Clostridioides difficile infections: Preventive strategies

Jon Edman Wallér

Department of Infectious Diseases

Institute of Biomedicine

Sahlgrenska Academy, University of Gothenburg

UNIVERSITY OF GOTHENBURG

Gothenburg 2023

Cover illustration: Dominoes by Jon Edman Wallér

Jon.edman.waller@gu.se

ISBN 978-91-8069-401-8 (PRINT)
ISBN 978-91-8069-402-5 (PDF)

Printed in Borås, Sweden 2023
Printed by Stema Specialtryck AB

To Linn, Liv & My

“An ounce of prevention is worth a pound of cure.”

Benjamin Franklin

-

ABSTRACT

Clostridioides difficile infections primarily affect elderly, hospitalised patients treated with antibiotics and are among the most common healthcare-related infections. This thesis aimed to improve the understanding of the best prevention strategies for this disease, particularly in a Swedish setting. In Paper I, we evaluated the effects of an antibiotic stewardship programme. *C. difficile* infection incidence fell after a substantial reduction of cephalosporin use at the hospital. No significant change in incidence was seen at a comparable hospital where no stewardship programme was implemented. In Paper II, we evaluated two surveillance algorithms intended to detect outbreaks. None of these could accurately discriminate transmission events. We combined different typing methods with epidemiological links to determine the frequency of intrahospital disease transmission. Transmissions occurred infrequently in our setting. In Paper III, we constructed a mathematical, compartmental model of *C. difficile* transmission dynamics, where the environmental reservoir of *C. difficile* spores was modelled alongside patient compartments. Antibiotic stewardship had the largest potential for decreasing infections, while improved cleaning and disinfection practices could best decrease colonisations and environmental spores. Improved isolation had modest effects overall.

In conclusion, antibiotic stewardship, directed primarily at cephalosporins, is effective to reduce *C. difficile* infections in a real-life as well as a modelled Swedish setting. For optimal surveillance and outbreak detection, there is a need to further develop and validate methods. Improved general cleaning and disinfection in hospitals can potentially prevent colonisation and infections if a substantially increased rate of spore reduction is achieved. Such measures may be more important than isolation of infected patients.

Keywords: *Clostridioides difficile*, prevention, antibiotic stewardship, infection surveillance, outbreaks, compartmental modelling, cleaning, disinfection

ISBN 978-91-8069-401-8 (PRINT)
ISBN 978-91-8069-402-5 (PDF)

SAMMANFATTNING PÅ SVENSKA

Clostridioides difficile (C. difficile) är en bakterie som kan finnas i tarmen hos människan och andra djur. Den kan övergå i en tålig sporform som gör att den kan överleva utanför kroppen och stå emot vanliga desinfektionsmedel. I samband med sjukhusvård, antibiotikabehandling och andra riskfaktorer kan den orsaka infektion genom att bilda toxiner som påverkar tarmslemhinnans celler. Toxinerna orsakar inflammation som leder till diarré, smärtor och ibland allvarliga komplikationer. I studierna som ingår i den här avhandlingen har vi utvärderat olika sätt att förebygga att infektioner med denna bakterie uppstår och sprids på sjukhus. Ett sätt är att minska användningen av antibiotika som ökar risken för infektion mest. I Studie I utvärderade vi effekten av att kraftigt minska användningen av antibiotikaklassen cefalosporiner till förmån för preparat med lägre risk. Vi fann att antalet *C. difficile*-infektioner minskade, medan antalet infektioner var oförändrat på ett annat sjukhus där samma åtgärd inte hade införts. I Studie II undersökte vi hur ofta *C. difficile*-infektion smittar från patient till patient i en svensk kontext. Smitta mellan patienter är ett välkänt problem och leder ibland till stora utbrott av infektioner. I studien hittade vi några fall av smittspridning, men det skedde inte särskilt ofta. Vi utvärderade också två övervakningsmetoder med syfte att upptäcka smittspridningar, som utgick från antalet fall på olika avdelningar under en 30-dagarsperiod. Ingen av metoderna fungerade särskilt bra, eftersom de smittade patienterna ofta hade hunnit byta avdelning innan de blev sjuka och provtogs. I den tredje studien byggde vi en matematisk modell för att simulera ett sjukhus där patienter skrivs in och ut, ibland får i sig *C. difficile* och ibland blir sjuka av det. Vi simulerade också mängden sporer i miljön som kunde stiga eller sjunka. Vi prövade olika förebyggande åtgärder i modellen och uppskattade deras maximala effekt. Förändrad antibiotikaanvändning hade störst potential att minska antalet infektioner, medan mängden sporer i miljön och hur många som tar upp bakterien utan att bli sjuka påverkades mest av städning och desinfektion.

Sammanfattningsvis har avhandlingen bidragit med ny kunskap kring effektiva åtgärder för att förebygga *C. difficile*-infektion, särskilt i en svensk kontext.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Karp J*, **Edman-Wallér J***, Toepfer M, Lundqvist A, Jacobsson G. *Clostridioides difficile* incidence related to in-hospital cephalosporin use: a tale of two highly comparable hospitals. *Journal of Antimicrobial Chemotherapy* 2019; 74(1):182-189. doi: 10.1093/jac/dky408.

II. **Edman-Wallér J**, Toepfer M, Karp J, Rizzardi K, Jacobsson G, Werner M. *Clostridioides difficile* outbreak detection: evaluation by ribotyping and whole genome sequencing of a surveillance algorithm based on ward-specific cut-offs. *Infection Control and Hospital Epidemiology* 2023; Jun 23;1-5, online ahead of print. doi: 10.1017/ice.2023.113

III. **Edman-Wallér J**, Rizzardi K, Jacobsson G, Gerlee P. *Clostridioides difficile* transmission: a compartmental model accounting for environmental spore persistence. Submitted manuscript.

* Shared first authorship.

CONTENTS

ABBREVIATIONS

ADP	Adenosine diphosphate
BA	Bile Acid
CCTA	Cell Culture Cytotoxicity Assay
CDC	Centers for Disease Control and Prevention, USA
C. difficile	*Clostridioides difficile*
CDI	*Clostridioides difficile* infection
CDT	*Clostridioides difficile* binary Toxin
CI	Confidence Interval
cgMLST	core genome Multi Locus Sequence Typing
DNA	Deoxyribonucleic Acid
ECDC	European Centre for Disease Prevention and Control, EU
EIA	Enzyme Immunoassay
FMT	Faecal Microbiota Transplantation
HMW	High Molecular Weight
GTP	Guanosine triphosphate
kDa	kiloDalton
MALDI-TOF MS	Matrix-Assisted Laser Desorption/Ionisation - Time of Flight Mass Spectrometry
MLST	Multi Locus Sequence Typing
NAAT	Nucleic Acid Amplification Test

OR	Odds Ratio
PaLoc	Pathogenicity Locus
PBS	Phosphate-buffered saline
PCR	Polymerase Chain Reaction
RNA	Ribonucleic Acid
RT	Ribotype
SNV	Single Nucleotide Variant
ST	Sequence Type
TC	Toxigenic Culture
TcdA	*Clostridioides difficile* Toxin A
TcdB	*Clostridioides difficile* Toxin B
WGS	Whole Genome Sequencing

DEFINITIONS IN SHORT

Clostridioides difficile infection	Clinical findings compatible with *C. difficile* infection, e.g. diarrhoea (3 or more loose stools within 24 hours) and microbiological findings representing toxigenic *C. difficile* in stool
C. difficile colonisation	Presence of *C. difficile* (toxigenic or non-toxigenic) in the intestines of a carrier without symptoms of infection
Toxigenic *C. difficile*	*C. difficile* with the ability to produce toxins A or B (or both)
Non-toxigenic *C. difficile*	*C. difficile* without the ability to produce toxins A and B
Healthcare facility-associated *C. difficile* infection	*C. difficile* infection with symptom onset either during in-patient stay at a healthcare facility >48 hours after admission, or within four weeks after discharge from a healthcare facility
Community-associated *C. difficile* infection	*C. difficile* infection with symptom onset >12 weeks after discharge from a healthcare facility
Sporulation	The process where vegetative *C. difficile* transform into the endospore state
Germination	The process where *C. difficile* endospores transform into the vegetative state
Antibiotic stewardship	A systematic effort to ensure that antibiotics are not overused or misused
Cleaning	Removal of organic or inorganic contaminations from surfaces or objects

Disinfection	A physical or chemical process with the intention to kill or inactivate microorganisms

1 INTRODUCTION

1.1 THE BACTERIUM

Clostridioides difficile was first described by Ivan C. Hall and Elizabeth O'Toole in 1935 [1] as *Bacillus difficilis*. They found it to be an anaerobic, gram-positive rod, present in the faeces from several newly born, healthy babies. The word "difficilis" refers to the difficulties encountered when trying to isolate and culture the bacteria at the time. The discoverers correctly concluded that the species had a pathogenic potential due to toxin production by injecting filtrates subcutaneously in guinea pigs, which then died with convulsions. Marshall L. Snyder [2] further studied the pathogenicity and found that the toxins produced were lethal for several animal species but not as potent as botulinum toxin or tetanus toxin. In 1938, Prévot reclassified the species as part of the genus *Clostridium* and renamed it *Clostridium difficile.* [3]

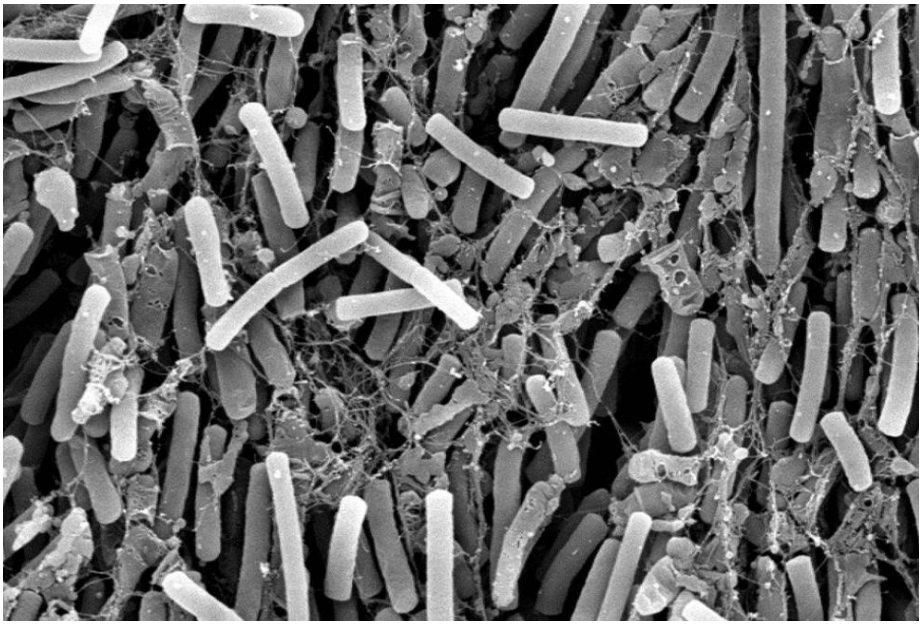


Figure 1. C. difficile bacteria as viewed in a scanning electron microscope. The bacteria are in the early stages (three days) of forming a biofilm. Work available in the Creative Commons Public Domains, produced by Semenyuk et al. PLoS One, 2014. [4]

Although a pathogenic potential of the species had been discovered, it had not yet been linked to any human disease. Anaerobic bacteria, in general, were not regarded as especially clinically interesting at the time of discovery. Furthermore, microbiological methods for routine culturing of anaerobic species at clinical laboratories were still to be developed. This all changed in the 1970s, with what has been called "the anaerobic renaissance". [5] Anaerobes were then found to be present in abscesses as well as abdominal and airway infections. The newly discovered antibiotics lincomycin and its derivative clindamycin, with effect on many anaerobes, were increasingly used to treat these infections. [6] In 1973, Cohen et al. [7] published a case reports series where three patients developed severe colitis after clindamycin treatment. This led other scientists to investigate the causes of pseudomembranous colitis, a disease first described in 1948. [8] By the second half of the 1970s, a group led by John G. Bartlett, through a series of studies, established the link between *C. difficile*, its toxins, and pseudomembranous colitis. [9-12] Bartlett's group also developed the first diagnostic test for *C. difficile* infection (CDI), the cell culture cytotoxicity assay, in the late 1970s. [13] Oral vancomycin, which had been used successfully for treatment of staphylococcal enterocolitis [14] and in animal models of *C. difficile* enterocolitis [10] was proven an effective treatment in 1980 [15] and metronidazole in 1983. [16]

During the 1980s and 1990s, diagnostic tests and antibiotic treatments for *C. difficile* infections were implemented at hospitals worldwide. It became clear that most *C. difficile* infections did not lead to pseudomembranous colitis but milder disease where diarrhoea was the main symptom. It also became established that most of the available antibiotic classes increased the risk of infection and that most cases were nosocomial. [17] Around the year 2000, a disturbing increase in incidence was observed in the United States and several European countries. Many of these cases were caused by a specific strain named BI/NAP/027 (depending on the kind of typing performed), were associated with severe disease, and more frequently affected people with few classic risk factors such as advanced age and comorbidities. [18] By the time of writing, this strain – now commonly referred to as Multilocus Sequence Type 1 (ST 1) or ribotype (RT) 027 – is now the dominant strain in several European countries. At the same time, it is still uncommon in countries such as Sweden. [19, 20]

Genetic studies in the age of whole genome sequencing made it clear during the 2010s that *C. difficile* is quite distantly related to other species within the

genus *Clostridium* and more closely related to the family *Peptostreptococcaceae*. A renaming to *Peptoclostridium difficile* was therefore suggested, [21] but other authors argued that a name that retained the initial "*C.*" would cause less practical problems. [22] The new name thus became *Clostridioides difficile* in 2016.

Although we cannot know how often *C. difficile* infections in humans occurred before the antibiotic era, it seems reasonable to believe that they were uncommon and that *C. difficile* was mainly a sporadic, harmless guest in our intestines before we began to disturb the gut microbiome with antimicrobials. [23] The epidemiology of *C. difficile* is constantly changing and will likely continue to change with our habits. The basis for this dissertation is that we can influence the direction, or at least rate, of these changes by wise choices.

1.1.1 LIFE CYCLE AND HABITAT

The life cycle of *Clostridioides difficile* comprises two distinct states: vegetative cells and spores, between which it alters depending on external conditions. Unlike true spores, which are multiple offsprings from a mother cell, the endospore is a transformed vegetative cell. For simplicity and according to current convention, *C. difficile* endospores will be referred to as spores in this thesis from here on. The spore has reduced its metabolism to a minimum, decreased its water content substantially, and modified the cell membrane and cell wall to a three-layered protective coat (Figure 2). [24] Vegetative *C. difficile* are strictly anaerobic and thus cannot survive for long in the oxygen-rich environment outside the gut. Conversely, spores are highly resistant to both oxygen and harsh environmental conditions, including exposure to heat, acid, ethanol, and other compounds commonly used to kill bacteria. The spore can germinate and convert back to a vegetative cell when the conditions are favourable. *C. difficile* spores can be found in most environments, inside and outside healthcare facilities. For instance, Janezic et al. [25] recovered *C. difficile* from surfaces in 70 % of investigated households, with the highest recovery rate from shoe soles.

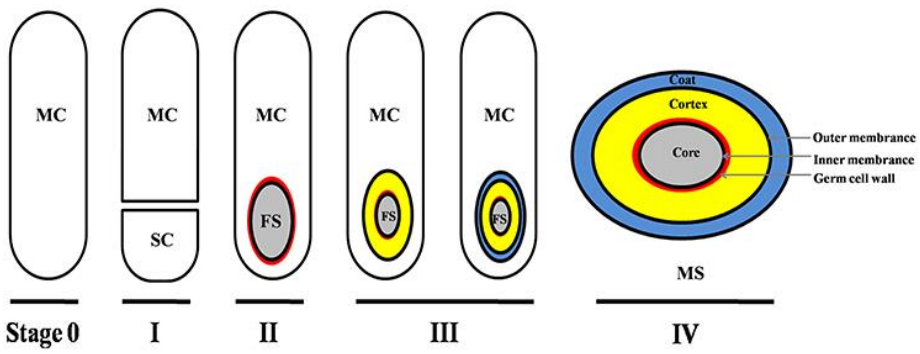


Figure 2. Different stages of sporulation and the final structure of the C. difficile spore. MC, mother cell compartment; SC, smaller compartment; FS, forespore compartment; MS, mature spore. Reproduced with permission from Zhu et al., Front Cell Infect Microbiol, 2018, Copyright Zhu, Sorg & Sun.

Clostridioides difficile has been found in the gut of a diverse range of hosts, including mammals such as horses, [26] sheep, [27] and pigs, [28] as well as at least some birds [29] and reptiles. [30] Regardless of species, the rate of gut colonisation is usually higher in the newly born individuals than in adults. [26-28] This pattern also holds for humans, where the colonisation rates can be as high as 60–70% in infants, [31] and the bacterium was indeed first discovered in infants under ten days of age. [1] This suggests that the ecological niche of vegetative *C. difficile* is in the gut of recently born animals. The spore state enables transfer between hosts as the former host ages and develops a more mature gut microbiome. This view is supported by the fact that germination (the transformation of spores to vegetative bacteria) is triggered not by sensing of nutrients such as sugars and amino acids, as is the case in many other spore-forming bacterial species (e.g., *Bacillus*) but by a particular composition of bile acids. [24] Human infant bile has high levels of, e.g., taurocholate, [32] a potent inducer of germination, while the germination inhibitor chenodeoxycholate [33] occurs in lower levels compared to adults. [32] (Figure 3a-b)

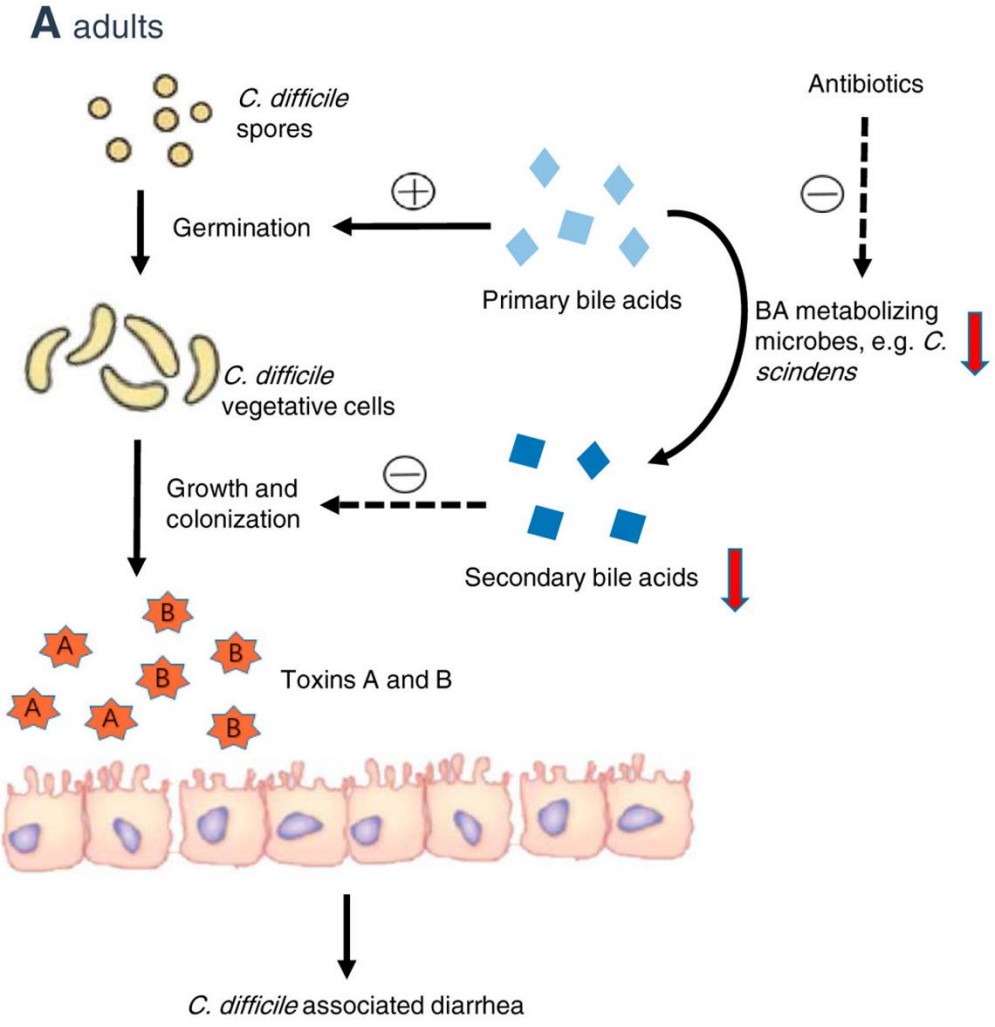


Figure 3a. Interactions between C. difficile and bile acids in the adult human gut. Antibiotic treatment leads to fewer bile acid metabolising microbes, which in turn leads to a bile acid composition that promotes C. difficile spore germination and growth of vegetative cells. C. difficile produces toxins that affect epithelial cells in the colon and cause symptoms. Reprinted with permission from Cheng et al., Physiol Genomics, 2019. [34]

