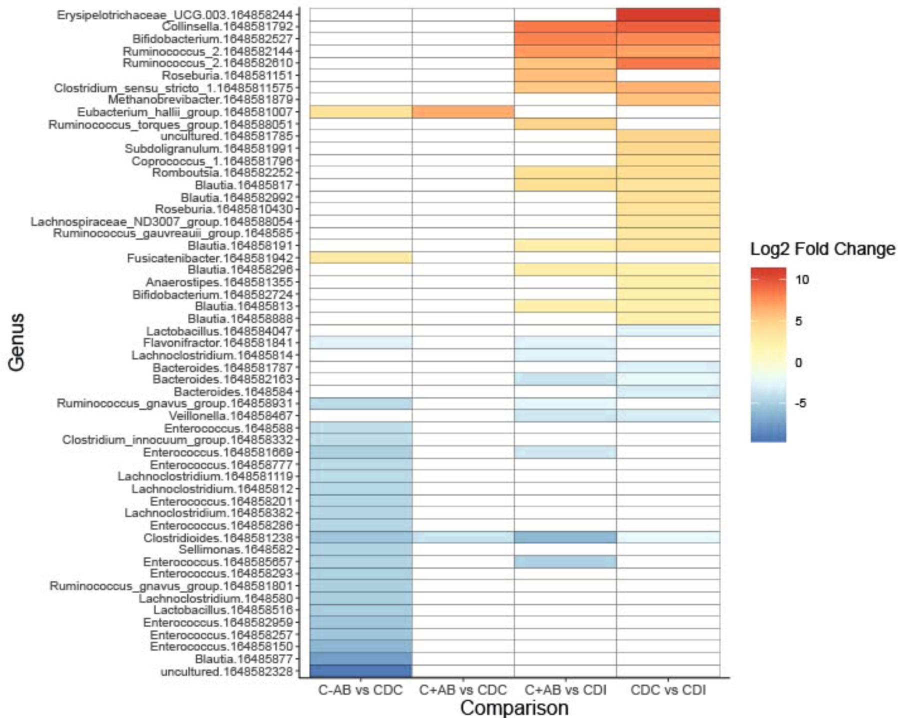

Figure 3. Heatmap showing differentially abundant bacterial taxa between without previous antibiotic use (C-AB), with previous antibiotic use (C+AB), CDC and CDI patients

Bacterial taxa with a Log2 fold change of at least (-)2.25 and a Benjamini-Hochberg corrected p-value ≤ 0.05 are shown on operational taxonomic unit (OUT)-level. OTU numbers are indicated as 164858xxxxxx. A full overview can be found in Table S2A. CDC, *Clostridioides difficile* colonization; CDI, *Clostridioides difficile* infection.

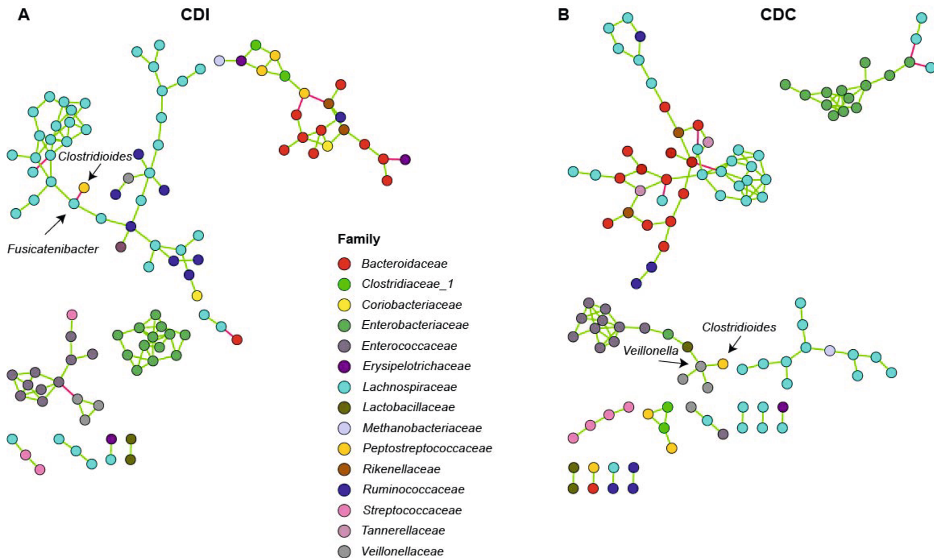

Figure 4. Association network analysis using SPIEC-EASI in CDI (A) and CDC (B) patients. Each node represents a single OTU and is coloured according to family-level taxonomy. Green edges indicate a positive association between nodes, red edges indicate a negative association between nodes. CDI, *Clostridioides difficile* infection; CDC, *Clostridioides difficile* colonization.

Discussion

It is generally accepted that CDI can develop due to a disturbed gut microbiota. In contrast, not much is known about the role of the gut microbiota in *C. difficile* colonization. In this study, the gut microbiota of patients with asymptomatic *C. difficile* colonization was characterized. CDC patients had unique gut microbiota signatures, and bacterial taxa could be identified that may be of relevance for further mechanistic studies. While 16S rRNA gene amplicon sequencing did not allow for identification of *Clostridioides* in all colonized and CDI patients, its relative abundance increased in a step-wise manner from controls to colonized patients and CDI patients.

Bacterial diversity was decreased in CDC and CDI patients, and microbiota composition was mostly patient group-specific. Interestingly, microbiota composition was associated with previous antibiotic use within the control group, but not within the CDC group. This may suggest that CDC patients already have a disturbed bacterial community prior to colonization, independent of antibiotic treatment, although the underlying reason remains unclear. Another explanation could be that, as 73.2% of CDC patients had previous

antibiotic use, too few CDC patients without antibiotic use were included to effectively identify an antibiotic treatment effect within this group.

Multiple differentially abundant genera were found between control, CDC patients, and CDI patients, and included *Eubacterium hallii*, *Fusicatenibacter*, and *Veillonella*. Bacterial network analysis showed that *C. difficile* was directly negatively associated with *Fusicatenibacter* in CDI patients, and directly positively associated with *Veillonella* in CDC patients. This may indicate that *Fusicatenibacter* may play a role in preventing CDI development, while *Veillonella* may play a role in *C. difficile* colonization, respectively.

It has previously been hypothesized that *Eubacterium* species are protective against CDI development in asymptomatic carriers. (10) In our study, *E. hallii* was more abundant in controls (with and without antibiotic use) than in CDC patients. *E. hallii* is known to produce the three main SCFAs (propionate, acetate, and butyrate (29, 30), and is increasingly being investigated for its potential benefit in metabolic disease. (31) This bacterium may contribute to colonization resistance against *C. difficile* through SCFAs production, although the role of SCFAs against *C. difficile* remains debated. (32, 33) Possibly, *E. hallii* contributes to colonization resistance through secondary bile-acid production. Secondary bile acids are known to inhibit *C. difficile* growth, and a secondary bile acid-producing bacterium, *Clostridium scindens*, enhances colonization resistance against *C. difficile*. (34, 35) *E. hallii* possesses *bsh* genes, which are necessary for deconjugation of conjugated bile acids, which is a crucial step prior to converting deconjugated bile acids into secondary bile acids. (31) However, although the most important enzyme for secondary bile acids conversion, 7α-dehydroxylase, was demonstrated to be present in *Eubacterium* species, no homologue has been detected in *E. hallii*'s genome. (31, 36-38)

Veillonella was more abundant in CDI patients than in CDC patients and controls with prior antibiotic use, and was positively associated with *Clostridioides* in colonized patients in our study. *Veillonella* is normally found in the oral cavity, where it can form dental plaques with *Streptococcus*, but is also found in atherosclerotic plaques and fecal samples from patients with atherosclerosis. (39, 40) *Veillonella and* streptococci may be metabolically linked through lactic acid, which also holds for other lactic-acid producing bacteria, like lactobacilli. (41, 42) *Lactobacillus* and *Veillonella* were indeed directly positively linked in our network analysis. While increased relative abundance of *Veillonella* may be a result of intrinsic resistance to multiple antibiotics, a recent in vitro study showed that *Veillonella* increases when a dysbiotic microbiota is co-cultivated with *C. difficile*.

(43) In addition, increased *Veillonella* abundance has been reported prior to CDI onset. (44) These studies, combined with our data, suggest that *Veillonella* is associated with *C. difficile* colonization and infection. It remains unclear whether *Veillonella* has a role in CDI development (e.g., via biofilm formation), or whether it simply outgrows as a result of altered metabolic pathways or unoccupied niches in the gut due to antibiotic use or *C. difficile* expansion.

Fusicatenibacter was differentially abundant between C-AB and CDC patients, and was negatively associated with *Clostridioides* in CDI patients in our study. This bacterium has only been cultured recently (2013), and we are the first to describe an association between *Fusicatenibacter* and *C. difficile* colonization or infection. (45) Previously, *Fusicatenibacter sacchivorans*, the only known species within the *Fusicatenibacter* genus, was shown to be increased in inactive ulcerative colitis (UC) patients and decreased in active UC, related to its positive association with IL-10 production. (46)

Our study had some limitations. Almost all diagnosed CDI patients (39 of 41) came from the LUMC, while CDC and controls were derived from both Amphia hospital and LUMC. As the LUMC is a university affiliated hospital instead of a general hospital, patient characteristics in these groups may not have been completely comparable. As such, solid organ transplants, previous hospitalization, immunosuppressant use, and chemotherapy were more frequent in LUMC. Several of these clinical variables significantly affected overall microbiota composition, which challenges studying the sole effect of CDI on microbiota composition. Another limitation is that a single stool sample was available. Therefore, it is impossible to determine if patients were transiently or persistently colonized by *C. difficile*. Patients classified as CDC might have included patients with only transient passage of spores. (2) Lastly, we have not performed functional characterization of the microbiota, e.g., by metabolomics or transcriptomics.

However, our study had multiple important strengths. Firstly, this is the first study that investigates microbiota composition of *C. difficile* colonized patients, as compared to controls and CDI patients, with more than 10 patients included per group. This allowed for more robust statistical analysis, and for detecting smaller and subtle changes within the composition. Secondly, controls in this study were not healthy controls. Instead, controls and CDC patients were selected from the same cohort of newly admitted patients, and all three groups were hospitalized on the same wards to make the comparisons more fair.

Thirdly, CDI was well defined. Although molecular testing is nowadays often used as a stand-alone test to diagnose CDI, these assays cannot discern colonization from infection. (47) In our study, all samples suspected of CDI were (also) tested with an assay detecting free toxins. Laboratory results were interpreted in conjunction with clinical symptoms. According to the Dutch sentinel surveillance program and the ECDC criteria, patients had to have diarrhea for at least 2 days and/or pseudomembranous colitis at endoscopy and no other apparent cause of diarrhea. Although milder cases may have been missed by using these strict criteria, we are quite confident that our CDI group consisted of clinical relevant CDI cases requiring CDI treatment. Fourthly, duplicates for DNA extraction and sequencing were included to detect potential bias. All these duplicates showed very high similarity in composition profiles, demonstrating the reproducibility of DNA extraction and sequencing procedures (Figure S1).

Conclusions

We demonstrated that colonization and infection by *C. difficile* are associated with decreased bacterial diversity in the gut and differences in relative abundance of specific bacterial taxa, including *Veillonella*, *Fusicatenibacter*, *Eubacterium hallii*, *Bacteroides*, and members of the *Lachnospiraceae* and *Ruminococcaceae* families. Future studies could focus on functional characterization of the microbiota, e.g., by metabolomics or transcriptomics, and on co-cultivation of specific bacteria, e.g., *Fusicatenibacter* with *C. difficile*, in light of *C. difficile* colonization and infection. In addition, it is relevant to determine if the observed gut microbiota changes are present before acquiring colonization and/or CDI, or merely as a consequence.

Author contributions

Conceptualization, M.J.T.C., Q.R.D., E.J.K., and R.D.Z.; Data curation, M.J.T.C. and K.M.V.; Formal analysis, M.J.T.C., Q.R.D., and R.D.Z.; Funding acquisition, M.J.T.C. and E.J.K.; Investigation, M.J.T.C., C.H., I.M.J.G.S., and R.D.Z.; Methodology, M.J.T.C., Q.R.D., and R.D.Z.; Project administration, M.J.T.C. and K.M.V.; Resources, M.J.T.C. and K.M.V.; Software, Q.R.D. and R.D.Z.; Supervision, E.J.K. and R.D.Z.; Validation, M.J.T.C., Q.R.D., and E.M.T.; Visualization, M.J.T.C. and Q.R.D.; Writing—original draft, M.J.T.C. and Q.R.D.; Writing—review and editing, M.J.T.C., Q.R.D., E.M.T., C.H., I.M.J.G.S., K.M.V., E.J.K., and R.D.Z. All authors contributed substantially; approved the submitted version; and agree to be personally accountable for their own contributions and for ensuring that questions related

to the accuracy or integrity of any part of the work, even parts in which the author was not personally involved, are appropriately investigated, resolved, and documented in the literature. All authors have read and agreed to the published version of this manuscript.

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Conflicts of interest

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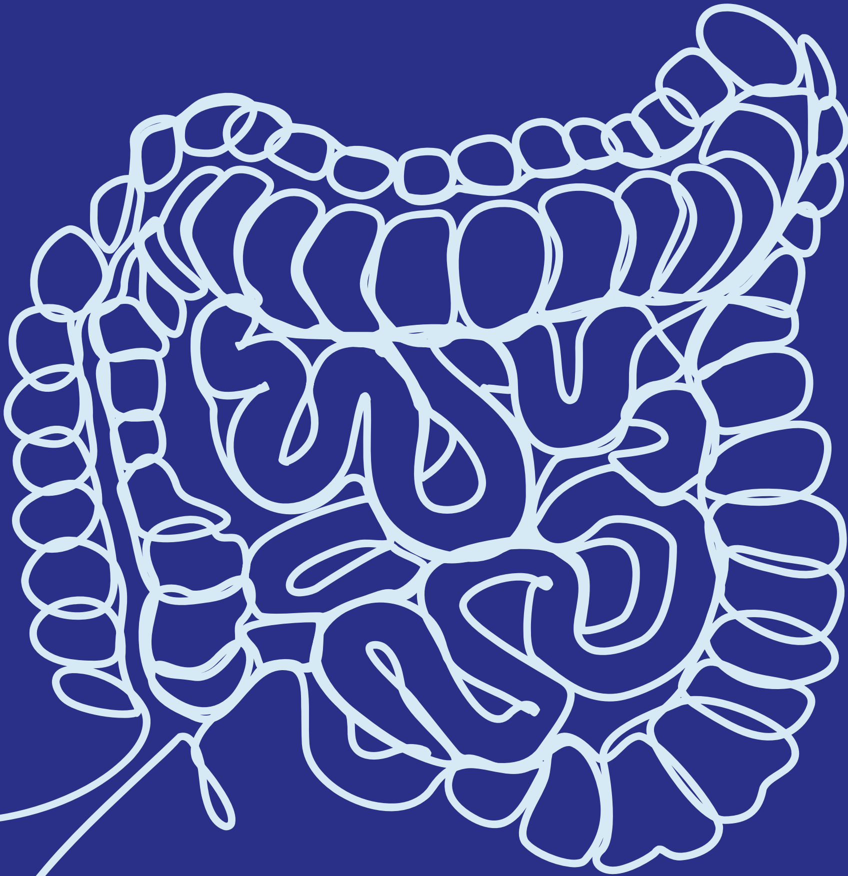
CHAPTER 6

Screening for *Clostridioides difficile* colonization at admission to the hospital: a multi-centre study



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Abstract

Objective. This study aimed to assess the value of *C. difficile* colonization (CDC) screening at hospital admission in an endemic setting.

Methods. A multi-centre study was performed in 4 hospitals located across the Netherlands. Newly admitted patients were screened for CDC. The risk to develop *C. difficile* infection (CDI) during admission and one-year follow-up was assessed for colonized and non-colonized patients. *C. difficile* isolates from colonized patients were compared with isolates from incident CDI cases using core genome multi locus sequence typing (cgMLST) to determine if onwards transmission had occurred.

Results. CDC was present in 108/2211 admissions (4.9%), while colonization with a toxigenic strain (tCDC) was present in 68/2211 (3.1%) of admissions. Among these 108 colonized patients, diverse PCR ribotypes were found and no 'hypervirulent' RT027 was detected ((95% CI, 0- 0.028). None of the colonized patients developed CDI during admission (0/49, 95% CI 0-0.073) or one-year follow-up (0/38, 95% CI 0-0.93). Core genome MLST identified 6 clusters with genetically related isolates from tCDC and CDI patients, but in these clusters only one possible transmission event from a tCDC to a CDI patient was identified by epidemiological data.

Conclusion. In this endemic setting with a low prevalence of 'hypervirulent' strains screening on CDC at admission did not detect any CDC patient who progressed to symptomatic CDI and only one possible transmission event from a colonized patient to a CDI patient. Thus, screening on CDC at admission is not useful in this setting.

Introduction

Clostridioides difficile infection (CDI) remains an important source of healthcare and antibiotic associated diarrhoea. However, not every individual will develop symptomatic CDI after contact with *C. difficile* spores: patients with asymptomatic *C. difficile* colonization (CDC) outnumber symptomatic CDI patients (1). CDC patients do not exhibit symptoms, but might progress to symptomatic CDI upon disturbance of their microbiota. Also, they do shed *C. difficile* spores in their environment thereby acting as a reservoir and potential source for *C. difficile* (2, 3). Although infection control measures focus currently on symptomatic cases only (4), literature has shown that isolation of CDC patients may help in preventing nosocomial transmission (5). Notably, most studies on the importance of CDC patients are conducted in settings with high CDI incidence rates and/or a high proportion of hypervirulent ribotypes (6, 7). The contribution of CDC patients to the epidemiology of CDI is less well known in other settings. In this study, we investigate the value of a CDC screening program on hospital admission in an endemic setting. Factors that determine the need for such a screening program including the prevalence of colonization, the risk of colonized patients to progress to CDI and the chance of onwards transmission from CDC to CDI patients were taken into account.

Methods

Study design and patients

The study was performed in 4 acute care hospitals (3 university-affiliated, 1 general) located across the Netherlands. In one of these hospitals the Dutch reference laboratory for *C. difficile* is housed and all hospitals participate in national sentinel CDI surveillance. In each of the 4 hospitals, patients were enrolled during a 6 to 8 month period between January 2015 and December 2016. Adult patients admitted to predefined wards (medical and surgical) were eligible. Patients with CDI at admission or CDI diagnosed within the first 72hrs of admission were excluded. Additional exclusion criteria are listed in S1. Patients could be enrolled more than once if readmitted during the study period. Consenting subjects had stool samples (and in 1 hospital partly rectal swabs) collected within 72hrs of admission. If patients were discharged before spending 72hrs in the hospital, stool samples could be collected at home and returned to the hospital, no time limit was imposed on collection of these samples. Patients with a positive *C. difficile* culture but no diagnosis of CDI were considered *C. difficile* colonized (CDC). The subset of CDC patients with

a toxigenic strain in their stool cultures were considered toxigenic *C. difficile* colonized (tCDC). CDC patients were included as cases in the case control study after obtaining written informed consent. For each case, 3 controls were selected from the cohort that tested negative for *C. difficile* in their stool samples obtained at admission. These controls were the 3 consecutive patients who submitted a study stool sample to the laboratory and agreed to participate in the case control study.

Toxigenic *C. difficile* isolates from tCDC and CDI patients were compared to determine if transmission from tCDC patients to CDI patients had occurred. CDI cases were all hospitalized patients diagnosed with CDI during the study period and 3 months thereafter in each of the participating hospitals. Isolates of these CDI cases were collected and sent to the Dutch CDI reference laboratory as part of the national sentinel CDI surveillance (8). All CDI cases had to comply with definitions valid in the surveillance protocol (see S2). Test methods for diagnosing CDI in the 4 hospitals are described in S3. Samples from recurrent (>2 but <8 weeks after initial episode) or new (≥8 weeks after the initial episode) CDI episodes were once more included.

Microbiological analysis

Stool culture for the presence of *C. difficile* was performed on a daily basis; during weekends or holidays samples were stored at 4^{0}C until the following working day. Culture methods are described in S4. All identified isolates from (enrichment) culture were ribotyped by resolution capillary gel-based electrophoresis PCR-ribotyping, using the Dutch national reference laboratory library (9). In addition, a multiplex PCR to detect toxin genes *tcdA*, *tcdB*, *cdtA* and *cdtB* was performed on cultured isolates (10). Strains positive for *tcdA*, *tcdB* or *cdtA/cdtB* were defined as toxigenic strains, all other strains were defined as non-toxigenic strains.

Data collection

Patient information was collected at baseline via a patient's questionnaire and medical electronical records. For each patient, the Charlson's Comorbidity Index was calculated (11). Follow-up by patient's questionnaires was scheduled at 30 days and 1 year after enrolment to determine how many patients developed CDI.

Transmission analysis using core genome MLST

Methods used for reculturing, sequencing and construction of the core genome MLST are described in S5. In short, genomes were assembled as previously described (12), annotated with Prokka (13), and alleles for the cgMLST were predicted with a method compatible with SeqSphere (14). Library preparation is described in S6. Based on previous publications (15), ≤ 2 different alleles in the cgMLST were considered to be the same strain if the time frame of sampling was less than 124 days, and ≤ 3 different alleles if it was less than 1 year. Ward movement data of CDI and colonized patients were investigated if their isolates were genetically related. Criteria for epidemiologic linkage are described in S7.

Statistical analysis

Characteristics of CDC patients and tCDC patients were compared to their respective controls. All analyses were performed using STATA SE statistical software version 15.1 (Statacorp, Texas, USA). A p-value of <0.05 was considered statistically significant.

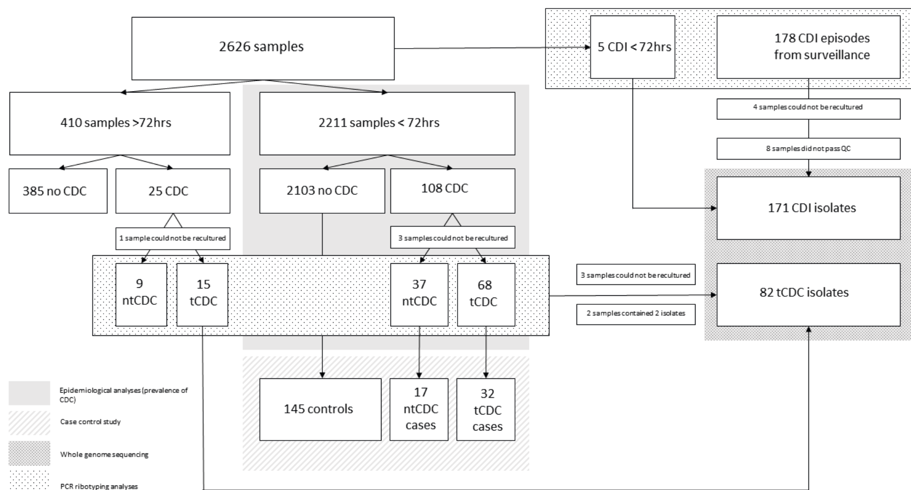

Figure 1. Flowchart of included samples.
CDI; *Clostridoides difficile* infection, CDC: *Clostridoides difficile* colonization, ntCDC: non-toxigenic *Clostridioides difficile* colonization, tCDC: toxigenic *Clostridoides difficile* colonizatiion

Ethical and Methodological Considerations

The study protocol was submitted to the Institutional Review Ethics Board that deemed that this research is not subject to the Medical Research Involving Human Subjects Act. They had no objection to the conduction of the research or collection of the stool samples on admission under verbal informed consent. *C. difficile* culture results were not disclosed to patients or treating physicians. Patients selected as cases and controls provided written informed consent. Stool samples from CDI patients were collected as part of routine care and PCR ribotyped for surveillance purposes. No additional consent was required for whole genome sequencing of samples.

Data availability

All genomic data has been uploaded to the European Nucleotide Archive under study number PRJEB25045.

Results

Included samples and prevalence of *C. difficile* colonization

In total 2626 samples were screened for CDC, ranging from 500 to 1011 samples per hospital (Table 1). 415 samples were excluded from epidemiological analyses (Figure 1). From the remaining 2211 samples, 1736 were stool samples, 467 were rectal swabs, and for 8 information about sampling method was lost. *C. difficile* was found in 108 samples, thus the prevalence of CDC at admission to the hospital was 4.9% (108/2211). Toxigenic strains were found in 68/108 samples. The prevalence of tCDC was therefore 3.1% (68/2211).

