CHAPTER 1

GENERAL INTRODUCTION
**INTRODUCTION**

*Clostridium difficile* is a Gram-positive, spore-forming, anaerobic bacterium that can act as a pathogen due to its ability to produce toxins. Toxin-producing *C. difficile* strains can merely colonise our bowels or cause gastrointestinal infection (*C. difficile* infection: CDI), with symptoms ranging from mild, self-limiting diarrhoea to severe pseudomembranous colitis and sepsis with high morbidity and mortality rates. CDI is a common and resilient cause of nosocomial diarrhoea. It is a major concern in hospitals and other healthcare facilities, since the spore-forming ability of *C. difficile* enhances transmission. Recurrence of CDI after an initial episode is common and adds further to the CDI –related burden of morbidity and healthcare costs in a fragile, mostly elderly population.\(^{1-3}\)

**Individual risk factors**

The most important risk factor for developing *C. difficile* infection is use of antibiotics. *C. difficile* enters the body via the faeco-oral route when spores and vegetative cells are ingested. Spores can survive the acid environment of the stomach and germinate after arrival in the small bowel. Here vegetative *C. difficile* cells can colonise the intestines and under the right circumstances cause infection, e.g. when as a result of antibiotic exposure a niche in the disrupted microbiota allows proliferation. Practically all antibiotics have been associated with CDI, with clindamycin, fluoroquinolones, and cephalosporin imposing the highest risk.\(^{1,4,5}\) There are several other factors that contribute to disturbed ‘colonisation resistance’: e.g. increasing age, extensive comorbidity or severe illness, abdominal surgery, chemotherapy and inflammatory bowel disease.\(^{6}\) Other well described risk factors for CDI include hospitalisation (due to contamination of the environment, with carriage rates increasing over time), acquisition of specific *C. difficile* strains (especially the ribotype 027 strain), inadequate serum IgG antitoxin immune response and probably the use of gastric acid suppressors.\(^{1,7,8}\)

**Morbidity, mortality and implications**

CDI has major implications for the individual patient and the healthcare system in general. Complications include severe pseudomembranous colitis (figure 1), toxic megacolon, perforation and sepsis, and death. After initial recovery, recurrent disease is common (on average 20-25% - but with large variation between 12% and 64% in observational studies) and recurrence risk increases with every episode.\(^{8,10}\) Mortality has been investigated in various studies in a heterogeneous range of CDI populations, with varying definitions, sample size and quality – estimating the attributable mortality at ≥6% of cases, again increasing with age.\(^{9,11}\) Attributable costs are estimated at $3,000 - $30,000 per
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case of hospital-acquired CDI, mostly due to extended length of stay (3-21 days).\(^{(3)}\) In the Netherlands approximately 3000 hospitalised patients develop CDI per year, of which an estimated 120 patients die.\(^{(12)}\)
Several infection control measures are recommended to minimise spread of spores within a hospital or healthcare facility, like protective clothing, hand hygiene, isolation precautions, environmental cleaning and cleaning of medical equipment, and good antibiotic stewardship.\(^{(13;14)}\)

Diagnosis

Early and rapid identification of \textit{C. difficile} infection is vital for the initiation of aforementioned infection control measures and treatment, and thus to limit transmission.\(^{(15)}\) Diagnostic tests are aimed at demonstrating either the toxin-producing (‘toxigenic’) bacterium itself, its antigens, its toxins or the genes coding for them. Numerous diagnostic tests are at hand.

![Figure 1: CT abdomen of a patient with severe pseudomembranous colitis due to \textit{C. difficile} infection during the 2013 ribotype 027 outbreak in VU University medical centre. The colon is diffusely dilated with extensive bowel wall thickening. The patient underwent total colectomy, but died several days later of persistent septic shock and cardiac failure.](image)
The traditional reference standard is the cell cytotoxicity assay (CCA).\textsuperscript{(16)} For this test a mammalian cell culture is exposed to faecal eluate. The cell culture disintegrates due to cytotoxicity when \textit{C. difficile} toxins are present in the stool sample, and the addition of antitoxin neutralizes the effects of the toxin on the cells (figure 2).\textsuperscript{(17;18)} Because of the test’s specialised requirements and turnaround time, most laboratories do not routinely use CCA anymore. Anaerobic culture on selective media, followed by testing cultured strains for the in vitro ability to produce toxins (toxigenic culture), is very sensitive and also referred to as a reference test, but is also time consuming.\textsuperscript{(17;18)} Moreover, demonstrating the presence of toxigenic \textit{C. difficile} strains in stool samples does not prove symptomatic infection since this also occurs in asymptomatic colonisation (limited specificity).