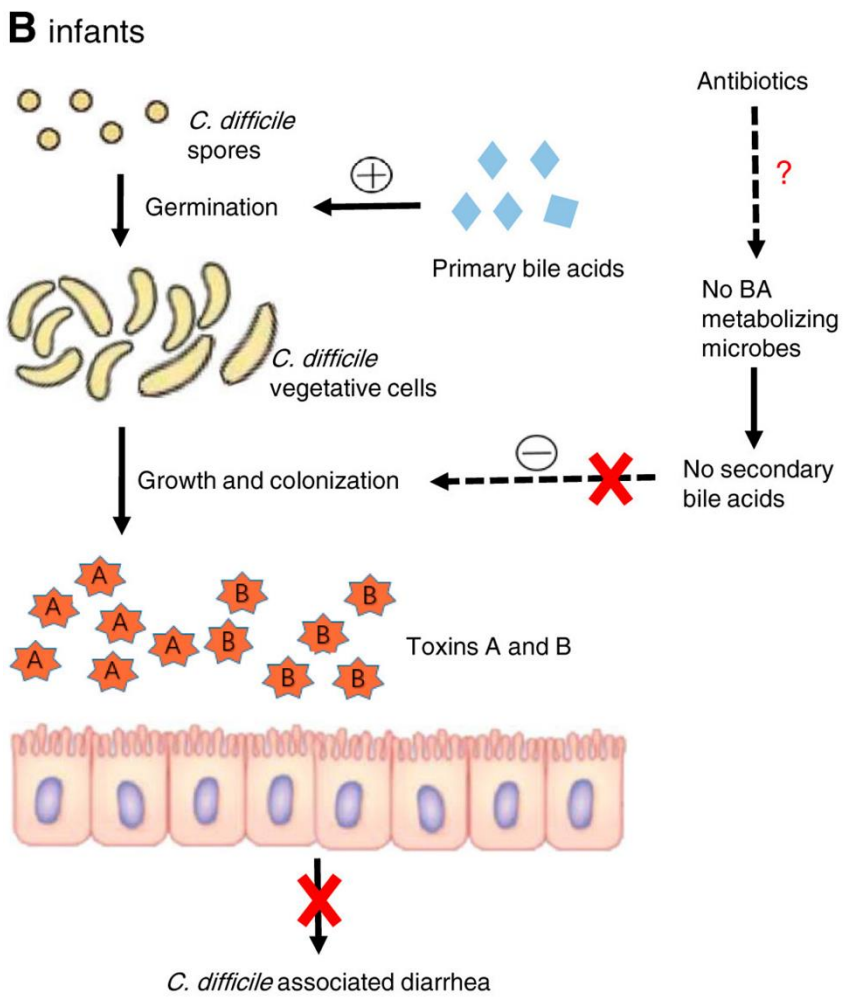


Figure 3b. Interactions between C. difficile *and bile acids in the infant human gut. All bile acids are primary, promoting* C. difficile *spore germination and growth of vegetative cells.* C. difficile *produces toxins that, for yet unknown reasons, do not affect the epithelial cells in the colon. The infant is heacily colonised but asymptomatic. Reprinted with permission from Cheng et al., Physiol Genomics, 2019. [34]*

Rates of asymptomatic *C. difficile* colonisation in healthy adults have varied between 0% and 15% in different studies. [35] In most studies, colonisation has been defined as the detection of *C. difficile* or *C. difficile* toxin in the faeces of individuals in the absence of diarrhoea and other symptoms associated with *C. difficile* infection. This may represent both long-term colonisation and more temporary presence. One study found, for instance, that 6.6% of healthy adults were positive for *C. difficile* in stool, but only a third of those tested positive for the same strain at follow-up testing one months later. [36] Patients with recent hospitalization or stay at a long-term healthcare facility generally have higher colonisation rates than the general population. [35] Thus, although infants have the highest colonisation rate, *C. difficile* can also be part of the adult microbiome without causing disease. This is especially true for non-toxigenic strains, which lack toxins and are protective against symptomatic *C. difficile* infection. [37]

1.1.2 TOXINS AND OTHER VIRULENCE FACTORS

The most important virulence factors of *C. difficile* are Toxin A (TcdA) and Toxin B (TcdB). Both have similar structures and belong to a family of high molecular weight (>250 kDa) bacterial toxins known as large clostridial cytotoxins. [38] They both consist of four subunits with different roles in the multi-step pathogenic process: binding to and uptake in the host cell, translocation from the endosome to the cytoplasm, and activating the enzymatic subunit. This subunit, the glucosyltransferase domain, inactivates GTP-binding proteins of the Rho family in the host cell by glycosylation. [39] GTP-binding proteins act as switches in the cell signaling pathways, in this case, the pathway of building actin fibers vital for the cytoskeleton. Thus, cells exposed to TcdA and TcdB lose their cytoskeletal structure, which can be seen under the microscope as a rounding of cells. [40] In recent years, studies have indicated that apart from the previously described mechanism of action, high concentrations of TcdB may also cause rapid cell death by inducing the intracellular production of reactive oxygen species. [41-43] To which degree this mechanism of action is relevant for the clinical manifestations of *C. difficile* infections is yet unclear.

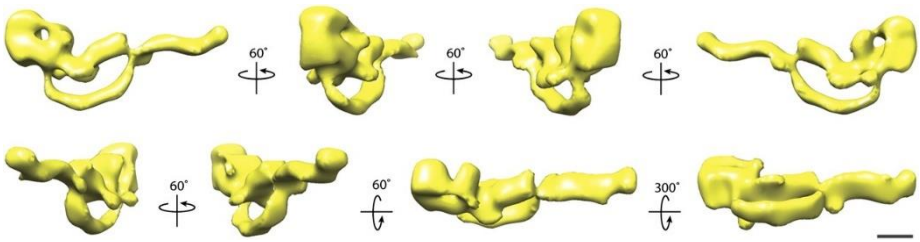


Figure 4. C. difficile *Toxin A as viewed from different angles. 3D reconstruction based on negative stain electron microscopy. Toxin B has a similar structure (not shown). The scale bar in the lower right represents 5 nanometers. Reproduced with permission from Pruitt et al., PNAS, 2010. [44]*

Some strains of *C. difficile*, notably the hypervirulent strain ribotype 027/sequence type 1 (RT027/ST1), produce another toxin. This binary toxin, CDT, was first described in 1997 [45] and belongs to a different family of toxins than TcdA and TcdB. The pathogenic effect is mediated by a direct effect on actin by ADP-ribosylation, inhibiting the proliferation of actin chains. [39] While the association with hypervirulent strains suggests relevance for causing severe infections, the importance of this toxin for pathogenicity is still unclear. Experiments in animal models have suggested that it might induce inflammation in the small intestine rather than the colitis induced by the other toxins. [46]

The genes encoding TcdA and TcdB are, when present, always found in the same genomic location. This chromosomal location is referred to as the Pathogenicity Locus (PaLoc) (Figure 5). The CDT toxin genes (*cdtA* and *cdtB*) are present at another genomic location. Besides the *tcdA* and *tcdB* genes, PaLoc includes three genes believed to have regulatory (*tcdR/tcdD* and *tcdC*) and secretory (*tcdE*) functions. [47] Despite being present in the chromosome, the presence or absence of the PaLoc varies even between closely related strains. Dingle et al. [48] showed, by comparing the core genome with the PaLoc genome of a large number of isolates, that this "gene package" has been acquired and lost frequently among different clades of *C. difficile* in recent history. Horizontal gene transfer of the PaLoc has also been demonstrated. [49] Thus, the PaLoc can be viewed as a mobile genetic element. [50]

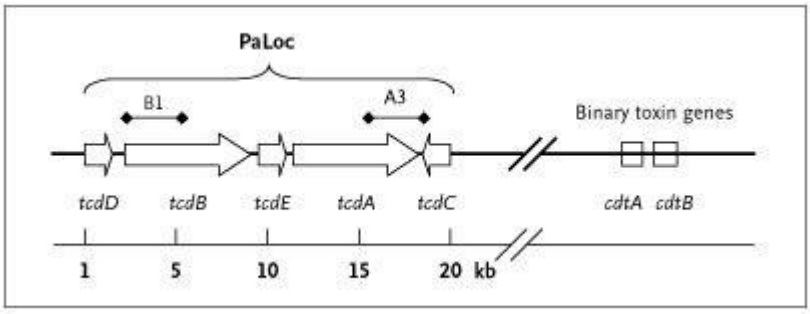


Figure 5. Genes within the Pathogenicity Locus (PaLoc) of the C. difficile *genome. The cdt genes are present in a different genomic location. Reproduced with permission from McDonald et al., N Engl J Med, 2005, [51] Copyright Massachusetts Medical Society.*

Interestingly, human infants and young individuals of most other species seem to be largely unaffected by *C. difficile* toxins. [31] The reason for this is yet to be wholly understood, but it has been proposed, based on animal studies, that the infant's gut lacks the receptors needed for toxin uptake. [52] Toxigenic strains still excrete toxins when they colonise the infant gut, [53] apparently to no benefit but at the expense of the nutrient resources required to build toxins. Given the assumption that the infant gut of animals and humans is their main niche, the benefit for the bacteria of producing toxins in these circumstances seems questionable. Toxin production in *C. difficile* might be better explained by evolutionary forces exerted on the PaLoc itself as a mobile element. [54]

In non-toxigenic strains, the PaLoc is replaced by a short, non-coding region. [47] Toxigenic strains can be divided into different toxinotypes based on the genetic sequence in the PaLoc and the *cdt* gene. [55] Most toxinotypes code for both TcdA and TcdB, but some only have the *tcdB* gene, and one toxinotype that is *tcdB* negative but *tcdA* positive has been described. [47]

Although toxins are the main virulence factors and non-toxigenic strains are considered apathogenic, a few other structures have been proposed as additional virulence factors. The surface layer protein of *C. difficile* consists of a high molecular weight and a low molecular weight subunit. It is vital in adherence to gastrointestinal cells. [56] The role in pathogenesis is still being

determined for other structures, such as fimbriae, pili, flagellae, and surface polysaccharides. [57]

1.2 *C. DIFFICILE* INFECTION

C. difficile spores enter the body through the oral route. Whether this occurs mainly by contaminated hands, food intake, or other ways has yet to be thoroughly investigated. However, as *C. difficile* spores are ubiquitous in most environments, [58] it can be presumed that we all ingest them from time to time by our regular habits. The spores, with their protective coat, can survive the gastric acid of the stomach. [59] They remain dormant at least until reaching the distal part of the small intestine, the ileum. Germination can occur in this part of the gut if the conditions are favourable. A combination of adequate levels of certain bile acids (e.g., taurocholic acid) and either amino acids (e.g., glycine) or divalent cations (e.g., calcium) induces the transformation from spores to active, vegetative bacteria. [60]

As previously stated, the bile acid composition in infant intestines is more likely to induce germination, which can explain the high colonisation rate of this group. Likewise, antibiotic treatment affects other bacterial species in the adult gut microbiome, which have a role in deconjugating and converting primary bile acids to secondary ones. This leads to a composition of bile salts resembling that in the infant gut and an increased chance of germination of *C. difficile* spores in intestines where the host has received antibiotics. [34]

Entering the colon in the vegetative state, *C. difficile* must compete for nutrients amongst countless species of commensal bacteria. The chance of survival and proliferation at this stage is assumed to increase significantly if the microbiome has been decimated by recent antibiotic treatment. During the growth phase, toxin production is inhibited. [61] This may explain the paradoxical finding that growth rate is inversely correlated to disease severity. [62] There is a trade-off between proliferation and toxin production, which consumes considerable resources given the large size of the toxins.

To achieve long-term colonisation, it is plausible that biofilm formation by the bacteria is vital. *C. difficile* biofilm was first described as late as 2012. [63] Subsequently, *C. difficile* has been shown to form biofilms with other

gut commensals in mouse intestines [64] and human intestine models. [65] Both spores and toxins can be found in mature biofilms. Furthermore, the resistance to metronidazole is increased 100-fold, [4] suggesting that surviving biofilms may be important for recurrences after a primary infection.

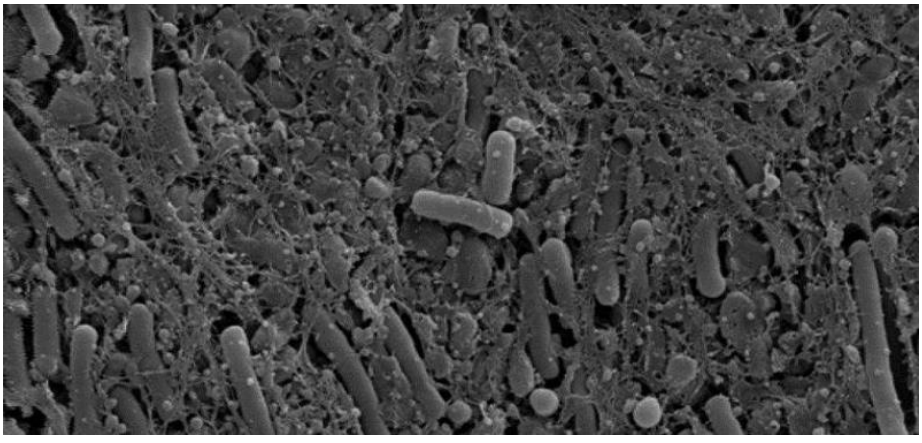


Figure 6. Six days old biofilm of C. difficile*, as viewed in a scanning electron microscope. Work available in the Creative Commons Public Domains, produced by Semenyuk et al. PLoS One, 2014. [4]*

After successful proliferation in the large intestine, some vegetative bacteria are transformed into dormant spores. The trigger for sporulation is not yet known, [66] but a proportion of vegetative bacteria are required to turn into the spore state to enable transmission to new hosts. The spores are excreted with the faeces and deposited in the environment, where they can survive for months or even years. [67, 68] If and when they, by chance, enter a new gastrointestinal tract, the cycle starts over.

1.2.1 CLINICAL PICTURE, TREATMENT, AND RECURRENCE

Clostridioides difficile infections manifest on a scale from mildly loose stools to life-threatening, fulminant pancolitis. Diarrhoea, defined as three or more loose stools within 24 hours, is the cardinal infection symptom. [69] Other symptoms include fever, nausea, and abdominal pain. In more severe cases,

the infection may progress to cause (but is not the sole cause of) pseudomembranous colitis. This condition is characterized by macroscopically visible yellow-white plaques on the colonic mucosa (Figure 7). [70, 71] Severe cases can also present with ileus, [72] where diarrhoea may be absent, or toxic megacolon, [73, 74] Signs of fulminant disease include elevated leukocyte counts, abdominal distension and pain, and hemodynamic instability. [75]

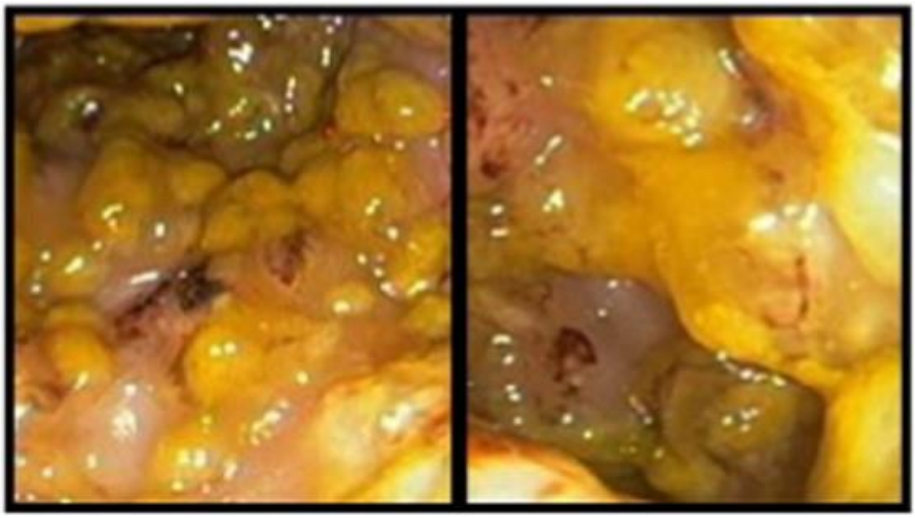


Figure 7. Pseudomembranes on the colonic mucosa, as viewed by flexible sigmoidoscopy. Reprinted with permission from Farooq et al 2015, [71] Copyright Elsevier.

In mild cases, discontinuation of the inciting antibiotic can be enough to resolve the infection. [76, 77] Otherwise, there are a few different choices for treating *C. difficile* infection. Metronidazole is an anaerobe-specific antibiotic with an effect on *C. difficile* and has been widely used historically for this purpose. However, randomized controlled studies have shown an inferior clinical success rates compared to vancomycin, especially in more severe disease. [78, 79] Recent European guidelines recommend metronidazole only in settings where the alternatives are unavailable. [77] Instead, vancomycin or fidaxomicin are recommended as first-line choices. Vancomycin is a glycopeptide antibiotic that affects the cell wall of gram-positive bacteria. Its clinical effect on *C. difficile* infection, with around 85–90% clinical cure rate, is similar to that of the newer drug fidaxomicin, [80] which was introduced in 2011. Fidaxomicin is a narrow-spectrum antibiotic that selectively targets *C.*

difficile, preventing further disruption of the gut microbiota. [81] This is thought to contribute to the lower frequency (around 30% lower [82]) of recurrences seen in fidaxomicin-treated patients compared to vancomycin-treated ones, [80, 83] which is its main advantage. The drawback in current clinical practice is its high cost, which so far has precluded wide use in most settings.

Recurrences occur in around 20% of cases treated with metronidazole or vancomycin. [84, 85] In these cases, symptoms re-emerge after finished treatment, usually within the first few weeks but sometimes after months. [86] The high rate of recurrences compared to other bacterial infections is assumed to be due to surviving spores in the environment or the colon, as these are unaffected by antibiotics. [87] The same strain as that involved in the primary infection causes most recurrences, but new strains are responsible in 10-40% of cases. [86, 88] After one recurrence, the risk of subsequent recurrences is increased, [89] sometimes leading to a hard-to-break cycle of recurrences and treatments. Restoration of a normal gut microbiome by transplantation to the colon of faecal contents from a donor (faecal microbiota transplantation, FMT) has proved to be an effective and relatively inexpensive treatment method to break this cycle. [90]

Aside from antibiotics and FMT, fluid restoration and other supportive care is often indicated. In the more severe cases of fulminant colitis, emergency surgical treatment such as colectomy should be considered. [91] Lastly, a monoclonal antibody directed at TcdB, bezlotoxumab, has recently been added to the arsenal of treatment options. It is recommended as additional therapy in some cases, such as recurrence after fidaxomicin treatment of the primary infection, in current European guidelines. [77]

1.2.2 DIAGNOSTICS

The cell culture cytotoxicity assay (CCTA), first developed by Chang et al. in the late 1970s, [13] was the first diagnostic test for *C. difficile* infection and is still considered a valid reference method for evaluating newer laboratory methods. [92] The test is performed by exposing cell cultures to a faecal filtrate from the patient with suspected infection, with and without antibodies directed at the toxin. If the test is positive, cell rounding caused by the toxin can be observed after 24–48 hours, but not in the cell culture where antitoxin was added. As the test requires cell culture facilities and experience in

assessing cell rounding, and takes several days to perform, it is no longer widely used in clinical practice.

Alternative diagnostic methods were developed during the 1980s, including selective media enabling *C. difficile* culturing [93] and enzyme immunoassays (EIAs) permitting the detection of TcdA or TcdB directly from faecal samples. [94] The main problem with the first approach was that non-toxigenic strains would grow in the cultures, decreasing the specificity of the analysis. The second approach had the reverse problem: high specificity but limited sensitivity, [95] especially for TcdB. [96] A combination of the two methods was eventually proposed: toxigenic culture, where culture is performed followed by an EIA on the cultured strains. [97] Toxigenic culture became an alternative reference standard, [92] but the main drawback in clinical practice was the long turnaround time of three or more days.

In the early 1990s, a latex test with high sensitivity but low specificity was developed, [98, 99] aiming at a glutamate dehydrogenase (GDH). This antigen is specific to *C. difficile* but is produced by non-toxigenic as well as toxigenic strains. The diagnostic performance is thus similar to culture, but with the advantage that the test is much faster.

Around the same time, polymerase chain reaction (PCR) methods for *C. difficile* began to be developed. [100] Targeting the toxin genes could circumvent the problem of false positives from non-toxigenic strains while the sensitivity remained high. [101] PCR analyses have later been joined by other types of nucleic acid amplification tests (NAATs). However, the presence of toxin genes in faeces does not necessarily mean that toxins are expressed and active (e.g., not neutralized by antibodies). Wilcox, [102] therefore, argued that, as toxin PCR as a standalone test fails to discriminate between *C. difficile* infection and asymptomatic colonisation, a multi-step approach should be used. Such an approach, where a highly sensitive test and a highly specific test are combined, followed by either a third test or clinical evaluation in ambiguous cases, is currently recommended in European [103] (Figure 8) and American [104] diagnostic guidelines for *C. difficile*. The American guidelines, however, state that a standalone NAAT may be used ”when there are pre-agreed institutional criteria for patient stool submission”, acknowledging the fact that diagnostic performance in terms of positive and negative predictive value is dependent on case selection (i.e., pre-test probability) [105].