Table 1. Included samples and prevalence of CDC and tCDC per hospital

Hospital	Enrolment period	N included samples	N CDC	N tCDC	Prevalence CDC (%)	Prevalence tCDC (%)	N patients enrolled in case control study
LUMC	Jan 2015-Jul 2015	453	19	10	4,19	2,21	44
Erasmus	Sept 2015-Apr 2016	581	36	24	6,20	4,13	50
Amphia	Oct 2015-Mar 2016	786	33	20	4,20	2,54	72
Radboud	April 2016-Nov 2016	391	20	14	5,12	3,58	28
Total		2211	108	68	4,88	3,08	194

Apr, April; CDC: *Clostridioides difficile* colonization; Jan, January; Jul, July; LUMC, Leiden University Medical Center; Mar, March; Nov, November; Oct, October; Sep, September; tCDC: toxigenic *Clostridioides difficile* colonization.

Patient characteristics

In total 194 patients were enrolled in the case-control study: 32 tCDC patients , 17 patients colonized by non-toxigenic strains (ntCDC) and 145 controls (Figure 1). Results from univariate analysis are shown in Table 2.

CDI during follow-up

None of 49 colonized (95% CI 0-0.073) or 145 control patients (95% CI 0-0.025) developed CDI during admission or within the month after enrollment. Questionnaires at one year follow-up were returned by 152 (85% of alive) patients (38 CDC patients and 114 controls). None of these patients reported to have developed CDI during follow-up (0/38 CDC, 95% CI 0-0.093 and 0/114 controls, 95% CI 0-0.032). Chart review of deceased patients showed than one control patient developed CDI 2 months after negative admission screen.

PCR ribotyping and sequence typing

Forty-four different (known) PCR ribotypes were identified among 129 colonized patients. Colonization with the 'hypervirulent' RT027 was not identified, 4 patients were colonized with the 'hypervirulent' RT078 (all from different hospitals). During the study period and 3 months thereafter, 183 CDI episodes were identified and these samples were included for comparison with tCDC isolates. RT027 was also not found among CDI patients.

In total 253 strains were available for WGS analysis (82 isolates from tCDC patients and 171 isolates from CDI patients (Figure 1). Sequence types were assigned to all isolates (Figure 2). ST11 (RT078, RT826 and related ribotypes) was more frequently found among CDI patients than among tCDC patients (19.9% vs 4.9%, $p<0.01$) (Figure 2).

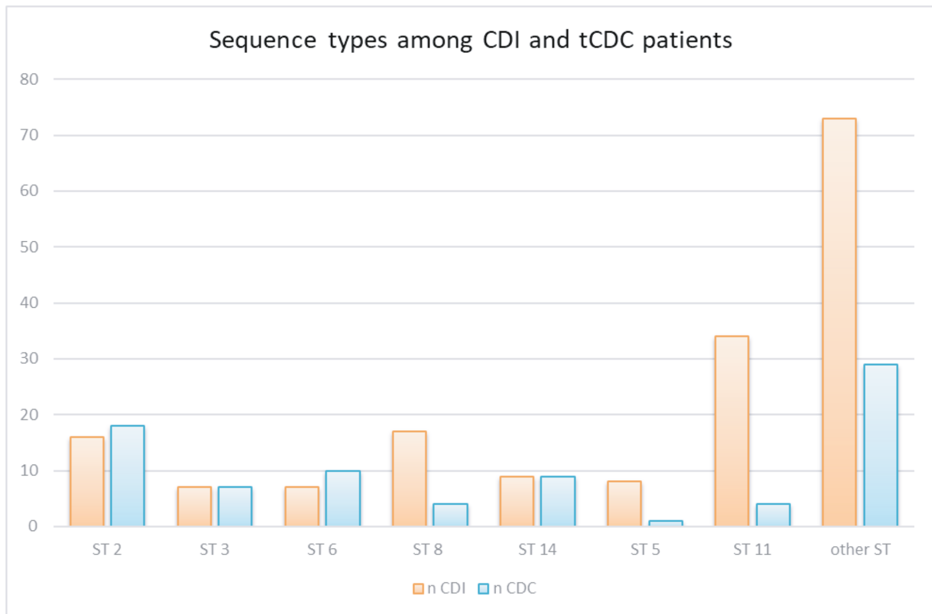

Figure 2. Sequence types among patients with *Clostridioides difficile* infection and toxigenic *Clostridioides difficile* colonization.
tCDC, toxigenic *Clostridoides difficile* colonization; CDI, *Clostridoides difficile* infection; ST, sequence type

Core genome MLST

Given the aforementioned cut-offs, in total 24 clusters could be identified (Figure S1). Six of these clusters (C1-C6) contained isolates from CDI and tCDC patients. Ward movement data for these clusters were investigated.

In cluster C1 (2 patients with RT020/220 from the same hospital), patients shared a ward 11 days before the first patient was found to be colonized and 37 days before the other patient was diagnosed with CDI (direction of transmission indeterminate). In cluster C2 (2 CDI and 2 CDC patients with RT265 from 2 different hospitals) a possible epidemiological link could be established between 2 of the patients: these patients were admitted to the same ward at the time of the first CDI positive sample and the second patient was found to be *C. difficile* colonized 42 days later at readmission (directional transmission from CDI to CDC).

In the other four clusters (C3-C6), no epidemiological link could be found (see legend Figure S1).

Thirteen clusters contained isolates from CDI episodes only (Figure S1). The largest of these clusters was earlier determined to be an outbreak of RT826 (7 samples RT826, one sample RT127) at a single ward in one of the hospitals (16).

Five pairs of genetically related isolates were detected in colonized patients (Figure S1). Four of these pairs were identified from the same hospital >20 days apart, but one of these pairs contained isolates from the same ward sampled only 2 days apart.

Discussion

In this multi-centre study, we screened 2211 patients on hospital admission and found that *C. difficile* colonization was present in 4.9% of admissions; colonization with toxigenic *C. difficile* strains was present in 3.1% of admissions, comparable to previously reported numbers (5, 17, 18). Identified strains among colonized patients were genetically diverse, indicating various reservoirs. Sixty-three percent of colonized patients were colonized by toxigenic strains, but in contrast to other studies (19, 20) not a single colonized patient developed CDI in the year after study enrolment. The reason why we could not confirm a high risk to develop CDI may either be that the number of colonized patients in our study was too low, and/or that there was truly no increased risk due to the local situation such as low numbers/absence of virulent strains circulating among colonized patients and low antimicrobial use (21).

We identified only one possible onwards transmission event from a colonized patient: a tCDC and CDI patient shared a ward before they tested positive for *C. difficile*. Our data are in contrast with published reports (6, 7), which could be explained by the low incidence setting in which our study was performed. During the study period, CDI incidence ranged from 1.87 to 4.59 CDI cases per 10,000 admission days among the hospitals (8, 22). Only one outbreak due to RT826 was detected (16). The hypervirulent RT027 was not detected in CDI nor in CDC patients. As higher transmission has been shown for certain lineages (23) the absence of these lineages may explain why transmission was infrequent in our study and also why no other large clusters between CDI patients were detected by cgMLST. Moreover, other local characteristics may play a role, like antimicrobial pressure and infection control policies.

Of note, we also detected a few genetically related pairs of isolates in colonized patients, suggesting a common source or transmission before admission, although the detection of genetical identical isolates on the same ward only 2 days apart raises the suspicion of transmission (either patient to patient or from the hospital environment) during admission in that particular case.

Our study had numerous strengths. We captured all CDI cases as all 4 hospitals participate in continuous sentinel CDI surveillance. Moreover, CDI diagnosis was not only based on laboratory tests, instead all cases underwent chart review by local infection control personnel and had clinical symptoms compatible with CDI. We included all CDI cases that occurred in the hospitals instead of CDI cases diagnosed on study wards only, as transmission may possibly extend beyond wards (24).

However, our study also has some limitations. First of all, we may have missed a substantial amount of *C. difficile* introductions into the hospitals due to study design (screening was performed on only a few specific wards per hospital) and difficulties in study execution (stool samples were only received from half of 5200 consenting subjects). During the study period, the total number of admissions in the four hospitals were 13987, 19424, 21220 and 25510, respectively, indicating that screening for colonization was not performed in the vast majority of these admissions. On the other hand, to account for *C. difficile* transmission extending beyond wards, all incident CDI cases from each entire hospital were included in cgMLST. Thereby, we are underestimating the contribution of colonized patients to CDI overall, as a source could possibly not be identified if a CDI case occurred on a ward where screening for colonization was not performed.

Furthermore, patients were only sampled once during the study. Consequently, we do not know how many patients were (a) transiently colonized, (b) persistent carriers, or (c) acquired colonization during admission, although this may affect both the risk for CDI progression and *C. difficile* transmission pressure. Moreover, we did only include hospital-onset CDI cases, thereby ignoring that transmission may not (directly) lead to symptomatic CDI. Patients that acquired *C. difficile* from a colonized patient during admission, but developed CDI only after discharge, have not been captured in our study.

Table 2. Univariate analysis of potential risk factors for CDC and tCDC.

	49 CDC patients	145 controls	CDC vs control (OR, 95% CI)	32 tCDC patients	95 controls	tCDC vs control (OR, 95% CI)
Male sex	23 (46.9%)	78 (53.8%)		16 (50.0)	56 (59.0%)	
Median age (IQR)	59 (47.5-67.5)	61 (52-68)		57 (48-71)	63 (52-70)	
Born in the Netherlands	45 (91.8%)	133 (91.7%)		30 (93.8)	88 (92.6%)	
Comorbidity						
Median Charlson Comorbidity Score (IQR)	3 (2-4)	3 (1-5)		3 (2-5)	3 (1-5)	
Solid organ transplant	12 (24.5%)	15 (10.3%)	2.8 (1.2-6.5)	10 (31.3)	11 (11.6%)	3.5 (1.3-9.2)
IBD	8 (16.3%)	7 (4.8%)	3.8 (1.3-11.2)	7 (21.9)	4 (4.2%)	6.4 (1.7-23.5)
Non-metastatic solid malignancy	9 (18.4%)	25 (17.2%)	1.1 (0.5-2.5)	5 (15.6)	18 (19.0%)	0.8 (0.3-2.3)
Metastatic solid malignancy	3 (6.1%)	16 (11.0%)	0.5 (0.1-1.9)	0 (0)	10 (10.5%)	-
Chronic kidney disease	15 (30.6%)	24 (16.6%)	2.2 (1.1-4.7)	11 (34.4)	17 (17.9%)	2.4 (0.98-5.9)
DM uncomplicated	7 (14.3%)	21 (14.5%)	1.0 (0.4-2.5)	5 (15.6)	11 (11.6%)	1.4 (0.5-4.4)
DM end-organ damage	3 (6.1%)	9 (6.2%)	1.0 (0.3-3.8)	2 (6.3)	7 (7.4%)	0.8 (0.2-4.3)
Myocardial infarction	5 (10.2%)	16 (11.0%)	0.9 (0.3-2.6)	3 (9.4)	11 (11.6%)	0.8 (0.2-3.0)
Peptic ulcer disease	3 (6.1%)	11 (7.6%)	0.8 (0.2-3.0)	2 (6.3)	9 (9.5%)	0.6 (0.1-3.1)
COPD	12 (24.5%)	19 (13.1%)	2.2 (0.95-4.8)	8 (25.0)	11 (11.6%)	2.5 (0.9-7.0)
Mild liver disease	4 (8.2%)	10 (6.9%)	1.2 (0.4-4.0)	4 (12.5)	7 (7.4%)	1.8 (0.5-6.6)
Severe liver disease	2 (4.1%)	5 (3.5%)	1.2 (0.2-6.3)	1 (3.3)	4 (4.2%)	0.7 (0.1-6.8)
HIV	0 (0%)	1 (0.7%)	-	0 (0)	1 (1.1%)	-
BMT or SCT	0 (0%)	2 (1.4%)	-	0 (0)	2 (2.1%)	-
Psychiatric disorder	6 (12.2%)	17 (11.7%)	1.1 (0.4-2.8)	4 (12.5)	10 (10.5%)	1.2 (0.4-4.2)
Previous diarrhea and CDI						
Diarrhea in previous 3 months	26 (53.1%)	59 (40.7%)	1.6 (0.9-3.2)	16 (50.0)	36 (37.9%)	1.6 (0.7-3.7)
Previous CDI	3 (6.1%)	1 (0.7%)	9.3 (0.9-91.9)	2 (6.3)	0 (0%)	-
Household member with previous CDI	0 (0%)	1 (0.7%)	1.0 (1.0-1.0)	0 (0)	1 (1.1%)	1.0 (1.0-1.0)

Table 2. *Continued.*

	49 CDC patients	145 controls	CDC vs control (OR, 95% CI)	32 tCDC patients	95 controls	tCDC vs control (OR, 95% CI)
Healthcare contact						
Previous hospital admission (last 12 months)	36 (73.5%)	72 (49.7%)	2.8 (1.4-5.7)	24 (75.0)	47 (49.5%)	3.1 (1.3-7.5)
Working in healthcare system	3 (6.1%)	17 (11.7%)	0.5 (0.1-1.8)	1 (3.1)	9 (9.5%)	0.3 (0.04-2.5)
Previous medication use (last 3 months)						
Antibiotics	34 (69.4%)	79 (54.5%)	1.9 (0.9-3.8)	23 (71.9)	51 (53.7%)	2.2 (0.9-5.3)
PPI or antacids	36 (73.5%)	87 (60.0%)	1.8 (0.9-3.8)	25 (78.1)	56 (59.0%)	2.5 (0.98-6.3)
Anti-cancer chemotherapy	3 (6.1%)	11 (7.6%)	0.8 (0.2-3.0)	2 (6.3)	7 (7.4%)	0.8 (0.2-4.3)
Immunosuppressants	25 (51.0%)	53 (36.6%)	1.8 (0.9-3.5)	19 (59.4)	34 (35.8%)	2.6 (1.2-6.0)
Animal contact						
Pet dog	22 (44.9%)	34 (23.5%)	2.7 (1.3-5.3)	17 (53.1)	21 (22.1%)	4.0 (1.7-9.3)
Pet cat	3 (6.1%)	18 (12.4%)	0.5 (0.1-1.7)	2 (6.3)	10 (10.5%)	0.6 (0.1-2.9)
Contact with livestock	3 (6.1%)	17 (11.7%)	0.5 (0.1-1.8)	3 (9.4)	11 (11.6%)	0.8 (0.2-3.0)
Children in household attending daycare	1 (2.0%)	5 (3.5%)	0.6 (0.1-5.1)	0 (0)	4 (4.2%)	-

BMT, bone marrow transplantation; CDC, *Clostridioides difficile* colonization; CDI, *Clostridioides difficile* infection; COPD, chronic obstructive pulmonary disease; DM, diabetes mellitus; HIV, human immunodeficiency virus; IBD, inflammatory bowel disease; IQR, interquartile range; OR, Odds ratio; PPI, proton pump inhitor; SCT, stem cell transplantation; tCDC, toxigenic *C. difficile* colonization

Environmental swabs were not taken during our study though colonized patients may contaminate the hospital environment with spores that can persist for long times. A direct transmission link can be missing when *C. difficile* acquisition occurs at a later moment from this contaminated hospital environment.

Criteria to determine epidemiologic linkage were quite strict and did not take into account transmission beyond wards. In our study, data about patients' movements to other hospital areas (like the radiology department) were not available.

Another limitation includes the applied criteria to consider isolates to be the same strain, as these were originally based on single nucleotide variant analysis instead of cgMLST. As it is not known if the discriminatory power of both approaches is similar, we checked all comparisons with 3 or less allele differences in cgMLST. In all besides one comparison, one allele was equal to 1 single nucleotide polymorphism (SNP). In the last comparison, where only one allele difference was predicted, this allele had 2 SNPs. We therefore think that the criteria are still applicable to our study.

Implementing screening was difficult and burdensome, while those tCDC patients that were detected did not have a high risk of progressing to CDI themselves and were not identified as an important direct source for incident hospitalized CDI cases. However, tCDC patients may still contribute to *C. difficile* transmission by transmitting *C. difficile* to other patients who remain asymptomatically colonized instead of (directly) progressing to CDI, or by contaminating the hospital environment. The hospital environment can however be contaminated in many other ways, for example by CDI patients whose isolation precautions are lifted after resolution of symptoms but who are still shedding spores. Therefore, we think that we should focus on decreasing CDI susceptibility (e.g. by antimicrobial stewardship programs) and complying with general infection prevention measures to prevent spread from *C. difficile* and other nosocomial pathogens. Sentinel surveillance to monitor CDI incidence rates and circulating ribotypes and the use of molecular typing in case of suspected transmission is of value to detect clusters and outbreaks (25). A very typical example of this approach was the finding of the RT826 cluster that was already detected via sentinel surveillance (16), and turned out to be the only transmission between multiple CDI patients detected by cgMLST in this study.

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Conflicts of interest

All authors report no conflicts of interest.

Authors' contributions

MC and EK designed the study. MC, ET, CV, MV and JH were local investigators at the four study hospitals. CH and IS performed the culturing of the isolates and DNA extraction. MS performed the DNA sequencing, genome assembly and annotation. MS, NK and BH performed the bioinformatics analysis. MC performed the epidemiological analyses. MC and BH wrote the manuscript with the help and feedback of all other authors.

Acknowledgements

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Supplementary material

S1. Additional exclusion criteria

Patients were excluded if:

- admitted from other wards within the same hospital
- admitted for palliative care
- hemodynamically unstable
- residency outside the Netherlands
- unable to speak or read Dutch or English
- unable to participate in the verbal informed consent process on their own behalf or represented by a surrogate

S2. Definition of CDI according to the Dutch sentinel surveillance program

Patients had to have clinical suspicion of CDI (which included either diarrhea -defined as at least 3 loose stools per 24hrs for 2 days- or toxic megacolon, and the absence of an alternative explanation for diarrhea) in combination with a positive *C. difficile* test or the presence of pseudomembranous colitis by endoscopy/histopathology.

S3. Test methods used for diagnosing CDI in the 4 hospitals

Toxin A/B EIA (1 hospital), a Nucleic Acid Amplification Assay (1 hospital), a Nucleic Acid Amplfication Assay in combination with a Toxin A/B EIA (1 hospital) or a GDH EIA in combination with a Toxin A/B EIA (1 hospital).

S4. *C. difficile* culture methods

Stool samples and rectal swabs were plated directly on CLO plates (selective *C. difficile* medium containing cefoxitin, amphotericin B and cycloserine, BioMérieux, The Netherlands) and after ethanol shock on CNA (Columbia blood-agar containing colistin and nalidixic acid, BioMérieux , The Netherlands) and CLO plates. After 48 and 96hrs, plates were read. Presumptive colonies were identified by Matrix-Assisted Laser Desorption Ionization–Time Of Flight (MALDI-TOF) Mass Spectrometry and sent to the national reference laboratory for GluD PCR to confirm *C. difficile* presence. For rectal swabs, an additional enrichment

culture was performed. Swabs were inoculated in *C. difficile* enrichment modified broth (*Clostridium difficile* enrichment broth with 0.1% sodium taurocholate moxalactam (32mg/L), norfloxacin (12mg/L) and cysteine hydrochloride (500mg/L), Mediaproducts BV, Groningen, the Netherlands) for 5 days, and then subcultured on CLO plates.

S5. Methods for DNA preparation, sequencing and core genome MLST

C. difficile strains were anaerobically (re)cultured for 48hrs on TSS plates (Trypcase Soy agar + 5% sheep blood, Biomérieux). A single colony was picked, suspended in 9ml BHI (Brain Heart Infusion broth, Media Products Groningen) and incubated anaerobically overnight at 37°C with gentle shaking at 100rpm. The culture was centrifuged afterwards at 4000rpm for 10 minutes. The pellet was then resuspended in 800µl PBS (Phosphate-buffered saline Media Products Groningen), and 24µl of lysozyme (50 g/l) was added to a final concentration of 1,3g/l. The solution was incubated for 20 minutes at 37°C. Afterwards 15µl of proteinase K (20g/l, Roche) were added to a final concentration of 1,3g/l, and the solution was again incubated for 20 minutes at 37°C. DNA was extracted with the QiaSymphony and the program Complex800_OBL_V4_DSP. The extracted DNA was used for whole genome sequencing and ribotyping. Ribotyping was performed according to (1), with the following changes: PCR was performed on a BIO-RAD MyCyclerTM and analyzed on the Applied Biosystems 3500xL Genetic Analyzer. All samples were sequenced on an Illumina HiSeq X10 machine in paired-end mode, with a read length of 151.

Genomes were assembled using Velvet v1.2.10 (2), SSPACE v2.0 (3) and GapFiller v1.1 (4) as described previously (5), and annotated with Prokka, version 1.5 (6) with options -M n -V b. MLST types were determined with MLSTcheck (7) version 2.1.1706216. All reads were mapped to their respective genomes with bowtie2 v. 2.3.4.1 (8) and further converted to sorted bam files with samtools v. 1.6 (9). Optical duplicates were marked with PicardTools v. 1.124, and SNPs were called with HaplotypeCaller from the GATK package, v.4. 1.2 (10), with ploidy set to 2, to detect possibly mixtures of two different strains. Results were visualized with af-plot v 0.2.1, https://doi.org/10.5281/zenodo.3238297. Additionally all samples were profiled with checkm v1.0.13 (11). The typing scheme, sequences of alleles, and other relevant information for *C. difficile* core genome MLST (cgMLST) were downloaded from cgmlst.org. A blastn search (with standard parameters; Blast v 2.11) with all predicted *C. difficile* genes was performed against the cgMLST database. A gene was assigned to an allel if it was 100% identical over at least 95% of its length. A gene was tentatively assigned to an allel if it was at least 99% identical over 95% of its length. Assignment of cluster types

was performed via the hamming distance as implemented in Numpy/SciPy (12), based on the predicted allels. A minimum spanning tree was constructed via the networkX library (13), and was visualized in Cytoscape 3 (14). A limited amount of samples was also analysed in SeqSphere (15), for validation purposes. All genomic data has been uploaded to the European Nucleotide Archive under study number PRJEB25045.

We applied a threshold of <= 3 alleles in cgMLST as recent data show that this threshold better identifies outbreaks than the current threshold of 6 alleles, especially in ribotypes with lower mean intra-ribotype allele differences (Baktash, submitted).

S6. Library preparation: IHTP WGS NEB UltraII library process – DUAL/QUAD processing using Sanger 168 tags and PE1 tag2 sets 1-4

- Samples quantified with Biotium Accuclear Ultra high sensitivity dsDNA Quantitative kit using Mosquito LV liquid platform, Bravo WS and BMG FLUOstar Omega plate reader and cherrypicked to 200ng / 120ul using Tecan liquid handling platform.
- Cherrypicked plates sheared to 450bp using a Covaris LE220 instrument.
- Post sheared samples purified using Agencourt AMPure XP SPRI beads on Agilent Bravo WS.
- Library construction (ER, A-tailing and ligation) using 'NEB Ultra II custom kit' on an Agilent Bravo WS automation system.
- PCR set-up using KapaHiFi Hot start mix and Sanger 168 tags (i7) and PE1.D1-PE1.D2 (Dual) or PE1.D1-PE1.D4 (Quad) tag2 (i5) tags on Agilent Bravo WS automation system.
- PCR cycles, 6 standard cycles,
- Post PCR plate purified using Agencourt AMPure XP SPRI beads on Beckman NX96 liquid handling platform.
- Libraries quantified with Biotium Accuclear Ultra high sensitivity dsDNA Quantitative kit using Mosquito LV liquid handling platform, Bravo WS and BMG FLUOstar Omega plate reader.
- 2 x 96 (Dual) or 4 x 96 (Quad) libraries pooled in equimolar amounts on a Beckman BioMek NX-8 liquid handling platform.
- Library pools normalised depending on sequencing platform. For NovaSeq, pools are normalised to 1.2nM (XP) or 2.25nM (Standard S4) and loaded on requested Illumina sequencing platform. For HiSeqX platforms pools normalised to 2.8nM.