In general, many clinical laboratories have moved to enzyme immunoassays (EIAs) detecting toxins or glutamate dehydrogenase (a cell-wall associated enzyme), and nucleic acid amplification tests (NAAT) because they are thought to be more cost-effective and offer turnaround times of minutes to hours instead of the 2 to 3 days required for CCA or toxigenic culture. Many of the EIAs are simple and easy to perform, but have limitations with regards to sensitivity and specificity.\textsuperscript{(17;18)} Nucleic acid amplification tests to demonstrate genes coding for toxin production have high sensitivity and short turnaround time, but are more costly and require more specialised equipment and expertise.\textsuperscript{(17)} Moreover, like culture alone, NAAT does not discriminate between infection and mere colonisation with a toxin producing \textit{C. difficile} strain (limited specificity). Recent studies demonstrated the clinical relevance of this finding: patients with toxigenic \textit{C. difficile} strains in their stool (as demonstrated by culture or NAAT) did not have an increased mortality unless there were toxins present in their stool.\textsuperscript{(19;20)} This indicates presence of toxins in faecal samples best defines true \textit{C. difficile} infection, or at least serious \textit{C. difficile} infection, 

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2.png}
\caption{Cell cytotoxicity assay (CCA) for the detection of \textit{C. difficile} toxins A and B is performed upon mammalian cell cultures. Prepared faecal samples from suspected patients are added onto a monolayer of cultured cells (in this case Vero cell monolayers). Cells are inspected after 24 and 48 hours of incubation: the presence of toxins exert a cytopathic effect characterized by rounding of cells due to disruption of tight junctions and destruction of the cytoskeleton (right panel, 2b). Parallel samples that are incubated with specific \textit{C. difficile} antitoxin do not show this effect (left panel, 2a). Source: dr. ir. J Corver and prof. dr. EJ Kuijper, Department of Medical Microbiology, Leiden University Medical Centre (LUMC), Netherlands.}
\end{figure}
and the significance of ‘only’ culture or NAAT positive samples is uncertain. Considering that the percentage of hospitalised patients asymptomatically colonised with *C. difficile* is up to 15-20%\[^{6,8,21}\], and that diarrhoea due to non-CDI causes is common in this population, using only NAAT could cause substantial overdiagnosis and overtreatment.

Molecular typing of *C. difficile* strains is important to gain a better understanding of the epidemiology of CDI, and specifically to identify outbreaks. Polymerase chain reaction (PCR) ribotyping is based on recognition of patterns of PCR products of the 16S-23S ribosomal ribonucleic acid (rRNA) intergenic spacer region, and is the standard typing method used in Europe. *C. difficile* ribotype 027 appears to be mostly associated with severe disease, high recurrence rates and hospital outbreaks.

Limited sensitivity and/or specificity of the current diagnostic options has led to the development of several diagnostic algorithms that combine tests to improve diagnostic accuracy.\[^{17,18}\] First and foremost the diagnosis CDI should only be made in patients with symptoms suggestive of CDI, and not by laboratory findings alone. Clinicians should be aware that a substantial proportion of infected patients can be missed or overtreated based on stand-alone tests. The current guidelines therefore recommend combining tests to optimise positive and negative predictive value.\[^{13,18}\]

In daily practice, on top of the limitations of the available tests, several factors delay the identification of *C. difficile* infections. These include patient’s and doctor’s delay (e.g., the doctor does not consider the possibility of a *C. difficile* infection, or decides to wait and see if symptoms pass), delays in sampling stool, and time required to process samples in the laboratory. As a result, almost 3-8 days pass between onset of symptoms to start of treatment.\[^{22,23}\]

**Treatment**

Considering that the disrupted gut microbiota plays a crucial role in CDI, discontinuing the causative antibiotic (if possible) is the most physiological treatment of CDI. In about 15% of (mild) cases cessation of the inciting antibiotic alone is sufficient to resolve symptoms.\[^{17}\] For mild to moderate CDI, metronidazole is usually recommended as the first line antibiotic treatment, largely because of its efficacy, limited costs (a 10-day course costs about €7,- in the Netherlands (2016)\[^{24}\]), and extensive experience.\[^{1,6,17,25,26}\] For the treatment of severe disease, vancomycin is better than metronidazole with regards to cure rates, but also significantly more expensive (€430,- for a course of 4 daily capsules of 250 mg; €80,- if the solution for infusion is taken orally in a dose of 4 x 125 mg\[^{24}\]); for milder cases vancomycin and metronidazole are considered equivalent.\[^{27}\] Fidaxomicin has similar cure rates as vancomycin, but recurrence rates appear to be lower with fidaxomicin, especially for those not infected with ribotype 027.\[^{28-30}\] A course of fidaxomicin currently costs over € 1700,-\[^{24}\]. Therefore, it is usually recommended to treat a first recurrence with the initial therapy (metronidazole or vancomycin), especially
in non-severe disease, although some guidelines favour vancomycin for all first recurrences. For those with multiple recurrences, the efficacy of antibiotic treatment further decreases, which is not surprising considering antibiotics do nothing to restore gut microbiota. Immunotherapy (e.g. the use of monoclonal antitoxin antibodies or prophylactic immunisation) could reduce the recurrence rate of CDI; but is not yet available for routine clinical use. Faecal microbiota transfer (FMT), although still relatively unconventional, unappealing and unstandardized, seems to be by far the most effective treatment for recurrent CDI, with few short-term side effects.