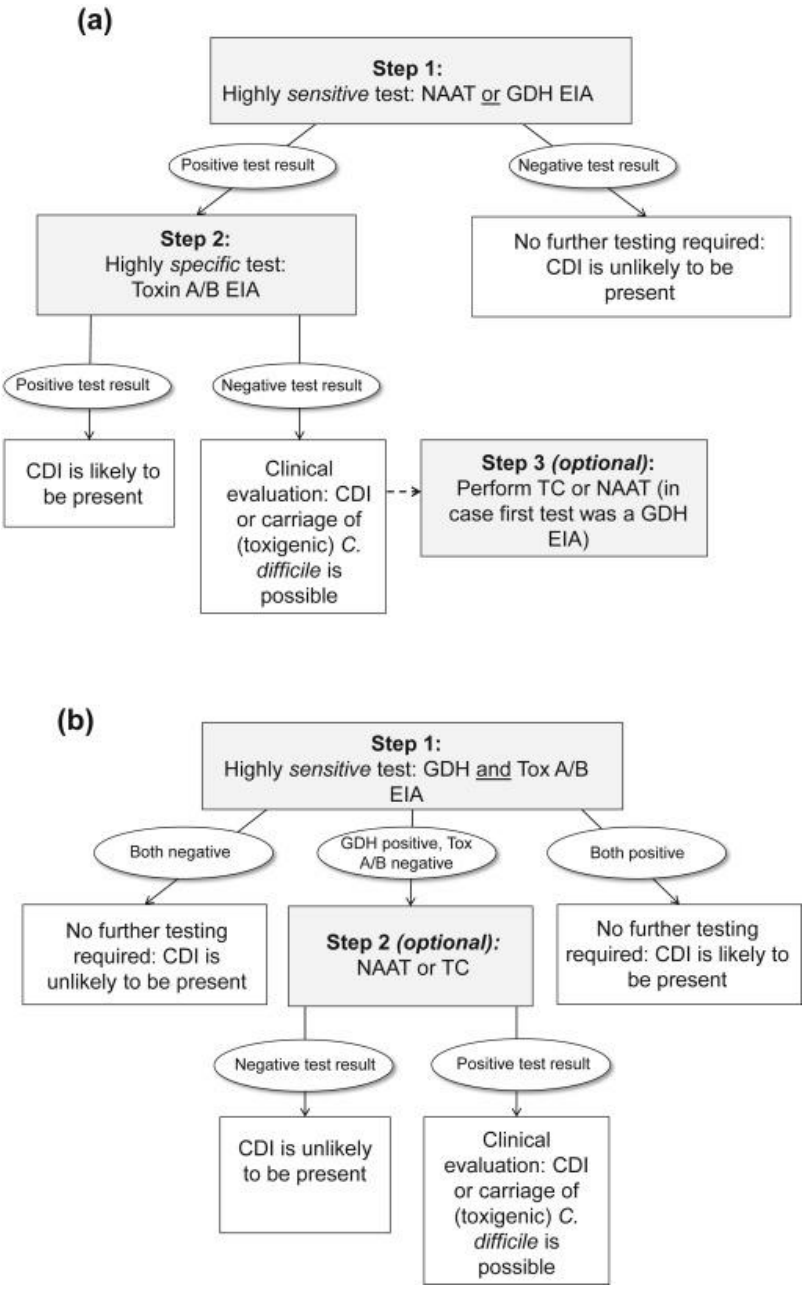


Figure 8. Two alternative (a and b) proposed algorithms in the current European guidelines for C. difficile *diagnostics, combining test of high sensitivity with tests of high specificity. Reproduced under the Creative Commons CC-BY-NC-ND license, from Crobach et al, CMI, 2016. [103]*

In Sweden, diagnostic methods vary. Most clinical laboratories shifted from EIAs to PCR during the 2010s. An evaluation of this shift showed no apparent signs of an increased number of false positives for PCR, which may be due to a clinical tradition in Sweden of only testing for *C. difficile* upon clinical suspicion. [106] Some laboratories use PCR as a stand-alone test, while other use the two-step approach described above.

1.2.3 TYPING METHODS

During the 1980s, clusters of pseudomembranous colitis and other manifestations of *C. difficile* infections started to be recognized, and the need for typing methods for outbreak investigations and surveillance became apparent. Early methods relied on identifying phenotypic characteristics such as soluble proteins, extracellular antigens, and toxins by different kinds of electrophoresis. [107]

The next step was to develop genotypic rather than phenotypic methods, improving the discriminatory resolution. DNA can be fragmented by restriction endonucleases, enzymes that cleave DNA at specific sequences (e.g., AAGCTT for endonuclease HindIII [108]). The fragments can then be separated by gel electrophoresis, resulting in an array of bands representing different DNA fragment sizes. The method described is called restriction endonuclease analysis (REA). [109] In a further development of the method, pulsed field electrophoresis (PFGE), the electric field changes direction, which makes it possible to separate larger DNA fragments and in many cases achieve a higher discriminatory resolution. [110] A third endonuclease-based method is ribotyping, where the separated DNA fragments are hybridized with labelled ribosomal RNA probes. The probed sequences present in the sample are thus visualised, and the pattern of visualised gene fragments enables the discrimination of different ribotypes. [111] In a comparison between these three methods in 1994, [112] REA and PFGE had similar discriminatory power, while ribotyping performed worse than the other two.

In the mid-1990s, the PCR technique was applied to the concept of ribotyping of bacteria (Figure 9). [113] Instead of relying on probe hybridization of ribosomal sequences present in the samples, suitable sequences could now be multiplied before electrophoresis. Later, the method's performance was improved by using high-resolution capillary gel-based electrophoresis. [114] Importantly, reproducibility across laboratories

was achieved through a centralized standardized protocol and reference database of ribotypes. [115] PCR ribotyping became a standard for *C. difficile* typing. However, with little more than a hundred ribotypes, of which a few are common in a given setting, the resolution is insufficient to confirm close relationships between strains. [116]

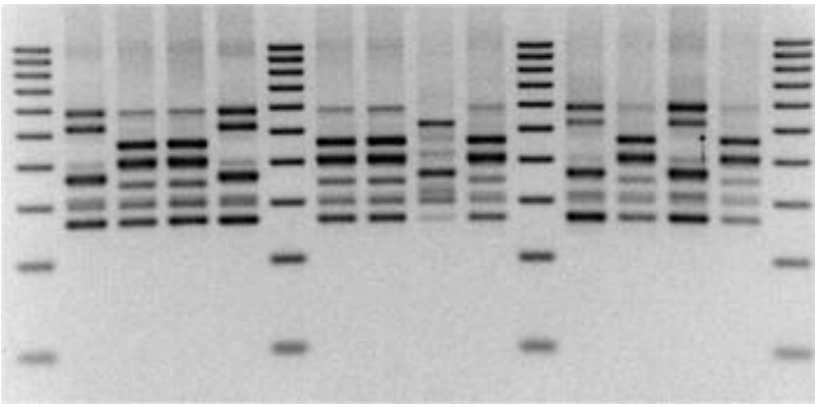


Figure 9. PCR ribotyping by agarosis gel electrophoresis, introduced in the mid-1990s. Ribosomal gene fragments are multiplied and then separated based on their size, resulting in different patterns for different strains. Reprinted with permission from Brazier, CMI, 2001. [117]

In 2006, the whole genome of *C. difficile* was first sequenced. [118] During the 2010s, sequencing methods increasingly became more efficient, affordable, and applied in epidemiological investigations of outbreaks. [119] Multilocus sequence typing (MLST), where around seven housekeeping genes are sequenced, was an early sequence-based typing method. [120] However, the resolution for *C. difficile* is comparable to that of PCR ribotyping. [121] Whole genome sequencing (WGS) offers the ultimate level of resolution, down to the single nucleotide, and has increasingly been applied in *C. difficile* outbreak investigations in the last decade. [116, 122] While WGS may be the ultimate way to compare two isolates, the high resolution becomes a data processing liability when large amounts of strains are to be compared to each other, and there is often also a need to divide similar strains into groups. To address this problem, core genome multilocus sequence typing (cgMLST) has been developed to combine high discriminatory power with manageable amounts of data and the ability to divide strains into defined groups. In this approach, a selection of a few thousand genes are sequenced, and the genomic data are converted to a series

of numbers [123] or letter strings (hashes) [124] that represent variants of each gene.

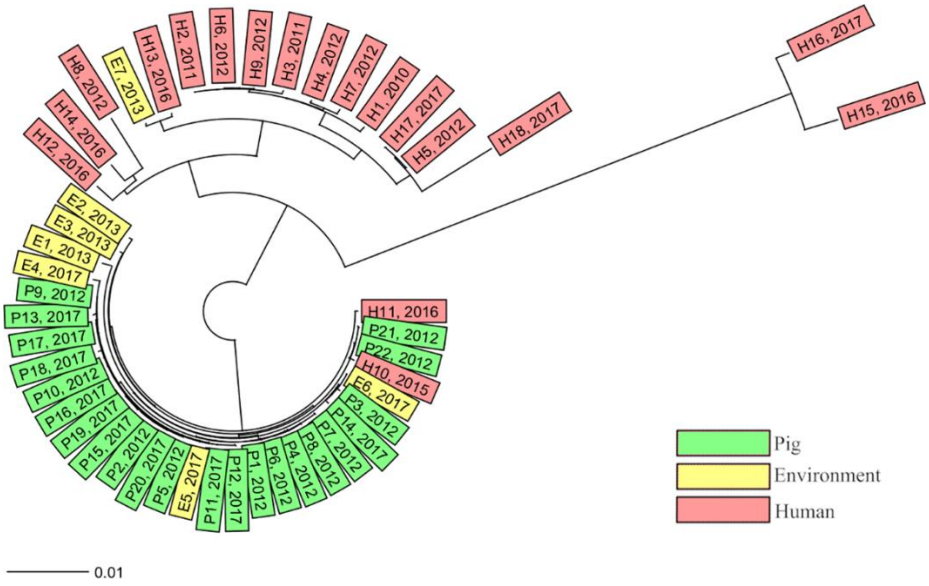


Figure 10. Visualisation of the genetic relatedness between isolates from a suspected cluster. All isolates are sequence type 35/ribotype 046, collected from humans as well as pigs and environmental sources between 2012 and 2017. Whole genome sequencing with cgMLST analysis revealed that some isolates were closely related while others were not. Available under the Creative Commons Attribution License, from Werner et al, PLoS One, 2020. [125]

Confirming close kinship between isolates demands typing methods with as high resolution as possible, but typing is also used to rule out suspected transmission. This is particularly useful when the investigation starts with an epidemiological link. To prove that two isolates are *not* closely related to each other, discriminatory resolution is less crucial. Instead, speed and affordability are of greater importance for the method's usefulness. A simple and inexpensive way of typing *C. difficile*, based on their high molecular weight surface proteins, was described in 2015 by Rizzardi et al. [126] The method employs matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS), using equipment already available at most clinical microbiology laboratories for routine species identification of bacteria. The typing corresponds roughly to PCR ribotyping but has a lower

resolution. A two-step approach can be employed in suspected outbreaks: first, MALDI-TOF typing to rule out some suspected connections, then, cgMLST or whole genome sequencing to confirm close kinship of still suspected strains. This strategy has been employed at the Swedish national reference laboratory for *C. difficile* since 2019. [127]

1.2.4 EPIDEMIOLOGY

The typical patient who develops *C. difficile* infection is an elderly individual with multiple comorbidities admitted to inpatient care and treated with antibiotics for an infection. Risk factors for infection can be divided into host-related, pharmacological, and related to clinical interventions.

Among host-related risk factors, advanced age is the most robust. [128] With increasing age, the gut microbiota changes and often becomes less diverse, [129] while the innate and adaptive immune responses against *C. difficile* and its toxins weaken. [130] Comorbidities such as chronic kidney disease, diabetes, malignancies, and inflammatory bowel disease have been identified as risk factors in meta-analyses. [131] A plausible mechanism might be that these conditions, like advanced age, affect immunity, the gut microbiome, or both.

Regarding pharmacological risk factors, antibiotic treatment is the strongest [128] and was noted in case series of pseudomembranous colitis even before their association with *C. difficile* was proven. [70] Other medications linked to a higher risk for *C. difficile* infection include proton pump inhibitors [132] and corticosteroids. [128]

Risk factors related to clinical interventions include hospital stay [132] and nasogastric tube feeding. [133] Hospital stay, however, is hard to evaluate separately due to the inevitable confounding with other risk factors. [131] Risk factors for recurrent disease are similar to those for primary infection. [89]

C. difficile infections can be divided into healthcare facility-associated cases, community-associated cases, and infections with unknown association, depending on the most probable origin. There are competing definitions on which infections should be regarded as associated to healthcare. For instance, the Swedish National Board of Health and Welfare regards all infections that

occur due to medical treatment, surgical, or diagnostic procedures as healthcare-associated. [134] By this definition, all *C. difficile* infections that are preceded by antibiotic treatment are regarded as healthcare-associated, regardless of any contact with hospitals. There are also a diverse set of terms in addition to the terms above that are used or previously have been used, such as nosocomial, hospital-associated, hospital-onset, et cetera.

The current definitions by the European Centre of Disease Control [135] regards *C. difficile* infections as healthcare facility-associated if the symptom onset occurs on day three or later, following admission to a healthcare facility on day one, or within four weeks after discharge. If the symptom onset occurs more than 12 weeks after the latest discharge, the case is defined as community-associated, and if symptom onset occurs between 4 and 12 weeks after discharge, it is defined as of unknown association. It is, however, important to note that these definitions are primarily intended to ensure comparable definitions for surveillance. [136] Hence, they are based on expert opinion and not validated against actual acquirement patterns.

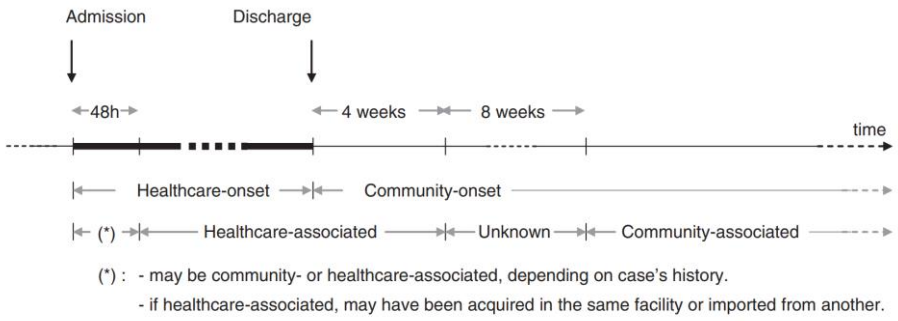


Figure 11. Current definitions recommended for surveillance of healthcare-facility-associated vs. community-associated C. difficile *infections, based on the time relation between symptom onset and hospital care. Reproduced with permission from Kuijper et al, CMI, 2006. [137]*

The incidence of *C. difficile* infections varies globally and has also varied over time. Most large studies have been performed in the Western world in the 21st century, where cumulative incidence has varied between 13 and 147 cases per 100,000 inhabitants per year. [138] In Sweden, this figure decreased from 85/100,000 inhabitants per year in 2012 to 66/100,000 in 2016. [139] In neighbouring countries, the corresponding figures were 92/100,000 per year in Finland in 2013 [140] and 85/100,000 per year in

Denmark in 2019. [141] In England, a remarkable decrease in incidence was seen between 2006 and 2013, [142] down to incidence figures below 20/100,000 inhabitants per year. [143] Thus, there seems to be room for further improvement of the Swedish incidence figures, given the proper measures.

The distribution of different *C. difficile* strains within different populations varies widely as well. In particular, countries differ regarding the prevalence of the epidemic strain ST 1/RT027, [19, 144] which has shown the potential to go from obscurity to the dominating strain in just a few years. For instance, in the English case, the proportion of ST 1/RT027 cases had risen to 25.1% in 2005 and 41.7% in 2006 [145] before the trend was reversed. The strain is usually resistant to fluoroquinolones, and the subsequent decrease in incidence in England has been attributed to the restriction of fluoroquinolone use. [142] The diversity of ribotypes in Sweden is comparably high, with RT014 as the most common, accounting for 10.5% of cases in 2016. [139] ST 1/RT027 is uncommon at <1% of nationally collected samples in 2011-2016, [106] and moxifloxacin resistance declined to <10% in 2016. [139] Only one more minor outbreak with ST 1/RT027 has been described. [146] Other more significant outbreaks in the 2010s have been caused by ST 35/RT046 [147] and a multi-drug resistant, toxin A-negative ST 37/RT017. [139]

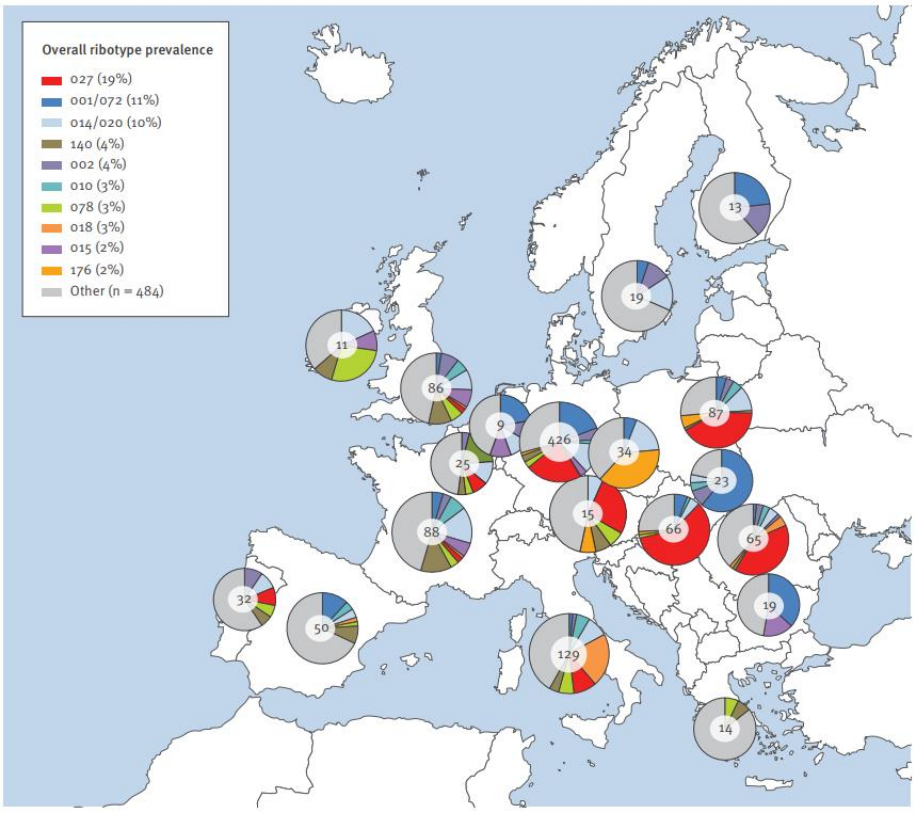


Figure 12. Distribution of ribotypes across Europe 2012-2013. Note the large differences between countries in the prevalence of RT027 (red). Available under the Creative Commons Attribution License, from Davies et al., Eurosurveill, 2016. [19]

1.3 PREVENTION

1.3.1 ANTIBIOTIC STEWARDSHIP

Antibiotic stewardship is a broad term that may include a variety of measures, such as antibiotic resistance surveillance, guidelines for antibiotic use, educational efforts toward healthcare workers or the public, et cetera. The aim is to ensure rational use of antibiotics: to avoid unnecessary use, thereby limiting the inevitable antibiotic resistance development associated with antibiotics.

Alexander Fleming, credited for discovering penicillin, warned against the induction of antimicrobial resistance due to misuse in his Nobel lecture in 1945. [148] New antibiotics were discovered or synthesized in a steady stream in the following two decades, and their use increased rapidly. However, around 1970, the discoveries began to dry up while resistant strains increased in frequency. [149] By the early 1990s, this development was recognized as a crisis, [150] and antibiotic stewardship programmes began to be implemented to ensure more prudent use of antibiotics to combat resistance. [151]

Around the same time, the choice of antibiotics was shown to have a substantial impact on the risk of *C. difficile* infection. [152-154] Clindamycin, cephalosporins, and fluoroquinolones were especially associated with an increased incidence of infections. In several cases, the increased risk coincided with the *C. difficile* strain being resistant to the antibiotic in question. [155] In addition, treatment with broad-spectrum antibiotics affects a larger share of the species in the human microbiome. In this way, they may induce intestinal dysbiosis as well as the selection of resistant strains to a higher degree than narrow-spectrum antibiotics. [156] Thus, antibiotic stewardship programmes aimed to reduce *C. difficile* infections were implemented and evaluated in the 2000s. [157] Today, preventing *C. difficile* infections is usually an integrated goal of antibiotic stewardship programmes. [158]

Meta-analyses have estimated the reduction in *C. difficile* incidence after the implementation of antibiotic stewardship programmes at hospitals to around 30-50%. [159, 160] However, strain distributions and the nature and degree of change of used antibiotics vary, making it difficult to predict the effect of a particular measure in different settings. Most studies have been performed in the UK and the USA, while studies have been scarce in the Nordic countries. A Swedish study published in 2011, where an antibiotic stewardship programme led to modest but statistically significant changes in antibiotic use, showed no change in *C. difficile* infection incidence. [161]

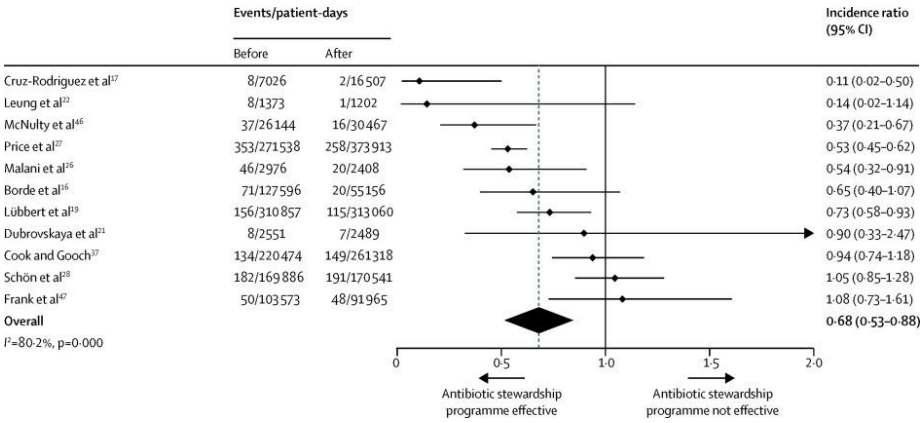


Figure 13. Forest plot of the effect of antibiotic stewardship programmes on C. difficile *infection incidence. The pooled incidence ratio results in an overall 32% decrease. Reproduced with permission from Baur et al., Lancet Infect Dis, 2017. [159]*

1.3.2 INFECTION CONTROL MEASURES

Infection control measures in the healthcare sector aim to prevent the transmission of pathogenic microorganisms to patients. The source can be other patients, healthcare workers, the hospital environment, or the patients themselves (e.g., translocation of *Staphylococcus aureus* from the nasal mucosa to a post-operative wound). Measures include (but are not limited to) isolation, hand hygiene, protective equipment such as gloves and gowns, and cleaning and disinfection of inanimate surfaces and medical equipment.