S7. Definitions for epidemiological linkage

Epidemiologically linkage was plausible if (a) the donor-recipient pair shared a ward after the donor tested positive and before the recipient tested positive or (b) shared a ward before either tested positive or (c) if the recipient was admitted to a ward that had been occupied by the donor patient before. A maximum infectious period of 8 weeks and incubation period of 12 weeks were allowed. Both CDI and colonized patient were assumed to contaminate the ward for 26 weeks after testing positive (16).

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Description belonging to FIgure S1:
Colonized patients are shown as circles, CDI patients are shown as diamonds.
Recurrent episodes with identical isolates in one patient are shown as larger diamonds (the number of retrieved isolates in the patient is given, e.g. 2 x 001 means that the patient had one initial episode and one recurrence with an identical isolate of RT001).
Colonization with identical isolates in one patient is shown as a larger circle.
Both colonization and infection with identical isolates in one patient is shown as a diamond/circle combination.
'?' means that the ribotype was not recognized in our reference database.
Clusters with isolates from both colonized and infected patients are numbered (C1-C6).
In cluster C3 (2 patients with RT014 from the same hospital), CDC was detected almost 5 months after CDI.
In cluster C4 (2 patients with RT002 from 2 different hospitals) CDC was detected 7 months after CDI in the other hospital.
In cluster C5 (2 patients with RT001 from the same hospital) community-onset CDI was diagnosed more than 5 months after detection of the colonized patient and the CDI patient had not been admitted to the hospital in the 12 weeks preceding CDI diagnosis.
Cluster C6 consisted of 5 patients with RT001 from 3 different hospitals. In the first hospital CDI was diagnosed 4 months after CDC; patients had not been admitted to the same ward in the 12 weeks preceding CDI diagnosis. The other CDI patient in this cluster derived from a different hospital and CDC patients from the third hospital were sampled 6 months apart.
CDI: *Clostridoides difficile* infection, tCDC: toxigenic *Clostridioides difficile* colonization

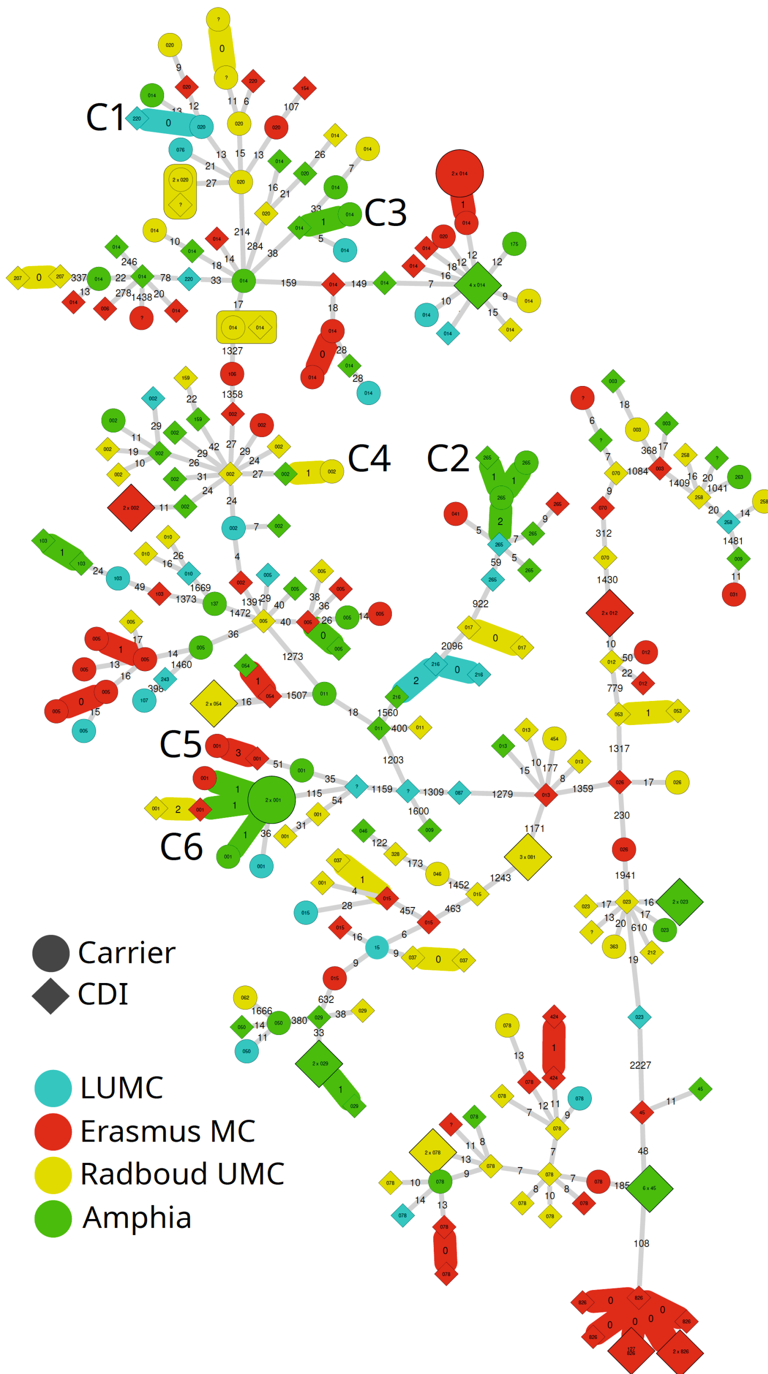

Figure S1. Core genome MLST of isolates from tCDC and CDI patients.
Description on left page.

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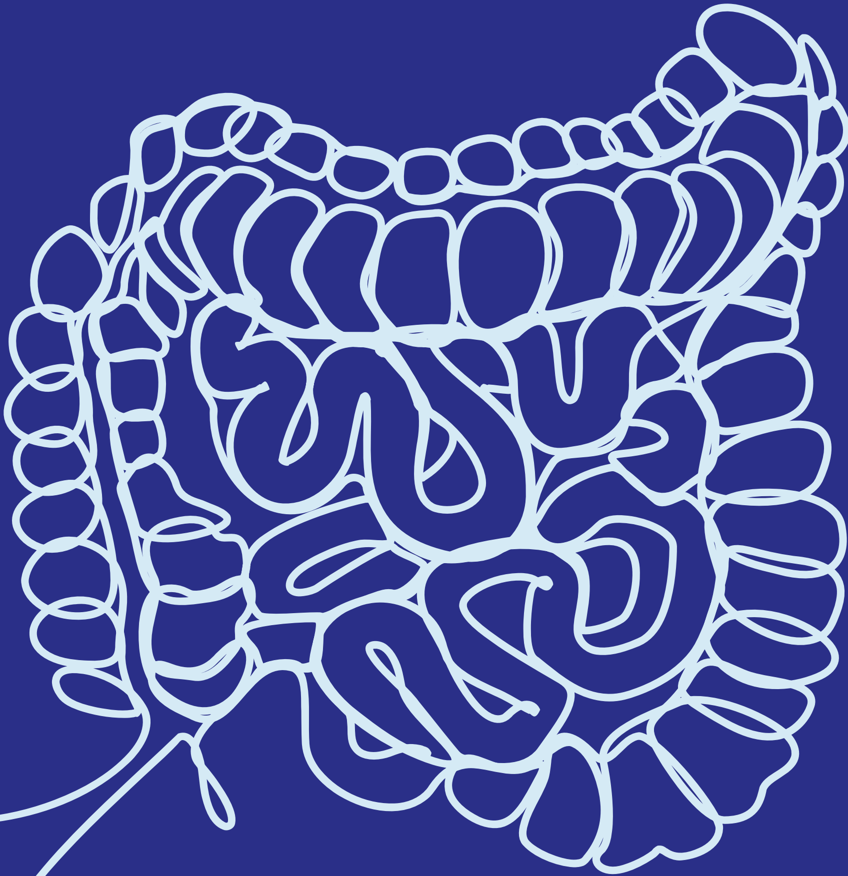
CHAPTER 7

An outbreak of *Clostridium difficile* infections due to a new PCR ribotype 826: epidemiological and microbiological analyses



Clinical Microbiology and Infection, 2018

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Abstract

Objectives. The aim was to investigate an unusual outbreak of 5 patients with in total 8 episodes of a *Clostridium difficile* infection (CDI) on a gastro intestinal surgical ward of a Dutch tertiary care university affiliated hospital.

Methods. Clinical case investigations and laboratory analyses were performed. Laboratory analyses included PCR ribotyping, MLVA typing, toxinotyping, antimicrobial susceptibility testing and whole genome sequencing.

Results. The outbreak was associated with recurrent and severe disease in 2 out of 5 patients. All episodes were due to a unique ribotype that was not recognized in the collection of an international network of reference laboratories and was assigned PCR ribotype 826. PCR ribotype 826 is a toxin A, toxin B and binary toxin positive ribotype which according to molecular typing belongs to clade 5 and resembles the so called “hypervirulent “ ribotype 078. The presence of a clonal outbreak was confirmed by whole genome sequencing, yet the source of this newly identified ribotype remained unclear.

Conclusion. This newly identified *C. difficile* PCR ribotype 826 is part of clade 5 and might as well have increased virulence. The recognition of this outbreak highlights the need of ongoing CDI surveillance to monitor new circulating ribotypes with assumed increased virulence.

Introduction

We identified an outbreak of eight episodes of CDI in five patients within a 4-month period (1 December 2015-31 March 2016). The outbreak occurred on a gastro-intestinal surgical ward of a Dutch tertiary care hospital. In this case series, we describe the clinical characteristics of affected patients and microbiological investigations that were performed on the identified strain.

Methods

The case series was conducted at a gastro-intestinal surgical ward of the Erasmus University Medical Center in Rotterdam, the Netherlands. The Erasmus MC participates in the national sentinel CDI surveillance program and therefore sends all samples from hospitalized CDI patients to the national Reference Laboratory for PCR ribotyping (I. K. Sanders *et al.*, paper presented at the 25th European

Congress of Clinical Microbiology and Infectious Diseases (ECCMID 2015), abstract P0793, 2015) (1). In case of an outbreak (defined as >2 isolates of the same type detected less than 7 days apart in one hospital either with onset of symptoms on the same ward, or accompanied by an increased CDI monthly incidence within the hospital: http://www.rivm.nl/Documenten_en_publicaties/Algemeen_Actueel/Uitgaven/Infectieziekten/CDiffNL/Tenth_Annual_Report_of_the_National_Reference_Laboratory_for_Clostridium_difficile_and_results_of_the_sentinel_surveillance), additional analyses can be performed by the Reference Laboratory. These include multiple-locus variable-number tandem-repeat analysis (2) , PCRs for toxin genes (3), PCRs for clade specific makers (4), antimicrobial susceptibility screening tests (E-test) and whole genome sequencing (5).

Patient information and medical history from all CDI cases during this outbreak were collected from the electronical medical records. Defined daily doses for all antibiotics used up to three months before development of CDI and Charlson co-morbidity scores were calculated. (6). CDI was classified as severe if one or more of the following conditions were present (attributable to CDI): fever (equal or above 38.5°C), rigors, hemodynamic instability, ileus, peritonitis, mental status changes, admission to ICU, end organ failure, leukocytosis (>15 x 10^9), leukopenia (<2 x 10^9), hypoalbuminemia (<30g/L), >1.5-fold increase in creatinine level above baseline, serum lactate >2.2mmol/L, pseudomembranous colitis,

colonic wall thickening, pericolonic fat stranding or ascites. All other cases were classified as mild CDI. (7, 8).

Written approval to conduct the case series was received from the medical ethics research committee of the Erasmus MC Rotterdam, the Netherlands (MEC-2015-306).

Results

The CDI incidence rate on the gastro-intestinal surgical ward was 3.3 per 10,000 patient days (July 2009-November 2015) and increased to 19.8 per 10,000 patient days (December 2015-March 2016). In total, 6 patients with CDI were diagnosed of which 5 had the same PCR ribotype.

The index case A of this outbreak was an 83-year old male patient who underwent a pancreaticoduodenectomy because of a carcinoma of the common bile duct one month earlier. In December 2015, during a readmission because of infected ascites, he developed diarrheal symptoms and was diagnosed with hospital-acquired CDI. Within one week after the start of his symptoms, two other patients (B and C) on the same ward were diagnosed with hospital-acquired CDI. All three patients were treated with a 7-11 day oral course of metronidazole and discharged.

In January 2016, a fourth hospital-acquired CDI case (D) on the ward was noticed. In February 2016, case A was readmitted because of a CDI recurrence and a fifth case (E) was reported. Case A was readmitted once more due to a second recurrence in February and case D was also diagnosed with a CDI recurrence in March. In total, 4 out of 8 CDI episodes (in 2 patients) were classified as severe CDI. None of the patients were admitted to the ICU due to CDI, and no CDI-related mortality (within 30 days) occurred. All patients had used antibiotics before acquiring CDI and total defined daily doses of antibiotics used before onset of CDI ranged from 21 to 63 (median 26.9). Four out of 5 patients had used proton pump inhibitors before the CDI diagnosis. The median Charlson co-morbidity score was 2, ranging from 0 to 8.

In accordance with local guidelines, all patients suspected of or having CDI were placed in a single room and were not allowed to use shared sanitation. Medical personnel wore protective disposable gowns and gloves when entering the room and handwashing with soap and water was endorsed. Isolation precautions were discontinued 48hours after

resolution of diarrheal symptoms. In reaction to this CDI outbreak, additional infection prevention measures were implemented on the ward during certain time periods (see Figure 1).These additional infection prevention measures included cleaning and disinfection using 1000ppm chlorine of the following items: automatic bedpan washer (daily), toilet chairs (after each use), utility room and sanitation (daily or twice daily) and all patients rooms of half the department (once, after recognition of the fifth case). Additionally, the metal bedpans were replaced by cardboard single use bedpans. Moreover, after the fifth case was diagnosed, 56 environmental swabs were taken on 2 different sampling days: February 19th and February 24th. Samples were taken from: sink, water tap, grip of cabinet, alarm system, dustbin, chairs/tables and bed curtains of a room that had been occupied by a CDI patient (before final cleaning); the same items in a clean room (after cleaning and disinfection with 1000pppm); and toilet, shower chair, sink, shower curtain, sack of laundry and towel dispenser of a shared bathroom (after cleaning and disinfection). Environmental swabs were inoculated in *C. difficile* enrichment modified broth (*Clostridium difficile* enrichment broth, Mediaproducts BV, Groningen, The Netherlands) for 1 week and subcultured on CLO plates (*Clostridium difficile* agar, Biomerieux, Marcy l'Etoile, France). No antibiotic restriction policy was implemented during this outbreak.

Stool samples of all 5 patients tested positive for toxin B and binary toxin genes in the Xpert *C. difficile*; however, the *TcdCΔ117* deletion specific for ribotype 027 was not identified. Investigations at the Reference Laboratory demonstrated the presence of *tcdA*, and confirmed the presence *tcdB* and the binary toxin genes. In addition, a 39-bp deletion in *tcdC* was detected.

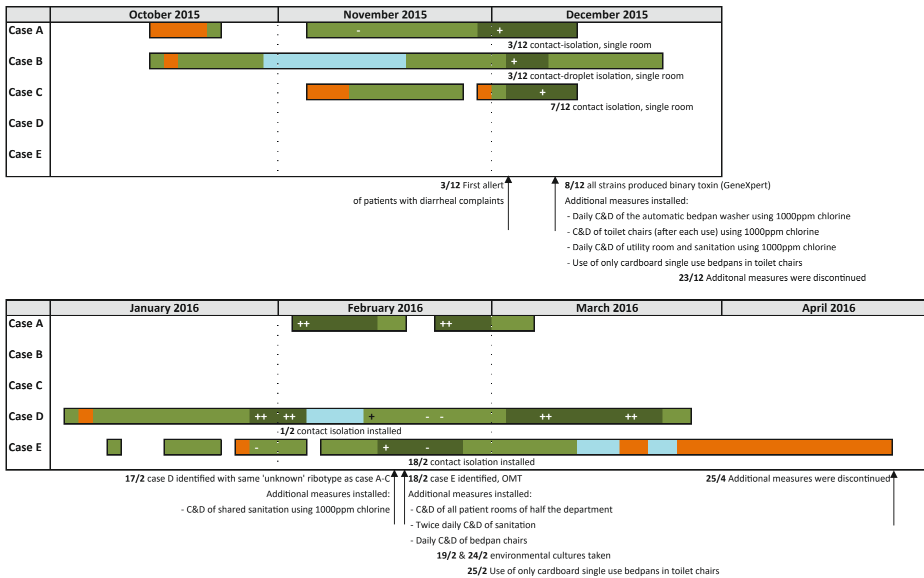

Figure 1. Epidemic curve of the 5 case patients infected with *C. difficile* caused by PCR ribotype 826
Green = outbreak non-ICU ward, Orange= other non-ICU ward, Blue= ICU, Dark green = diarrhoeal episode, White + = positive culture for *C. difficile* and mild *C. difficile* infection, White ++= positive culture for *C. difficile* and severe *C. difficile* infection, Black + = Positive *C. difficile* culture without diarrhoea, White - = Negative culture for *C. difficile*. C&D, cleaning and disinfection; ICU, intensive care unit; OMT, outbreak management team.

All 5 isolates and one isolate obtained from an environmental culture (taken from the sack of laundry in the shared bathroom after cleaning and disinfection) displayed the same PCR ribotyping profile. The profile was not recognized in the Dutch Reference Library (which is able to recognize 221 different PCR ribotypes) but resembled the profile of ribotypes 078, 126 and 066 most (all belonging to clade 5) (Figure 2a). A dataset of sized fragments obtained by capillary gel-based electrophoresis PCR ribotyping (1) was sent as FSA-file to international *C. difficile* reference laboratories (including the Leeds collection encompassing more than 800 PCR ribotypes , the WEBRIBO system, the CDC database and databases from Sweden, Portugal, Belgium, and Canada), but no match was found. The new strain was assigned as ribotype 826 by the Leeds Ribotyping reference network. PCR analysis of a clade 5 specific DNA marker (4) revealed that all ribotype 826 isolates were positive for the marker, confirming that ribotype 826 is part of clade 5.

According to Clinical and Laboratory Standards Institute (CLSI) breakpoints, all isolates were susceptible for erythromycin (MIC<2mg/L), clindamycin (MIC<2mg/L), metronidazole

(MIC=<2mg/L) and vancomycin (MIC<2mg/L), but resistant to ciprofloxacin (MIC>32mg/L) and moxifloxacin (MIC>32mg/L) (9).

The isolates were 100% identical with 0 summed tandem-repeat differences (STRD), thereby confirming a clonal complex according to MLVA.

In addition, whole genome sequencing was performed (Figure 2b). To provide phylogenetic context, reference strains 078, 126/078, 045, 033 and 066 and 4 patient samples from confirmed 078 cases were included. In total, 1678 SNPs were identified within this sample selection which is the expected variation between different ribotypes of one clade. Within the outbreak isolates, only 2 SNPs were identified (there was 1 SNP difference between the isolate from the recurrence in case A compared to the initial case A isolate and 1 SNP difference between the case D /case E isolates and the initial case A isolate). Clonality of these cluster isolates was thus confirmed by whole genome sequencing as the commonly used cut-off for classifying isolates as clonal is 0-2 SNPs (5).

Discussion

The occurrence of this CDI outbreak was uncommon as it occurred on a ward where transmission of *C. difficile* was rare, as proven by sentinel CDI surveillance. Also, two out of five patients had recurrent disease and were severely affected. Cases were due to a newly identified ribotype 826. Additional investigations showed that ribotype 826 belongs to clade 5 with a characteristic clade 5 specific DNA marker and a 39bp deletion in *TcdC*. Whole genome sequencing revealed that ribotype 826 resembles the ribotype 078 quite well. CDI cases due to clade 5 ribotypes have been reported to be associated with the highest 14-day mortality (10). We therefore assume that this new ribotype also has increased virulence, explaining the occurrence of this outbreak.

Whole genome sequencing results demonstrated clonality thereby confirming transmission, but still unanswered questions are what the source of this ribotype was and how transmission occurred. The index patient could have introduced this ribotype into the ward, although no unusual profession, recent travel or other remarkable expositions were reported. Alternatively, an undetected asymptomatic carrier might have introduced the ribotype and spread it to other patients. Transmission could have occurred via shared items as contamination was demonstrated in one of the environmental cultures, but unfortunately environmental swabs were only taken after the last patient was detected. The outbreak

ceased with the implementation of additional infection prevention measures, suggesting that these cleaning and disinfection measures were effective, probably together with a raised awareness among the healthcare workers. Since most PCR ribotypes of clade 5 are also found in animals, it is tempting to speculate that the newly recognised ribotype 826 derives from animals. The lack of this PCR ribotype in the databases of human collections supports this hypothesis. Unfortunately, reference laboratories for animal associated *C. difficile* infections are not available that could be used to match our isolates. To the best of our knowledge, no additional 826 isolates have been detected since this outbreak.

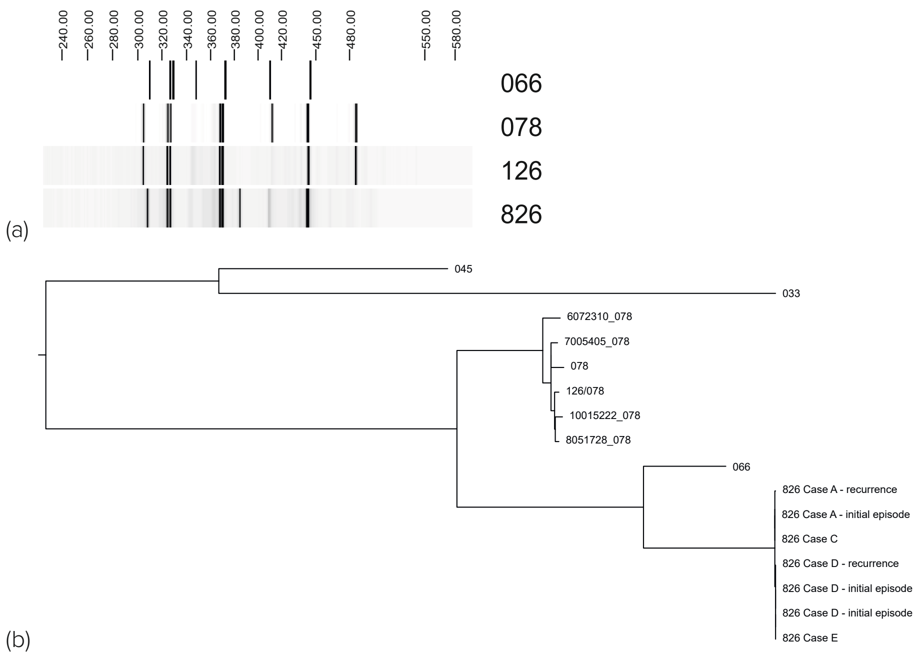

Figure 2. (a) PCR ribotyping patterns for ribotypes 066, 078, 126 and 826. Upper row indicates fragment sizes. **(b) Phylogenetic tree of ribotype 826 outbreak isolates and related ribotypes.** 078, reference ribotype 078 strain; 066, reference ribotype 066 strain; 045, reference ribotype 045 strain; 126078, reference ribotype 126/078 strain; 7005405_078/10015222_078, 8051728_078, 6072310_078, clinical patient CDI samples with confirmed ribotype 078; 4_826, sample from case A (recurrent episode); 3_826, sample from case A (initial episode); 6_826, sample from case C; 1_826, sample from case D (recurrent episode); 2_826, sample from case D (initial episode); 8_826, sample from case D (initial episode, repeat sample); 5_826, sample from case E. Isolate from case B could not be sequenced.

This outbreak indicates that new *C. difficile* ribotypes with increased virulence still emerge, at unexpected locations and without a clear source. Given the increased virulence and still unknown source of this newly identified ribotype, ongoing CDI surveillance remains essential and other institutions should now be aware of ribotype 826.

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Declarations of interest

MC, AV, CK, SvD, WB, CH, EK, MV: Nothing to declare.

Author's contribution

Epidemiological investigation and analysis: MC, AV, WB, EK, MV. Whole genome sequencing: CK. Outbreak management: WB, MV. All other laboratory investigations: CH. Surveillance data: SvD. Coordinated and supervised the study: EK, MV. All authors commented and agreed upon the final manuscript.

Part of this work was presented at ECCMID 2017, Vienna (oral presentation #OS0223).