The scent of CDI

In 2010 our nurses and medical staff were discussing the clinical patients admitted to the internal medicine ward of our hospital during the weekly grand rounds. It was reported that one of the patients had diarrhoea and on the suggestion whether it could be a *C. difficile* infection, an older nurse answered that she didn’t think so – “it doesn’t have that particular smell”. Ever since the 1970s, when *C. difficile* was first identified as the cause of pseudomembranous colitis, the diarrhoea has often been described as having a characteristic smell. Several studies were performed to determine the scent detection skills of nursing staff, and found large differences in their abilities. Considering that dogs have a far superior sense of smell, we hypothesised it should be possible for the canine nose to detect CDI with better than human accuracy.

Animal scent detection

The use of animals as scent detectors is far from new. Since the early 18th century, monks living in the Swiss Alps kept Saint Bernard dogs to guide them on their rescue missions following bad snowstorms. The dogs were not trained by the monks, but younger dogs learned how to perform search missions from their elders. Similarly, dogs have been used for prey hunting for centuries. The traditional way of ‘hunting’ for truffles is with the help of pigs and trained dogs alike. Other examples include Bedouins’ camels that use their sense of smell to find water by detecting geosmin (a bacterial product found in wet dirt) from up to 50 miles away, which is a real asset when travelling through the desert. Homing pigeons, used for pigeon post since before the time of Christ, are thought to at least partly depend on their sense of smell by navigating through odours they pick up from different wind directions. With regards to the medical field, our sense of smell has traditionally been one of our most readily available diagnostic tools. Well known examples are the smell of acetone in ketoacidotic patients in the emergency room, or of gas gangrene in wounds on the battle field.

Chapter 2 gives an overview of use of scent in diagnosing disease.
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Early and rapid identification of CDI is important to prevent transmission by initiating adequate isolation measures and treatment. Studies have, however, shown that the mean time between the onset of symptoms and treatment is about 3-8 days. Despite the availability of a wide range of diagnostic tests, CDI is still a widespread healthcare-related infection. The potential of using a dog for detection is the possibility to screen hospital wards for infected patients. Such screening could overcome common delays in diagnosis and thus help to control and prevent CDI outbreaks. In Chapter 3 the results of a proof of principle study are presented, in which we trained a dog to detect *C. difficile*, both in stool samples and in infected patients on hospital wards.

In Chapter 4 we assessed how accurate the trained detection dog was in identifying CDI patients in a ‘real-life’ outbreak setting, rather than in a case-control pilot design. We wondered whether performing regular surveillance rounds of the hospital during this outbreak (like a “pet scan”), could lead to earlier CDI detection compared to conventional diagnostic testing.

Chapter 5 describes a case-control study of an outbreak caused by a ribotype 027 *C. difficile* strain: which hospital-associated factors drove the outbreak of this particular strain?

In Chapter 6 we focus on olfactory detection by an electronic nose, i.e. profiling of volatile (organic) compounds (VOCs) in the headspace of faecal samples by means of Field Asymmetric Ion Mobility Spectrometry (FAIMS). FAIMS analyses the chemical composition of a gaseous mixture, for instance the VOCs emanating from a stool sample, based on how the molecules move through an oscillating electrical field. It creates a profile of the chemical composition of a sample (the “chemical fingerprint”) rather than identifies individual components. Can FAIMS be used to distinguish *C. difficile* - positive from - negative stool samples?

The FAIMS profile of gas-phase biomarkers emanating from stool samples mainly represents the gut microbiota. CDI patients with recurrent disease have more disturbed gut microbiota than those without. Chapter 7 describes whether FAIMS profiling can not only be used to diagnose *C. difficile* infection, but whether it can also predict recurrence risk by analysing the stool sample of the initial episode.

Chapter 8 is the final part of the thesis, which contains the summary, general discussion and recommendations.
Reference List

(9) Bagdasarian N, Rao K, Malani PN. Diagnosis and treatment of Clostridium difficile infection in adults: 2010 update by the society for healthcare epidemiology of America (SHEA) and the infectious diseases society of America (IDSA). Infect Control Hosp Epidemiol 2010;31:431-55.
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