The resilience of *C. difficile* spores constitutes a challenge for effective hygienic measures. Alcohol-based disinfectants are effective against many bacteria and viruses and are widely used in healthcare facilities for hand hygiene and disinfection of inanimate surfaces. *C. difficile* spores, however, are hardly affected by alcohols. Handwashing with water and soap is more effective than alcohol-based hand rub [162] simply because of its mechanical removal of spores. Transmission of *C. difficile* by the hands of healthcare workers has been implied. [163, 164] In the case of inanimate surfaces, a 2012 study showed that physical removal alone could reduce the spore burden by 2.9 $\log_{10}$ (i.e., 99,8%). [165] More significant 3–6 $\log_{10}$ reductions could be achieved with sporicidal agents such as sodium hypochlorite. However, sodium hypochlorite and other oxidising agents, which can kill off *C. difficile* spores at adequate concentrations, also have potential adverse

side effects on users [166] and the environment. [167] They are not suitable for hand disinfection due to their corrosive effects on the skin. In real-life settings, using sporicidal agents in cleaning protocols tends to reduce, but not eliminate, environmental contamination of *C. difficile* spores. [168, 169]

Inanimate surfaces in the hospital environment have been acknowledged as a reservoir for transmission since the late 1970s. [170] Spore contamination can be found in hospital rooms harbouring patients with and without *C. difficile* in their faecal samples. [171] Increased risk for *C. difficile* infection has been associated with staying in a room where a previous patient was infected, [172] or even, merely treated with antibiotics. [173] Besides symptomatically infected patients, around 3–10% of patients can be expected to be asymptomatically colonised by toxigenic *C. difficile* at hospital admission [174-176] and may act as additional sources of spores. Spores can also enter and spread within hospitals in other ways, such as by contaminated shoe soles from healthcare workers, patients, and visitors. [177, 178] Thus, there is a constant influx of spores to the hospital environment, not limited to excretions from symptomatically infected patients. The spores can then survive for months or years [67, 68] before they may end up in the gastrointestinal canal of a new patient, which in many cases makes transmission pathways very hard to follow.

Transmission dynamics of *C. difficile* have often been viewed as dominated by indirect patient-to-patient transmission with symptomatic patients within the healthcare facility as the primary source. This view has been reflected in recommended infection control measures directed at symptomatic patients: early diagnosis, single-room care, use of gloves and gowns, handwashing with soap, et cetera. [179] However, once these measures are implemented and overt outbreaks are prevented, only a small proportion of healthcare facility-associated *C. difficile* cases can be traced back to another symptomatic case. [180] Even without any contact precautions for symptomatic patients with non-hypervirulent strains, transmission to other patients occurred in only 1.3% of cases in a Swiss study. [181] The sources of the remaining cases have yet to be well known. Even when environmental samples can be linked genetically to infections, it is often impossible to determine which came first: gut colonisation or environmental contamination.

Figure 14. CDC poster promoting contact precautions and hand-washing when caring for patients with C. difficile *infection. Source: Centers for Disease Control and Prevention.*

Although it is not known what proportion of *C. difficile* infections are caused by spores acquired from the hospital environment, it is established that colonisation rates increase during hospital care. [174, 182] Given that spores are dispersed in the hospital environment and not only in rooms of symptomatically infected patients, improved general cleaning practices may potentially reduce healthcare-facility-associated *C. difficile* infections. The literature on this subject is sparse. Chau et al. [183] performed a systematic review and meta-analysis of the effects of environmental cleaning bundles. They found only ten eligible studies, of which one [184] was a randomised controlled trial. Chau et al. concluded that, while the bundles improved the thoroughness of cleaning as measured by removing surface markers or environmental *C. difficile* contamination, no statistically significant effect was found on the *C. difficile* infection incidence. However, several bundles studied only included rooms where symptomatic *C. difficile* infected patients were cared for, not general interventions. The bundles also varied in content, making it difficult to draw definite conclusions on the potential effects of improved cleaning practices for reducing *C. difficile* infections in healthcare facilities.

1.3.3 OUTBREAK SURVEILLANCE

C. difficile outbreaks range from transmission from one patient to another to country-wide outbreaks involving multiple hospitals. Outbreaks can be sudden and dramatic, as in the Växjö outbreak in Sweden, [146] or slow and insidious, such as the Eksjö outbreak. [147] The latter case constitutes a challenge as an increased incidence on the regional, hospital, or ward level may not be evident until many patients have suffered unnecessarily, if even then.

Dubberke et al. described in 2009 [185] a surveillance strategy based on the incidence of hospital-onset *C. difficile* infections. They showed that tracking other cases, such as community-onset cases, did not give any additional valuable information to detect outbreaks. This study is still the basis for the recommendation by the Infectious Disease Society of America to track hospital-onset *C. difficile* infectons as the minimum surveillance effort. [104] Interestingly, the outbreak definition used by Dubberke et al. was solely based on statistical anomalies and not validated by any typing of the strains involved in the suspected outbreaks.

Proposed cut-offs for suspecting outbreaks are often based on simple time-space heuristics, such as “two or more cases of the same microorganism at the same ward within a month” or computer-assisted and based on more detailed statistical calculations. [186] Regardless, they have seldom been validated against an objective assessment of actual transmission. PCR ribotyping has been used for such validation, [187] but the limitation in the resolution of this method precludes definitive conclusions about the performance of the suggested surveillance method. Now that whole genome sequencing is available, with the ability to determine genetic similarity down to the single nucleotide, it is about time to properly validate the methods used for the early detection of outbreaks.

1.3.4 OTHER PREVENTIVE MEASURES

Giving patients probiotics, i.e., viable microorganisms that may benefit the host´s intestinal microbiome and possibly prevent overgrowth of *C. difficile*, has been studied with various species and patient groups. A 2017 Cochrane meta-analysis concluded that there is moderate evidence that probiotics are effective for this purpose when the base-line risk for *C. difficile* infection is greater than 5%. [188] As this is a higher percentage than can be expected in most settings and patient groups, and probiotics may come with adverse side effects, probiotic prevention has so far not been recommended in European guidelines. [77]

C. difficile in animal hosts, and thereby its zoonotic potential, has attracted attention in recent years. Both domestic animals, such as cats and dogs, and foodstuff contaminated with *C. difficile* spores from livestock, are potential sources of transmission to humans. [189] Some researchers have therefore called for a holistic “One Health” perspective on the infection, where clinicians collaborate with veterinarians, environmentalists, and policymakers. [190, 191]

2 AIMS

The overall aim of this thesis was to contribute to the knowledge of how *C. difficile* infections best can be prevented, with a focus on the Swedish setting.

Specific aims were:

I. To determine the effects of a hospital antibiotic stewardship programme restricting cephalosporin use on the incidence of healthcare-facility associated *C. difficile* infections in a Swedish hospital.

II. To evaluate the performance of an early-warning algorithm, based on ward-specific incidence cut-offs, for detecting *C. difficile* transmission in hospitals, and to determine the frequency of intrahospital *C. difficile* transmission in a Swedish setting.

III. To develop a compartmental mathematical model for *C. difficile* transmission within a hospital, encompassing a separate compartment for the environmental spore reservoir, and to use the model to evaluate the effect of possible interventions.

3 PATIENTS AND METHODS

3.1 STUDY DESIGNS

Paper I and Paper II were both observational studies. Paper I had a retrospective design. It can best be viewed as a retrospective cohort study of admitted patients, although data on patients without *C. difficile* infection was limited to aggregated data on the number of admissions and antibiotics consumed. The primary exposure studied was care at a hospital where the antibiotic stewardship programme had been implemented. This intervention had already occurred when we started the data collection. In Paper II, the collection of isolates was prospective. It was primarily a study of the diagnostic performance of two proposed tools for detecting intra-hospital transmission. Paper III was a mathematical modelling study without patient data collection but with assumptions based on results from previously performed studies available in the literature.

3.2 PATIENTS AND SETTING

In Paper I, we evaluated the effect of an antibiotic stewardship programme initiated at Södra Älvsborg Hospital, Borås, Sweden, in 2008. The nearby Skaraborg Hospital, where no antibiotic stewardship programme was implemented, served as a control. Both hospitals are part of the same healthcare administrative region and serve populations of approximately the same size with secondary care. We included patients with a positive *C. difficile* toxin enzyme immunoassay or PCR (depending on the method used at the time and place) who were > 18 years old and fulfilled the healthcare-facility-associated *C. difficile* infection criteria. We based these criteria on those proposed by McDonald et al.; [136] symptom onset either during inpatient care >48 h after admission or symptom onset after discharge but within four weeks after an inpatient care episode. As tests are performed based on clinical symptoms in our setting, we assumed symptoms to be present the day the test was taken, if no information on symptom onset was available. Diagnostic tests were performed at two different clinical microbiology laboratories. We excluded recurrent cases, defined as a diagnosed *C. difficile* infection during the previous eight weeks. Patients fulfilling the criteria in 2007 (before the intervention) as well as in 2012 and

2015 (after the intervention) were studied. The total number of patients was 398.

In Paper II, we studied patients at the same hospitals as in Paper I, but during 2020 and 2021. As we were interested in all cases of possible transmission, we included all patients (regardless of symptoms) with a positive toxin PCR, including community-acquired cases, recurrent cases, and cases where the patient was <18 years old. The total number of patients was 673.

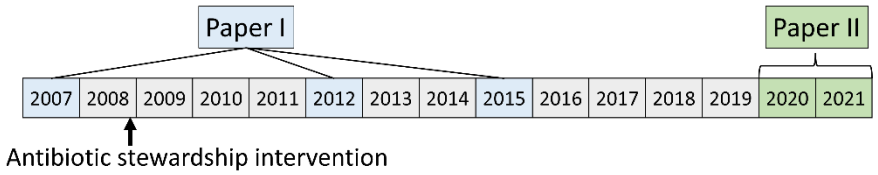
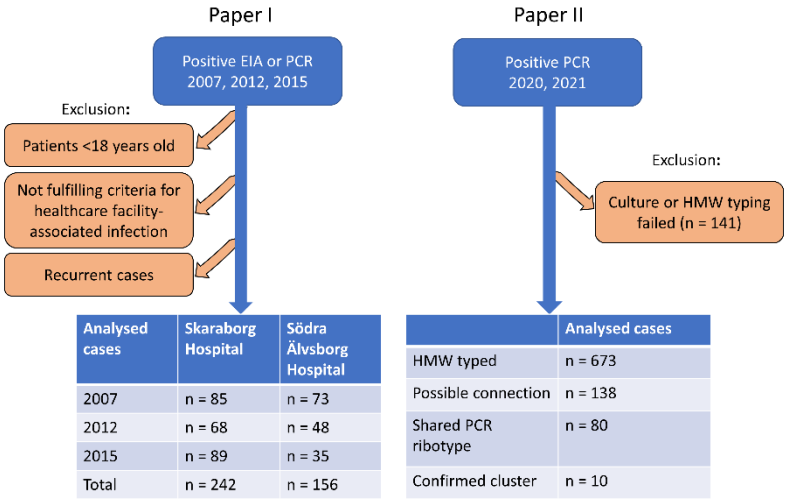


Figure 15. Years studied in Paper I and Paper II.

3.3 CHART REVIEW

In Paper I, we used chart review as the primary data source. Jon Edman-Wallér reviewed medical records and collected data at Södra Älvsborg Hospital, and Johan Karp did the same at Skaraborg Hospital. We had continuous contact during data collection to ensure that we assessed the information in the same way. We recorded several variables: age, sex, number of inpatient days in the last 30 days, and known risk factors for *C. difficile* infection. These included host-specific (inflammatory bowel disease, haematological malignancies, diabetes mellitus, chronic renal failure) and pharmacological (antibiotics, corticosteroids, proton pump inhibitors). We obtained the administration route and a detailed recording of the number of defined daily doses for each class of antibiotics received within 30 days before the positive diagnostic test. We also recorded the number of days since antibiotic treatment had started (if symptom onset occurred during treatment) or since the last antibiotic dose and the positive *C. difficile* test (if symptom onset occurred after treatment). Finally, we collected outcome measures: number of days of care (if any) at an intensive care unit, the number of days of *C. difficile* infection treatment, and whether death had occurred within 30 days of the positive test.

In Paper II, Jon Edman-Wallér and Johan Karp performed the chart review. We recorded age, sex, and death within 30 days after the positive test for each patient, as well as the ward history (inpatient care) within 60 days before and 30 days following the positive test. We used the ward history combined with MALDI-TOF typing (see section 3.8) to identify potential transmission clusters.



Analysed cases	Skaraborg Hospital	Södra Älvsborg Hospital
2007	n = 85	n = 73
2012	n = 68	n = 48
2015	n = 89	n = 35
Total	n = 242	n = 156

	Analysed cases
HMW typed	n = 673
Possible connection	n = 138
Shared PCR ribotype	n = 80
Confirmed cluster	n = 10

Figure 16. Overview of cases included and excluded in Paper I and Paper II. Patients from Skaraborg Hospital and Södra Älvsborg Hospital were studied in both papers.

3.4 HOSPITAL-WIDE DATA COLLECTION

To relate the defined daily doses of antibiotics given to *C. difficile* patients in Paper I to the general consumption of the same antibiotics, we used data from the hospital pharmacy (Södra Älvsborg Hospital) and the Swedish eHealth Agency (Skaraborg Hospital). To get a denominator for the incidence of healthcare facility-associated *C. difficile* infections, we used IBM Cognos (IBM, Armonk, NY, USA) for data on the number of admissions each year.

3.5 ATTRIBUTION OF CASES TO DIFFERENT ANTIBIOTICS

To estimate to which degree different antibiotics caused *C. difficile* infections in Paper I, we used the data for each patient of antibiotics given during the 30 days before the positive test. Each case was attributed to the antibiotics given during this time, based on the amount of defined daily doses of each drug. If only one antibiotic had been given, the whole case was attributed to it. If a patient had received, e.g., four defined daily doses of each of two antibiotics, 0.5 cases were attributed to each drug. This way, we could estimate the number of attributable cases for each antibiotic in the different years and hospitals. We could also relate the attributed cases for a given antibiotic to the total amount of defined daily doses prescribed at the hospital the same year, resulting in a risk estimate for each drug with the unit healthcare-facility-associated *C. difficile* infection cases per 10,000 defined daily doses.

To estimate the potential of an antibiotic stewardship programme in a Swedish setting in Paper III, we combined Swedish data on hospital antibiotics consumption [192] with a recent meta-analysis on the risk increase for *C. difficile* infections associated with different antibiotics. [193] Point estimates of the Odds Ratio were used, except for antibiotics where the confidence intervals overlapped 1; in this case, it was set to 1. The yearly consumption was then multiplied by $OR - 1$ to estimate the share of attributable excess cases for each antibiotic. Excess cases for all antibiotics were added to estimate the potential decrease in infections if all antibiotics were changed to low-risk agents.

3.6 TOXIN ENZYME IMMUNOASSAY

Enzyme Immunoassays were used clinically, and hence, for inclusion in the study for some of the patients studied in Paper I. Premier® A&B (Meridian Bioscience, Charlotte, NC, USA) was used at both hospitals in 2007 and at Skaraborg Hospital in 2012. In this method, microwells are coated with toxin-specific antibodies. A faecal specimen from the patient is added, and if toxin A or B is present, they bind to the antibodies in the coating. In the next step, other antibodies are added to the well and bind to the toxins. These antibodies are conjugated with an enzyme (horseradish peroxidase) that catalyses the conversion of a chromogenic substrate which is added in the last step. The presence of toxins thus results in a colour shift. [194]

In 2012, the clinical microbiology laboratory at Södra Älvsborg Hospital had changed methods to Vidas® A&B (bioMérieux, Marcy-l'Étoild, France). This is an automated system, formally an Enzyme-linked Fluorescent Assay (ELFA), based on the same principles as described above. Wells are coated with anti-toxin antibodies to which toxins in the faecal samples bind. Additional antibodies directed at the toxins are then added, which are conjugated with biotin. Biotin binds strongly to streptavidin, which is added in conjunction with alkaline phosphatase in the next step. In the last step, alkaline phosphatase catalyses a substrate to a fluorescent product, and the fluorescence is measured. [195] As this test can give borderline positive results, in contrast to the Premier test which is always positive/negative, we included patients with an equivocal result if their medical records revealed that they were treated for clinically suspected *C. difficile* infection or had diarrhoea unexplained by other causes that did not spontaneously resolve. This was a way to limit the effects of the inevitable bias introduced by the different diagnostic methods at the two compared hospitals at different times. The two tests had similar sensitivity and specificity against cell culture cytotoxicity assay in a 2008 evaluation. [196]

3.7 TOXIN PCR

Relevant to Paper I, in 2015, the clinical microbiology laboratory at Skaraborg Hospital had changed their diagnostic test for *C. difficile* from the Premier EIA to a duplex PCR for tcdA and tcdB. The method was based on de Boer et al. [197] with a modification in the form of using eSwab samples at 1:10 dilution in phosphate-buffered saline (PBS) before extraction instead of a direct assay from faeces. Again, this may have introduced bias in Paper I, where incidence figures were compared between hospitals with different diagnostic platforms. However, we compared the positivity rate before and after the change from EIA to PCR at Skaraborg Hospital and found no increase after the change but rather a slight decrease.

In Paper II, Södra Älvsborg Hospital had also changed to a PCR-based method: the BDMax™ Cdiff Assay (Becton, Dickinson & Co, Franklin Lakes, NJ, USA), which is directed at a conserved region within the *tcdB* gene. At Skaraborg Hospital, the in-house PCR step had been substituted by the commercial kit AmpliDiag *C. difficile*+027 (MobiDiag, Espoo, Finland). The potential bias introduced by different diagnostic platforms was a lesser problem in this paper, as there was no aim to make comparisons between hospitals.

3.8 CULTURING AND MALDI-TOF TYPING

In Paper II, all samples positive for toxin PCR were further examined by culturing and MALDI-TOF typing. The analyses were performed at the Unilabs clinical microbiology laboratory at Skaraborg Hospital, Skövde, Sweden. Briefly, a selective *C. difficile* plate made of fastidious anaerobe agar with cycloserine and cefoxitin as additives was used for cultures. The plates were anaerobically incubated in 36 ^{O}C for 2-3 days, after which identified *C. difficile* cultures were spread on a blood agar plate and incubated for another 24 hours. The HMW typing was then performed as described by Rizzardi et al. [126]

3.9 PCR RIBOTYPING

In Paper II, capillary gel electrophoresis-based PCR ribotyping was performed as described by Indra et al. [114] with modifications as described by Rizzardi. [126] The analyses were performed at the Public Health Agency of Sweden, Solna, Sweden. We performed PCR ribotyping on isolates where a transmission event was suspected based on shared ward history and identical MALDI-TOF type.

3.10 WHOLE GENOME SEQUENCING

In Paper II, we performed whole genome sequencing on isolates where transmission was suspected, and their PCR ribotypes were found identical. Whole genome sequencing was performed at the Public Health Agency of Sweden on the Ion Torrent platform, as described by Harvala et al. [198] A mean coverage of 41.1x (standard deviation 17.0x) was obtained. We compared the genomes of isolates from suspected clusters and determined single nucleotide variations between them. We used a cut-off of <3 single nucleotide variations to judge whether the strains were closely related, which we based on earlier studies on the mutation rate for *C. difficile* and proposed cut-offs for suspecting transmission. [180, 199]

3.11 SURVEILLANCE ALGORITHMS

Paper II was initially conceived when I was put in charge of the surveillance of *C. difficile* infections at the Infection Prevention and Control unit at Södra Älvsborg Hospital in 2018. Our surveillance strategy at the time was based

on a manual review of cases at different wards, without any set cut-offs for suspecting outbreaks. We had tried using a simple heuristic rule of "two or more cases at the same ward within the same month" but found that, at some wards, this happened frequently. Reviewing historical data, we found that the baseline incidence could vary tenfold or more between different wards and concluded that ward-specific cut-offs would be more reasonable. We based such cut-offs on the Poisson distribution and described our approach in a short paper. [200] The study presented in Paper II was performed to validate whether this surveillance strategy would be successfully identify transmission events. As a comparison, we also evaluated the simpler rule of two or more cases described above.

3.12 COMPARTMENTAL INFECTIOUS DISEASE MODELLING

Paper III resulted from a desire to understand *C. difficile* infection dynamics better. In this work, we collaborated with a mathematician to design a transmission model of *C. difficile* in a hospital. We decided to use a compartmental model based on the classic susceptible – infected – recovered (SIR) model, which has roots in the early 20th century. [201] In such models, a population is divided into different compartments. A set of differential equations defines the change in the number of individuals in each compartment over time. These changes depend on the number of individuals in other compartments, e.g. the number of patients being infected at a given time interval depends on the number of already infected patients, as they can transmit the infection to susceptible individuals. In our model, we tried to model a typical hospital in a Swedish setting. All assumptions in the model input were as far as possible based on Swedish circumstances, and the final model was calibrated to reflect Swedish incidence numbers and reasonable colonisation rates. The modelled hospital has 500 beds, and patients are admitted and discharged at a certain rate. Most patients admitted do not harbour *C. difficile*, but some are asymptomatically colonised, and a few have a symptomatic infection. Unexposed patients may become exposed during their care, and colonised patients may become infected. A novelty with our model was that we included a separate compartment for *C. difficile* spores, which affects the colonisation rate of unexposed patients. This environmental reservoir may, as the other compartments, increase or decrease depending on the dynamics of the model.

3.13 STATISTICAL METHODS

In Paper I, we compared two hospitals at three different periods, resulting in six groups being compared. As the quantitative variables were not normally distributed according to the D'Agostino and Pearson normality test, we used the non-parametric Kruskal-Wallis test instead of ANOVA for these variables. For categorical variables, the Chi^2 test was performed. When significant differences were detected in these analyses, follow-up post-hoc tests were performed. To limit the number of statistical analyses and avoid mass significance problems, we performed these post-hoc tests only between different hospitals in the same year and different years at the same hospital. The tests employed were the Mann-Whitney U-test for quantitative variables and Fisher´s exact test for categorical ones (for comparisons between hospitals in the same year) or a second round of Kruskal-Wallis or Chi^2 (for comparisons at the same hospitals in different years). Finally, Bonferroni correction was used to correct for multiple comparisons. The study´s statistical significance limit was set at 0.05; this was thus adjusted to 0.01 by Bonferroni correction.