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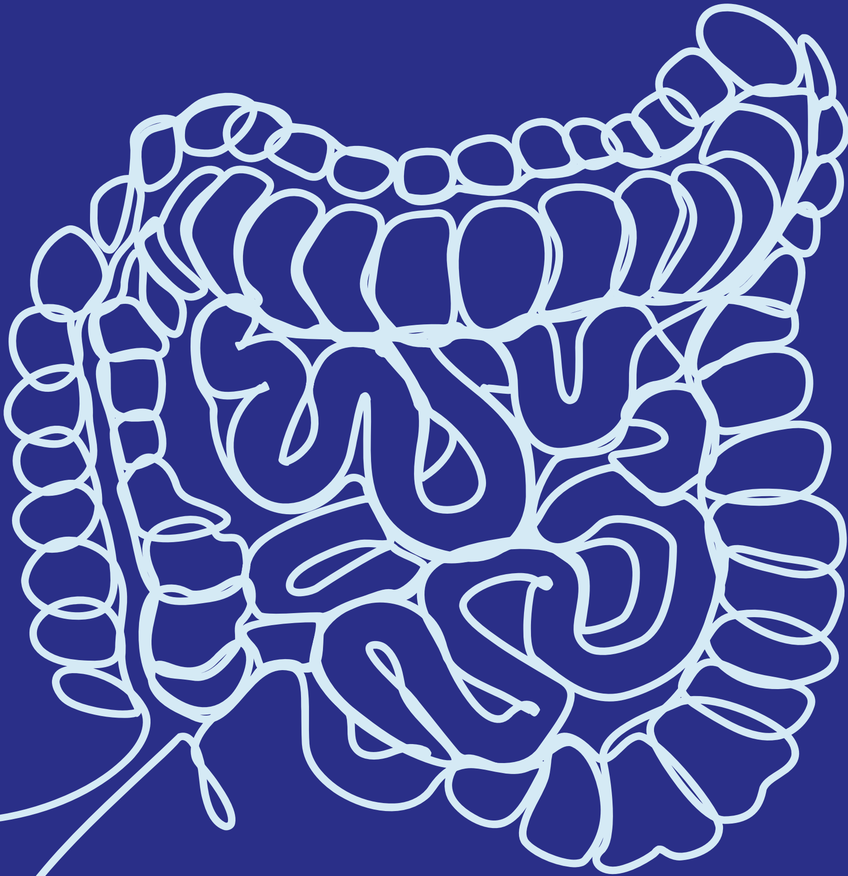
CHAPTER 8

Community-onset *Clostridioides difficile* infection in hospitalized patients in The Netherlands



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Abstract

Background. *Clostridioides difficile* infection (CDI) is increasingly reported in the community. The aim of this study was to analyze characteristics of hospitalized patients with community-onset CDI (CO-CDI).

Methods. In the Netherlands, 24 hospitals (university-affiliated and general hospitals) participate in the sentinel CDI surveillance program. Clinical characteristics and 30-day outcomes of hospitalized patients >2 years old diagnosed with CDI are registered. Samples of these patients are sent to the national reference laboratory for polymerase chain reaction ribotyping. Data obtained for this surveillance from May 2012 to May 2018 were used to compare CO-CDI with hospital-onset (HO)-CDI episodes.

Results. Of 5405 registered cases, 2834 (52.4%) were reported as HO-CDI, 2174 (40.2%) were CO-CDI, and 339 (6.3%) had onset of symptoms in another healthcare facility (eg, nursing home). The proportion of CO-CDI increased over the years and was lower during winter months. Hospitalized patients with CO-CDI were younger (63.8 vs 68.0 years, $P < .001$) and more often females (53.0% vs 49.6%, $P = .02$) than patients with HO-CDI. Median time between onset of symptoms and CDI testing was longer in CO-CDI (4 vs 1 day, $P < .001$). Similar ribotypes were found in CO-CDI and HO-CDI, but ribotype 001 was more frequent among HO-CDI, whereas ribotype 023 was more frequent in CO-CDI. Six of 7 (85.7%) surgeries due to CDI, 27 of 50 (54%) ICU admissions due to CDI, and 48 of 107 (44.9%) of CDI-associated deaths were attributable to CO-CDI.

Conclusions. Our study demonstrates that patients hospitalized with CO-CDI contribute substantially to the total number of CDI episodes and CDI-associated complications in hospitals, stressing the need for awareness and early testing for CDI in community and outpatient settings and also in patients admitted from community with diarrhoea. Surveillance programs that also target nonhospitalized CDI patients are needed to understand the true burden and dynamics of CDI.

Clostridioides difficile, formerly named *Clostridium difficile* (1), is a Gram-positive, anaerobic bacterium. Once a subject encounters *C difficile* spores, colonization and subsequent establishment of asymptomatic carriership or progression to symptomatic *C. difficile* infection (CDI) is possible, especially when the gut microbiota is altered and protective humoral immunity is diminished. (2, 3) During the last decades, *C. difficile* epidemiology has changed, and outbreaks due to so-called "hypervirulent" strains were reported in many countries. (4) Although CDI is often a healthcare-associated disease, it has recently been recognized that onset of symptoms is often in the community, after hospital discharge. Moreover, community-associated CDI, in patients without recent hospital admission, is increasingly recognized. (5, 6) In response to the outbreaks in the beginning of this millennium, many countries implemented compulsory or voluntary surveillance programs, to monitor CDI incidence rates and circulating ribotypes. (7) In the Netherlands, a voluntary CDI surveillance program has been implemented since 2009. Data from this sentinel surveillance program were used to compare characteristics and outcomes between patients with reported community-onset symptoms and patients with hospital-onset of symptoms.

Materials and methods

The Dutch sentinel CDI surveillance program is conducted by the Centre for Infectious Disease Control of the National Institute for Public Health and the Environment (RIVM) in collaboration with the Leiden University Medical Centre, where the national *C. difficile* reference laboratory is housed. Between 2012 and 2018, 24 acute care hospitals located across the country have been participating in the voluntary surveillance, both university-affiliated hospitals (n = 6) and general hospitals (n = 18). In these hospitals, all CDI cases in hospitalized patients >2 years old are registered. Patients who are not admitted to the hospital, but treated as outpatients or by their general practitioner, are not included in our surveillance program. Each year, an annual report is compiled, which can be found on the website of the RIVM. (8) For this analysis, all patients registered for the Dutch sentinel CDI surveillance from May 2012 to May 2018 were considered.

Definitions

Definitions valid for our surveillance program were also used for this study and are shown in Table 1.

Table 1. Definitions used in this study.

CDI	*Clostridoides difficile* infection. Diarrhea OR toxic megacolon in combination with a test positive for *C. difficile* toxins or toxigenic *C. difficile* and no alternative explanation for the diarrhea, or evidence of pseudomembranous colitis diagnosed by endoscopy, surgery or histopathology
Severe CDI	CDI with (1) bloody diarrhea, (2) pseudomembranous colitis, (3) dehydration and/or hypoalbuminemia (<20g/L) or (4) fever >38.0°C and leukocytosis (>15.0 X 10^9/L)
Community-onset CDI (CO-CDI)	CDI in all patients who had onset of symptoms at home and were admitted because of or with these symptoms , regardless of whether there had been a previous hospital admission or not
Hospital-onset CDI (HO-CDI)	CDI in all patients who developed symptoms during hospital admission and were consequently diagnosed with CDI
Outbreak	>2 isolates of the same type detected less than 7 days apart in one hospital either with onset of symptoms on the same ward, or accompanied by an increased CDI monthly incidence within the hospital
Recurrence	A CDI episode occurring between 2 to 8 weeks after a previous episode
Complicated course	If ICU admission or surgery due to CDI or any mortality including CDI attributable mortality within the first 30 days after CDI diagnosis occurred
CDI attributable mortality	Mortality in a CDI patient in the absence of other comorbidities that would normally have led to death

CDI, *Clostridioides difficile* infection; ICU, intensive care unit.

Data collection

Clinical characteristics of all CDI cases were collected via a web-based questionnaire by the local medical microbiologist or infection control practitioners. Collected data included age, gender, date of sample submission, location of symptom onset (ie, community, hospital, nursing home, or other healthcare facility), severity of CDI, and 30-day outcomes. Antibiotic use at the moment of hospital admission or during admission preceding the submission of the stool sample was also registered (in terms of yes/no).

Microbiological analyses

At each participating hospital, CDI was diagnosed per local protocol. Diagnostic strategies changed over the years. In May 2013, 58% of participating hospitals relied on stand-alone Tox A/B enzyme immune-assay (Tox A/B EIA), whereas 26% of hospitals relied on a stand-

alone nucleic acid amplification test (NAAT). By May 2017, 54% of the participating hospitals had switched to the use of an ESCMID-recommended algorithm (either NAAT or glutamate dehydrogenase [GDH] EIA followed by confirmatory Tox A/B EIA or Tox A/B and GDH EIA as a first step optionally followed by NAAT or toxigenic culture) (9), whereas 33% of hospitals now (2017) relied on NAAT as stand-alone assay. Cultured *C. difficile* isolates identified by matrix-assisted laser desorption/ionization time-of- flight analyzer (MALDI-TOF) or fecal samples (if no culture was performed at the local hospital) were sent to the national reference laboratory. At the national reference laboratory, samples were recultured on *C. difficile* selective agar supplemented with cefoxitin amphotericin B and cycloserine (CLO medium; bioMérieux), with and without ethanol shock pre- treatment. (10) After incubation in an anaerobic environment at 37°C for 48 hours, suspicious colonies were tested by an in-house GDH polymerase chain reaction (PCR) to confirm the presence of *C. difficile*. (10) Polymerase chain reaction ribotyping was performed as previously described. (11) At the moment, the national reference laboratory is able to recognize 263 different ribotypes and collaborates with Leeds University hospital (Dr. Warren Fawley and Prof. Dr. Mark Wilcox) to expand the collection with clinical relevant types.

Statistical analysis

Characteristics between patients with community-onset CDI (CO-CDI) and hospital-onset CDI (HO-CDI) were compared with t test, $\chi 2$ test, or Mann-Whitney U test where appropriate. *Clostridioides difficile* infection incidence and HO-CDI incidence were calculated as the number of cases per 10 000 patient-days. The proportion of patients with CO-CDI and HO-CDI was calculated, also over the different years and seasons. Five hospitals (2 university-affiliated and 3 general hospitals) from different regions in the Netherlands with monthly admission data available were selected to study seasonal trends in CDI and HO-CDI for the years 2012/2013, 2014/2015, 2016/2017, and 2017/2018. A logistic regression analysis with CO-CDI as outcome variable and year, respectively, and season as covariate was performed to determine whether there was any annual or seasonal variation. For this regression analyses, 2 groups were created: all patients with CO-CDI were counted as such, and patients with HO-CDI or onset in another healthcare facility were counted as non-CO- CDI. Patients with missing location of onset were excluded for this analysis. In addition, a logistic regression analysis with CO-CDI as outcome variable was performed with age, gender, recurrence (yes/no), season, and year as covariates to determine factors independently associated with CO-CDI. A sensitivity analysis was performed in which the definition of CO-CDI was extended to include all patients who developed CDI symptoms

within the first 2 days of hospital admission. $P < .05$ were considered statistically significant. All analyses were performed using STATA SE statistical software, version 15.1 (StataCorp, College Station, TX).

Results

Between May 2012 and May 2018, a total of 5405 CDI cases were registered in 24 participating hospitals. The annual mean incidence of CDI in hospitalized patients was 3.11 cases per 10 000 patient-days, ranging from 3.06 to 3.26 cases per 10 000 patient- days over the 5 years. The mean incidence of HO-CDI was 1.63 (95% confidence interval [CI], 1.57–1.69) and ranged from 1.75 (95% CI, 1.59–1.92) in 2012–2013 to 1.53 (95% CI, 1.39–1.68) in 2017–2018. During the 6-year period, the prevalence of the so-called hypervirulent PCR ribotype 027 decreased from 3.4% (95% CI, 2.0–4.8) in 2012–2013 to 0.6% (95% CI, 0.1–1.1%) in 2016–2017. In this sentinel surveillance program, only 1 outbreak (7 patients) due to ribotype 027 was reported over the study period, in May 2013. Other outbreaks reported during the study period were due to ribotype 001 (2 outbreaks), ribotype 015 (1 outbreak), and ribotype 826 (1 outbreak). (12) The outbreaks affected 3 to 33 patients (median 4.5 patients). Of 5405 episodes, location of CDI symptoms onset was missing for 58 episodes. Of the remaining 5347 episodes, 2174 (40.2% of total) were CO-CDI, 2834 (52.4%) were HO-CDI, and 339 (6.3%) had onset of symptoms in a healthcare facility other than a hospital (162 in a nursing home and the other 177 not specified). Over the years, the proportion of CO-CDI increased from 35.4% in 2012–2013 to 44.9% in 2017–2018 (Figure 1). In univariate analysis, the proportion of CO-CDI cases was significantly higher in the last 4 years compared to the first year of the study period: odds ratio (OR) = 1.24 (95% CI, 1.01–1.51) for 2014–2015, OR = 1.28 (95% CI, 1.05–1.56) for 2015–2016, OR = 1.22 (95% CI, 1.00–1.48) for 2016–2017, and OR = 1.43 (95% CI, 1.17–1.75) for 2017–2018, respectively.

During winter months, the proportion of HO-CDI was 60.7%: this fell to 53.1% in spring, 51.3% in summer, and 52.2% in autumn (data from 1045 CDI cases from 5 hospitals) (Figure 2). The seasonal variation in proportions of HO-CDI, CO-CDI, and episodes with onset in another healthcare facility over the years is shown in Figure 3. In univariate analysis, the proportion of CO-CDI was significantly lower in winter compared to the 3 other seasons: OR = 1.28 (95% CI, 1.10–1.49) for spring, OR = 1.42 (95% CI, 01.22–1.66) for summer, and OR = 1.27 (95% CI, 1.08–1.48) for autumn.

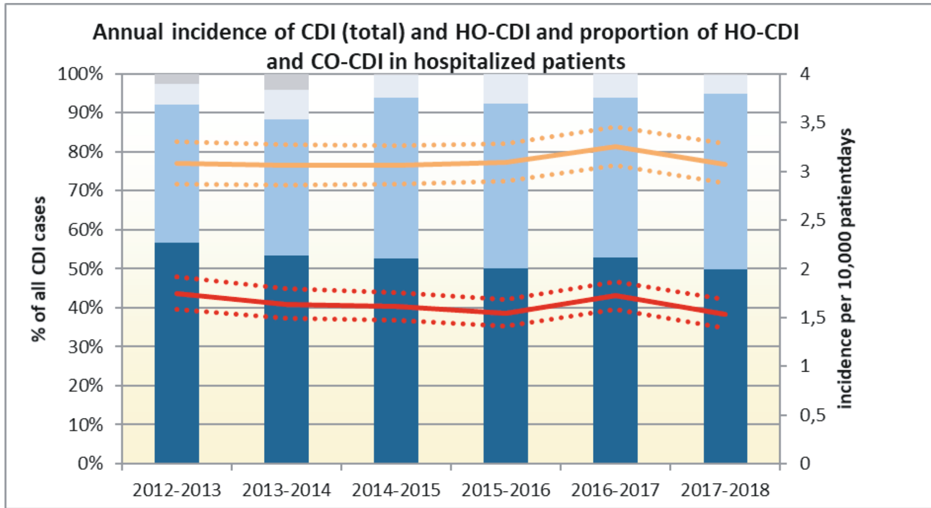

Figure 1. Proportions of hospital-onset *Clostridioides difficile* infection ([HO-CDI], dark blue), community-onset *C. difficile* infection ([CO-CDI], middle blue) and episodes with onset in another healthcare facility (light blue) in hospitalized patients over the years, and incidence of CDI (total, orange line) and HO-CDI (red line) in hospitalized patients. (Data are from all 5405 cases, 2012-2018)

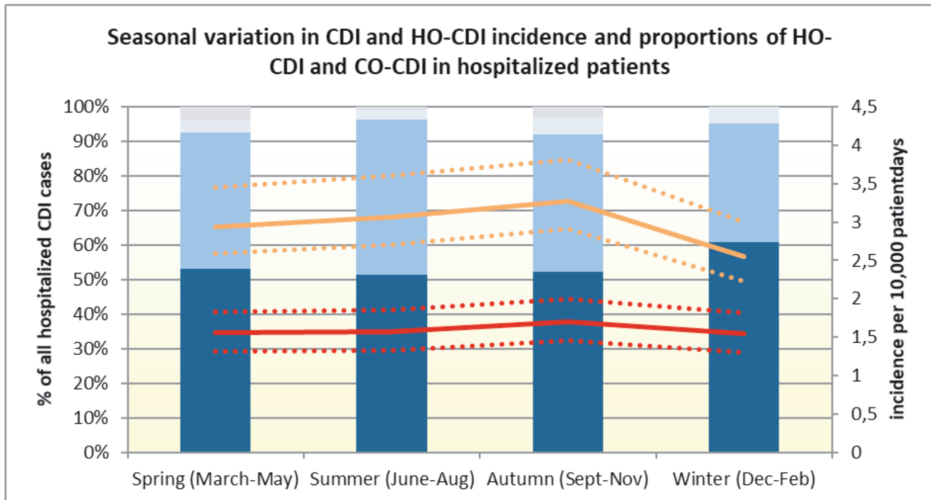

Figure 2. Proportions of hospital-onset *Clostridioides difficile* infection ([HO-CDI],dark blue), community-onset *C. difficile* infection ([CO-CDI], middle blue) and episodes with onset in another healthcare facility (light blue) in hospitalized patients over the seasons, and incidence of CDI (total, orange line) and HO-CDI (red line) in hospitalized patients. (Data are from 5 hospitals, 1045 cases, for 2012/2013, 2014/2015, 2016/2017 and 2017/2018)

During the study period, 953 episodes were reported to be severe CDI: 662 (69.5%) of these were CO-CDI and 331 (34.7%) of these were HO-CDI. In total, 7 patients required surgery due to CDI; 6 of 7 (85.7%) of these surgeries were performed in CO-CDI episodes. Twenty-seven of the 50 (54%) intensive care unit (ICU) admissions due to CDI was reported in CO-CDI, and 48 of 107 (44.9%) of all CDI-associated mortality could be attributed to CO-CDI episodes.

When characteristics were compared between CO-CDI and HO-CDI, we found that patients with CO-CDI were younger than patients with HO-CDI (63.8 vs 68.0 years, P = .000) and more often females (53.0% vs 49.6%, P = .02) (Table 2). Of CO-CDI episodes, 472 of 1409 (33.5%) were classified as a recurrence. In contrast, only 16.2% of patients with HO-CDI had a CDI episode in the previous 2–8 weeks (P = .000 compared with CO-CDI). The median time between onset of symptoms and submission of a stool sample for CDI testing was 4 days (interquartile range [IQR], 2–11) in CO-CDI patients and 1 day (IQR, 0–2) in HO-CDI patients. Antibiotics were used by 2096 of 2517 (83.3%) HO-CDI patients, and 49.5% of patients with CO-CDI were using antibiotics at admission (P = .000).

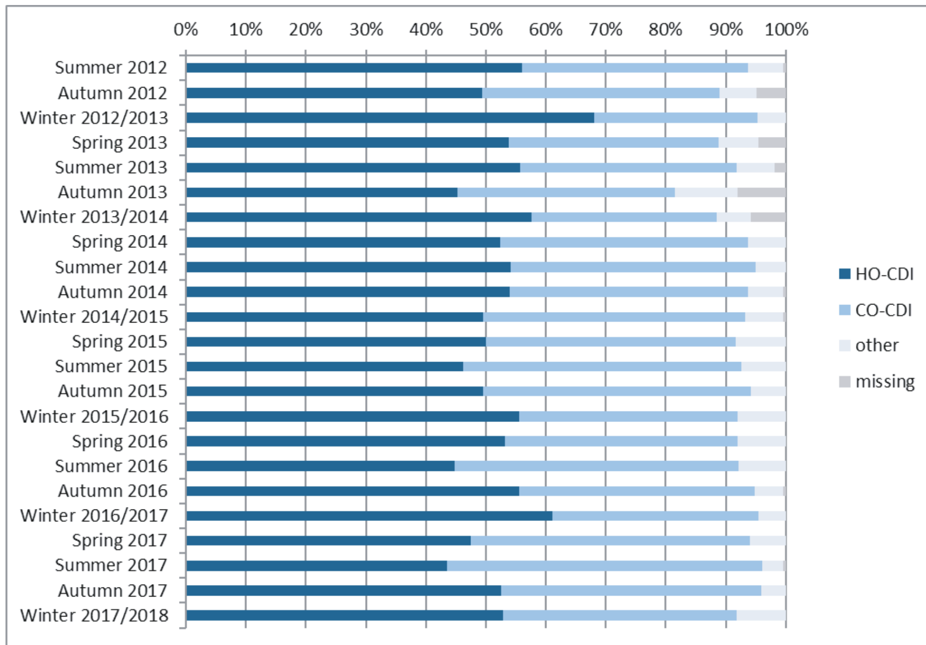

Figure 3. Proportions of hospital-onset *Clostridioides difficile* infection (HO-CDI), community-onset *C. difficile* infection (CO-CDI) episodes with onset in another healthcare facility over the seasons and years in hospitalized patients. (Data are from 5186 cases, 2012-2018)

Clostridioides difficile infection was more frequently reported to be severe in CO-CDI episodes (30.2% vs 12.8%, P = .000). The percentage of patients who required an ICU admission due to CDI was comparable among the 2 groups (1.4% vs 1.0%, respectively; P = .15), and CDI-associated mortality was also similar (2.6% vs 2.5%, P = .86). However, HO-CDI episodes more often had a complicated course than CO-CDI episodes (13.8% vs 10.5%, P = .001), and this was mainly due to a higher overall mortality in the HO-CDI group (12.8% vs 8.7% in the CO-CDI group, P = .000).

In multivariate logistic regression, younger age, spring/ summer/autumn season, female gender, and recurrent episode were associated with CO-CDI (Table 3). In multivariate analysis, study year was not associated with CO-CDI.

Table 2. Characteristics of CDI episodes in community-onset *Clostridoides difficile* infection ([CO-CDI], n=2174) and hospital-onset *C. difficile* infection ([HO-CDI], n=2834)

	CO-CDI	HO-CDI	p-value
Mean age (SD)	63.8 (20.5)	68.0 (17.5)	<0.001
Male (%)	1021/2173 (47.0)	1429/2834 (50.4)	0.02
Previous CDI <8 weeks (%)	472/1409 (33.5)	304/1575 (16.2)	<0.001
Median days onset symptoms-sample submission (IQR)	4 (2-11)	1 (0-2)	<0.001
Severe CDI (%)	622/2057 (30.2)	331/2262 (12.8)	<0.001
colitis	97/2057 (4.7)	47/2593 (1.8)	<0.001
dehydration	321/2057 (15.6)	164/2429 (6.3)	<0.001
bloody diarrhea	170/2057 (8.3)	40/2593 (1.5)	<0.001
fever	207/2057 (10.1)	135/2593 (5.2)	<0.001
Complicated course (%)	197/1879 (10.5)	331/2392 (13.8)	0.001
Surgery due to CDI	6/1879 (0.3)	1/2392 (0.04)	0.03
ICU admission due to CDI (%)	27/1879 (1.4)	23/2392 (1.0)	0.15
CDI-associated 30-day mortality (%)	48/1879 (2.6)	59/2392 (2.5)	0.86
Overall 30-day mortality (%)	164/1879 (8.7)	307/2392 (12.8)	<0.001

IQR, interquartile range; SD, standard deviation

Ribotyping results were available for 4068 episodes. The most common ribotypes in CO-CDI episodes were 014/020, 078/126, and 002. The most common ribotypes in HO-CDI were 014/020, 078/126, and 001 (Figure 4). Ribotype 001 was more frequently found in HO-CDI episodes than in CO-CDI episodes (10.0% vs 6.1%, P = .000), and ribotype 023 was more frequently found in CO-CDI (3.5% vs 2.1%, P = .006). After excluding the year 2016–2017 (in which a RT001 outbreak occurred), the results remained significant. Ribotype 027

was found in 20 of 1758 (1.1%) CO-CDI samples and 42 of 2310 (1.8%) of HO-CDI samples (P = .08).

In the sensitivity analysis, 440 patients who developed CDI symptoms within the first 2 days of admission were categorized as CO-CDI instead of HO-CDI. Just like in the main analysis, patients with CO-CDI were younger and more often females and experienced severe and recurrent CDI more frequently (data not shown).