In Paper II, we evaluated the ability of the early-warning algorithms to detect transmission events by calculating the sensitivity, specificity, and positive and negative predictive value with 95% confidence intervals based on the binomial distribution. This calculation was based on individual cases and whether they were part of a cluster. We used Student's t-test for comparisons between groups for continuous variables, as these were approximately normally distributed, and Fisher's exact test for categorical variables. We also calculated the Simpson index (D) for assessing strain diversity. This was calculated by the formula $D = \sum(\frac{n}{N})^2$, where n is the number of a particular strain, and N is the total number of isolates. The Simpson diversity index was then calculated by the formula $1 - D$.

In Paper I, we used GraphPad Prism version 7 (GraphPad Software, San Diego, CA, USA) for statistical analyses and for producing graphs. In Paper II, we used Microsoft Excel 365 (Microsoft, Redmond, WA, USA) and SPSS version 28 (IBM, Armonk, NY, USA) for statistical calculations and graphs. In Paper III, the model was solved in Python using the Scipy-method odeint.

3.14 ETHICAL CONSIDERATIONS

In Paper I and Paper II, the main ethical considerations were related to the personal integrity of the patients involved. As part of the studies, we had to access the patients' medical records, which contain sensitive information regarding the health of individuals. Obtaining informed consent from each patient would have rendered both studies unfeasible. By performing the studies, we argued that the potential gain for *C. difficile* patients overall would outweigh the harm caused by accessing the medical records. The regional ethics board of Göteborg (Paper I, decision number T599-18) and the ethics review authority of Sweden (Paper II, decision number 2019-03298) agreed with this, and ethics approvals were obtained. Ethics approval was not applicable in Paper III, as no actual patients were studied.

4 RESULTS

4.1 IMPACT OF ANTIBIOTIC STEWARDSHIP

At Södra Älvsborg Hospital, where the antibiotic stewardship programme was implemented in 2008, we showed in Paper I that the use of cephalosporins decreased from 19,343 DDDs in 2007 to 2,566 DDDs (-87%) in 2012 and further to 2,389 DDDs (-88%) in 2015. As substitutes, the consumption of other antibiotics, such as piperacillin-tazobactam, benzylpenicillin, and cloxacillin, surged. Changes in the same direction but much less pronounced were noted at Skaraborg Hospital. Parallel to the changed antibiotic use, healthcare-facility-associated *C. difficile* infections decreased substantially at Södra Älvsborg Hospital, from 2.25 cases/1,000 admissions in 2007 to 1.48 (-34%, p = 0.0029) in 2012 and 1.16 cases (-48%, p = 0.0014) in 2015. At Skaraborg Hospital, no statistically significant changes in incidence were seen. Comparing hospitals, the difference in incidence between them was statistically significant in 2015 (1.16 vs. 2.38 cases/1,000 admissions, p = 0.0002).

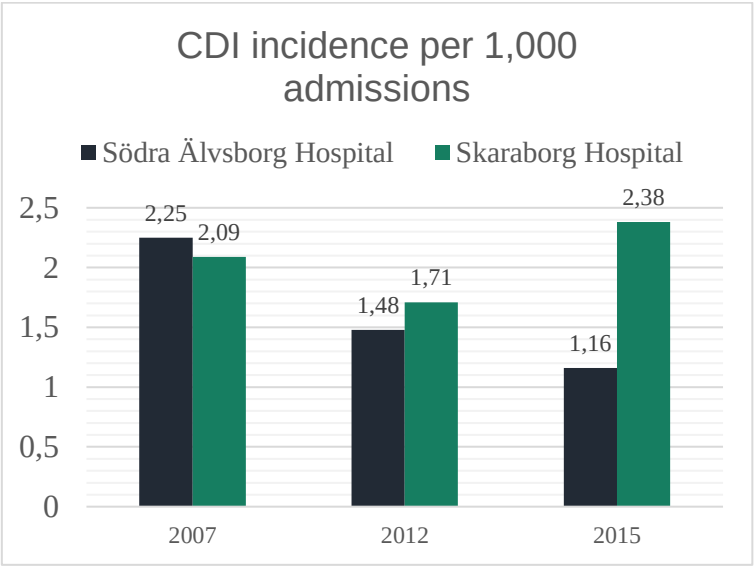


Figure 17. Incidence per 1,000 admissions of C. difficile *infections at the two hospitals in the years studied in Paper I.*

To model a Swedish hospital in Paper III, we combined national data on antibiotic consumption in Swedish hospitals [192] with risk increase estimates of *C. difficile* infections associated with different antibiotics from a recent meta-analysis. [193] By this calculation, we estimated that 30.6% of cases could be prevented if all high-risk antibiotics were substituted for agents with a low risk of causing *C. difficile* infection. Cephalosporins and beta-lactamase-resistant penicillins were estimated to have the largest shares of excess cases due to a combination of elevated risk and high consumption (Figure 18).

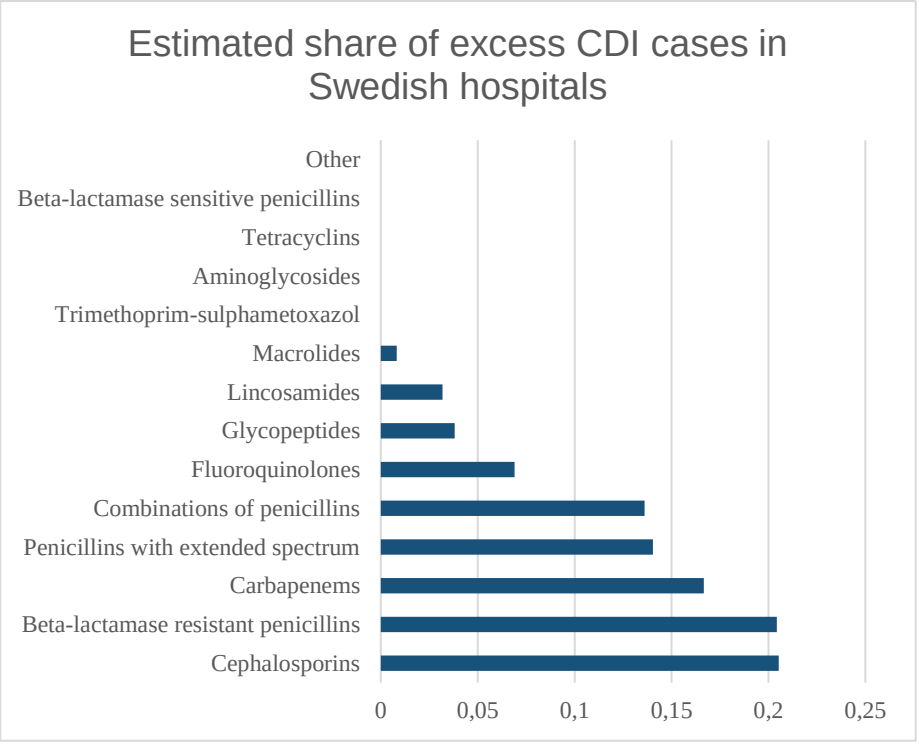


Figure 18. Estimated share of excess CDI cases in Swedish hospitals, based on Swedish data on hospital consumption in 2021 [192] and a meta-analysis of the risk increase of each type of antibiotic. [193] This estimation was used in Paper III for modelling the effects of an antibiotic stewardship programme.

4.2 ANTIBIOTIC-SPECIFIC ATTRIBUTION OF CASES

In Paper I, we compared the number of cases attributable to different antimicrobial agents used within 30 days before the positive test to the total consumption of the same drug at both hospitals. The resulting unit was attributed healthcare-facility-associated cases/10,000 defined daily doses, and the results confirmed the propensity of cephalosporins to increase the risk of *C. difficile* infection more than other antibiotics in our setting (Figure 19). Cefotaxime had the highest number of attributable cases/10,000 DDD at 23.7, followed by clindamycin and cefuroxime at 14.0. While the corresponding number for piperacillin-tazobactam was quite high at 9.2, other substitutes, such as benzylpenicillin (1.5) and cloxacillin (2.2), had substantially lower numbers. Doxycycline had the lowest number at 1.4 cases/10,000 DDD.

We found that, at both hospitals together in 2007, the number of healthcare-facility-associated *C. difficile* infections was 2.16 per 1,000 admissions. Of these, 0.65 cases per 1,000 admissions (30%) could be attributed to cephalosporins. This pattern prevailed at Skaraborg Hospital in 2012 and 2015, with 0.51 cases per 1,000 admissions (30%) in 2012 and 0.79 cases per 1,000 admissions in 2015 (33%) attributable to cephalosporins. At Södra Älvsborg Hospital, in contrast, cases attributable to cephalosporins were 0.05 per 1,000 admissions in both 2012 and 2015. Attributable cases for other antibiotics substituting cephalosporins were found to increase little or not at all over the years studied. The largest effect was seen for piperacillin-tazobactam, which increased in use by 803% between 2007 (1,291 defined daily doses) and 2015 (11,661 defined daily doses), while attributed cases increased by 110% from 0.10 to 0.21 attributable cases per 1,000 admissions.

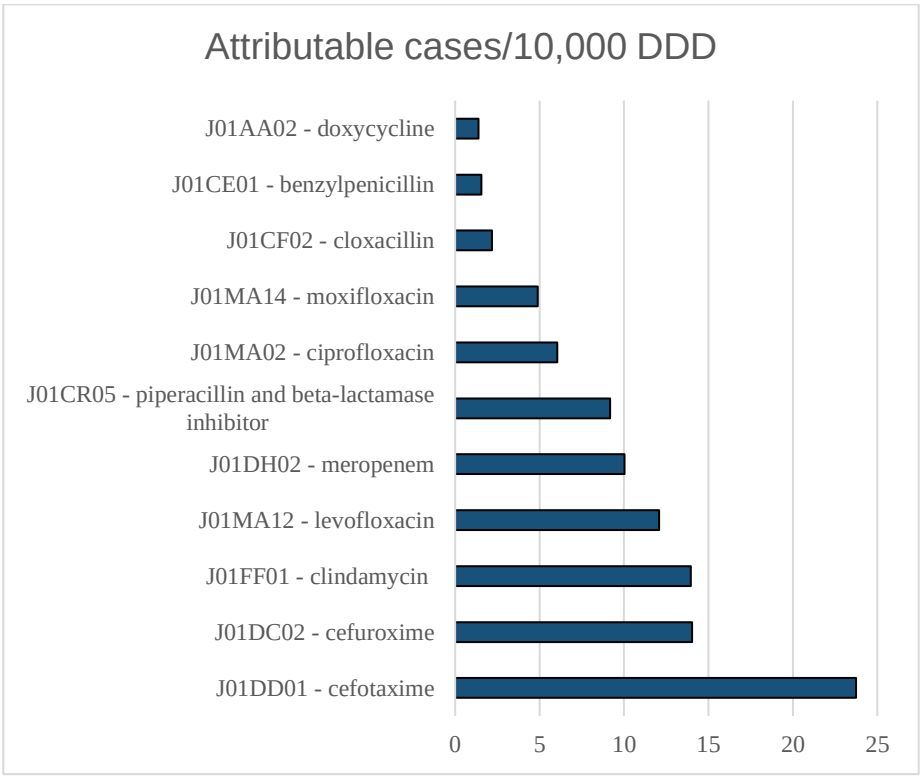


Figure 19. Healthcare-facility-associated C. difficile *infection cases attributable to a selection of different antibiotics related to their total consumption at Skaraborg Hospital and Södra Älvsborg Hospital in 2007, 2012, and 2015.*

4.3 PERFORMANCE OF SURVEILLANCE ALGORITHMS

In Paper II, we found that the surveillance algorithm based on ward-specific cut-offs that we had proposed had a sensitivity of 30.0% and a specificity of 83.7%. The alternative "two-case algorithm" had, as could be expected, a higher sensitivity (50.0%) but a lower specificity (70.3%). The confidence intervals overlapped those expected by chance for both algorithms.

4.4 POTENTIAL OF MODELLED INTERVENTIONS

We found in Paper III that an intervention where patients with symptomatic *C. difficile* infections were isolated to the degree that they shedded no spores to their environment led to moderate decreases in colonisations (-12.2%), infections (-8.6%), and the environmental spore reservoir (-21.8%). Antibiotic stewardship had a greater potential of decreasing the number of infections (-30.6%), while the decrease in spores (-6.2%) and colonisations were modest (-2.5%). Improved removal of spores (+30%) from the hospital environment resulted in substantial decreases in colonised patients (-22.5%), infections (-16.0%), and the environmental spore reservoir (-39.7%).

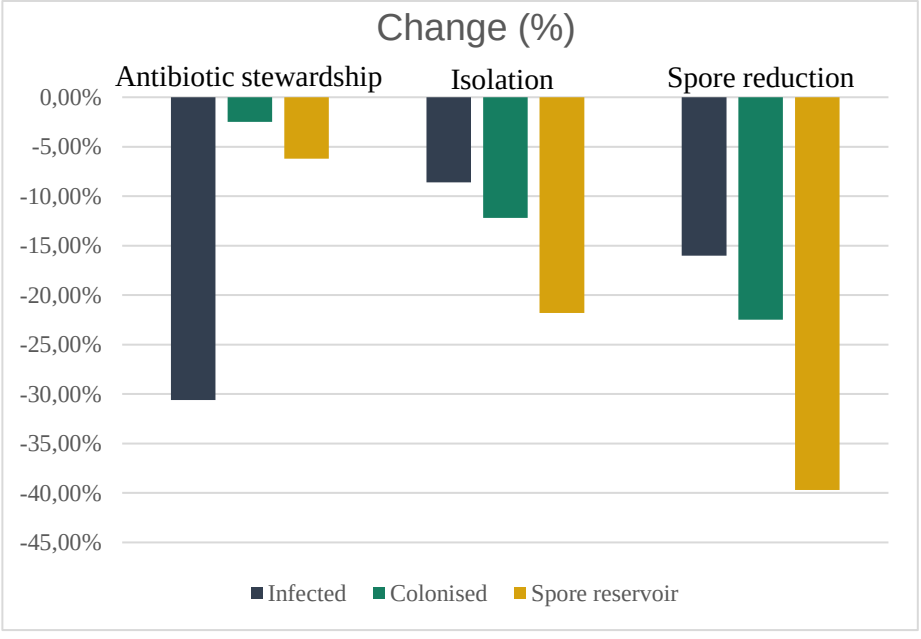


Figure 20. Changes in the number of infected and colonised patients and the environmental spore reservoir level after the interventions modelled in Paper III.

4.5 TIME BETWEEN ANTIBIOTIC TREATMENT AND SYMPTOM ONSET

An incidental finding in Paper I was that there were differences between hospitals and years regarding the time for symptom onset in relation to antibiotic therapy. At Södra Älvsborg Hospital, the proportion of cases where symptom onset occurred during antibiotic therapy was 48% in 2007 and decreased to 44% in 2012 and 34% in 2015. A reverse trend was observed at Skaraborg Hospital, where 60% onset during antibiotic treatment in 2007 increased to 69% in 2012 and 76% in 2015. Furthermore, the proportion of cases without known antibiotic treatment within the last 30 days increased at Södra Älvsborg Hospital from 5% in 2007 to 13% in 2012 and 23% in 2015, while the corresponding figures at Skaraborg Hospital remained essentially unchanged in the years studied. As these results were unexpected, and the study was not designed to investigate this further, we do not know the reasons for the differences. A possible explanation could be that cefotaxime induces *C. difficile* infection more rapidly than other agents. However, there could be other explanations, such as longer treatment times at Skaraborg Hospital. Our research group has planned a follow-up study to investigate this finding more closely.

4.6 TRANSMISSION EVENTS

In Paper II, we found 10 cases (1.5%) that were part of confirmed transmission chains. These were divided into four clusters, with 2–3 patients in each cluster. The patients involved in transmission had a mean age of 77 years; 7/10 were women, and 1/10 died within 30 days of the test. Each cluster had different ribotypes: RT014 (ST 2), RT020 (ST 2), RT001 (ST 3), and RTx231 (ST 11). Each main hospital had two clusters each. The shared wards where transmission likely occurred were two surgical wards and two medical nephrology wards. Only two patients had their *C. difficile* test taken at the same ward as the shared ward where transmission likely occurred.

Table 1. Shared wards and diagnosing wards of patients in confirmed clusters.

Cluster	Patient	Shared ward	Ward where the test was taken
A	S088	Surgical (general)	Gastrointestinal surgery ward
	S117		Medical (general)
	S245		Infectious diseases (outpatient)
B	S225	Medical (nephrology)	Medical (nephrology
	S238		Medical (general)
C	B125	Medical (nephrology)	Infectious diseases
	B145		Medical (nephrology)
	B166	-	Neurology (stroke)
D	B209	Surgical ward (mixed)	Medical (lung)
	B225		Medical (general)

4.7 STRAIN DIVERSITY

The 673 culturable and HMW-typable isolates were distributed over 34 different HMW types. The Simpson diversity index was calculated to be 0.9. The most common HMW type was HMW14, corresponding to two of the most common ribotypes in Sweden, RT014 and RT020. [139] HMW24, corresponding to the epidemic strain ST 1/RT027, occurred only in two cases (0.3%). Among the 138 cases where ribotyping was performed, 33 different ribotypes were identified, of which RT014 and RT020 were the most common.

5 DISCUSSION

Preventive measures have the potential to reduce harm before it has occurred. This idea is the basis for public health science and has led to important achievements for humanity in the last centuries, such as greatly reducing infectious diseases by vaccinations, clean water supplies, and disinfection of instruments and hands in healthcare. However, there are multiple challenges involved in implementing preventive measures in healthcare. First, the preventive effect of a specific measure needs to be demonstrated through scientific studies. Second, managers need to be convinced that resources should be allocated to the preventive measure instead of some other pressing issue. Third, healthcare workers and service personnel also need to be motivated as the intervention often involves changed work tasks. At all these stages, preventive efforts have the disadvantage that the beneficiaries are unidentified, future patients rather than identifiable patients suffering here and now. [202] The physician may prefer a low-toxicity, broad spectrum antibiotic over penicillin to secure that the treatment will be effective in the case that the suspected pneumonia unexpectedly turns out to be a urinary tract infection. The hospital management may prefer to allocate resources to new cancer treatments over building surveillance systems for outbreaks that seem to rarely occur. Research funders may be more interested in projects aiming at specific patient groups rather than projects investigating the effects of improved general cleaning measures. A recurring theme in this discussion section will be that more research is needed.

5.1 ANTIBIOTIC STEWARDSHIP

The potential effects of antibiotic stewardship are dependent on the setting. Sweden has had low frequencies of antimicrobial resistance compared to most countries. [203] Antibiotic stewardship has been implemented to different degrees since 1995 in a national collaboration network called Strama (Swedish strategic programme against antibiotic resistance), with an initial focus on primary care prescriptions and a broader scope over time. [204] Antibiotic use in primary care and hospitals is less extensive and has a higher proportion of narrow-spectrum agents than in most European countries. [205]

The intervention in Paper I was performed in the context of, and was indeed part of, a shift in antibiotic choices for in-hospital patients. Cefuroxime had

been among the most used antibiotics in Swedish hospitals until 2009, when national experts argued for phasing out its use in favour of penicillins (for streptococcal and staphylococcal infections) or cefotaxime (for infections with *Enterobacterales*). [206] Accordingly, at Skaraborg Hospital, cefuroxime use had been reduced to a minimum while cefotaxime had taken its place as the main cephalosporin used by 2012. At Södra Älvsborg Hospital, the change was taken one step further. Critics of the shift from cefuroxime to cefotaxime warned that the higher degree to which active metabolites of cefotaxime are excreted in bile could lead to an increased incidence of *C. difficile* infections. [207] This scenario did not come true regarding increased *C. difficile* incidence nationally, [139] but our findings in Paper I align with the notion that cefotaxime increases the risk for *C. difficile* infection more than cefuroxime. The incidence at Skaraborg Hospital remained essentially unchanged over the years studied, despite a substantially decreased (-36%) overall consumption of cephalosporins.

The setting also affects the potential of antimicrobial stewardship interventions modelled in our Paper III. Based on the Swedish hospital consumption of different antibiotics and risk estimates from a meta-analysis, our estimation is a theoretical calculation and does not consider secondary effects such as lower transmission rates. Still, a 31% decrease seems reasonable, given our results in Paper I and other previous studies on antibiotic stewardship programmes. The strength of the approach is that a similar calculation can be made for any other setting where the estimated potential of antibiotic stewardship programmes would be different. In Paper I, the cases attributable to specific antibiotics were calculated in another way, yet there is a high degree of similarity between the results. This is true for the total share of excess cases as well as the estimations for specific antibiotic classes. However, in the calculation in Paper I, there may be a bias of indication as broad-spectrum, intravenous antibiotics are more often administered to more severely ill patients than oral, narrow-spectrum agents. These patients are more likely to have other risk factors for *C. difficile* infection, such as advanced age and co-morbidities, which are not considered in our calculation.

Most previous studies on *C. difficile* infections related to antibiotic stewardship programmes have been performed in North America and the United Kingdom, where antibiotic consumption and ribotype distributions differ from the Swedish setting. Some antibiotic stewardship programmes in the meta-analysis by Baur et al. [159] were implemented at the hospital level,

others at a ward or unit level. Furthermore, the antibiotics targeted for reduction varied considerably. In one case, cephalosporin use even increased as part of the stewardship programme. [208] Thus, antibiotic stewardship is a heterogeneous concept, and there is reason to continue evaluating the effects of specific interventions in specific settings.

As mentioned in the methods section, differences in the diagnostic platforms used at the time may have influenced the results in Paper I. In 2015, when the difference between hospitals was statistically significant, Skaraborg Hospital used a PCR toxin test while Södra Älvsborg Hospital still used an enzyme immunoassay as a stand-alone test. The ~50% lower incidence figure at Södra Älvsborg Hospital in 2015 may, therefore, be an exaggerated result. However, the attribution of cases to different inciting antibiotics per 10,000 defined daily doses was very well aligned to risk estimates for hospital-acquired *C. difficile* infections in other studies. [193] Around 30% of cases at Skaraborg Hospital could be attributed to cephalosporins in all years studied, while these cases almost vanished at Södra Älvsborg Hospital.

While a decrease in incidence occurred after an antibiotic stewardship intervention, there may have been other factors involved. For instance, a new hospital building was inaugurated at Södra Älvsborg Hospital in 2010. This may have led to a "reset" of environmental spore levels in parts of the hospital, and thus contributed to a decrease in *C. difficile* infection incidence. In a 2022 Japanese study, relocation to a new hospital building was associated with a significant decrease of *C. difficile* infections. [209] Other possible confounders include differences in the compliance to infection control routines, cleaning, and disinfection.

5.2 IN-HOSPITAL TRANSMISSION

Our results in Paper II show that *C. difficile* transmission within wards from symptomatic patients to patients who subsequently fall ill is an infrequent event. These results are in line with the other findings in the last decade, where the high discriminatory power of whole genome sequencing has revealed that many apparent clusters based on earlier typing methods were, in many cases, quite distant genetically. [125, 180, 210] The results also align with a study from another Swedish region published in 2018. [146] Among 1000 isolates over almost four years in two counties (total population ~338,000), three clusters with a total of 24 isolates (2.4%) were confirmed by WGS to be closely related. However, no more than 29 isolates (2.9%) were

sequenced, compared to 80/673 (11.8%) in our study. The largest cluster (12 cases) was the so far only occurrence of an ST 1/RT027 outbreak in Sweden.

Taken together, in a Swedish setting, with a diverse type distribution and strong antibiotic stewardship practice, *C. difficile* clusters appear infrequently. Still, we found one cluster per hospital and year, which perhaps could have been prevented. On the other hand, the resources needed to prevent these few transmission events may not be proportional to the effects, as there is an alternative cost for every measure taken.

Neither our study nor that by Ortega et al. [146] was designed to capture transmission from asymptomatically colonised patients. This patient group was suggested as a potentially important reservoir in the 2000's [164] and has been increasingly investigated in the last ten years. Eyre et al. [174] followed a cohort of 132 medical inpatients, of which 14 (11%) were asymptomatically colonised at baseline. Their *C. difficile* isolates were whole genome sequenced and compared to isolates from clinical cases during approximately the same time. Based on genetic similarity and epidemiological connections, the authors found no clear cases where asymptomatic colonisation later resulted in a symptomatic infection in another patient. However, the authors concluded this does not rule out asymptomatically colonised patients as a substantial reservoir since the cohort studied was rather small.

In a 2018 study by Donskey et al., [211] transmissions from symptomatic infections and asymptomatic colonisations were identified within a hospital and its associated long-term healthcare facility. They used a combination of REA typing and WGS. The authors found seven suspected transmissions out of 37 cases (18.9%), of which 5 (13.5%) were from asymptomatically colonised patients. This high frequency of identified transmissions compared to other studies might be partially due to the inclusion of both a hospital and its associated long-term healthcare facility in the analysis. However, it is notable that all confirmed transmissions were REA typed to BI, corresponding to the epidemic strain ST 1/RT027. No transmissions were identified for other strains.

In a Danish study from 2017, Blixt et al. [212] took a different approach. A cohort of patients were screened for asymptomatic *C. difficile* colonisation at admission. One of the exposures studied was to be exposed to an asymptomatically colonized patient in the same room. The authors found that

this exposure increased the risk of developing *C. difficile* infection from 2.6% to 4.6%. Again, however, these results have been strongly influenced by the prevalence of ST 1/RT027: While this strain was prevalent in 9.7% of patients colonised at admittance, it accounted for 70% of the infections in previously unexposed patients. Thus, most infections originating from asymptomatically colonised patients were likely of this strain.

The jury is still out, but transmission from asymptomatically colonised patients seems relevant mainly to ST 1/RT027 infections. For other strains, they likely account for only a minor part of transmissions.

5.3 SURVEILLANCE ALGORITHMS

We showed in Paper II that our proposed algorithm, as well as a simpler algorithm based on two or more cases at a ward in a month, failed to discriminate actual transmission from sporadic cases. The main reason was the time lag between acquiring spores and symptomatic infection. In most cases, patients had either been moved to a different ward or discharged and readmitted during this time span. Most contagious diseases have some time lag between acquisition and symptoms, which means that ward-based surveillance may also be unsuitable for other infections. A way to ensure that all in-hospital transmissions are detected is to perform whole genome sequencing of all infections, but this is so far not feasible or cost-effective in most settings. A combination of validated surveillance algorithms and whole genome sequencing of selected cases may be more reasonable. Improved surveillance could also be achieved by better use of software assistance, where data collected from multiple sources (e.g., laboratory data paired with antibiotic prescription data and ward history data) could be analysed, and warning signals triggered. Still, such systems should also be validated against confirmed transmission events to ensure that they produce the intended results.

In our study, like the study by Ortega et al., [146] we used the inexpensive HMW typing method to sort out possible clusters. Since its development, it has been introduced in several Swedish regions where *C. difficile* is cultured as part of the diagnostics process – especially in counties that have experienced *C. difficile* outbreaks in the recent past. Together with other surveillance and epidemiological linking, it can be used to sort out potential clusters to analyse further by whole genome sequencing.

5.4 MODELLED EFFECTS

In Paper III, we investigated the effect of three possible intervention strategies in our model. In the antibiotic stewardship intervention, we modelled decreased use of antibiotics with increased risk for *C. difficile* infection as a decreased flow from the compartment of colonised to infected. Based on our understanding of the infectious process, it could be argued that antibiotic use also affects the risk of colonisation. For instance, in a trial with twelve healthy volunteers taking oral third-generation cephalosporins, eleven became asymptomatically colonised with *C. difficile* after treatment. [213] However, other types of antibiotics, such as betalactam-betalactamase inhibitor combinations, can decrease the risk of colonisation. [182] In a meta-analysis, antibiotic treatment overall did not have a statistically significant impact on the risk of *C. difficile* colonisation. [214] Hence, our results where antibiotic stewardship substantially affects infections but has modest effects on colonisation are reasonable. Still, these dynamics may be impacted by the choice of antibiotic classes targeted in an antibiotic stewardship intervention. Real-life studies on the effects of antibiotic stewardship have focused mainly on *C. difficile* infections rather than colonisation. More research on the effects on colonisation would be useful to make more detailed assumptions in this area.

In the isolation intervention, we modelled a case where infected patients cannot disseminate spores to the hospital environment, for instance, by being moved to a separate building. The effects of this intervention were moderate. These results highly depend on our assumptions of the rate of spore contamination for infected versus colonised patients. The assumption was that infected patients contribute four times as many spores per time unit to the environment, based on a study of 44 infected and 35 colonised patients and the environmental samples taken in their rooms. [215] Further studies on the subject would be useful to make more accurate estimations. The assumption is also complicated by the fact that infection control measures (e.g., isolation, glove use, oxidative disinfection) are taken when patients are diagnosed with an infection, but usually not when patients are only colonised. Thus, the dissemination of spores is modified by these measures. Furthermore, there may be significant differences between individuals regarding to which degree they contribute spores to the environment.

In the intervention where an increased reduction of spores modelled an improved general level of cleaning and disinfection, we found substantial

effects, especially on the colonisation prevalence and the environmental spore level. As a result, infections also decreased when fewer patients were colonised during their stay. In this case, the challenge is determining what would be needed to increase the reduction rate of spores by 30%, as we modelled. To uphold a higher reduction rate of spores over time, the measures would need to be both effective and sustainable. Such measures may be resource-intensive and the results not immediately obvious as environmental spore levels change slowly. The amount of research on the effectiveness of cleaning and disinfection on *C. difficile* incidence is, so far, limited. [183, 216] Most studies have been uncontrolled, with various risks for bias, and widely varying regarding the nature of the interventions, methods, follow-up time, et cetera. This makes it hard to draw conclusions on the potential effect of general cleaning and disinfection interventions.

6 CONCLUSIONS AND FUTURE PERSPECTIVES

We showed in Paper I that an antibiotic stewardship programme affected the *C. difficile* infection incidence in a Swedish setting. In Paper III, we calculated that there is a potential for a ~31% decrease in *C. difficile* cases by changing antibiotic prescription patterns at an average Swedish hospital based on antibiotic consumption in 2021. Our results strengthen the case that rational use of antibiotics is among the most important ways to decrease the incidence of *C. difficile* infections. Antibiotic stewardship interventions targeting *C. difficile* infections should ideally consider not only the prescription patterns of the local setting, but also the local *C. difficile* epidemiology. Which types are most prevalent and are there strains in circulation with resistance to certain antibiotics?

Improving isolation routines for infected patients may only have moderate effects, according to our mathematical model in Paper III. Other studies point in the same way, such as the Swiss study, [181] where isolation and contact precautions were discontinued for non-hypervirulent strains, and transmissions still occurred infrequently. More rigorous measures for infected patients or asymptomatic carriers seem unlikely to substantially affect the *C. difficile* infection incidence in settings with a low prevalence of epidemic strains. However, ST 1/RT027 has shown how fast and effectively it can spread in hospitals, in individual studies [211, 212] and national surveillance data. [19] While precautions were put in place for this strain in the Swiss study, ST 35/RT046 caused a severe outbreak in Sweden despite the strain not being widely recognized as an epidemic or hypervirulent strain. [147] There is no clear-cut dividing line between endemic and epidemic *C. difficile* strains. Relaxing infection control measures might then be a gamble that risks inviting strains with a high propensity to spread, especially as these strains also tend to cause severe disease.

Improved surveillance practices are essential to detect outbreaks early, but Paper II showed that transmission clusters constitute only a minor part of the total incidence in our setting. Like the infection control measures, active surveillance with appropriate tools is good insurance against severe outbreaks but not something that substantially decreases the number of infections in a non-epidemic setting. However, outbreaks can become very severe in terms of both human suffering and long-term healthcare costs. Given this, it is

striking that methods for outbreak detection have been studied and, especially, validated to such a low degree. This lack of evidence not only exists for *C. difficile* surveillance, but for surveillance of infectious diseases overall, as concluded in a 2016 systematic literature review on early infectious disease outbreak detection. [217]

In our model in Paper III, increasing the rate of environmental spore reduction had large effects, primarily on colonisation but also on the infection prevalence. An increased overall spore reduction rate targets spores regardless of source: infected patients, asymptomatically colonised patients, previous patients, visitors, healthcare workers, et cetera. The question is: how do we effectively improve the spore reduction rate? So far, studies on improved cleaning and disinfection practices have not been too convincing. [183] However, this research area is far from exhausted. Besides the more traditional approaches, interesting alternatives to labour-intensive manual cleaning and disinfection routines include automated systems using ultraviolet light or vaporised hydrogen peroxide. [218]

Over the years, research on *C. difficile* infections and its prevention has mainly been focused on hospitals and other healthcare facilities. This thesis is no exception to that rule. However, given the high diversity of strains in healthcare facility-associated cases, disclosed in recent years by sequencing methods, it may now be time to look up and see the bigger picture. Can *C. difficile* infections be prevented by measures in the community, such as better control of spore contamination of meat, vegetables, and other foodstuff? Could improved cleaning of domestic areas be an effective preventive measure for individuals at high risk? Tracing the infections to their diverse sources, including in the community, is challenging. Still, it may be what is needed to further advance our understanding of effective preventive measures for this disease.

ACKNOWLEDGEMENTS

I would like to thank the following persons for their contributions to this thesis.

Gunnar Jacobsson, my main supervisor, for continuous support and sensible advice through the whole process.

Maria Werner, co-supervisor, for continuous support and for encouraging me to pursue research despite its inevitable ups and downs.

Kristina Rizzardi, co-supervisor, for your support, collaboration, and valuable expertise in diagnostic and typing methods.

Anders Lundqvist, co-author, for great collaboration and for initiating the project which would later become Paper I.

Johan Karp, co-author, for fruitful collaboration on several papers and mutual assistance in the often very tedious data collection.

Philip Gerlee, co-author, for inspirational collaboration and discussions where infectious disease epidemiology meets applied mathematics.

Michael Toepfer, co-author, for invaluable bacteriological assistance as well as much needed language reviews of manuscripts.

Ia Adlerberth and **Martina Wahllöf**, for great feedback at my half-time seminar.

Mattias Molin and **Salmir Nasic**, for consultations and discussions on statistical methods.

My current and former managers **Eva-Marie Boman**, **Markus Håkansson**, **Susann Skovbjerg**, **Bodil Jönsson,** and **Ann-Sofie Gering**, for letting me take the necessary time off for research.

All people involved in the collection, transport, and analysis of samples for Paper II at the Clinical Microbiology laboratories at Södra Älvsborg Hospital, Unilabs Skövde, and the Public Health Agency of Sweden in Solna.

Maria Hedelin, **Ola Bratt**, **Martin Gellerstedt**, and the late **Gunnar Steineck**, for great teaching in epidemiology, research methods, and statistics at the Clinical Research School in Göteborg.

All my co-students at the Clinical Research School for great fun as well as great learning during our two years of courses. Also, **Anna Lindblom**, for inspiring me to apply to the programme.

Agnes Wold and (again) **Ia Adlerberth**, for providing a great platform for teaching: the Nordic master programme in Infection Prevention and Control at the University of Gothenburg.

All current and former students at said programme, as well as all members of the course development committee, for teaching me a lot about teaching since 2019. A special thanks to **Ingemar Qvarfordt** and **Leif Dotevall** for great collaboration on planning the third course of the programme in the middle of the Covid-19 pandemic.

Torbjörn Norén and the other co-members of the group that updated the Swedish recommendations for *C. difficile* infections 2023, a work that served as a good foundation for the thesis writing.

Annika Ljung, for being toastmaster at the dissertation defense party and for lots of laughs in our shared rooms at the clinical microbiology laboratories, both in Göteborg and Borås. Always remember that the Lord works in mysterious ways.

Johan Lindström, for being toastmaster at the dissertation defense party and for fun and clever discussions through the years about research as well as work and family life.

All other colleagues and co-workers that I have had the pleasure to get to know through the years, at the Infectious Diseases and Infection Prevention and Control departments and the Research unit at Södra Älvsborg Hospital, as well as the Clinical Microbiology departments at Södra Älvsborg and Sahlgrenska Hospitals.

Johannes Ohvo, for taking my mind off research by countless of hours of smacking a ball on the squash court.

Anne Filipsson, my mother, for showing by example that anything is possible if you want it and are willing to work for it. Also, for the collaboration on our fiction book “Dödlig smitta” where (spoiler alert) *C. difficile* had a main role.

Per Edman, my father, for showing by example how to balance ambitions and aspirations with pure enjoyment of life.

Liv & **My Wallér**, my beloved daughters, for being who you are and for embracing your father´s interests by learning the names of several bacterial species (including, of course, the tongue twister *Clostridioides difficile*).

Linn Wallér, my wife, for endless support and for reminding me to take a break now and then when I get too caught up in research.

Finally, I would like to thank the following institutions for their financial support of the projects involved in this thesis.

The Local Research and Development Council, Södra Älvsborg.

The Healthcare committee, Region Västra Götaland.

The Centre for Antibiotic Resistance Research at the University of Gothenburg.

Gothenburg Medical Society, Göteborg.

Sparbanken Sjuhärad Foundation, Borås.

Alice Swenzon Foundation for Scientific Research.

Agreement concerning research and education of doctors (ALF).

REFERENCES

1. Hall IC, O'toole E. INTESTINAL FLORA IN NEW-BORN INFANTS: WITH A DESCRIPTION OF A NEW PATHOGENIC ANAEROBE, BACILLUS DIFFICILIS. JAMA Pediatrics. 1935;49:390-402.
2. Snyder ML. Further Studies on Bacillus Difficilis (Hall and O'Toole). The Journal of Infectious Diseases. 1937;60(2):223-31.
3. Prévot A. Études de systématique bactérienne. IV. Critique de la conception actuelle du genre Clostridium. Annales de l'Institut Pasteur. 1938;61:72-91.
4. Semenyuk EG, Laning ML, Foley J, Johnston PF, Knight KL, Gerding DN, et al. Spore formation and toxin production in Clostridium difficile biofilms. PLoS One. 2014;9(1):e87757.
5. Phillips I. Past and current use of clindamycin and lincomycin. Journal of Antimicrobial Chemotherapy. 1981;7(suppl_A):11-8.
6. Dhawan VK, Thadepalli H. Clindamycin: a review of fifteen years of experience. Rev Infect Dis. 1982;4(6):1133-53.
7. Cohen LE, McNeill CJ, Wells RF. Clindamycin-Associated Colitis. JAMA. 1973;223(12):1379-80.
8. CF Dixon RW. Acute pseudomembranous enteritis or enterocolitis; a complication following intestinal surgery. Surg Clin North Am. 1948;28:999-1023.
9. Bartlett JG, Chang TW, Gurwith M, Gorbach SL, Onderdonk AB. Antibiotic-associated pseudomembranous colitis due to toxin-producing clostridia. N Engl J Med. 1978;298(10):531-4.
10. Bartlett JG, Onderdonk AB, Cisneros RL. Clindamycin-associated colitis in hamsters: protection with vancomycin. Gastroenterology. 1977;73(4 Pt 1):772-6.
11. Bartlett JG, Onderdonk AB, Cisneros RL, Kasper DL. Clindamycin-associated colitis due to a toxin-producing species of Clostridium in hamsters. J Infect Dis. 1977;136(5):701-5.
12. Chang TW, Bartlett JG, Gorbach SL, Onderdonk AB. Clindamycin-induced enterocolitis in hamsters as a model of pseudomembranous colitis in patients. Infect Immun. 1978;20(2):526-9.
13. Chang TW, Lauermann M, Bartlett JG. Cytotoxicity assay in antibiotic-associated colitis. J Infect Dis. 1979;140(5):765-70.
14. Khan MY, Hall WH. Staphylococcal enterocolitis--treatment with oral vancomycin. Ann Intern Med. 1966;65(1):1-8.
15. Mogg GA, Arabi Y, Youngs D, Johnson M, Bentley S, Burdon DW, et al. Therapeutic trials of antibiotic associated colitis. Scand J Infect Dis Suppl. 1980(Suppl 22):41-5.

16. Teasley DG, Gerding DN, Olson MM, Peterson LR, Gebhard RL, Schwartz MJ, et al. Prospective randomised trial of metronidazole versus vancomycin for Clostridium-difficile-associated diarrhoea and colitis. Lancet. 1983;2(8358):1043-6.
17. Gerding DN, Johnson S, Peterson LR, Mulligan ME, Silva J, Jr. Clostridium difficile-associated diarrhea and colitis. Infect Control Hosp Epidemiol. 1995;16(8):459-77.
18. O'Connor JR, Johnson S, Gerding DN. Clostridium difficile infection caused by the epidemic BI/NAP1/027 strain. Gastroenterology. 2009;136(6):1913-24.
19. Davies KA, Ashwin H, Longshaw CM, Burns DA, Davis GL, Wilcox MH. Diversity of Clostridium difficile PCR ribotypes in Europe: results from the European, multicentre, prospective, biannual, point-prevalence study of Clostridium difficile infection in hospitalised patients with diarrhoea (EUCLID), 2012 and 2013. Euro Surveill. 2016;21(29).
20. Clostridioides (Clostridium) difficile infections - Annual Epidemiological Report for 2016-2017: European Centre for Disease Prevention and Control; 2022 [Available from: https://www.ecdc.europa.eu/sites/default/files/documents/clostridioides-clostridium-difficile-infections.pdf]
21. Yutin N, Galperin MY. A genomic update on clostridial phylogeny: Gram-negative spore formers and other misplaced clostridia. Environ Microbiol. 2013;15(10):2631-41.
22. Lawson PA, Citron DM, Tyrrell KL, Finegold SM. Reclassification of Clostridium difficile as Clostridioides difficile (Hall and O'Toole 1935) Prévot 1938. Anaerobe. 2016;40:95-9.
23. Langdon A, Crook N, Dantas G. The effects of antibiotics on the microbiome throughout development and alternative approaches for therapeutic modulation. Genome Med. 2016;8(1):39.
24. Paredes-Sabja D, Shen A, Sorg JA. Clostridium difficile spore biology: sporulation, germination, and spore structural proteins. Trends Microbiol. 2014;22(7):406-16.
25. Janezic S, Mlakar S, Rupnik M. Dissemination of Clostridium difficile spores between environment and households: Dog paws and shoes. Zoonoses Public Health. 2018;65(6):669-74.
26. Weese JS, Slovis N, Rousseau J. Clostridioides (Clostridium) difficile in neonatal foals and mares at a referral hospital. J Vet Intern Med. 2021;35(2):1140-6.
27. Knight DR, Riley TV. Prevalence of gastrointestinal Clostridium difficile carriage in Australian sheep and lambs. Appl Environ Microbiol. 2013;79(18):5689-92.