Table 3. Multivariate analysis of variables associated with community-onset *Clostridioides difficile* infection (CO-CDI)

Variable	Odds ratio (95% CI)
Age (each year increase)	0.99 (0.98-0.99)
Gender	
male	1 (reference)
female	1.19 (1.03-1.36)
Recurrence	
no	1 (reference)
yes	2.08 (1.78-2.44)
Season	
Winter	1 (reference)
Spring	1.43 (1.18-1.74)
Summer	1.57 (1.29-1.92)
Autumn	1.26 (1.04-1.54)
Year	
2012-2013	1 (reference)
2013-2014	0.76 (0.57-1.01)
2014-2015	1.01 (0.77-1.31)
2015-2016	0.96 (0.73-1.24)
2016-2017	0.94 (0.72-1.21)
2017-2018	1.03 (0.79-1.34)

CI, confidence interval

Discussion

In this 6-year period of sentinel CDI surveillance in 24 acute care hospitals in the Netherlands, we found that 40.2% of 5405 hospitalized patients with CDI reported onset of symptoms in the community. This observation is in line with earlier studies; proportions ranging from 36% to 58% have been described, although studies in the higher range included patients with symptom onset in the first 48 hours of admission as CO-CDI. (10, 13-15) When we extended our definition of CO-CDI to all patients with symptoms in the community or within the first 2 days of admission, 48% of patients were classified as CO-CDI, and CO-CDI was thereby more frequent than HO-CDI. The incidence of HO-CDI was relatively stable over the seasons, but CO-CDI was less often observed during winter. The higher proportions in spring, summer, and autumn for CO-CDI might indicate that CO-CDI is less related to the winter peak in antibiotic use or that CO-CDI development is delayed in these patients. Antibiotic use was indeed less frequently reported in CO-CDI compared with HO-CDI. However, only antibiotic use at moment of admission was included, and no data about previous antibiotic treatment (eg, in the last 3 months) were available.

In our study, we only analyzed hospitalized patients, because this is the targeted group of our CDI surveillance program, similar to surveillance programs in many other countries. Therefore, our cohort consisted of all patients with HO-CDI and patients with CO-CDI who were subsequently admitted to the hospital. It is understandable that mostly patients with severe or difficult-to-treat CO-CDI were admitted to the hospital. The restriction to admitted patients introduces collider bias, a specific form of selection bias. Included patients were either patients with HO-CDI or patients with severe or recurrent CO-CDI, necessitating admission. Patients with mild or moderate CO-CDI are therefore underrepresented in the cohort. Despite this limitation, we think that our study demonstrates several important findings.

The majority (65.3%) of severe CDI episodes in hospitalized patients had a community-onset. This demonstrates that although we do not know which proportion of CO-CDI episodes requires hospital admission, their share in severe cases in the hospital is substantial. *Clostridioides difficile* infection- related complications were often reported in CO-CDI episodes: the majority (6 of 7, 85.7%) of all surgery due to CDI could even be attributed to CO-CDI episodes. A direct comparison of CO-CDI versus HO-CDI episodes shows that in these hospitalized CO-CDI episodes, CDI was more often reported to be severe and that CDI attributable mortality was comparable between CO-CDI and HO-CDI. However, it is

unclear to what extent these observations would change if all CO-CDI episodes would have been included. For example, a recent US study, in which both inpatients and outpatients were enrolled (instead of only patients with CO-CDI and subsequent hospital admission), did find a higher disease severity in patients with HO-CDI, on the contrary. (16)

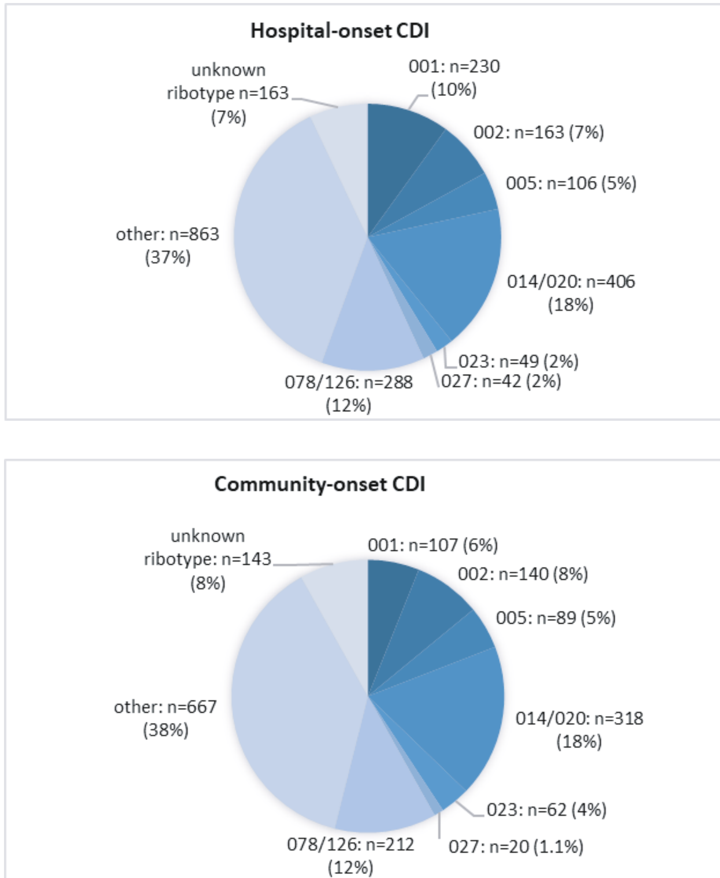

Figure 4. Ribotype distribution in hospital-onset Clostridioides difficile infection (HO-CDI) (upper) and community-onset C. difficile infection (CO-CDI) (lower) in hospitalized patients. (data are from 4068 samples with PCR ribotyping result available, 2012-2018: 1758 CO-CDI episodes and 2310 HO-CDI episodes)
CO-CDI; community onset CDI, HO-CDI: hospital-onset CDI, CDI; *Clostridium difficile* infection.

Patients with CO-CDI were younger and more often females than patients with HO-CDI, and we thereby confirm findings from previous studies. (13, 14, 17) The younger age in CO-CDI may reflect younger mean age in the community than in the hospital, but once more indicates that not all patients with CDI have the traditional risk factors such as age >65 years. The higher proportion of females in the CO-CDI group has been suggested to be a reflection of a more care-seeking behavior in females compared with males and, therefore, a higher chance of being diagnosed and hospitalized with CDI. (17, 18) Similar to other studies (13), the median delay between onset of symptoms and submission of a stool sample was higher in CO-CDI (median 4 days) than in HO-CDI (1 day) in our cohort. Of note, the delay may both be caused by a patient's delay in seeking medical attention and lack of clinical CDI suspicion by healthcare workers.

During the study period, CDI was found at an endemic rate with a low rate of RT027 and only a few (mostly limited) outbreaks, thereby minimizing the impact of hospital outbreaks on the proportions of CO-CDI versus HO-CDI and minimally impacting retrieved ribotypes in both groups. Although similar ribotypes were identified in CO-CDI and HO-CDI, ribotype 001 was more frequently found in HO-CDI. Polymerase chain reaction ribotype 001 is one of the most common types found in the Dutch surveillance program (ranging from 3.4% to 10.2% in the last 5 years). On the other hand, RT023 was more frequently found in CO-CDI. RT023 contains all 3 toxins (TcdA, TcdB, and CDT) and was recently reported to be associated with severe disease (Shaw HA, Preston MD, Vendrik KEW *et al.*, unpublished observations, 2019). However, a community source for RT023 has not been identified yet and among asymptomatic *C difficile* carriers, RT023 is not frequently found, making them an unlikely reservoir for RT023 (Crobach MJT, manuscript in preparation). (19)

The proportion of CO-CDI seemed to be increasing over the years, although this was not significant in multivariate analysis. It is unfortunate that in the Dutch CDI surveillance, no information about previous healthcare contact is registered. Therefore, we do not know whether the reported (increasing) CO-CDI cases were due to (an increase in) true community-acquired cases (CA-CDI, defined as CDI and no previous healthcare admission within the previous 12 weeks) or community-onset healthcare facility-associated cases (CO-HCFA-CDI, defined as CDI with onset of symptoms in the community or within 48 hours of admission and a healthcare admission within the previous 4 weeks). (7, 20, 21) In the Netherlands, the mean length of stay (LOS) in hospitals has dropped from 8.5 days in 2000 to 5.2 days in 2012, and it remained relatively stable from 2012 onwards. Because hospital LOS has decreased over the years, adverse events including hospital-acquired

CDI (HA-CDI) presenting as diarrhea with a community onset may increasingly become apparent only after discharge. Studies have shown that HA-CDI is especially frequently reported within the 4 weeks after discharge (15, 18) when patients have been exposed to *C. difficile* spores in the hospital environment (22) and possible inpatient and postdischarge antibiotic treatment may have led to a disruption of the protective microbiota. A large-scale US study including data from 2000 to 2007 demonstrated that HA-CDI was the reason for readmission in 1.8% of 170 995 readmissions and was thereby even more reported than HA-CDI that occurred during the initial admission, suggesting that most HA-CDI cases may now be occurring after discharge. (18) Another US study performed in 2011 confirmed that a large proportion of HA-CDI occurs postdischarge, by demonstrating that of 188 900 HA-CDI cases, 57% had onset of symptoms during hospitalization, whereas 43% developed symptoms in the community. (23) Of note, a large proportion of these HA-CDI cases (31%) were due to the NAP1/RT027 strain, which may have been a major reason why many patients developed CDI after being exposed to *C. difficile* during admission. In addition, in Denmark, 46% of HA-CDI cases registered in 1 Capital Region in Denmark in 2010–2015 had community-onset of symptoms. (24) However, lower proportions of CO-HA-CDI have been reported in other studies (16, 25), indicating local differences in frailty of the studied population, hospital LOS, CDI awareness in the hospitals or community, circulating ribotypes, and differing healthcare systems. Besides the reported increase in HA-CDI occurring postdischarge, CA-CDI incidence has also been reported to have increased over the last years. (26) The reported rate of CA-CDI in hospitalized patients was 14% in a European study in 2008. (27) In 2016, 5756 of 7711 (74.6%) of episodes registered for European CDI surveillance led by European Centre for Disease Prevention and Control (ECDC) were HA-CDI, whereas the remainder were either CA-CDI or CDI of unknown origin. (28) It is interesting to note that CA-CDI is often reported in patients who lack specific risk factors for CDI. (5, 29) Whether the increase in CA-CDI is a true increase, or also partly determined by a higher awareness of CDI in the community in patients without typical risk factors, is difficult to ascertain.

Conclusions

In conclusion, in an endemic CDI setting, we found that 40% of hospitalized CDI patients reported community-onset symptoms. A substantial proportion of CDI complications is caused by CO-CDI. Therefore, healthcare workers, especially general practitioners, should be aware of CDI (recurrences) with onset of symptoms in the community and consider CDI testing. Because only hospitalized CDI patients are included in our (and many other

including ECDC) sentinel surveillance programs, the importance of CO-CDI (which includes both CA-CDI and CO-HA-CDI) as a public health concern remains unclear. Therefore, we think that additional studies in the community and amongst outpatients are needed to elucidate this.

Acknowledgments

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Potential conflicts of interest

All authors: no reported conflicts of in- terest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest.

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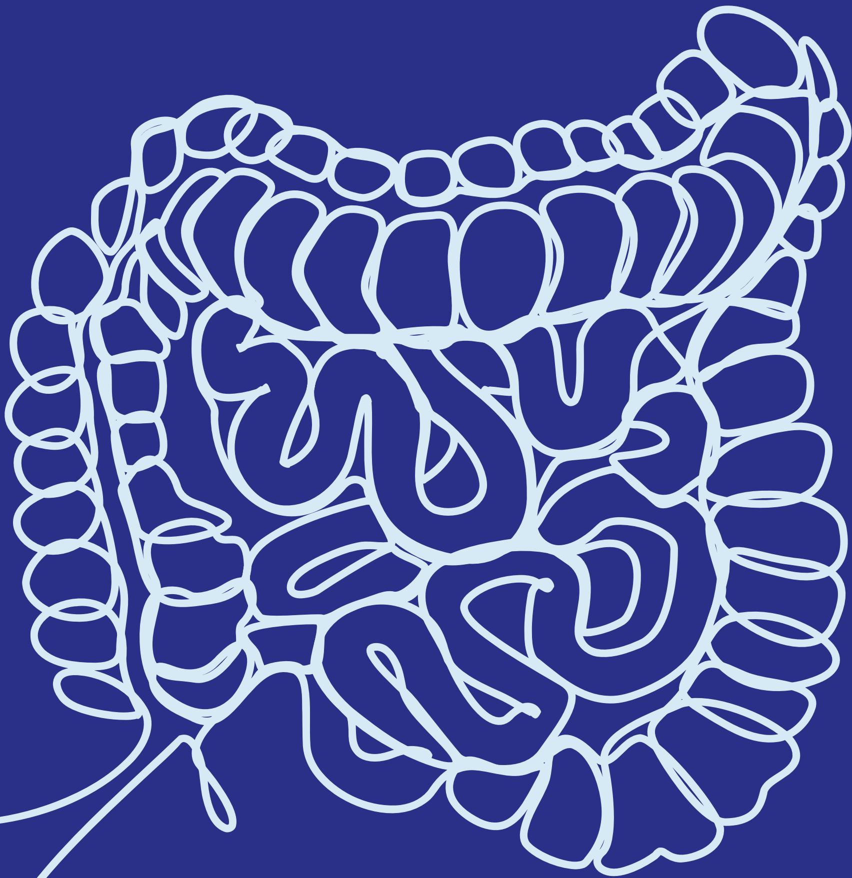
8

CHAPTER 9

Summary and general discussion



Before putting the findings from this thesis in perspective, we will summarize our main findings.

Main Findings

In CHAPTER 2, an extensive review of the literature on diverse aspects of *C. difficile* colonization (CDC) is provided. As there are no uniform criteria for CDC, we started our review by clearly defining CDC, also to be able to discern colonization from infection (Table 1). As patients with continuous or prolonged colonization may very well differ from patients who are found to carry *C. difficile* only at one point in time, we further subdivided colonization in transient and persistent colonization (Table 1).

Table 1. Definitions for *C. difficile* infection (CDI) and *C. difficile* colonization (CDC)

C. difficile infection (CDI)	presence *of C. difficile* toxin (ideally) or a toxigenic strain type and clinical manifestations of CDI[1]
C. difficile colonization (CDC)	detection of the organism in the absence of CDI symptoms
transient	*C. difficile* detected at one point in time
persistent	*C. difficile* detected at several points in time

[1] Clinical presentations compatible with *Clostridioides difficile* infection include diarrhea (defined as Bristol stool chart types 5 to 7 plus a stool frequency of three stools in 24 or fewer consecutive hours, or more frequently than is normal for the individual), ileus (defined as signs of severely disturbed bowel function, such as vomiting and absence of stool with radiological signs of bowel distention), and toxic megacolon (defined as radiological signs of distention of the colon, usually to ≥10 cm in diameter, and signs of a severe systemic inflammatory response)

Not only definitions, but also the diagnostic methods to establish CDC are not clearly described in the literature. When considering how to test for CDC, it is important to first assess how CDI should be diagnosed, as incorrect use of laboratory assays for CDI detection can cause overdiagnosis of CDI and underestimation of CDC. In CHAPTER 3, updated recommendations for diagnosing CDI are provided. After publication of the first ESCMID recommendations for diagnosing *C. difficile* infections (1), NAATs had become commercially available and were more and more used as the test of choice, often as stand-alone assay. To better define the role of NAATs in CDI diagnosis, there was a need for updated recommendations. These recommendations were based on a literature review and meta-analysis of studies evaluating test performances of commercial CDI assays. Results showed that although NAATs are highly specific, their positive predictive values (PPV) will be too low at low pre-test probability (as will be the case in stool samples submitted for CDI testing). We therefore recommended against the use of NAATs for stand-alone use. In

line with the previous ESCMID guidelines, we recommended to combine two assays in an algorithm to decrease the percentage of false-positive results. The algorithm should start with an assay with a high negative predictive value (NPV) (i.e. a highly sensitive test) that reliably classifies samples with a negative test result as non-CDI. All samples with a first positive test result will be tested with a second assay with a high PPV (i.e. a highly specific test) that reliably classifies samples with a positive test as CDI. Both GDH EIA and NAAT are suitable as the first assay in the algorithm as they are sensitive assays. The second assay could preferably be a Toxin A/B EIA as these are among the most specific assays and have the additional benefit of detecting free toxins (thought to correspond to clinical disease). (2) Some pitfalls in CDI diagnosis were not addressed in the ESCMID guidelines. These include for example the awareness that PCR RT023 produces colourless colonies on the chromogenic agar ChromID and the possibility of CDI due to *C. difficile* strains that are positive for binary toxin only (and hence will not be detected with most commercial NAATs). (3) These exceptions once again illustrate that interpretation of CDI test results may not always be straightforward.

Although algorithmic testing offers the most accurate CDI testing method, the drawback is that samples need to be tested by multiple assays which may delay diagnosis. Therefore, in CHAPTER 4 we determined whether quantitative results of a NAAT (when used as a first assay in a two-step algorithm) could predict the result of the subsequent Toxin A/B EIA assay. In NAAT, lower cycle quantification (*Cq*) values correspond to higher bacterial counts, and we hypothesized that lower *Cq* values would also be predictive of the presence of toxins. For the analysis, we used a collection of samples submitted for CDI testing to two hospital laboratories (n=2669 and n=1718, respectively). Moreover, we included samples from patients with asymptomatic CDC on hospital admission (samples were derived from the CDD study, see CHAPTER 6). We found significantly lower *Cq* values in stool samples that tested positive for toxins. Using receiver operating characteristic curve analysis, we demonstrated that with the optimal *Cq* cutoff values, prediction of the toxin A/B EIA results was accurate for 78.9% and 80.5% of samples in hospital 1 and 2, respectively. We concluded that *Cq* values can indeed serve as predictors of toxin status, and might possibly aid in establishing a preliminary diagnosis. Yet, due to the suboptimal correlation between the two tests, additional toxin testing is still needed. Interestingly, comparable *Cq* levels were found in CDC patients and diarrheal patients testing negative for toxins, suggesting that these groups are indeed only colonized by *C. difficile* and that the latter group represented patients colonized by *C. difficile* with diarrhea due to another cause

than CDI. Our results also show that reliance on NAAT as stand-alone test may lead to overdiagnosis of CDI and underestimation of CDC.

In **CHAPTER 2**, we clarified which assays that are traditionally used for diagnosing CDI can also be applied to detect CDC. A specific concern when assessing CDC is that bacterial counts may be lower than in CDI, and therefore the most sensitive methods – like culture- should be used. Also, it is very important to confirm the absence of symptoms suggestive of CDI to prevent confusion between these two conditions. Further in this discussion, we will elaborate more on the risks of confusion between CDC and CDI depending on test methodology and the consequences hereof.

With the realization that there are patients who are colonized with *C. difficile*, but do not demonstrate symptoms, a question arose: which mechanisms allow for colonization but protect against symptomatic disease? The immune system is thought to play an important role. Antibodies directed to the proteins on the surface of *C. difficile* may protect against colonization. (4-6) On the other hand, antibodies directed to the toxins of *C. difficile* may protect against disease by neutralization of toxins. (7) By limiting the detrimental effects of the toxins on the gut epithelium, these latter antibodies may also add in a faster restoration of colonization resistance. (8) Besides the immune system, the gut microbiota seems to play an important role in the susceptibility for *C. difficile* colonization and infection. At the start of this thesis, only a few small studies had characterized the microbiota of patients with asymptomatic CDC. (9, 10) These studies and mouse study results suggested that colonization could be established in microbiota with a decreased species richness and decreased microbial diversity. The presence of specific bacterial taxa was thought to protect from progression to CDI. The paucity of available data on this topic made us decide to characterize the microbiota of colonized patients. In addition, we wanted to compare the microbiota of colonized patients with that of non-colonized patients and symptomatic CDI patients, to be able to identify which microbiota composition is associated with resistance or susceptibility to CDC and CDI. For this analysis, we used a subset of samples obtained during the CDD study (see **CHAPTER 6**): 41 samples from colonized patients and 43 samples from controls. The third group consisted of 41 samples of symptomatic CDI patients obtained for sentinel surveillance purposes. Gut microbiota composition in these three groups was determined using 16S rRNA gene amplicon sequencing of which the results are described in **CHAPTER 5** of this thesis. Bacterial diversity was decreased both in CDC and CDI patients, but the microbiota composition in CDC patients differed from that in CDI patients. The genus *Veillonella* was more abundant in CDI patients, and

also found to be positively associated with *C. difficile* in colonized patients. *Veillonella* may therefore indicate susceptibility to colonization and infection by *C. difficile*. *Eubacterium hallii* and *Fusicatenibacter* were more abundant in control patients than in colonized patients. Also, *Fusicatenibacter* was negatively associated with *C. difficile* in CDI patients. Thus, *Eubacterium hallii* and *Fusicatenibacter* may indicate resistance against CDC and subsequent infection. In CHAPTER 5, we also speculated on the underlying mechanisms for these findings, i.e. the influence of these microbiota members on bile acid metabolism. Although we could not demonstrate causality in this study, the identification of these specific genera may be useful for future studies.

Another part of this thesis focuses on the implications of CDC. Prior to the start of my research, a number of studies had shown that a considerable part of new CDI cases could not be explained by transmission from other known CDI cases. (11, 12) In one study 29% of new CDI cases were associated with asymptomatic *C. difficile* carriers, although this may have been an overestimation as the typing method in this study (MLVA) may have been not discriminative enough. (13) However, the interest in other possible *C. difficile* reservoirs and specifically *C. difficile* colonized patients as the source for new CDI cases grew. Patients who are already colonized at hospital admission gained special interest as it was already known that these patients can introduce *C. difficile* into the hospital and transmit it to other patients. (14) In studies, rates of asymptomatic CDC among patients at hospital admission were reported to range from 3% to 21% (CHAPTER 2). Risk factors for CDC at hospital admission that were identified in these studies included recent hospitalization, chronic dialysis, corticosteroid/ immunosuppressant use, gastric acid suppressant use and antibodies against toxin B. (15-17) Apart from being a potential source for onwards transmission, several studies also pointed towards a higher risk for patients colonized by toxigenic strains to subsequently develop CDI. (18) In order to estimate the prevalence of CDC in the Netherlands and study onwards transmission from asymptomatic carriers, we designed the CDD study (*Clostridoides difficile* colonization study, ***C**lostridioides **d**ifficile* **d**ragerschap studie in Dutch). We thought that this study would provide valuable information on CDC prevalence and *C. difficile* transmission in a setting where CDI is endemic with a stable CDI incidence and low prevalence of 'hypervirulent' strains. Result from the CDD study are described in CHAPTER 6 of this thesis. In this multicentre study, we were able to screen 2211 patients in 4 hospitals within 72hrs of hospital admission. We found that CDC was present in 4.9% (108/2211) of admissions, while colonization with toxigenic *C. difficile* strains (tCDC) was present in 3.1% (68/2211) of admissions. To evaluate the consequences of CDC, patient were followed up for progression to CDI. None of the colonized patients

developed CDI during admission or one-year follow-up (one-year follow-up available for 38 colonized patients). In addition, isolates from patients colonized by toxigenic strains were compared with isolates from patients that were diagnosed with CDI during the study period. Core genome MLST (cgMLST) demonstrated no definite transmission from tCDC patients to CDI cases. Only one probable onwards transmission event from a CDC patient was detected as two patients with genetically identical strains shared a ward before the first patient was found to be colonized and the other was diagnosed with CDI. Moreover, the only transmission between multiple symptomatic patients detected by cgMLST was a PCR RT826 cluster that was already detected via sentinel surveillance. This unusual outbreak is described as an outbreak report in **CHAPTER 7** of this thesis. The outbreak involved five patients, of whom two had recurrent disease. Clinical case investigations and microbiological analyses including whole genome sequencing showed that all episodes were due to clonal spread of a unique ribotype that was never recognized before. The newly identified ribotype was assigned PCR RT826. This new ribotype resembles the 'hypervirulent' PCR RT078, belongs to clade 5 and carries all three toxin genes (*tcdA*, *tcdB* and binary toxin genes). No definitive source for this newly identified strain could be demonstrated. Yet, the absence of this ribotype in international databases of *C. difficile* strains found in humans and the observation that most ribotypes of clade 5 can also be found in animals (19), made us speculate that this newly identified ribotype might have derived from an animal source. Either the index patient, an undiagnosed CDI patient or an asymptomatic carrier might have introduced this strain into the ward, where it was further transmitted among susceptible patients. Since this outbreak, *C. difficile* PCR RT826 has neither been found in sentinel surveillance samples or in outbreak studies in the Netherlands, indicating that rapid recognition and early implementation of additional infection control measures are important to prevent further spread within the hospital and to other healthcare facilities.

As discussed in **CHAPTER 6**, screening for CDC on admission like we performed in the CDD study was time-consuming and burdensome but did not detect patients that contributed to *C. difficile* epidemiology by progression to symptomatic CDI. At most one patient contributed to *C. difficile* epidemiology by onwards transmission. Therefore, we concluded that screening for CDC at hospital admission is of little value in an endemic setting with low prevalence of 'hypervirulent' ribotypes.

C. difficile transmission extends beyond the hospital and CDI is increasingly reported in the community (20, 21).

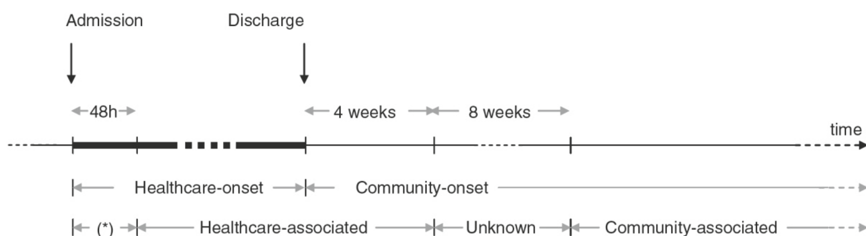


Figure 1. Definitions for community-associated and healthcare-associated *Clostridioides difficile* infection (CDI).
Adopted from Kinross *et al.* (22)

In CHAPTER 8, we used data obtained for our national sentinel CDI surveillance program from 2012 to 2018 to analyse characteristics of CDI patients with community-onset of symptoms (CO-CDI) and subsequent hospitalization. CO-CDI can be community-associated or healthcare-associated (see Figure). (22) In total 2174/5405 (40.2%) of hospitalized patients with CDI had onset of their symptoms in the community. This proportion increased over the years. There was a delay in testing for CDI in community-onset episodes compared to hospital-onset episodes (median time between start of symptoms and CDI test four days vs one day, $P < .001$). PCR RT001 was more frequently found in patients with hospital-onset episodes, while RT023 was more frequently found in community-onset episodes. Although results from this study should be interpreted with caution as the design (i.e. only including patients with hospital-onset CDI or patients with CO-CDI who necessitated hospital admission) led to collider bias, we did demonstrate that CO-CDI episodes contribute considerably to the total CDI burden in hospitals as 6/7 surgeries, 27/50 ICU admissions and 48/107 CDI-associated deaths were reported in community-onset episodes. Surveillance programs that also target non-hospitalized patients will give a better impression of the true burden of CDI inside and outside the hospital. Extending surveillance to the community may also provide valuable information about transmission patterns and shed new light on other *C. difficile* sources, including colonized patients.

Challenges in *C. difficile* colonization studies

When performing or interpreting studies on *C. difficile* colonization, several challenges are encountered, including diagnostic procedures, representativeness of study cohorts and duration of carriage.

Diagnostic procedures

When studying CDC in humans, the first hurdle to overcome is to establish a correct diagnosis of CDC.

The various assays that are available for diagnosing CDI are also applied in studies where CDC is the condition of interest. Culture has been applied in studies investigating CDC, with or without determination of the toxin-producing potential of the recovered isolate (ie, toxigenic culture). As bacterial counts may be lower than in CDI, sensitive culture media should be used or a broth enrichment culture should be applied. (23) A major advantage of using culture is that recovered isolates can be used for (ribo)typing and transmission investigations. Nowadays most studies that do not focus on transmission patterns use NAAT as the assay of choice to detect CDC. NAAT has a good sensitivity and specificity compared to the gold standard toxigenic culture and is therefore a reasonable faster and less labor-intensive method. Most NAATs detect conserved regions within *tcdB*, and hence only toxigenic strains are detected. As both TC and NAAT detect the presence of a toxigenic *C. difficile* strain, the presence of diarrhea should be ruled out to be confident that these patients are not actual CDI patients.

Colonized patients are assumed to have toxin negative stools. Therefore, Tox A/B EIA and CCNA are an illogical choice if CDD is the condition of interest as these assays detect free toxins instead of the presence of the organisms. However, it is not always as clear-cut as that. In infants, a positive CCNA in the absence of clinical symptoms may be indicative of CDC as toxin presence does not always seem to correlate with clinical symptoms in this age group. (24) But also in adult patients with asymptomatic CDC, positive CCNA results can be found: in one study 30/77 rectal swabs from CDC patients tested positive by CCNA. (25) Despite these observations, there is still no rationale for the use of a Tox A/B EIA or CCNA in studies investigating CDD as has been previously done (26): although some colonized patients with a positive assay but absence of diarrhea can be diagnosed in this way, the majority of colonized patients will have no toxin production and will go unnoticed.

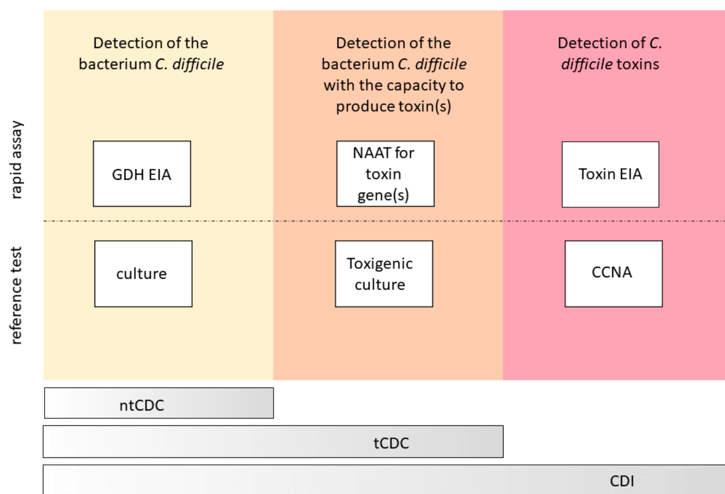


Figure 2. Assays used for detection of *C. difficile* colonization and diagnosing *C. difficile* infection. The lower bars indicate which category of assays will give positive results in these three conditions. CDI, *Clostridioides difficile* infection; CCNA, cell cytotoxicity neutralization assay; EIA, enzyme immunoassay; GDH, glutamate dehydrogenase; NAAT, nucleic acid amplification test; ntCDC, *C. difficile* colonization with a non-toxigenic strain, TC, toxigenic culture; tCDC, *C. difficile* colonization with a toxigenic strain.

In many studies investigating CDC rectal or even perirectal swabs are used instead of stool samples. The use of (peri)rectal swabs has the advantage of obtaining samples in a timely manner without the need to wait for bowel movements. As testing for CDC in studies is often performed as part of a screening program timely results are needed, especially if isolation precautions are imposed on colonized patients. (27) Several studies have demonstrated acceptable sensitivity when culture (28, 29), or NAAT (30-32) are performed on (peri)rectal swabs. Swabs in these studies were however taken from symptomatic patients or even swabbed from *C. difficile* positive stool samples. As the mean density of *C. difficile* in stool sof asymptomatic carriers has been reported to be 100-fold lower than for CDI patients, perirectal swabs might be less suitable for detecting CDC. (33) In a screening study in asymptomatic patients, NAAT on perirectal swabs was shown to have a positive predictive value of 75.2% compared to toxigenic culture on the same perirectal swabs, with part of the NAAT positive toxigenic culture negative results explained by the receipt of *C. difficile* inhibitory antimicrobials. (34) An alternative method to enhance *C. difficile* detection from perirectal swabs in low density samples is an enrichment broth prior to NAAT testing. (35) Compared to toxigenic culture from stool samples, sensitivity and specificity for this test method from perirectal swabs were 100% and 99.1% respectively. (35) At the moment, large

studies that compare direct NAAT or TC on perirectal swabs versus on stool samples in asymptomatic patients are lacking. In many studies, the advantages of rapid and more complete sampling by the use of (peri)rectal swabs will outweigh the drawback of their possible suboptimal sensitivity. Choices for testing methods will therefore merely depend on study aims and resources, but when appraising the literature it is important to be aware of these limitations. Of note, the procedure for taking rectal swabs is often not specified in studies which makes it even more difficult to interpret if true rectal swabs (e.g., placing the swab into the rectum, rotating and removing the swab) or (non-invasive) perirectal swabs were taken.

In studies comparing infected with colonized patients, reliance on NAAT testing could unintentionally deplete the colonized group of less healthy subjects. In these studies, the absence of diarrhea is often a prerequisite for being classified as colonized instead of infected. However, CDC patients may very well develop diarrhea due to non-CDI reasons and excluding these patients from the colonized group may affect microbiota and epidemiological studies.

When estimating the risk for colonized patients to progress to CDI, a correct diagnosis of CDI is also essential. Although diagnostic guidelines have been published **(CHAPTER 3)** (36), recommended CDI testing algorithms are frequently not in place, neither in daily practice nor in studies. Data from ECDC CDI surveillance show that in 2016, ESCMID recommended algorithms were not used in 28.5% of surveillance periods in participating hospitals (ECDC 2018). In the Netherlands, most recent data show that 41% of hospitals participating in national CDI surveillance do not use an ESCMID recommended algorithm; 36% of hospitals relied on stand-alone NAAT. (37) Also in studies, reliance on stand-alone NAAT testing is common, although large studies have shown that NAAT positivity (ie without toxin positivity) does not correlate with clinical outcome and is therefore not indicative of true CDI. (2, 38) Although low cycle threshold (CT) values by NAAT testing could indicate toxin positive patients, toxin testing is still needed due to the suboptimal specificity of this approach **(CHAPTER 4).** (39) Refraining from free toxin testing can introduce misclassification of colonized patients as CDI and can inflate incidence rates. This has been shown in the LUCID study in which CDI testing policies across 60 hospitals in three European countries (UK, France, Italy) were investigated (40). In hospitals that used methods that do not detect free toxin, mean CDI positivity rates were 2.5-fold higher than in hospitals using a recommended algorithm for CDI testing. Annual CDI incidence rates were also significantly higher: 5.2/10,000 patient-days in hospitals not detecting toxin

versus 2.0/10,000 patient-days in hospitals using a recommended algorithm. (40) When CDC patients are monitored for development of subsequent CDI, omitting free toxin testing in case of suspected CDI may very well overestimate the risk to develop CDI. Still, several studies using NAAT or toxigenic culture for detection of CDC permit a positive result with the same assay to be diagnostic for CDI once the patient develops diarrhea. (41-43) For example, in the study by Blixt and colleagues in which 3605 patients were screened for CDC on admission, a high risk of developing CDI during hospitalization was found for patients colonized by toxigenic *C. difficile* (9.4% versus 2.3% in non-colonized patients). (43) However, both *C. difficile* colonization and infection were based on a positive NAAT, with the only difference the absence or presence of diarrhea at moment of testing. Studies that omit free toxin testing for CDI diagnosis mostly require that other reasonable explanations for diarrhea are absent before considering diarrhea due to *C. difficile*, but clinical judgment can be burdensome especially in situations where there is a high chance of developing diarrhea. In a study performed in an ICU setting, CDC at ICU admission was associated with a strongly increased risk for development of CDI (relative risk 10.3 compared to non-colonized patients), but as both CDC and CDI were based on NAAT testing, there is a chance that these figures are inflated due to the many other reasons for diarrhea in the ICU setting, which were probably not apparent. (44)

Given the above, it is clear that the wide range of available assays and testing strategies induce heterogeneity between studies. Interpreting study results therefore should always include a thorough assessment of methods used to diagnose CDC (and CDI). The above also highlights that not only CDI diagnosis should be based on laboratory assays in combination with clinical symptoms, but that the same holds for CDC diagnosis. Studies that are not only based on laboratory data but also include clinical evaluation have added value.

Representativeness of study cohorts

Collecting fecal samples for studies on CDC can be burdensome. In our CDD study (CHAPTER 6), over 5000 patients agreed to participate in the study, but we only received stool samples in 42% of them. Similar figures are reported in other studies that tried to obtain stool samples at hospital admission: a UK and US study managed to obtain samples in 132/227 (58%) and 320/729 (43%) of consenting patients, respectively. (15, 16) These suboptimal collection rates may impact the representativeness of study cohorts. In the US study by Leekha, patients who submitted a stool sample were older and had more often a history of recent hospitalization, antibiotic use or residency in a long-term care

facility. (16) On the other hand, in the study by Eyre, age was not different among patients who did or did not submit a stool sample, but patients who did submit stool samples more often had loose stools, a non-hematological malignancy or use of gastric acid suppressant medication. (15) To be less dependent on participants' bowel habits, the use of rectal swabs has been applied in several studies. In a large Canadian study investigating colonization on admission, rectal swabs were used in case no stool sample could be obtained. (17) With this approach, they were able to collect a sample from 5232/5422 (96%) of consenting subjects. Although this rate is significantly higher than in studies collecting stool samples, only 5422/9502 (57%) of eligible patients agreed to participate in the study. If this was due to the invasive character of the rectal swab procedure or venipuncture that was scheduled on the day of admission, remains speculative.

Moreover, a greater or smaller subset of patients of interest is classified ineligible depending on the exclusion criteria of the specific study. For example, patients admitted for palliative care or with hemodynamic instability are generally excluded. In studies using rectal swabs, additional exclusion criteria like neutropenia or thrombocytopenia are often applied. In most studies, patients with an anticipated short hospital stay are excluded, although definitions of short stay may range from 24 hours to 5 days. (27, 45) Given the subset of patients deemed ineligible and the difficulties in obtaining consent for rectal swabs or obtaining stool samples, one should consider that in most studies only a small proportion of patients on hospital admission are included in the final study cohort. In the aforementioned Canadian and US study, 5232/12304 (43%) and 320/1464 (22%) of newly admitted patients were finally included, respectively. (16, 17) Colonization rates may be influenced by this selection, although the magnitude remains unclear. Except from a possible effect on colonization rates, suboptimal inclusion rates may hamper the interpretation of studies that are investigating transmission patterns. In studies that investigate onwards transmission from colonized to symptomatic patients, new *C. difficile* introductions into the ward will be missed if not all newly admitted patients can be screened on admission. Hence, the contribution of colonized patients to *C. difficile* epidemiology may be underestimated.

The difficulties in obtaining consent and samples can be overcome if *C. difficile* screening can be implemented in another obligatory screening program. This was done in one study where patients undergoing VRE surveillance testing were also screened for *C. difficile*. (13) The drawback of this approach, however, is that screening was only performed in patients with known risk factors for VRE -which may not be the same for *C. difficile*- i.e., if admitted from another healthcare facility or if admitted to the ICU. In 2013, an intervention consisting

of detecting and isolating of CDC patients was endorsed in the Quebec Heart and Lung Institute in Quebec in response to high healthcare-associated CDI incidence rates. (27) Patients admitted directly to the ward or with an anticipated stay <24hrs were ineligible due to logistical reasons. Aside from these patients, 7599/8218 (93%) of eligible patients could be screened during the intervention. Although a comparable approach seems the most promising to achieve high inclusion rates, implementing such a program for study purposes cannot be justified in most situations.

Apart from the low inclusion rates encountered in most studies, another issue that may impact the representativeness of the results is the in- or exclusion of patients with previous CDI. In- or exclusion criteria for patients with previous CDI differ between studies and this may effect representativeness and induce heterogeneity between studies. These differing criteria may very well impact colonization rates and the risk for patients to progress to CDI among studies, as patients with previous CDI do shed spores for prolonged times and are at risk for developing recurrent CDI. (46, 47)

In the CDD study **(**CHAPTER 6**)**, we also encountered the difficulties in obtaining samples. For example, we decided not to exclude patients with an anticipated short hospital stay as our goal was to estimate the total number of colonized patients at admission and to include all isolates that could be transmitted to other patients. However, our attempt to enroll all these patients was not successful as many patients were already discharged before a stool sample could be obtained. We therefore think that our work cannot be used to make definitive conclusions on the precise contribution of colonized patients in *C. difficile* epidemiology in our setting. We do think however, that we demonstrated that *C. difficile* screening in an endemic situation like ours does not detect a significant amount of introductions of *C. difficile* in the ward that are the source for new *C. difficile* infections.

Duration of carriage and study design

CDC is not a static condition, instead studies have shown that colonization is often lost without targeted interventions. (45, 48) Also, a surveillance study in healthy subjects in the community showed that among those subjects who remained *C. difficile* positive, this was often not due to retainment of the same strain. (49)

In fact, the detection of *C. difficile* in stools may not even imply true colonization, but may also be indicative of pass-through of the bacterium without it establishing true colonization.

In an experimental study, administration of a suspension of non-toxigenic *C. difficile* spores after oral vancomycin pretreatment led to persistent CDC in 44% of healthy volunteers 14-21 days after the last dose of spores. (50) However, *C. difficile* was also temporarily found in stools of patients who were not pretreated with oral vancomycin 2 days after their last dose but not beyond, indicating transient pass-through of ingested *C. difficile* rather than colonization in this group. Or, alternatively, these patients without antibiotic pretreatment did develop less intense colonization below the threshold of detection. As most studies accept a single *C. difficile* positive sample diagnostic of CDC, the group of CDC patients in most studies constitutes a heterogenic population of patients with colonization, transient carriage and pass-through. This has implications when risk factors for CDC are investigated, as risk factors for truly colonized patients may very well differ from risk factors for patients with pass-through – who might not have risk factors at all.

Also, when appraising the risk of colonized patients to progress to CDI, the case-mix of patients in studies may impact the perceived risk as patients with transient CDC might have another risk for progression than patients with longstanding CDC.

For transmission studies, this heterogeneity of the study population is probably less important. When studying introductions of *C. difficile* strains into the hospital environment and their onward transmission, the total burden of patients carrying *C. difficile* at that moment should be included, as all these patients contribute to the shedding of spores. However, infection pressure will depend on the duration of colonization and subsequent shedding of spores, too.

C. difficile colonization revisited

Data from this thesis will be combined with recent literature to elaborate on the role of *C. difficile* colonization in the epidemiology of *C. difficile.*

Role of colonized patients in *C. difficile* epidemiology: progression to CDI

Previous studies have shown that patients colonized by toxigenic strains on hospital admission have a higher risk to progress to CDI during admission, with a pooled 5.9-times higher risk than non-colonized patients. (18) In a recent large study on this topic 19112 patients were screened for CDC on admission (as part of a *C. difficile* admission screening

and isolation program) and development of CDI during or after discharge was retrieved for all colonized patients. (51) In total 7.6% of 513 colonized patients developed CDI during hospital stay (median onset of symptoms 4 days after admission), and an additional 3.6% of patients who did not develop CDI during admission eventually developed CDI after admission. Another large study in which 3605 patients were screened on admission also found that patients with tCDC were at a higher risk of developing CDI: 20/213 (9.4%) patients with tCDC versus 76/3251 (2.3%) of non-colonized patients developed CDI. (43)

In our study, we could not confirm this high risk for tCDC patiens to develop CDI. This could be due to the fact that we were only able to include a limited number of colonized patients. However, differences in study setting may also impact the risk of colonized patients to progress to CDI and should be considered here.

In contrast to the two large studies mentioned before, our study was performed in a setting with a low CDI incidence overall, and more specifically a low PCR RT027 incidence: during the study, this ribotype was not found among infected or colonized patients. In the context of high PCR RT027 infection rates, colonization rates by this ribotype appear to be increased, too. (33, 52) Patients who acquire *C. difficile* PCR RT027 during admission will more often develop symptomatic CDI than remain asymptomatically colonized. (52), indicating that the virulence of the acquired strain can affect the likelihood to develop colonization or infection. If this also implies that once colonized with PCR RT027 there is still a higher chance to progress to CDI is unclear. However, as RT027 is often acquired during hospital admission (52, 53), patients colonized by PCR RT027 may represent a subgroup of patients with recent acquisition of this strain during a previous admission. Recent acquisition of *C. difficile* in itself was shown to be more strongly associated with development of CDI than pre-existing colonization. (54)

In our study, patients were only enrolled from regular medical and surgical wards; intensive care units or bone marrow transplant units were not included. On these latter wards, several risk factors that were found to increase the risk to progress to CDI may be more prevalent, including increasing length of stay, exposure to multiple classes of antibiotics, use of opioids and cirrhosis. (51) Overrepresentation of these risk factors may explain the high risk of progression to CDI in these studies. However, the fact that a substantial part of patients admitted to these wards will eventually develop diarrhea during the course of admission (often due to a variety of causes other than CDI) may also interfere with assessing

progression to CDI and may have inflated numbers, especially if less specific methods to diagnose CDI are used.

From the above we can speculate that colonization with toxigenic *C. difficile* poses patients at a higher risk to progress to CDI if certain factors are present. These factors might either be pathogen factors like the virulence of the acquired strain (i.e. PCR RT027) or host factors like microbial perturbation through use of antibiotics.

Role of *C. difficile* colonized patients: onwards transmission

Skin and environmental contamination with *C. difficile* spores is frequently found in asymptomatic carriers. (33) Therefore, onwards transmission from these asymptomatic patients might occur, but the importance of this transmission route is also very likely to depend on the setting. In the CDD study – although hampered by methodological constraints as explained before- colonized patients did not seem to play an important role in onwards transmission. This finding contrasts with studies where 6% to 29% of new CDI cases could be linked to asymptomatic carriers (13, 55), or where the amount of exposure to colonized patients correlated with the risk to develop CDI. (43)

From the literature, it is known that different *C. difficile* ribotypes have different modes of transmission. Within hospitals, PCR RT027 strains of CDI patients are often clonal or genetically related indicating higher levels of in-hospital transmissibility. (56) Onwards transmission from colonized to infected patients was also found to be frequently due to PCR RT027. (55, 57) This may be explained by a higher transmissibility of this strain due to more profuse shedding and more effective persistence of spores in the hospital environment. (58) Alternatively, the observed increased onwards transmission for this strain may be explained by the fact that patients who acquire this strain more often develop symptoms, thereby increasing detection.

In situations where infection pressure due to PCR RT027 diminishes, one might imagine that other transmission patterns become more important, like transmission from patients who are colonized on admission with non-healthcare-associated strains. In our endemic setting, patients on hospital admission were colonized with a variety of *C. difficile* strains, indicating diverse reservoirs. However, onwards transmission from these colonized patients was not detected. Possibly, these strains may less often be transmitted or may not (directly) lead to symptoms once transmitted. When diarrhea develops after discharge, CDI may not be

captured as patients might refrain from consulting their general practitioner or may not be tested for CDI in the community setting. (59) Alternatively, our study observation may be due to incomplete sampling of admitted patients in combination with the low transmission rate for a single asymptomatic carrier. (15, 60)

When putting the above in perspective, we acknowledge that colonized patients can be a source for onwards *C. difficile* transmission especially when ribotypes with higher transmissibility are prevalent among colonized patients. In the setting with high PCR RT027 rates, studies have however shown that symptomatic patients still contribute more to transmission than colonized patients. (55) If this is also true for settings with lower PCR RT027 rates needs to be elucidated. A study performed in a setting with a low PCR RT027 rate demonstrated that new CDI cases were three times more likely to be acquired from a previous CDI case than from a colonized but fecal toxin-negative patient with diarrhea. (61) Due to the design of the study, asymptomatically colonized patients (i.e. without diarrhea) were not included. Interestingly, the overall proportion of new cases that could be explained by transmission from CDI patients or colonized diarrheal patients was low in this setting: only 4% of new CDI cases were genetically related and shared the same ward simultaneously or within 28 days. Although it is not known to what extent transmission from colonized patients could explain new CDI cases, suggesting that asymptomatic carriers accounted for the remaining 96% of new CDI cases would be overstated. Hence, based on this study and from our own experience, we assume that in endemic settings sources other than direct contact with CDI patients or asymptomatic carriers may also play an important role. One of the most important sources could be the hospital environment. For PCR RT027, it was shown that transmission is even more often transmitted via ward contamination (66%) than via direct donor-recipient contact (34%). (58) Colonized patients can contaminate their hospital environment without directly transmitting *C. difficile* to another patient and spores can persist for prolonged times. Direct onward transmission will not be detected, but this is still an important route in which colonized patients can contribute to *C. difficile* transmission.