28. Norén T, Johansson K, Unemo M. Clostridium difficile PCR ribotype 046 is common among neonatal pigs and humans in Sweden. Clin Microbiol Infect. 2014;20(1):O2-6.
29. Abdel-Glil MY, Thomas P, Schmoock G, Abou-El-Azm K, Wieler LH, Neubauer H, et al. Presence of Clostridium difficile in poultry and poultry meat in Egypt. Anaerobe. 2018;51:21-5.
30. Ramos CP, Santana JA, Morcatti Coura F, Xavier RGC, Leal CAG, Oliveira Junior CA, et al. Identification and Characterization of Escherichia coli, Salmonella Spp., Clostridium perfringens, and C. difficile Isolates from Reptiles in Brazil. Biomed Res Int. 2019;2019:9530732.
31. Jangi S, Lamont JT. Asymptomatic colonization by Clostridium difficile in infants: implications for disease in later life. J Pediatr Gastroenterol Nutr. 2010;51(1):2-7.
32. Murphy GM, Signer E. Bile acid metabolism in infants and children. Gut. 1974;15(2):151-63.
33. Sorg JA, Sonenshein AL. Chenodeoxycholate is an inhibitor of Clostridium difficile spore germination. J Bacteriol. 2009;191(3):1115-7.
34. Cheng S, Zhu L, Faden HS. Interactions of bile acids and the gut microbiota: learning from the differences in Clostridium difficile infection between children and adults. Physiol Genomics. 2019;51(6):218-23.
35. Furuya-Kanamori L, Marquess J, Yakob L, Riley TV, Paterson DL, Foster NF, et al. Asymptomatic Clostridium difficile colonization: epidemiology and clinical implications. BMC Infectious Diseases. 2015;15(1):516.
36. Galdys AL, Nelson JS, Shutt KA, Schlackman JL, Pakstis DL, Pasculle AW, et al. Prevalence and duration of asymptomatic Clostridium difficile carriage among healthy subjects in Pittsburgh, Pennsylvania. J Clin Microbiol. 2014;52(7):2406-9.
37. Gerding DN, Sambol SP, Johnson S. Non-toxigenic Clostridioides (Formerly Clostridium) difficile for Prevention of C. difficile Infection: From Bench to Bedside Back to Bench and Back to Bedside. Front Microbiol. 2018;9:1700.
38. Schirmer J, Aktories K. Large clostridial cytotoxins: cellular biology of Rho/Ras-glucosylating toxins. Biochim Biophys Acta. 2004;1673(1-2):66-74.
39. Kordus SL, Thomas AK, Lacy DB. Clostridioides difficile toxins: mechanisms of action and antitoxin therapeutics. Nat Rev Microbiol. 2022;20(5):285-98.
40. von Eichel-Streiber C, Boquet P, Sauerborn M, Thelestam M. Large clostridial cytotoxins--a family of glycosyltransferases modifying small GTP-binding proteins. Trends Microbiol. 1996;4(10):375-82.
41. Beer LA, Tatge H, Reich N, Tenspolde M, Olling A, Goy S, et al. Early cell death induced by Clostridium difficile TcdB: Uptake and Rac1-

glucosylation kinetics are decisive for cell fate. Cell Microbiol. 2018;20(10):e12865.
42. Farrow MA, Chumbler NM, Lapierre LA, Franklin JL, Rutherford SA, Goldenring JR, et al. Clostridium difficile toxin B-induced necrosis is mediated by the host epithelial cell NADPH oxidase complex. Proc Natl Acad Sci U S A. 2013;110(46):18674-9.
43. Frädrich C, Beer LA, Gerhard R. Reactive Oxygen Species as Additional Determinants for Cytotoxicity of Clostridium difficile Toxins A and B. Toxins (Basel). 2016;8(1).
44. Pruitt RN, Chambers MG, Ng KK-S, Ohi MD, Lacy DB. Structural organization of the functional domains of Clostridium difficile toxins A and B. Proceedings of the National Academy of Sciences. 2010;107(30):13467-72.
45. Perelle S, Gibert M, Bourlioux P, Corthier G, Popoff MR. Production of a complete binary toxin (actin-specific ADP-ribosyltransferase) by Clostridium difficile CD196. Infect Immun. 1997;65(4):1402-7.
46. Kuehne SA, Collery MM, Kelly ML, Cartman ST, Cockayne A, Minton NP. Importance of Toxin A, Toxin B, and CDT in Virulence of an Epidemic Clostridium difficile Strain. The Journal of Infectious Diseases. 2013;209(1):83-6.
47. Monot M, Eckert C, Lemire A, Hamiot A, Dubois T, Tessier C, et al. Clostridium difficile: New Insights into the Evolution of the Pathogenicity Locus. Sci Rep. 2015;5:15023.
48. Dingle KE, Elliott B, Robinson E, Griffiths D, Eyre DW, Stoesser N, et al. Evolutionary history of the Clostridium difficile pathogenicity locus. Genome Biol Evol. 2014;6(1):36-52.
49. Brouwer MS, Roberts AP, Hussain H, Williams RJ, Allan E, Mullany P. Horizontal gene transfer converts non-toxigenic Clostridium difficile strains into toxin producers. Nat Commun. 2013;4:2601.
50. Mullany P, Allan E, Roberts AP. Mobile genetic elements in Clostridium difficile and their role in genome function. Res Microbiol. 2015;166(4):361-7.
51. McDonald LC, Killgore GE, Thompson A, Owens RC, Jr., Kazakova SV, Sambol SP, et al. An epidemic, toxin gene-variant strain of Clostridium difficile. N Engl J Med. 2005;353(23):2433-41.
52. Eglow R, Pothoulakis C, Itzkowitz S, Israel EJ, O'Keane CJ, Gong D, et al. Diminished Clostridium difficile toxin A sensitivity in newborn rabbit ileum is associated with decreased toxin A receptor. J Clin Invest. 1992;90(3):822-9.
53. Kociolek LK, Espinosa RO, Gerding DN, Hauser AR, Ozer EA, Budz M, et al. Natural Clostridioides difficile Toxin Immunization in Colonized Infants. Clin Infect Dis. 2020;70(10):2095-102.

54. Haudiquet M, de Sousa JM, Touchon M, Rocha EPC. Selfish, promiscuous and sometimes useful: how mobile genetic elements drive horizontal gene transfer in microbial populations. Philos Trans R Soc Lond B Biol Sci. 2022;377(1861):20210234.
55. Rupnik M, Janezic S. An Update on Clostridium difficile Toxinotyping. J Clin Microbiol. 2016;54(1):13-8.
56. Calabi E, Calabi F, Phillips AD, Fairweather NF. Binding of Clostridium difficile surface layer proteins to gastrointestinal tissues. Infect Immun. 2002;70(10):5770-8.
57. Awad MM, Johanesen PA, Carter GP, Rose E, Lyras D. Clostridium difficile virulence factors: Insights into an anaerobic spore-forming pathogen. Gut Microbes. 2014;5(5):579-93.
58. Curry S. Clostridium difficile. Clin Lab Med. 2010;30(1):329-42.
59. Rao A, Jump RL, Pultz NJ, Pultz MJ, Donskey CJ. In vitro killing of nosocomial pathogens by acid and acidified nitrite. Antimicrob Agents Chemother. 2006;50(11):3901-4.
60. Kochan TJ, Shoshiev MS, Hastie JL, Somers MJ, Plotnick YM, Gutierrez-Munoz DF, et al. Germinant Synergy Facilitates Clostridium difficile Spore Germination under Physiological Conditions. mSphere. 2018;3(5).
61. Hundsberger T, Braun V, Weidmann M, Leukel P, Sauerborn M, von Eichel-Streiber C. Transcription analysis of the genes tcdA-E of the pathogenicity locus of Clostridium difficile. Eur J Biochem. 1997;244(3):735-42.
62. Tschudin-Sutter S, Braissant O, Erb S, Stranden A, Bonkat G, Frei R, et al. Growth Patterns of Clostridium difficile - Correlations with Strains, Binary Toxin and Disease Severity: A Prospective Cohort Study. PLoS One. 2016;11(9):e0161711.
63. Donelli G, Vuotto C, Cardines R, Mastrantonio P. Biofilm-growing intestinal anaerobic bacteria. FEMS Immunol Med Microbiol. 2012;65(2):318-25.
64. Semenyuk EG, Poroyko VA, Johnston PF, Jones SE, Knight KL, Gerding DN, et al. Analysis of Bacterial Communities during Clostridium difficile Infection in the Mouse. Infect Immun. 2015;83(11):4383-91.
65. Engevik MA, Danhof HA, Auchtung J, Endres BT, Ruan W, Bassères E, et al. Fusobacteriumnucleatum Adheres to Clostridioides difficile via the RadD Adhesin to Enhance Biofilm Formation in Intestinal Mucus. Gastroenterology. 2021;160(4):1301-14.e8.
66. Zhu D, Sorg JA, Sun X. Clostridioides difficile Biology: Sporulation, Germination, and Corresponding Therapies for C. difficile Infection. Front Cell Infect Microbiol. 2018;8:29.

67. Deng K, Plaza-Garrido A, Torres JA, Paredes-Sabja D. Survival of Clostridium difficile spores at low temperatures. Food Microbiol. 2015;46:218-21.
68. Setlow P. I will survive: DNA protection in bacterial spores. Trends Microbiol. 2007;15(4):172-80.
69. Bagdasarian N, Rao K, Malani PN. Diagnosis and treatment of Clostridium difficile in adults: a systematic review. Jama. 2015;313(4):398-408.
70. Cammerer RC, Anderson DL, Boyce HW, Jr, Burdick GE. Clinical Spectrum of Pseudomembranous Colitis. JAMA. 1976;235(23):2502-5.
71. Farooq PD, Urrunaga NH, Tang DM, von Rosenvinge EC. Pseudomembranous colitis. Dis Mon. 2015;61(5):181-206.
72. Kelly CP, Pothoulakis C, LaMont JT. Clostridium difficile colitis. N Engl J Med. 1994;330(4):257-62.
73. Mogg GA, Keighley MR, Burdon DW, Alexander-Williams J, Youngs D, Johnson M, et al. Antibiotic-associated colitis--a review of 66 cases. Br J Surg. 1979;66(10):738-42.
74. Sayedy L, Kothari D, Richards RJ. Toxic megacolon associated Clostridium difficile colitis. World J Gastrointest Endosc. 2010;2(8):293-7.
75. Girotra M, Kumar V, Khan JM, Damisse P, Abraham RR, Aggarwal V, et al. Clinical predictors of fulminant colitis in patients with Clostridium difficile infection. Saudi J Gastroenterol. 2012;18(2):133-9.
76. Olson MM, Shanholtzer CJ, Lee JT, Jr., Gerding DN. Ten years of prospective Clostridium difficile-associated disease surveillance and treatment at the Minneapolis VA Medical Center, 1982-1991. Infect Control Hosp Epidemiol. 1994;15(6):371-81.
77. van Prehn J, Reigadas E, Vogelzang EH, Bouza E, Hristea A, Guery B, et al. European Society of Clinical Microbiology and Infectious Diseases: 2021 update on the treatment guidance document for Clostridioides difficile infection in adults. Clin Microbiol Infect. 2021;27 Suppl 2:S1-s21.
78. Johnson S, Louie TJ, Gerding DN, Cornely OA, Chasan-Taber S, Fitts D, et al. Vancomycin, metronidazole, or tolevamer for Clostridium difficile infection: results from two multinational, randomized, controlled trials. Clin Infect Dis. 2014;59(3):345-54.
79. Zar FA, Bakkanagari SR, Moorthi KM, Davis MB. A comparison of vancomycin and metronidazole for the treatment of Clostridium difficile-associated diarrhea, stratified by disease severity. Clin Infect Dis. 2007;45(3):302-7.
80. Louie TJ, Miller MA, Mullane KM, Weiss K, Lentnek A, Golan Y, et al. Fidaxomicin versus vancomycin for Clostridium difficile infection. N Engl J Med. 2011;364(5):422-31.

81. Louie TJ, Cannon K, Byrne B, Emery J, Ward L, Eyben M, et al. Fidaxomicin preserves the intestinal microbiome during and after treatment of Clostridium difficile infection (CDI) and reduces both toxin reexpression and recurrence of CDI. Clin Infect Dis. 2012;55 Suppl 2(Suppl 2):S132-42.
82. Liao JX, Appaneal HJ, Vicent ML, Vyas A, LaPlante KL. Path of least recurrence: A systematic review and meta-analysis of fidaxomicin versus vancomycin for Clostridioides difficile infection. Pharmacotherapy. 2022;42(11):810-27.
83. Al Momani LA, Abughanimeh O, Boonpheng B, Gabriel JG, Young M. Fidaxomicin vs Vancomycin for the Treatment of a First Episode of Clostridium Difficile Infection: A Meta-analysis and Systematic Review. Cureus. 2018;10(6):e2778.
84. Karp J, Edman-Wallér J, Toepfer M, Jacobsson G. Risk factors for recurrent healthcare-facility associated Clostridioides difficile infection in a Swedish setting. Anaerobe. 2023;81:102738.
85. Kelly CP, LaMont JT. Clostridium difficile--more difficult than ever. N Engl J Med. 2008;359(18):1932-40.
86. Figueroa I, Johnson S, Sambol SP, Goldstein EJ, Citron DM, Gerding DN. Relapse versus reinfection: recurrent Clostridium difficile infection following treatment with fidaxomicin or vancomycin. Clin Infect Dis. 2012;55 Suppl 2(Suppl 2):S104-9.
87. Chilton CH, Pickering DS, Freeman J. Microbiologic factors affecting Clostridium difficile recurrence. Clinical Microbiology and Infection. 2018;24(5):476-82.
88. Alfayyadh M, Collins DA, Tempone S, McCann R, Armstrong PK, Riley TV, et al. Recurrence of Clostridium difficile infection in the Western Australian population. Epidemiol Infect. 2019;147:e153.
89. Song JH, Kim YS. Recurrent Clostridium difficile Infection: Risk Factors, Treatment, and Prevention. Gut Liver. 2019;13(1):16-24.
90. Baunwall SMD, Lee MM, Eriksen MK, Mullish BH, Marchesi JR, Dahlerup JF, et al. Faecal microbiota transplantation for recurrent Clostridioides difficile infection: An updated systematic review and meta-analysis. EClinicalMedicine. 2020;29-30:100642.
91. Khanna S, Pardi DS. Clostridium difficile infection: management strategies for a difficult disease. Therap Adv Gastroenterol. 2014;7(2):72-86.
92. Planche T, Wilcox M. Reference assays for Clostridium difficile infection: one or two gold standards? Journal of Clinical Pathology. 2011;64(1):1-5.
93. Carroll SM, Bowman RA, Riley TV. A selective broth for Clostridium difficile. Pathology. 1983;15(2):165-7.

94. Aronsson B, Granström M, Möllby R, Nord CE. Enzyme immunoassay for detection of Clostridium difficile toxins A and B in patients with antibiotic-associated diarrhoea and colitis. Eur J Clin Microbiol. 1985;4(2):102-7.
95. Vargas SO, Horensky D, Onderdonk AB. Evaluation of a new enzyme immunoassay for Clostridium difficile toxin A. J Clin Pathol. 1997;50(12):996-1000.
96. Bartlett JG. Historical Perspectives on Studies of Clostridium difficile and C. difficile Infection. Clinical Infectious Diseases. 2008;46:S4-S11.
97. Delmée M, Van Broeck J, Simon A, Janssens M, Avesani V. Laboratory diagnosis of Clostridium difficile-associated diarrhoea: a plea for culture. J Med Microbiol. 2005;54(Pt 2):187-91.
98. Jung K, Aronsson B. Rapid diagnosis of Clostridium difficile - associated diarrhoea using a latex agglutination test. APMIS. 1990;98(7-12):652-4.
99. Lyerly DM, Barroso LA, Wilkins TD. Identification of the latex test-reactive protein of Clostridium difficile as glutamate dehydrogenase. J Clin Microbiol. 1991;29(11):2639-42.
100. Kato N, Ou C-Y, Kato H, Bartley SL, Luo C-C, Killgore GE, et al. Detection of Toxigenic Clostridium difficile in Stool Specimens by the Polymerase Chain Reaction. The Journal of Infectious Diseases. 1993;167(2):455-8.
101. Guilbault C, Labbé AC, Poirier L, Busque L, Béliveau C, Laverdière M. Development and evaluation of a PCR method for detection of the Clostridium difficile toxin B gene in stool specimens. J Clin Microbiol. 2002;40(6):2288-90.
102. Wilcox MH. Overcoming barriers to effective recognition and diagnosis of Clostridium difficile infection. Clinical Microbiology and Infection. 2012;18(s6):13-20.
103. Crobach MJ, Planche T, Eckert C, Barbut F, Terveer EM, Dekkers OM, et al. European Society of Clinical Microbiology and Infectious Diseases: update of the diagnostic guidance document for Clostridium difficile infection. Clin Microbiol Infect. 2016;22 Suppl 4:S63-81.
104. McDonald LC, Gerding DN, Johnson S, Bakken JS, Carroll KC, Coffin SE, et al. Clinical Practice Guidelines for Clostridium difficile Infection in Adults and Children: 2017 Update by the Infectious Diseases Society of America (IDSA) and Society for Healthcare Epidemiology of America (SHEA). Clin Infect Dis. 2018;66(7):e1-e48.
105. Monaghan TF, Rahman SN, Agudelo CW, Wein AJ, Lazar JM, Everaert K, et al. Foundational Statistical Principles in Medical Research: Sensitivity, Specificity, Positive Predictive Value, and Negative Predictive Value. Medicina (Kaunas). 2021;57(5).

106. Rizzardi K, Åkerlund T, Norén T, Matussek A. Impact of ribotype on Clostridioides difficile diagnostics. Eur J Clin Microbiol Infect Dis. 2020;39(5):847-53.
107. Wüst J, Sullivan NM, Hardegger U, Wilkins TD. Investigation of an outbreak of antibiotic-associated colitis by various typing methods. J Clin Microbiol. 1982;16(6):1096-101.
108. Tang D, Ando S, Takasaki Y, Tadano J. Mutational analyses of restriction endonuclease—HindIII mutant E86K with higher activity and altered specificity. Protein Engineering, Design and Selection. 2000;13(4):283-9.
109. Wren BW, Tabaqchali S. Restriction endonuclease DNA analysis of Clostridium difficile. J Clin Microbiol. 1987;25(12):2402-4.
110. Goering RV. Pulsed field gel electrophoresis: A review of application and interpretation in the molecular epidemiology of infectious disease. Infection, Genetics and Evolution. 2010;10(7):866-75.
111. Bowman RA, O'Neill GL, Riley TV. Non-radioactive restriction fragment length polymorphism (RFLP) typing of Clostridium difficile. FEMS Microbiol Lett. 1991;63(2-3):269-72.
112. Kristjánsson M, Samore MH, Gerding DN, DeGirolami PC, Bettin KM, Karchmer AW, et al. Comparison of restriction endonuclease analysis, ribotyping, and pulsed-field gel electrophoresis for molecular differentiation of Clostridium difficile strains. J Clin Microbiol. 1994;32(8):1963-9.
113. Kostman JR, Alden MB, Mair M, Edlind TD, LiPuma JJ, Stull TL. A universal approach to bacterial molecular epidemiology by polymerase chain reaction ribotyping. J Infect Dis. 1995;171(1):204-8.
114. Indra A, Huhulescu S, Schneeweis M, Hasenberger P, Kernbichler S, Fiedler A, et al. Characterization of Clostridium difficile isolates using capillary gel electrophoresis-based PCR ribotyping. J Med Microbiol. 2008;57(Pt 11):1377-82.
115. Fawley WN, Knetsch CW, MacCannell DR, Harmanus C, Du T, Mulvey MR, et al. Development and validation of an internationally-standardized, high-resolution capillary gel-based electrophoresis PCR-ribotyping protocol for Clostridium difficile. PLoS One. 2015;10(2):e0118150.
116. Seth-Smith HMB, Biggel M, Roloff T, Hinic V, Bodmer T, Risch M, et al. Transition From PCR-Ribotyping to Whole Genome Sequencing Based Typing of Clostridioides difficile. Front Cell Infect Microbiol. 2021;11:681518.
117. Brazier JS. Typing of Clostridium difficile. Clinical Microbiology and Infection. 2001;7(8):428-31.

118. Sebaihia M, Wren BW, Mullany P, Fairweather NF, Minton N, Stabler R, et al. The multidrug-resistant human pathogen Clostridium difficile has a highly mobile, mosaic genome. Nat Genet. 2006;38(7):779-86.
119. Parcell BJ, Gillespie SH, Pettigrew KA, Holden MTG. Clinical perspectives in integrating whole-genome sequencing into the investigation of healthcare and public health outbreaks - hype or help? J Hosp Infect. 2021;109:1-9.
120. Maiden MC, Bygraves JA, Feil E, Morelli G, Russell JE, Urwin R, et al. Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. Proc Natl Acad Sci U S A. 1998;95(6):3140-5.
121. Griffiths D, Fawley W, Kachrimanidou M, Bowden R, Crook DW, Fung R, et al. Multilocus sequence typing of Clostridium difficile. J Clin Microbiol. 2010;48(3):770-8.
122. Eyre DW, Cule ML, Walker AS, Crook DW, Wilcox MH, Peto TEA. Hospital and community transmission of Clostridium difficile: a whole genome sequencing study. The Lancet. 2012;380:S12.
123. Bletz S, Janezic S, Harmsen D, Rupnik M, Mellmann A. Defining and Evaluating a Core Genome Multilocus Sequence Typing Scheme for Genome-Wide Typing of Clostridium difficile. J Clin Microbiol. 2018;56(6).
124. Eyre DW, Peto TEA, Crook DW, Walker AS, Wilcox MH. Hash-Based Core Genome Multilocus Sequence Typing for Clostridium difficile. J Clin Microbiol. 2019;58(1).
125. Werner A, Mölling P, Fagerström A, Dyrkell F, Arnellos D, Johansson K, et al. Whole genome sequencing of Clostridioides difficile PCR ribotype 046 suggests transmission between pigs and humans. PLoS One. 2020;15(12):e0244227.
126. Rizzardi K, Åkerlund T. High Molecular Weight Typing with MALDI-TOF MS - A Novel Method for Rapid Typing of Clostridium difficile. PLoS One. 2015;10(4):e0122457.
127. New routine for Clostridioides difficile typing (in Swedish): Public Health Agency of Sweden; 2019 [Available from: https://www.folkhalsomyndigheten.se/slim/nyheter/ny-rutin-for-typning-av-clostridioides-difficile/]
128. Furuya-Kanamori L, Stone JC, Clark J, McKenzie SJ, Yakob L, Paterson DL, et al. Comorbidities, Exposure to Medications, and the Risk of Community-Acquired Clostridium difficile Infection: A Systematic Review and Meta-analysis. Infection Control & Hospital Epidemiology. 2015;36(2):132-41.
129. Ghosh TS, Shanahan F, O'Toole PW. The gut microbiome as a modulator of healthy ageing. Nat Rev Gastroenterol Hepatol. 2022;19(9):565-84.-

130. Shin JH, High KP, Warren CA. Older Is Not Wiser, Immunologically Speaking: Effect of Aging on Host Response to Clostridium difficile Infections. J Gerontol A Biol Sci Med Sci. 2016;71(7):916-22.
131. Eze P, Balsells E, Kyaw MH, Nair H. Risk factors for Clostridium difficile infections - an overview of the evidence base and challenges in data synthesis. J Glob Health. 2017;7(1):010417.
132. Bignardi GE. Risk factors for Clostridium difficile infection. J Hosp Infect. 1998;40(1):1-15.
133. Vardakas KZ, Konstantelias AA, Loizidis G, Rafailidis PI, Falagas ME. Risk factors for development of Clostridium difficile infection due to BI/NAP1/027 strain: a meta-analysis. Int J Infect Dis. 2012;16(11):e768-73.
134. Healthcare-associated infections, HAI (in Swedish): National Board of Health and Welfare; 2023 [Available from: https://patientsakerhet.socialstyrelsen.se/risker-och-vardskador/vardskador/vri--vardrelaterade-infektioner/]
135. European Surveillance of Clostridioides (Clostridium) difficile infections. Surveillance protocol version 2.4. Stockholm, Sweden: European Centre for Disease Prevention and Control; 2019.
136. McDonald LC, Coignard B, Dubberke E, Song X, Horan T, Kutty PK. Recommendations for surveillance of Clostridium difficile-associated disease. Infect Control Hosp Epidemiol. 2007;28(2):140-5.
137. Kuijper EJ, Coignard B, Tüll P. Emergence of Clostridium difficile-associated disease in North America and Europe. Clinical Microbiology and Infection. 2006;12:2-18.
138. Balsells E, Shi T, Leese C, Lyell I, Burrows J, Wiuff C, et al. Global burden of Clostridium difficile infections: a systematic review and meta-analysis. J Glob Health. 2019;9(1):010407.
139. Rizzardi K, Norén T, Aspevall O, Mäkitalo B, Toepfer M, Johansson Å, et al. National Surveillance for Clostridioides difficile Infection, Sweden, 2009-2016. Emerg Infect Dis. 2018;24(9):1617-25.
140. Kotila SM, Mentula S, Ollgren J, Virolainen-Julkunen A, Lyytikäinen O. Community- and Healthcare-Associated Clostridium difficile Infections, Finland, 2008-2013. Emerg Infect Dis. 2016;22(10):1747-53.
141. Persson S, Nielsen HL, Coia JE, Engberg J, Olesen BS, Engsbro AL, et al. Sentinel surveillance and epidemiology of Clostridioides difficile in Denmark, 2016 to 2019. Euro Surveill. 2022;27(49).
142. Dingle KE, Didelot X, Quan TP, Eyre DW, Stoesser N, Golubchik T, et al. Effects of control interventions on Clostridium difficile infection in England: an observational study. Lancet Infect Dis. 2017;17(4):411-21.

143. King A, Mullish BH, Williams HRT, Aylin P. Comparative epidemiology of Clostridium difficile infection: England and the USA. International Journal for Quality in Health Care. 2017;29(6):785-91.
144. Freeman J, Vernon J, Pilling S, Morris K, Nicolson S, Shearman S, et al. Five-year Pan-European, longitudinal surveillance of Clostridium difficile ribotype prevalence and antimicrobial resistance: the extended ClosER study. Eur J Clin Microbiol Infect Dis. 2020;39(1):169-77.
145. Brazier JS, Raybould R, Patel B, Duckworth G, Pearson A, Charlett A, et al. Distribution and antimicrobial susceptibility patterns of Clostridium difficile PCR ribotypes in English hospitals, 2007-08. Euro Surveill. 2008;13(41).
146. Ortega L, Ryberg A, Johansson Å. HMW-profiling using MALDI-TOF MS: A screening method for outbreaks of Clostridioides difficile. Anaerobe. 2018;54:254-9.
147. Magnusson C, Mernelius S, Bengnér M, Norén T, Serrander L, Forshell S, et al. Characterization of a Clostridioides difficile outbreak caused by PCR ribotype 046, associated with increased mortality. Emerg Microbes Infect. 2022;11(1):850-9.
148. Fleming A. Penicillin. Nobel Lecture, December 11, 1945: Nobel Prize organisation; 1945 [Available from: https://www.nobelprize.org/uploads/2018/06/fleming-lecture.pdf]
149. Hutchings MI, Truman AW, Wilkinson B. Antibiotics: past, present and future. Current Opinion in Microbiology. 2019;51:72-80.
150. Neu HC. The crisis in antibiotic resistance. Science. 1992;257(5073):1064-73.
151. Louie TJ, Read RR. Antibiotic Stewardship, Calgary 1995-1997: Regionalization, Restructuring and Retrenchment. Canadian Journal of Infectious Diseases. 1998;9:602652.
152. Gaynes R, Rimland D, Killum E, Lowery HK, Johnson TM, 2nd, Killgore G, et al. Outbreak of Clostridium difficile infection in a long-term care facility: association with gatifloxacin use. Clin Infect Dis. 2004;38(5):640-5.
153. Johnson S, Samore MH, Farrow KA, Killgore GE, Tenover FC, Lyras D, et al. Epidemics of diarrhea caused by a clindamycin-resistant strain of Clostridium difficile in four hospitals. N Engl J Med. 1999;341(22):1645-51.
154. Settle CD, Wilcox MH, Fawley WN, Corrado OJ, Hawkey PM. Prospective study of the risk of Clostridium difficile diarrhoea in elderly patients following treatment with cefotaxime or piperacillin-tazobactam. Aliment Pharmacol Ther. 1998;12(12):1217-23.
155. Gerding DN. Clindamycin, Cephalosporins, Fluoroquinolones, and Clostridium difficile–Associated Diarrhea: This Is an Antimicrobial Resistance Problem. Clinical Infectious Diseases. 2004;38(5):646-8.

156. Cižman M, Plankar Srovin T. Antibiotic consumption and resistance of gram-negative pathogens (collateral damage). GMS Infect Dis. 2018;6:Doc05.
157. Valiquette L, Cossette B, Garant M-P, Diab H, Pépin J. Impact of a Reduction in the Use of High-Risk Antibiotics on the Course of an Epidemic of Clostridium difficile-Associated Disease Caused by the Hypervirulent NAP1/027 Strain. Clinical Infectious Diseases. 2007;45(Supplement_2):S112-S21.
158. The Core Elements of Hospital Antibiotic Stewardship Programs: 2019: Centers for Disease Control and Prevention; 2019 [Available from: https://www.cdc.gov/antibiotic-use/healthcare/pdfs/hospital-core-elements-H.pdf]
159. Baur D, Gladstone BP, Burkert F, Carrara E, Foschi F, Döbele S, et al. Effect of antibiotic stewardship on the incidence of infection and colonisation with antibiotic-resistant bacteria and Clostridium difficile infection: a systematic review and meta-analysis. Lancet Infect Dis. 2017;17(9):990-1001.
160. Feazel LM, Malhotra A, Perencevich EN, Kaboli P, Diekema DJ, Schweizer ML. Effect of antibiotic stewardship programmes on Clostridium difficile incidence: a systematic review and meta-analysis. J Antimicrob Chemother. 2014;69(7):1748-54.
161. Schön T, Sandelin LL, Bonnedahl J, Hedebäck F, Wistedt A, Brudin L, et al. A comparative study of three methods to evaluate an intervention to improve empirical antibiotic therapy for acute bacterial infections in hospitalized patients. Scand J Infect Dis. 2011;43(4):251-7.
162. Jabbar U, Leischner J, Kasper D, Gerber R, Sambol SP, Parada JP, et al. Effectiveness of alcohol-based hand rubs for removal of Clostridium difficile spores from hands. Infect Control Hosp Epidemiol. 2010;31(6):565-70.
163. Jullian-Desayes I, Landelle C, Mallaret MR, Brun-Buisson C, Barbut F. Clostridium difficile contamination of health care workers' hands and its potential contribution to the spread of infection: Review of the literature. Am J Infect Control. 2017;45(1):51-8.
164. Riggs MM, Sethi AK, Zabarsky TF, Eckstein EC, Jump RL, Donskey CJ. Asymptomatic carriers are a potential source for transmission of epidemic and nonepidemic Clostridium difficile strains among long-term care facility residents. Clin Infect Dis. 2007;45(8):992-8.
165. Rutala WA, Gergen MF, Weber DJ. Efficacy of different cleaning and disinfection methods against Clostridium difficile spores: importance of physical removal versus sporicidal inactivation. Infect Control Hosp Epidemiol. 2012;33(12):1255-8.

166. Chung I, Ryu H, Yoon SY, Ha JC. Health effects of sodium hypochlorite: review of published case reports. Environ Anal Health Toxicol. 2022;37(1):e2022006-0.
167. Parveen N, Chowdhury S, Goel S. Environmental impacts of the widespread use of chlorine-based disinfectants during the COVID-19 pandemic. Environ Sci Pollut Res Int. 2022;29(57):85742-60.
168. Alfa MJ, Lo E, Wald A, Dueck C, DeGagne P, Harding GK. Improved eradication of Clostridium difficile spores from toilets of hospitalized patients using an accelerated hydrogen peroxide as the cleaning agent. BMC Infect Dis. 2010;10:268.
169. Schroeder CP, Hengel RL, Nathan RV, Ritter TE, Obi EN, Lancaster C, et al. Appropriate cleaning reduces potential risk of spore transmission from patients with Clostridioides difficile infection treated in outpatient infusion centers. Anaerobe. 2022;77:102617.
170. Ellis Mulligan M, Rolfe RD, Finegold SM, George WL. Contamination of a hospital environment byClostridium difficile. Current Microbiology. 1979;3(3):173-5.
171. Reigadas E, Vázquez-Cuesta S, Villar-Gómara L, Onori R, Alcalá L, Marín M, et al. Role of Clostridioides difficile in hospital environment and healthcare workers. Anaerobe. 2020;63:102204.
172. Sood G, Truelove S, Dougherty G, Landrum BM, Qasba S, Patel M, et al. Clostridioides difficile infection (CDI) in a previous room occupant predicts CDI in subsequent room occupants across different hospital settings. Am J Infect Control. 2022;50(12):1352-4.
173. Freedberg DE, Salmasian H, Cohen B, Abrams JA, Larson EL. Receipt of Antibiotics in Hospitalized Patients and Risk for Clostridium difficile Infection in Subsequent Patients Who Occupy the Same Bed. JAMA Intern Med. 2016;176(12):1801-8.
174. Eyre DW, Griffiths D, Vaughan A, Golubchik T, Acharya M, O'Connor L, et al. Asymptomatic Clostridium difficile colonisation and onward transmission. PLoS One. 2013;8(11):e78445.
175. Säll O, Johansson K, Norén T. Low colonization rates of Clostridium difficile among patients and healthcare workers at Örebro University Hospital in Sweden. Apmis. 2015;123(3):240-4.
176. Terveer EM, Crobach MJ, Sanders IM, Vos MC, Verduin CM, Kuijper EJ. Detection of Clostridium difficile in Feces of Asymptomatic Patients Admitted to the Hospital. J Clin Microbiol. 2017;55(2):403-11.
177. Büchler AC, Wicki M, Frei R, Hinic V, Seth-Smith HMB, Egli A, et al. Matching Clostridioides difficile strains obtained from shoe soles of healthcare workers epidemiologically linked to patients and confirmed by whole-genome sequencing. J Hosp Infect. 2022;126:10-5.

178. Janezic S, Blazevic I, Eyre DW, Kotnik Kevorkijan B, Remec T, Rupnik M. Possible contribution of shoes to Clostridioides difficile transmission within hospitals. Clin Microbiol Infect. 2020.
179. Vonberg RP, Kuijper EJ, Wilcox MH, Barbut F, Tüll P, Gastmeier P, et al. Infection control measures to limit the spread of Clostridium difficile. Clin Microbiol Infect. 2008;14 Suppl 5:2-20.
180. Eyre DW, Cule ML, Wilson DJ, Griffiths D, Vaughan A, O'Connor L, et al. Diverse sources of C. difficile infection identified on whole-genome sequencing. N Engl J Med. 2013;369(13):1195-205.
181. Widmer AF, Frei R, Erb S, Stranden A, Kuijper EJ, Knetsch CW, et al. Transmissibility of Clostridium difficile Without Contact Isolation: Results From a Prospective Observational Study With 451 Patients. Clinical Infectious Diseases. 2016;64(4):393-400.
182. Dubberke ER, Reske KA, Seiler S, Hink T, Kwon JH, Burnham CA. Risk Factors for Acquisition and Loss of Clostridium difficile Colonization in Hospitalized Patients. Antimicrob Agents Chemother. 2015;59(8):4533-43.
183. Chau JPC, Liu X, Lo SHS, Chien WT, Wan X. Effects of environmental cleaning bundles on reducing healthcare-associated Clostridioides difficile infection: a systematic review and meta-analysis. J Hosp Infect. 2020;106(4):734-44.
184. Ray AJ, Deshpande A, Fertelli D, Sitzlar BM, Thota P, Sankar CT, et al. A Multicenter Randomized Trial to Determine the Effect of an Environmental Disinfection Intervention on the Incidence of Healthcare-Associated Clostridium difficile Infection. Infect Control Hosp Epidemiol. 2017;38(7):777-83.
185. Dubberke ER, Butler AM, Hota B, Khan YM, Mangino JE, Mayer J, et al. Multicenter study of the impact of community-onset Clostridium difficile infection on surveillance for C. difficile infection. Infect Control Hosp Epidemiol. 2009;30(6):518-25.
186. Huang SS, Yokoe DS, Stelling J, Placzek H, Kulldorff M, Kleinman K, et al. Automated detection of infectious disease outbreaks in hospitals: a retrospective cohort study. PLoS Med. 2010;7(2):e1000238.
187. Faires MC, Pearl DL, Ciccotelli WA, Berke O, Reid-Smith RJ, Weese JS. Detection of Clostridium difficile infection clusters, using the temporal scan statistic, in a community hospital in southern Ontario, Canada, 2006–2011. BMC Infectious Diseases. 2014;14(1):254.
188. Goldenberg JZ, Yap C, Lytvyn L, Lo CK, Beardsley J, Mertz D, et al. Probiotics for the prevention of Clostridium difficile-associated diarrhea in adults and children. Cochrane Database Syst Rev. 2017;12(12):Cd006095.

189. Tsai CS, Hung YP, Lee JC, Syue LS, Hsueh PR, Ko WC. Clostridioides difficile infection: an emerging zoonosis? Expert Rev Anti Infect Ther. 2021;19(12):1543-52.
190. Lim SC, Knight DR, Riley TV. Clostridium difficile and One Health. Clinical Microbiology and Infection. 2020;26(7):857-63.
191. Usui M. One Health approach to Clostridioides difficile in Japan. J Infect Chemother. 2020;26(7):643-50.
192. SWEDRES SVARM 2021: Sales of antibiotics and occurence of antibiotic resistance in Sweden. Public Health Agency of Sweden and National Veterinary Institute of Sweden; 2022.
193. Slimings C, Riley TV. Antibiotics and hospital-acquired Clostridium difficile infection: update of systematic review and meta-analysis. J Antimicrob Chemother. 2014;69(4):881-91.
194. Premier(R) Toxins A&B Enzyme Immunoassay for the Detection of Clostridium difficile Toxin A and Toxin B in Stool Specimens - Package Insert: Meridian Bioscience; [Available from: https://www.meridianbioscience.com/uploads/616096_pi.pdf]
195. VIDAS(R) C. difficile Toxin A & B (CDAB) - Package Insert: Biomérieux SA; [Available from: https://fardavar.com/Upload/%D9%85%D8%B4%D8%AE%D8%B5%D8%A7%D8%AA%20%D9%85%D8%AD%D8%B5%D9%88%D9%84/%D8%AF%D9%81%D8%AA%D8%B1%DA%86%D9%87%20%DA%A9%DB%8C%D8%AA%20%D9%88%D8%A7%DB%8C%D8%AF%D8%A7%D8%B3%202021/%D8%AF%D9%81%D8%AA%D8%B1%DA%86%D9%87TOXIN%20AB.pdf]
196. Eastwood K, Else P, Charlett A, Wilcox M. Comparison of nine commercially available Clostridium difficile toxin detection assays, a real-time PCR assay for C. difficile tcdB, and a glutamate dehydrogenase detection assay to cytotoxin testing and cytotoxigenic culture methods. J Clin Microbiol. 2009;47(10):3211-7.
197. de Boer RF, Wijma JJ, Schuurman T, Moedt J, Dijk-Alberts BG, Ott A, et al. Evaluation of a rapid molecular screening approach for the detection of toxigenic Clostridium difficile in general and subsequent identification of the tcdC Δ117 mutation in human stools. J Microbiol Methods. 2010;83(1):59-65.
198. Harvala H, Alm E, Åkerlund T, Rizzardi K. Emergence and spread of moxifloxacin-resistant Clostridium difficile ribotype 231 in Sweden between 2006 and 2015. New Microbes New Infect. 2016;14:58-66.
199. Didelot X, Eyre DW, Cule M, Ip CLC, Ansari MA, Griffiths D, et al. Microevolutionary analysis of Clostridium difficile genomes to investigate transmission. Genome Biology. 2012;13(12):R118.

200. Edman-Wallér JE, Werner M. Detecting Clostridium difficile outbreaks with ward-specific cut-off levels based on the Poisson distribution. Infect Control Hosp Epidemiol. 2019;40(2):265-6.
201. Ross R. An application of the theory of probabilities to the study of a priori pathometry. —Part I. Proc R Soc Lond. 1916;92:204-30.
202. Hemenway D. Why we don't spend enough on public health. N Engl J Med. 2010;362(18):1657-8.
203. Antimicrobial resistance surveillance in Europe 2022 - 2020 data: WHO Regional Office for Europe/European Centre for Disease Prevention and Control; 2022 [
204. Struwe J. Fighting antibiotic resistance in Sweden – past, present and future. Wiener klinische Wochenschrift. 2008;120(9):268-79.
205. Antimicrobial consumption in the EU/EEA (ESAC-Net): European Centre for Disease Prevention and Control; 2022 [2023-08-22]. Available from: https://www.ecdc.europa.eu/sites/default/files/documents/ESAC-Net_AER_2021_final-rev.pdf]
206. Hanberger H OI, Giske CG, Kahlmeter G. Time to abolish "the house wine" (in Swedish): Läkartidningen (Swedish Medical Journal); 2009 [Available from: https://lakartidningen.se/klinik-och-vetenskap-1/2009/01/dags-att-slopa-ehusets-vin/]
207. Gårdlund B KM. On the to be or not to be of cefuroxime (in Swedish): Läkartidningen (Swedish Medical Journal); 2009 [Available from: https://lakartidningen.se/wp-content/uploads/OldWebArticlePdf/1/12742/LKT0938s2382_2384.pdf]
208. Dubrovskaya Y, Papadopoulos J, Scipione MR, Altshuler J, Phillips M, Mehta SA. Antibiotic Stewardship for Intra-abdominal Infections: Early Impact on Antimicrobial Use and Patient Outcomes. Infection Control & Hospital Epidemiology. 2012;33(4):427-9.
209. Shiode J, Fujii M, Nasu J, Itoh M, Ishiyama S, Fujiwara A, et al. Correlation between hospital-onset and community-onset Clostridioides difficile infection incidence: Ward-level analysis following hospital relocation. Am J Infect Control. 2022.
210. Eyre DW, Golubchik T, Gordon NC, Bowden R, Piazza P, Batty EM, et al. A pilot study of rapid benchtop sequencing of Staphylococcus aureus and Clostridium difficile for outbreak detection and surveillance. BMJ Open. 2012;2(3).
211. Donskey CJ, Sunkesula VCK, Stone ND, Gould CV, McDonald LC, Samore M, et al. Transmission of Clostridium difficile from asymptomatically colonized or infected long-term care facility residents. Infection Control & Hospital Epidemiology. 2018;39(8):909-16.
212. Blixt T, Gradel KO, Homann C, Seidelin JB, Schønning K, Lester A, et al. Asymptomatic Carriers Contribute to Nosocomial

Clostridium difficile Infection: A Cohort Study of 4508 Patients. Gastroenterology. 2017;152(5):1031-41.e2.
213. Chachaty E, Depitre C, Mario N, Bourneix C, Saulnier P, Corthier G, et al. Presence of Clostridium difficile and antibiotic and beta-lactamase activities in feces of volunteers treated with oral cefixime, oral cefpodoxime proxetil, or placebo. Antimicrob Agents Chemother. 1992;36(9):2009-13.
214. Anjewierden S, Han Z, Brown AM, Donskey CJ, Deshpande A. Risk factors for Clostridioides difficile colonization among hospitalized adults: A meta-analysis and systematic review. Infect Control Hosp Epidemiol. 2021;42(5):565-72.
215. Warren BG, Turner NA, Addison R, Nelson A, Barrett A, Addison B, et al. The Impact of Infection Versus Colonization on Clostridioides difficile Environmental Contamination in Hospitalized Patients With Diarrhea. Open Forum Infect Dis. 2022;9(4):ofac069.
216. Schoyer E, Hall K. Environmental Cleaning and Decontamination to Prevent Clostridioides difficile Infection in Health Care Settings: A Systematic Review. J Patient Saf. 2020;16(3S Suppl 1):S12-s5.
217. Steele L, Orefuwa E, Dickmann P. Drivers of earlier infectious disease outbreak detection: a systematic literature review. Int J Infect Dis. 2016;53:15-20.
218. Kato H, Hagihara M, Asai N, Shibata Y, Yamagishi Y, Iwamoto T, et al. A systematic review and meta-analysis of decontamination methods to prevent hospital environmental contamination and transmission of Clostridioidesdifficile. Anaerobe. 2022;73:102478.