Screening for CDC and isolation of *C. difficile* colonized patients

At the moment, infection control measures focus on symptomatic cases only. Guidelines on infection prevention in *C. difficile* do not include colonized patients (62), or mention that there are insufficient data to (a) recommend screening for asymptomatic colonization and (b) placing colonized asymptomatic patients on contact precautions. (63) Patients in the community can acquire *C. difficile* from a diverse reservoirs, including (but not limited

to) food, humans, animals and soil. When these colonized patients are admitted to the hospital, they introduce these diverse *C. difficile* strains into the hospital environment. Spores of *C. difficile* can persist in the hospital environment for long times and are difficult to kill. (64) Therefore, there seems to be a rationale for screening and isolating colonized patients at admission to prevent further spread of their persistent *C. difficile* spores into the hospital environment.

The most well-known example of a *C. difficile* screening program comes from a Canadian tertiary hospital. In this hospital, screening for CDC and isolation of colonized patients has been performed in response to high endemic CDI incidence rates. (27) All patients admitted through the emergency department were screened for CDC on admission using rectal swabs. Patients identified as colonized were placed under infection control measures resembling those for CDI. A significant decline in healthcare-associated CDI and infections due to PCR RT027 was noted after implementation of this strategy.

With the identification of risk factors for CDC on admission, screening may also be targeted to high risk groups only, making this approach less labor- and cost intensive. Previous hospitalization, gastric acid suppression, tube feeding, and corticosteroid use were identified as independent predictors of CDC in a recent meta-analysis (65), but risk factors for community-onset colonization (i.e. being colonized at hospital admission) were not separated from risk factors for hospital acquired colonization, though only the former are of interest for an admission screening program. Also, a distinction between colonization by toxigenic versus non-toxigenic strains was not made. Only a few studies have specifically investigated risk factors for tCDC on admission and reported recent hospitalization, chronic dialysis, corticosteroid use (16), older age, higher frailty scores (66), higher number of comorbidities, female sex and residential proximity to livestock farms as independent risk factors. (67) Risk factors for tCDC on admission will however largely depend on the community surrounding the hospital and case-mix of patients that are admitted. Knowledge of these specific risk factors may enable institutions to tailor screening to risk groups in their hospitals. This approach was applied in four US hospitals where admission screening for *C. difficile* was performed as an infection control initiative. (68) Only patients who had been previously hospitalized within two months, and/or had a history of CDI, and/or were in a long-term care facility in the prior six months were screened for tCDC on admission using perirectal swabs. Based on a pilot study in one of the participating hospitals the authors anticipated that they would detect 78% of all colonized patients while only testing 30% of admissions. Colonized patients were placed under infection control measures. A

statistically significant decline in hospital-onset CDI rates in these hospital was noted after implementation of this screening program. (68)

However, a major shortcoming of studies investigating *C. difficile* screening programs is that these do not show how the decrease in CDI incidence rates was achieved. (69) In these studies, isolates from CDI patients and colonized patients have not been investigated by whole genome sequencing or another discriminative typing method to determine their relatedness. Also, the numbers of colonized patients progressing from colonization to CDI have not been reported. Therefore, it is unclear if decreasing CDI incidence rates were due to (1) less progression from colonization to symptomatic CDI, (2) less onwards transmission from colonized patients, or (3) less transmission from known CDI cases. (Figure 3) As colonization status was disclosed to treating physicians, they might have restricted antibiotic use in colonized patients to decrease the chance of progression to CDI. Also, compliance with infection control measures may have increased as a result of an increased awareness associated with the introduction of the screening program, thereby diminishing spread from CDI patients. In the study by Longtin, an increased hand hygiene compliance was reported during the intervention period (suggesting that this but probably also other unmeasured confounders may have contributed to the decline in CDI rates). (27) It is crucial to understand which mechanisms led to the decrease in CDI incidence rates and decrease in NAP1/027 cases, especially to determine if CDC patients really need to be isolated, or whether increased awareness or only identifying CDC patients (but not isolating them) may suffice. The usefulness of a screening and isolation program will also largely depend on the epidemic setting. As discussed before, we did not detect colonized patients to be an important source for onwards *C. difficile* transmission in an endemic setting. (**CHAPTER 6**) Screening and isolation of colonized patients in an endemic setting may however still (indirectly) affect CDI incidence rates if less contamination of the hospital environment leads to less *C. difficile* acquisition and development of CDI in vulnerable patients. However, the question is if interventions intended to reduce transmission within hospitals are the most important in reducing CDI incidence rates. Two large multicentre studies have shown disappointing results from enhanced environmental disinfection on CDI incidence rates (70-72), despite a decreased recovery of *C. difficile* from high-touch surfaces in CDI rooms. (72) Even more, the believe in contact precautions for CDI patients as an important tool in CDI prevention may not always be justified. In a Swiss hospital with endemic CDI rates and a low proportion of hypervirulent ribotypes, contact precautions for CDI patients who were not severely incontinent were discontinued, except for the use of dedicated toilets. (73) Only two proven *C. difficile* transmission events were identified over a decade of experience in this hospital.

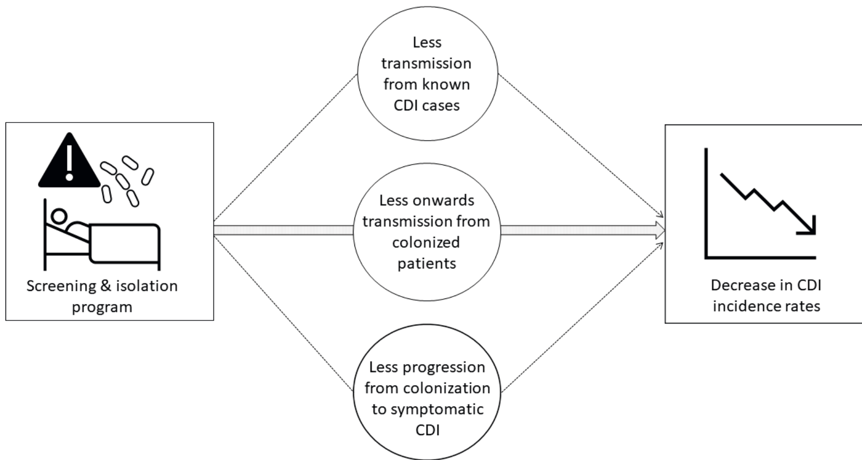


Figure 3. Mechanisms that may lead to a decrease in CDI incidence rates after implementation of screening for *C. difficile* colonization and isolation of colonized patients.
CDI, *Clostridoides difficile* infection

Detecting colonized patients may still aid in lowering CDI incidence rates, if targeted interventions for this group other than isolation precautions may decrease CDI incidence rates. This intervention may consist of antimicrobial stewardship programs (ASPs) focused specifically on colonized patients. ASPs that reduce inappropriate antimicrobials use are effective in reducing rates of CDI, especially in geriatric settings. (74) However, ASPs targeted at CDC patients only have not been studied. A subset of colonized patients is comprised of patients with previous CDI who remained colonized afterwards. These patients are known to be at a higher risk for (recurrent) CDI when treated with antibiotics. (75) ASPs focused specifically on these patients may thus be effective. However, another subset of colonized patients comprises patients who are long-term colonized, and who may not have a high risk to progress to CDI. Therefore, ASPs targeted on colonized patients as a whole group may not be effective. Of note, recent acquisition of *C. difficile* has been shown to be a risk factor for CDI. (54) Shifting the focus of ASPs to colonized patients only may therefore lead to unsatisfactory results, as non-colonized patients who acquire *C. difficile* during admission are not targeted by these programs, while indeed these patients may be at an increased risk to develop CDI. Other strategies that are currently investigated as prophylactic interventions for prevention of *C. difficile* infection in at-risk patients might also be suitable to prevent CDI progression in colonized patients, for example a toxin-based *C. difficile* vaccine (phase III CLOVER trial, NCT03090191, study completed December

2021, results awaited). If microbiota-based interventions like FMT capsules during antibiotic treatment or agents that bind and inactivate concomitantly administered antibiotics (such as ribaxamase and DAV-132), are also helpful in already colonized patients has to be awaited.

Apart from the fact that the relevance and mode of action of screening programs needs to be established better among different situations, there are also some unintended consequences of a screening and isolation program that need to be taken into account.

Implementing a screening and isolation program will lead to more patients cared for under contact precautions, with adverse outcomes like less patient- healthcare worker contact and a decreased patient satisfaction with care. (76) Also, the knowledge of CDC status may urge physicians to treat colonized patients with antibiotics once they develop diarrhea, despite the fact that diarrhea can be due to a variety of other causes. Treatment of colonized patients may however be more harmful than beneficial. Already in 1992, it was discovered that vancomycin treatment of colonized patients was temporarily effective, but also associated with a significantly higher rate of *C. difficile* carriage two months after treatment compared to placebo treated carriers. (48) In a recent small trial, NAAT-positive, toxin enzyme immunoassay (EIA)-negative patients were randomized to oral vancomycin or placebo. Oral vancomycin did not result in long-term clearance of *C. difficile*, but did disturb the microbiota, and was associated with colonization/shedding of vancomycin-resistant enterococci. (77)

In conclusion, reports have demonstrated decreases in CDI rates after implementation of screening and isolation of CDC patients. However, these reports did not show how this decrease was achieved. Also, the generalizability to endemic settings has not been shown. Although detection of colonized patients may also enable ASPs focused specifically on colonized patients, this approach has not been studied yet, and may in fact be less effective than universal ASPs. Given these limitations and the possible adverse consequences of a screening and isolation program, screening for asymptomatic CDC at hospital admission should not be implemented in routine care.

Instead, we should accept that *C. difficile* is abundant both in the hospital environment and the community setting, and can be acquired easily. In my opinion, it is better to focus on decreasing CDI susceptibility (e.g. by general ASPs) while complying with general infection prevention measures to prevent further spread from *C. difficile*, especially in settings with many susceptible patients like healthcare facilities.

Future Perspectives

In this paragraph, I will use the data from this thesis and the literature to elaborate on:

- Future CDI surveillance
- Disturbed microbiota: associated diseases and drug efficacy
- Microbiota modulating interventions

Future CDI surveillance

In this thesis, we have mainly discussed the role of colonized patients in epidemiology of CDI in the hospital. We have concluded that in an endemic setting, focusing on colonized patients will not have a major impact on CDI incidence rates. Should we then focus on other sources for *C. difficile* acquisition? Many *C. difficile* reservoirs exist, including domestic animals, farm animals, wild animals, food, water and soil. Previously undetected ribotypes can still emerge from these reservoirs, including more virulent strains or strains with higher transmissibility. Once a patient introduces such a *C. difficile* strain into the hospital, it may be detected through the observation of higher CDI incidence rates or suspected transmission events. The PCR RT826 cluster that we detected in our multicenter study is a good example of this and confirms the importance of sentinel CDI surveillance (supplemented with molecular typing in case of suspected transmission events). However, as sentinel surveillance is restricted to the hospital and CDI awareness in the community is still limited (59), CDI cases and even clusters of CDI cases in the community may be overlooked. This is problematic when studying transmission, as exposure to most *C. difficile* reservoirs (like animals, soil and food) occurs in the community. If transmission events close to the source are not detected, tracing back the source and transmission route once that specific strain is introduced into the hospital is almost impossible. Extending *C. difficile* surveillance to the community setting might enable detection of transmission within the community and possibly point to specific sources. However, the usefulness of a surveillance program in the community will depend on the capture rate of all CDI events. As most patients do not consult their general physician for short-term diarrhea and testing for pathogens in patients with diarrhea for less than 10 days or who are not severely ill is discouraged in The Netherlands (NHG standaard acute diarree 2014), we suspect that only a minority of CDI cases will be detected. Hence, suboptimal tracking of *C. difficile* transmission would occur thereby limiting the yield of a community surveillance program. Thus, we should settle for the second-best option of continuing surveillance in healthcare settings to detect increases in CDI incidence rates, clusters or outbreaks. In unforeseen

events, like community-acquired CDI with 'hypervirulent' or new ribotypes, additional investigations should be performed to evaluate potential transmission in the community or detect potential community sources.

Disturbed microbiota: associated diseases and drug efficacy

In this thesis we have previously focused on the risk for colonized patients to develop CDI, but what other consequences does colonization have for the involved individual? In this paragraph we will focus on conditions that are associated with a disturbed microbiota and on drugs whose efficacy may depend on the gut microbiota composition.

CDI is the textbook example of a disease that is caused by a disturbed microbiota. The overall microbiota composition of colonized patients differs from that of patients with symptomatic CDI, but it is also characterized by a decreased species richness and decreased microbial diversity compared to non-colonized patients or healthy controls. (9) (**CHAPTER 5**) I therefore believe that the presence of CDC can be regarded as an indicator of a certain degree of a disturbed microbiota. In addition, colonization in itself may possibly further disturb the microbiota, as was demonstrated using an *in vitro* model. (78) In this model, co-cultivation of *C. difficile* strains with fecal microbiota led to a decrease in richness and diversity, with a more pronounced effect in already disturbed microbiota. (78) 'Gut dysbiosis' has gained a lot of attention during the last decade, and was reported to be associated with intestinal disorders like inflammatory bowel disease and irritable bowel syndrome and the development of colorectal cancer. (79-82) Moreover, 'gut dysbiosis' is thought to exert effects beyond the gut, for example by influencing the risk factors for metabolic syndrome. (83) In addition, neurological diseases like Parkinson's disease and multiple sclerosis and psychiatric disorders are thought to be linked to 'gut dysbiosis' via the so-called hypothetical gut-brain axis. (84, 85) Although the causal direction of association between a disturbed microbiota and disease cannot be derived from observational studies in humans, animal models suggest that the microbiota is truly involved in pathogenesis of most of above mentioned conditions. (86) Assuming that a disturbed microbiota actually has a role in pathophysiology, one might wonder if patients with CDC are more prone to develop other 'dysbiosis' associated conditions, as they already have a disturbed microbiota. However, the exact microbiota changes will determine which conditions might be associated. A very typical example of this is the possible association between colibactin-producing *E. coli* and colorectal carcinogenesis. Colibactin-producing *E. coli*, also known as polyketide synthase-positive (pks+) *E. coli* are suspected to contribute to

colorectal carcinogenesis by the production of the genotoxin colibactin which induces double-strand DNA breaks. These pks+ *E. coli* are abundant in patients with recurrent CDI (87), but it is still unknown if this also holds for CDC patients.

Besides playing a possible role in the pathophysiology of certain diseases, the microbiota can also play a role in the effectiveness of certain drugs, for example by providing the required metabolism, such as for lactulose. (88) Another important illustration of altered drug efficacy is the potential influence of gut microbiota composition on anticancer activity of checkpoint inhibitors. Checkpoint inhibitors are immunomodulators that have dramatically changed the therapeutic landscape in several cancer types during the last decade. Checkpoint inhibitors work by blocking inhibitory checkpoints (e.g. programmed cell death-1 or programmed cell death ligand-1) thereby promoting immune-mediated elimination of tumor cells. Studies have shown that recent antibiotic use before starting checkpoint inhibitor therapy is associated with decreased progression free survival and overall survival (89-92), indicating that antibiotic induced disturbances modulate the immune response and dampen the effect of checkpoint inhibitors. Evidence for a causal relationship comes from studies in tumor bearing germ free mice: fecal microbiome transplantation (FMT) demonstrated that mice transplanted with stool from patients responding to checkpoint inhibitors had significantly reduced tumor growth compared to those transplanted with stool from patients who were non-responders. (93, 94) Recently, studies have been undertaken to evaluate which gut microbiota members are associated with clinical response in patients treated with checkpoint inhibitors. Although variable results were obtained, some bacterial species including *Akkermansia muciniphilia* and *Ruminococcace*ae were repeatedly found to be associated with favorable outcomes. (95) Given the disturbed gut microbiota composition and lower bacterial diversity in CDC patients, it would be interesting to assess if these patients represent a group that is at risk for decreased effectiveness of checkpoint inhibitors.

Microbiota modulating interventions

The evident association of CDI with a perturbed gut microbiota prompts the question if actions to restore the gut microbiota could be beneficial prior to the development of overt disease. One method to restore the perturbed microbiota in CDC subjects could be fecal microbiota transplantation (FMT), i.e. the transfer of faecal material from a healthy donor to the colonized subject. At the moment, FMT is well-known for its high efficacy in the treatment of recurrent CDI (rCDI). (96, 97) Administration of stools of healthy donors

results in a dramatic increase in microbiota diversity and resolution of symptoms in the majority of these patients, with reported success rates of more than 90%. (98). A drawback of FMT is the potential transfer of multidrug resistant bacteria, procarcinogenic bacteria and unrecognized pathogens. Therefore, there is a need for live biotherapeutic products, which are standardized and reproducible agents composed of defined consortia of isolated microbial strains. However, development of an effective live biotherapeutic product to replace FMT is not straightforward as not all mechanisms underlying the efficacy of FMT are well known. Although reconstitution of a robust and diverse gut microbiota is thought to explain at least part of the success of FMT, other components of the donor stool infusion like bacteriophages, metabolites and small molecules may also contribute to its effect. (99) Of note, the administration of simply a variety of bacterial strains without complete understanding of their function may have unanticipated adverse effects, as was reported in some studies on probiotics. (100, 101) At the moment, two live biotherapeutic products, RBX2660 and SER109, have been investigated in phase III trials for use in patients with recurrent CDI who had resolution of symptoms after treatment with standard-of-care antibiotics. Subsequent administration of the live biotherapeutic product reduced the risk of recurrence compared to placebo. (102, 103) Another live biotherapeutic product containing eight *Clostridia* strains was shown to be safe and well-tolerated and able to colonize the gut of healthy volunteers in a phase 1a/b study, but studies on efficacy have to be awaited. (104) In the future, these products may become an alternative for FMT in the treatment of rCDI. Yet, it is unclear if FMT is able to protect colonized patients from progression to CDI. The existence of patients with persistent colonization suggest that these patients' microbiomes are already less permissive towards CDI. Further elucidation of the gut microbiota composition and associated metabolic characteristics in preferably persistent carriers may be used to more specifically develop probiotic or prebiotic therapeutics against (progression to) CDI. A recent study compared not only the gut microbiota but also the gut metabolome of CDI patients and colonized subjects. (105) Compared to CDI patients, colonized patients' microbiota was enriched with species in the class *Clostridia* and their metabolomes were enriched with the carbohydrates sucrose, rhamnose and lactulose, which are non-utilizable by *C. difficile*. Therefore, they concluded that carbohydrate metabolism by other commensal *Clostridia* may prevent CDI by inhibiting *C. difficile* proliferation. (105) Hypothetically, this commensal metabolism can be used as a more specific tool against CDI, for example by administrating 'microbial accessible carbohydrates' to prevent *C. difficile* proliferation and decrease the risk of CDI in colonized subjects.

The successful implementation of FMT as a therapy for rCDI has encouraged research into FMT as a potential therapy for many other 'dysbiosis' related conditions like inflammatory bowel disease, irritable bowel syndrome, hepatic encephalopathy and autism. (106) Also, several clinical trials are currently performed to study the effect of FMT in metastatic melanoma, lung cancer and renal cell cancer for patients who are not (anymore) responding to checkpoint inhibitor therapy. Depending on the results of the currently performed trials, FMT could possibly be used to treat other conditions beyond rCDI in the future. At the moment, there is no place for FMT/microbiota modulating interventions in CDC patients, as it is yet unknown if restoration of their gut microbiota protects against CDI or other conditions that are associated with a disturbed microbiota. However, the detection of CDC should prompt awareness of a probable associated disturbed microbiota and I suggest that medication that could further disturb the microbiota should cautiously be prescribed in these patients.

C. difficile colonized patients remain a group of special interest for future research. Further mechanistic studies may analyze which factors beyond the gut microbiota composition (e.g. gut metabolites, immunologic factors) allow for colonization whilst protecting from infection. Possibly, this could lead to new treatment or preventive modalities for CDI. On the other hand, epidemiologic studies may shed light on the long-term consequences of CDC and may elucidate possible relations with conditions that are associated with a disturbed gut microbiota.

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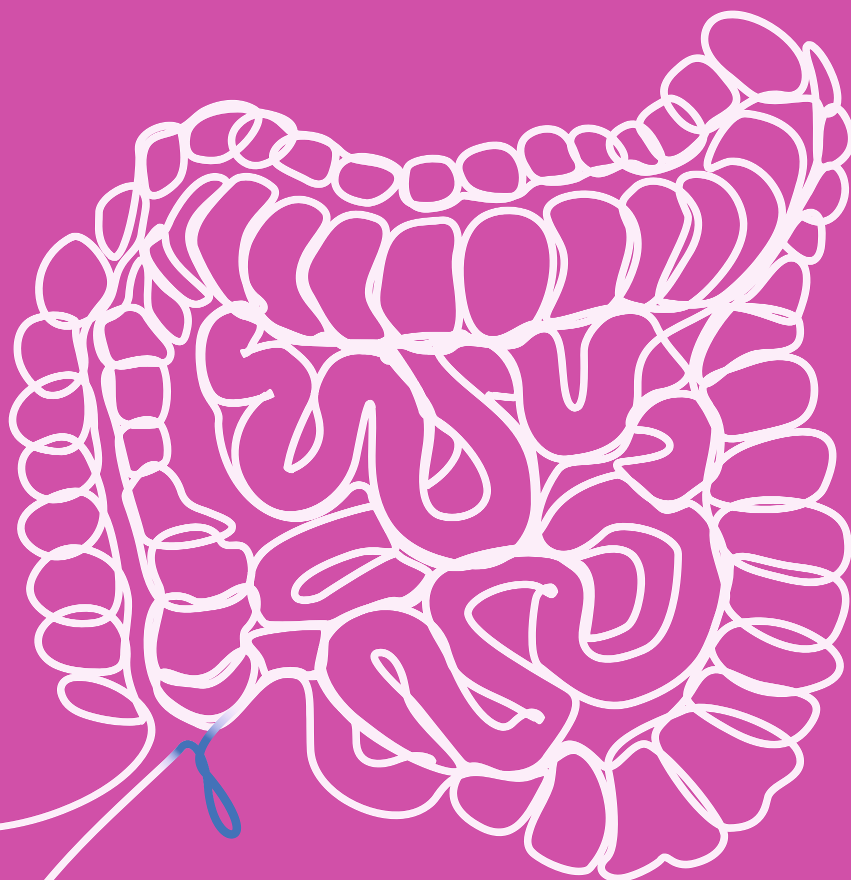
Appendices



Nederlandse samenvatting

Van alle micro-organismen die zich in en op het menselijk lichaam bevinden, herbergt de dikke darm er veruit de meeste. Deze darmmicrobiota vervult verschillende functies voor de menselijke gastheer. Zo biedt de microbiota bijvoorbeeld weerstand tegen ziekteverwekkers door concurrentie om voedingsstoffen en plaats (de zogenaamde kolonisatie resistentie). Een verstoring van de darmmicrobiota kan leiden tot een afname van deze kolonisatie resistentie en een verhoogde gevoeligheid voor infecties. Het bekendste voorbeeld hiervan is een infectie met de darmbacterie *Clostridoides difficile* die kan ontstaan na verstoring van de darmmicrobiota door antibioticagebruik. Terwijl bij gezonde mensen de groei van deze bacterie onderdrukt zou worden door een ingewikkeld samenspel van hun darmmicrobiota, kan *C. difficile* zich in een verstoorde darmmicrobiota flink gaan vermeerderen en toxines (gifstoffen) gaan produceren. Deze toxines zorgen voor de klinische symptomen van een *C. difficile* infectie (CDI), welke kunnen variëren van milde diarree tot soms een levensbedreigende darmontsteking. Ook zijn er mensen waarbij C. *difficile* zich wel weet te nestelen in de darm, maar (nog) geen symptomen veroorzaakt. Deze mensen noemen we asymptomatische dragers van *C. difficile*.

Sinds het veelvuldig gebruik van antibiotica is CDI uitgegroeid tot de meest voorkomende ziekenhuis-geassocieerde infectie in Europa en Noord-Amerika. Lange tijd werd gedacht dat de overdracht met name plaatsvond binnen ziekenhuizen via symptomatische patiënten. Aan het begin van deze eeuw kwam deze zienswijze in een ander daglicht te staan doordat nieuwe typeringstechnieken het mogelijk maakten om de overdracht van *C. difficile* vele malen beter in kaart te brengen. Een groot deel van de CDI gevallen bleek niet verklaard te kunnen worden door onderlinge overdracht tussen symptomatische patiënten. Daarmee begon de zoektocht naar andere mogelijke *C. difficile* bronnen, en realiseerde men zich dat ook asymptomatische *C. difficile* dragers een bron kunnen zijn voor verdere verspreiding. Met name asymptomatische dragers die opgenomen worden in een ziekenhuis zouden de bacterie het ziekenhuis binnen kunnen brengen en vervolgens verspreiden onder vatbare patiënten.

Het onderzoek dat beschreven wordt in dit proefschrift had tot doel om beter inzicht te krijgen in asymptomatisch *C. difficile* dragerschap. De uitdagingen die er zijn bij het detecteren van *C. difficile* dragerschap en het onderscheid maken tussen dragerschap en infectie worden beschreven. Daarnaast werden de verschillen tussen de darmmicrobiota van dragers en CDI patiënten onderzocht om beter te kunnen begrijpen welke factoren

bepalen of zich dragerschap of infectie ontwikkeld. Een andere onderzoeksvraag betrof het nut van het screenen op *C. difficile* dragerschap bij opname in het ziekenhuis. Hiertoe werd in een viertal Nederlandse ziekenhuizen onderzocht hoeveel patiënten bij opname in het ziekenhuis dragers zijn en wat hun betekenis is bij het ontstaan van nieuwe CDI gevallen.

C. difficile dragerschap: definitie en detectie

In **hoofdstuk 2** wordt de reeds bestaande literatuur omtrent *C. difficile* dragerschap gestructureerd samengevat.

Onderzoek doen naar *C. difficile* dragerschap begint bij een heldere definitie van wat hiermee bedoeld wordt. De criteria die gebruikt worden om *C. difficile* dragerschap te definiëren blijken in de literatuur echter aanzienlijk te verschillen, zoals wij in **hoofdstuk 2** van dit proefschrift beschrijven. Wij definiëren *C. difficile* dragerschap als de detectie van de bacterie in afwezigheid van CDI symptomen. Er kan onderscheid gemaakt worden in dragerschap van een toxinogene stam (een *C. difficile* stam die in staat is om toxines te produceren en dus ziekte kan veroorzaken) en dragerschap van een niet-toxinogene stam (een *C. difficile* stam die niet in staat is om toxines te produceren en dus geen ziekte veroorzaakt). Verder kunnen we dragerschap opsplitsen in voorbijgaand dragerschap, waarbij op één moment in de tijd dragerschap aangetoond wordt, en persisterend dragerschap, waarbij *C. difficile* op meerdere momenten in de tijd wordt aangetoond. Deze laatste opsplitsing is van belang omdat de risicofactoren en onderliggende mechanismen voor persisterend dragerschap anders kunnen zijn dan voor voorbijgaand dragerschap. Ook de kans dat dragerschap zich ontwikkelt tot een daadwerkelijke infectie kan verschillend zijn tussen deze groepen, en beide groepen spelen mogelijk een andere rol in de verdere verspreiding van *C. difficile.*

Niet alleen de definities, maar ook de methodes waarop *C. difficile* dragerschap gedetecteerd wordt verschillen aanzienlijk tussen gepubliceerde studies. In **hoofdstuk 2** wordt beschreven welke testen die normaliter gebruikt worden voor CDI diagnostiek ook ingezet kunnen worden voor de detectie van dragerschap. Omdat de bacterie-aantallen bij dragers lager kunnen zijn dan bij patiënten met een infectie, is het belangrijk om de meest sensitieve methodes te kiezen.

Verder is het van belang ons te realiseren dat CDI en dragerschap gemakkelijk met elkaar verward kunnen worden, omdat veel testen die gebruikt worden in de routine diagnostiek van CDI geen onderscheid kunnen maken tussen deze twee situaties. Om er zowel in de dagelijkse praktijk als in studies voor te zorgen dat CDI en dragerschap goed uit elkaar gehouden kunnen worden, is het van belang om de diagnostiek van CDI zo optimaal mogelijk te maken. Dat was het onderwerp van **hoofdstuk 3** en **hoofdstuk 4** van dit proefschrift.

Eén van de testen die veel gebruik wordt in de routine diagnostiek voor CDI is een toxine nucleic acid amplification assay (NAAT). Deze test detecteert in feite alleen de aanwezigheid van de genen die coderen voor de toxines, maar niet of deze toxines ook echt geproduceerd worden. Zowel bij dragerschap als bij CDI zal deze test dus een positieve uitslag geven en – indien gebruikt in de routine CDI diagnostiek- tot een overschatting van het aantal CDI gevallen en een onderschatting van het aantal dragers leiden. Een toxine enzyme immunoassay (EIA, een test gebaseerd op het aantonen van een antigeen, in dit geval het toxine) is de enige snelle test die vrije toxines detecteert en dus het beste correleert met een daadwerkelijke infectie, maar de suboptimale sensitiviteit van dit type testen is een groot nadeel. Om de diagnostiek van CDI te optimaliseren, werd in 2016 een literatuurreview en meta-analyse van de sensitiviteit en specificiteit van de beschikbare CDI testen verricht. Op basis hiervan werden nieuwe European Society of Clinical Microbiology and Infectious Diseases (ESCMID) aanbevelingen gedaan, welke beschreven worden in **hoofdstuk 3** van dit proefschrift. We concludeerden dat geen van de beschikbare snelle testen specifiek genoeg is om bij een lage voorafkans op CDI (zoals vaak het geval is in de ingezonden ontlastingsmonsters) nog een adequate positief voorspellende waarde te houden. Met andere woorden, als deze testen ingezet zouden worden als op zichzelf staande test om CDI te diagnosticeren zou dit tot veel fout-positieve resultaten leiden. Daarom adviseerden we – conform de vorige ESMCID aanbevelingen- het gebruik van een meerstaps algoritme. Zo'n algoritme zou moeten starten met een screeningstest met een hoge negatief voorspellende waarde (een zeer sensitieve test, zoals een EIA gericht op het enzym glutamaat dehydrogenase dat in alle *C. difficile* stammen voorkomt of een toxine NAAT) waardoor monsters met een negatieve testuitslag daadwerkelijk als negatief beschouwd kunnen worden. Alleen de monsters met een eerste positieve testuitslag zouden vervolgens opnieuw getest moeten worden met een tweede test. Omdat de voorafkans op CDI in deze geselecteerde groep monsters nu hoger is, wordt met een tweede specifieke test nu wel een voldoende hoge positief voorspellende waarde bereikt. Bij voorkeur wordt als tweede test een toxine EIA gebruikt,

omdat deze niet alleen specifiek is, maar ook het voordeel heeft van het detecteren van vrije toxines, die correleren met daadwerkelijke infectie.

Een nadeel van het gebruik van een meerstaps algoritme is dat er meerdere testen uitgevoerd moeten worden voordat de diagnose gesteld kan worden, wat kan leiden tot uitstel van de behandeling. Derhalve onderzochten wij in **hoofdstuk 4** van dit proefschrift of bij het gebruik van een 2-staps algoritme (test 1 NAAT, test 2 toxine EIA) al een voorlopige diagnose gesteld kan worden op basis van het kwantitatieve resultaat van de eerste NAAT test. Een lagere quantification cylce (*Cq*) waarde in de NAAT test (corresponderend met minder verdubbelingscycli die nodig zijn voordat het genetisch materiaal aangetoond kan worden) correspondeert met de aanwezigheid van een grotere hoeveelheid bacterie. Wij verwachtten dat samples met een lage *Cq* waarde overeen zouden komen met daadwerkelijk infectie/de aanwezigheid van toxines, en dus ook vaker positief zouden testten in de toxine EIA. Om dit onderzoeken voerden we een analyse uit op twee grote collecties ontlastingsmonsters (n=2669 en n=1718) die werden ingestuurd voor CDI diagnostiek naar de laboratoria van 2 Nederlandse ziekenhuizen. Beide ziekenhuizen gebruiken voor de diagnostiek van CDI een dergelijk 2-staps algoritme waarin monsters eerst getest worden met een NAAT en vervolgens met een toxine EIA. Van alle ontlastingsmonsters testten respectievelijk 208 en 113 positief met de NAAT. We vonden significant lagere *Cq* waarden in de monsters die ook positief testten in de toxine EIA dan in samples die negatief testten in de EIA (gemiddelde *Cq* waarde 24.4 versus 30.4 en 26.8 versus 32.2, $p<0.001$ voor beide cohorten). Bij het optimale afkappunt zou in 78.9% (ziekenhuis 1) en 80.5% (ziekenhuis 2) van de gevallen op basis van de kwantitatieve NAAT uitslag al een juiste voorspelling van de toxinestatus gedaan kan worden. Omdat wij dit een suboptimale voorspelling vinden, concludeerden wij dat de tweede test van dit algoritme (de toxine EIA) echt noodzakelijk is en niet achterwege gelaten kan worden na de initiële positieve NAAT uitslag.

De darmmicrobiota en *C. difficile* dragerschap

Het besef dat asymptomatisch dragerschap van *C. difficile* bestaat doet de vraag opkomen welke mechanismen mogelijk maken dat kolonisatie kan ontstaan, maar vervolgens wel bescherming bieden tegen het ontstaan van symptomatische ziekte. Naast het immuunsysteem lijkt vooral de samenstelling van de darmmicrobiota hier een belangrijke rol in te spelen. Verschillende processen in de levenscyclus van *C. difficile*, van de ontkieming van de sporen in het maagdarmkanaal en het ontstaan van kolonisatie tot

aan de productie van de toxines, worden immers beïnvloed door de darmmicrobiota. Het bestuderen van de verschillen in darmmicrobiota samenstelling van patiënten met CDI, met asymptomatisch *C. difficile* dragerschap of geen van deze beiden kan duidelijker maken welk veranderingen maken dat dragerschap of infectie kan ontstaan. In **hoofdstuk 5** van dit proefschrift vergeleken we de samenstelling van de darmmicrobiota van patiënten met dragerschap (n=41), met CDI (n=41) en zonder dragerschap of CDI (controles, n=43) door middel van 16S rRNA gene amplicon sequencing. In zowel dragers als CDI patiënten was de bacteriële diversiteit verlaagd in vergelijking met controles. De exacte samenstelling van de microbiota verschilde wel tussen CDI patiënten en dragers. Er werden verschillende bacteriegroepen geïdentificeerd die mogelijk geassocieerd zijn met gevoeligheid voor kolonisatie en infectie (*Veillonella)* of juist met resistentie tegen kolonisatie (*Eubacterium halii* en *Fusicatenibacter*), waar vervolgonderzoeken verder op in zouden kunnen gaan.

C. difficile screening bij ziekenhuisopname in de Nederlandse situatie

Voorafgaand aan de start van het onderzoek dat in dit proefschrift beschreven wordt, waren enkele publicaties verschenen die suggereerden dat asymptomatische dragers een belangrijke rol zouden kunnen spelen in de overdracht van *C. difficile*. Vooral dragers die opgenomen worden in het ziekenhuis lagen onder een loep, omdat dit een manier is waarop *C. difficile* in de ziekenhuisomgeving geïntroduceerd wordt en vervolgens verder verspreid kan worden. Ook zouden dragers tijdens ziekenhuisopname door verscheidene factoren die hun darmmicrobiota verstoren (zoals antibiotica gebruik) een grotere kans hebben dat hun dragerschap overgaat in een infectie. In **hoofdstuk 2** wordt besproken dat studies tonen dat 3 tot 21% van de patiënten bij opname in het ziekenhuis reeds drager is van *C. difficile*. Een meta-analyse van verschillende studies liet zien dat patiënten die bij opname drager zijn van een toxinogene stam inderdaad een hoger risico hebben op het ontwikkelen van CDI; voor hen geldt een relatief risico van 5.86. Indien asymptomatische dragers ook daadwerkelijk een belangrijke rol spelen in de overdracht van *C. difficile* zou het zinvol kunnen zijn om deze dragers te identificeren en isoleren zodat verdere verspreiding van *C. difficile* niet plaatsvindt. Een Canadees experiment had aangetoond dat deze aanpak inderdaad het aantal nieuwe CDI gevallen dat optreedt in het ziekenhuis kan verlagen. In Canada was op dat moment echter sprake van een hoge CDI incidentie en frequent voorkomen van een meer virulent *C. difficile* subtype (ribotype 027), een situatie niet vergelijkbaar met Nederland. Ook was niet duidelijk of de afname kwam doordat er minder overdracht was via dragers of doordat dragers minder vaak CDI ontwikkelden door meer alertheid op microbiota verstorende factoren. In Nederland is gedurende de laatste

jaren sprake van een stabiele lage CDI incidentie met slechts sporadische uitbraken. De meer virulente subtypes komen weinig voor. Omdat veel gegevens over dragerschap in een dergelijke situatie nog ontbraken, zetten wij de *Clostridioides difficile* dragerschap (CDD) studie op. De resultaten hiervan staan beschreven in **hoofdstuk 6**. Het doel van deze studie was om de waarde van *C. difficile* dragerschap screening bij opname in het ziekenhuis in een situatie met een lage CDI incidentie te bepalen. Hiertoe werd een multicenter onderzoek uitgevoerd in 4 ziekenhuizen verspreid door Nederland (Amphia Ziekenhuis te Breda, Radboud UMC te Nijmegen, Erasmus MC te Rotterdam en LUMC te Leiden). Nieuw opgenomen patiënten werden gescreend op *C. difficile* dragerschap door binnen 72 uur een ontlastingsmonster te verzamelen en te kweken op *C. difficile*. *C. difficile* dragerschap was aanwezig in 108/2211 (4.9%) van de opnames. Dragerschap van een toxinogene stam werd gevonden in 68/2211 (3.1%) van de opnames. Het meer virulente subtype ribotype 027 kwam niet voor onder dragers. Geen van de dragers ontwikkelde CDI tijdens opname of tijdens de 1-jaars follow-up. Door middel van een specifieke typeringsmethode (core genome multilocus sequence typing, cgMLST) werden *C. difficile* stammen van dragers vergeleken met stammen van CDI gevallen die optraden tijdens de studieperiode in deze ziekenhuizen, met als doel om te kunnen bepalen of overdracht vanaf dragers had plaatsgevonden. Op deze manier werd één mogelijke overdracht van een drager naar CDI patiënt gevonden: deze twee patiënten met een genetisch identieke stam waren voorafgaand aan hun beider diagnoses opgenomen geweest op dezelfde afdeling. Een belangrijke beperking van ons onderzoek was de suboptimale inclusie van patiënten doordat het verzamelen van ontlastingsmonsters moeizaam verliep. De screening voor *C. difficile* dragerschap bij opname in het ziekenhuis was tijdrovend en belastend, terwijl wij geen patiënten detecteerden die vanuit hun dragerschap CDI ontwikkelden en hoogstens 1 patiënt die een stam had overgebracht op een CDI patiënt. Wij concludeerden derhalve dat screening in een situatie zoals die in Nederland niet zinvol is. Mogelijk dat de *C. difficile* subtypes die in Nederland circuleren minder overgedragen worden en/of minder vaak tot ziekte leiden. Dit wil overigens niet zeggen dat dragers helemaal geen rol spelen in de epidemiologie van CDI. Waarschijnlijk contamineren dragers toch in meer of mindere mate de (ziekenhuis)omgeving, van waaruit op enig moment weer overdracht plaatsvindt naar patiënten die vervolgens dragerschap danwel een infectie kunnen ontwikkelen. Een directe link zal op deze manier echter vaak ontbreken.

In de cgMLST analyse die we in de studie verrichten bleek wel sprake van een groep CDI patiënten die allen geïnfecteerd waren met een genetische gerelateerde stam. Dit was een uitbraak die ook al opgevallen was in de CDI sentinel surveillance (een continue

surveillance om de incidentie van CDI en circulerende *C. difficile* subtypes te monitoren). Deze ongebruikelijke uitbraak wordt in nader detail beschreven in **hoofdstuk 7.** De uitbraak betrof 5 patiënten met in totaal 8 episodes van CDI op een afdeling gastro-enterologische chirurgie van een universitair ziekenhuis. In twee van de vijf patiënten was sprake van recidiverende en ernstige symptomen. Alle gevallen werden veroorzaakt door een uniek subtype dat niet bekend was in internationale referentie laboratoria en daarom het nieuwe PCR ribotype 826 (RT826) toegewezen kreeg. Het is een ribotype welke naast de gebruikelijke toxines A en B ook een derde toxine (het binaire toxine) bevat en lijkt op de virulente stam RT078. De bron voor deze nieuw stam kon niet achterhaald worden, maar gezien het ontbreken van deze stam in de humane internationale collecties en het feit dat RT078 en hiermee geassocieerde stammen vaak ook bij dieren gevonden worden deed ons speculeren dat er mogelijk sprake is geweest van overdracht vanuit een dierlijke bron. De eerste patiënt of een asymptomatische drager kan de stam geïntroduceerd hebben in het ziekenhuis, waarna verdere verspreiding plaatsvond. Sinds deze uitbraak is RT826 niet meer gevonden in de Nederlandse surveillance of bij ons bekende uitbraken.

CDI wordt de laatste jaren steeds vaker buiten het ziekenhuis gerapporteerd. De Nederlandse CDI sentinel surveillance richt zich alleen op in het ziekenhuis opgenomen patiënten. Bij een aanzienlijk deel van deze patiënten (40%) ontstaan de eerste klachten echter al buiten het ziekenhuis. In **hoofdstuk 8** werden gegevens van de CDI sentinel surveillance gebruikt om die gevallen van CDI beter te karakteriseren. Surveillance programma's die ook de CDI gevallen buiten het ziekenhuis includeren zouden niet alleen een completer beeld kunnen geven van de ware ziektelast van CDI, maar ook waardevolle informatie kunnen verschaffen over de manier waarop overdracht buiten de ziekenhuisomgeving plaatsvindt en wat – naast asymptomatische dragers- mogelijke andere bronnen van *C. difficile* zouden kunnen zijn. Echter, omdat de meeste patiënten met kortdurende diarree de huisarts niet bezoeken, danwel niet getest worden op CDI, zal een groot deel van de CDI gevallen buiten het ziekenhuis niet gedetecteerd worden. Daardoor zal de waarde van zo'n uitgebreidere surveillance te beperkt zijn.

Discussie en vooruitblik

In het laatste hoofdstuk van dit proefschrift worden de resultaten van ons onderzoek samengevat. Ook wordt besproken welke uitdagingen spelen bij het uitvoeren of interpreteren van onderzoek naar *C. difficile* dragerschap. Zo blijft het belangrijk ons te

realiseren dat *C. difficile* dragerschap gemakkelijk verward kan worden met CDI, vooral als er geen optimale diagnostiek ingezet wordt voor CDI.

De resultaten van ons onderzoek werden in context geplaatst met de recente literatuur en er werd vooruit gekeken naar mogelijk toekomstige ontwikkelingen. Als we kijken naar de rol die dragers spelen in het ontstaan van nieuwe CDI gevallen dan lijkt in een situatie vergelijkbaar met die in Nederland (lage incidentie van CDI, weinig circulerende virulente subtypes) directe overdracht vanuit dragers geen belangrijke besmettingsroute te zijn. Ook hadden dragers geen hoog risico op het ontwikkelen van CDI. Het screenen op asymptomatisch dragerschap bij ziekenhuisopname zal in een dergelijke situatie niet veel bijdragen aan het voorkomen van nieuwe CDI gevallen. Wel kunnen dragers zorgen voor verdere verspreiding van *C. difficile* binnen maar ook buiten de ziekenhuisomgeving en we dienen ons te beseffen dat *C. difficile* sporen wijdverspreid zijn. Iedereen kan dus gemakkelijk in contact komen met *C. difficile* sporen. Het lijkt daarom logischer om te focussen op het verminderen van de vatbaarheid voor CDI (door bijvoorbeeld antibiotic stewardship programma's) in combinatie met algemene infectiepreventie maatregelen om nog verdere verspreiding van *C. difficile* te voorkomen, vooral op plekken waar veel vatbare patiënten komen, zoals ziekenhuizen.

C. difficile dragers vormen een interessante groep voor verder onderzoek. Zo zou nader onderzocht kunnen worden welke mechanismen (naast de microbiota samenstelling) ervoor zorgen dat dragers geen infectie ontwikkelen. Hierbij kan gedacht worden aan immunologische factoren, of de metabolieten (stofwisselingsproducten) die aanwezig zijn in de darm. Mogelijk zou dit kunnen leiden tot de ontwikkeling van nieuwe CDI behandelmethodes of preventieve behandelingen ter voorkoming van CDI.

Aangezien *C. difficile* dragerschap gepaard gaat met een verstoorde darmmicrobiota, speculeerden wij of dragers ook meer risico hebben op het ontwikkelen van andere aandoeningen die geassocieerd worden met een verstoorde darmmicrobiota, zoals inflammatoire darmziekten (ziekte van Crohn en colitis ulcerosa), prikkelbare darm syndroom en metabool syndroom. Ook de effectiviteit van bepaalde medicatie wordt beïnvloed door de darmmicrobiota. Een voorbeeld hiervan is een verminderde activiteit van checkpoint remmers (immuuntherapie voor de behandeling van kanker) bij patiënten die een verstoorde darmmicrobiota hebben door recent antibioticagebruik. Het zou interessant zijn om te bekijken of de effectiviteit van checkpoint inhibitors ook verminderd is in *C. difficile* dragers.

Het herstellen van een verstoorde darmmicrobiota (door bijvoorbeeld een fecestransplantatie) is inmiddels een effectieve behandeling gebleken voor patiënten met terugkerende CDI. Of het ook zinvol is de darmmicrobiota van *C. difficile* dragers te herstellen om het risico op het ontwikkelen van CDI (of andere aan een verstoorde microbiota gelinkte aandoeningen) te voorkomen is nog onbekend.

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Curriculum vitae

Monique Jacqueline Theresia Crobach werd op 25 oktober 1982 geboren in Leiderdorp. Zij behaalde in 2001 haar eindexamen gymnasium aan het Rijnlands Lyceum in Oegstgeest. Hetzelfde jaar startte zij met haar studie Geneeskunde aan de Universiteit Leiden. Als student heeft zij sinds 2006 meegewerkt aan onderzoek naar *Clostridoides difficile* infecties op de afdeling Medische Microbiologie van het Leids Universitair Medisch Centrum (LUMC) bij prof. dr. E.J. Kuijper. Na het (cum laude) behalen van haar artsexamen in 2008 heeft zij enkele maanden op de afdeling Medische Microbiologie gewerkt als arts-onderzoeker om namens de European Society for Clinical Microbiology and Infectious Diseases (ESCMID) aanbevelingen op te stellen voor de diagnostiek van *C. difficile* infecties. Vervolgens is zij als arts-assistent gaan werken in het Bronovo Ziekenhuis te Den Haag op de afdeling interne geneeskunde. In 2009 startte zij in ditzelfde ziekenhuis met haar opleiding tot internist (opleider dr. J.W. van 't Wout). In 2012 zette zij haar opleiding voort in het LUMC (opleiders prof. dr. J.W.A. Smit, prof. dr. J.T. van Dissel en prof. dr . J.W. de Fijter). Van 2014 tot 2018 werd de opleiding onderbroken om promotieonderzoek te doen op de afdeling Medische Microbiologie van het LUMC onder supervisie van prof. dr. E.J. Kuijper. De resultaten van dat onderzoek zijn beschreven in dit proefschrift. Tijdens deze onderzoeksperiode coördineerde zij gedurende enige tijd de activiteiten van het nationaal referentielaboratorium voor *C. difficile* infecties, een samenwerkingsverband tussen het LUMC en het Rijksinstituut voor Volksgezondheid en Milieu (RIVM). In 2018 vervolgde zij haar opleiding tot internist met aandachtsgebied oncologie (opleider prof. dr. J.E.A. Portielje). Zij voltooide deze opleiding in april 2021 en is sindsdien werkzaam als internist-oncoloog in het Alexander Monro Ziekenhuis in Bilthoven. Monique is getrouwd met Sharan Kaulesar Sukul en samen hebben zij een zoon (Olivier, 2013) en een dochter (Annemijn, 2015).

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