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***Clostridium difficile* infection**

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Abstract

Infection of the colon with the Gram-positive bacterium *Clostridium difficile* is potentially life threatening, especially in elderly people and in patients who have dysbiosis of the gut microbiota following antimicrobial drug exposure. *C. difficile* is the leading cause of health-care-associated infective diarrhoea. The life cycle of *C. difficile* is influenced by antimicrobial agents, the host immune system, and the host microbiota and its associated metabolites. The primary mediators of inflammation in *C. difficile* infection (CDI) are large clostridial toxins, toxin A (TcdA) and toxin B (TcdB), and, in some bacterial strains, the binary toxin CDT. The toxins trigger a complex cascade of host cellular responses to cause diarrhoea, inflammation and tissue necrosis — the major symptoms of CDI. The factors responsible for the epidemic of some *C. difficile* strains are poorly understood. Recurrent infections are common and can be debilitating. Toxin detection for diagnosis is important for accurate epidemiological study, and for optimal management and prevention strategies. Infections are commonly treated with specific antimicrobial agents, but faecal microbiota transplants have shown promise for recurrent infections. Future biotherapies for *C. difficile* infections are likely to involve defined combinations of key gut microbiota.

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Author contributions

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Competing interests statement

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Clostridium difficile is a Gram-positive obligate anaerobic bacterium that was originally identified as part of the flora of healthy infants in 1935, described as an “actively motile, heavy-bodied rod with elongated subterminal or nearly terminal spores” (REF. 1) (FIG. 1). At that time, the strain was named *Bacillus difficilis* to reflect the difficulty experienced in isolating and culturing it. Despite the organism being present as a commensal bacterium in neonates, researchers noted that it could induce disease in animals, probably due to the production of a secreted toxin¹. Subsequent work established the high molecular weight clostridial toxins toxin A (TcdA) and/or toxin B (TcdB) as the main virulence (disease-causing) factors of *C. difficile*^{2,3}. A hallmark feature of *C. difficile* that sets it apart from other species in the class Clostridia is its ability to decarboxylate parahydroxyphenylacetic acid to produce *p*-cresol, which gives *C. difficile* its characteristic tar-like or pig-like smell⁴.

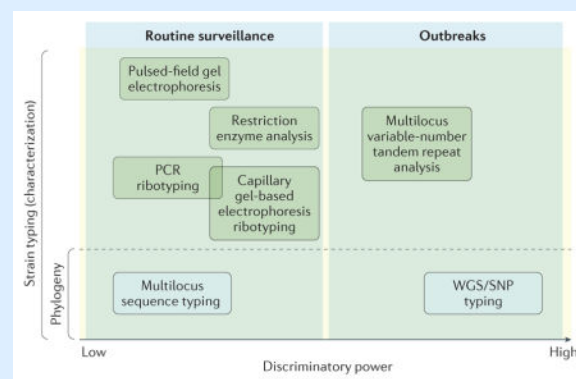
It was not until the 1970s that a detailed characterization of the bacterium, then called *C. difficile*, revealed its involvement in human disease⁵. This disease became widely known as *C. difficile*-associated disease/diarrhoea (CDAD); more recently, the term *C. difficile* infection (CDI) is preferred. In the early 2000s, an increase in severe cases of CDI was noted in Canada, the United States and Europe⁶, which was attributed to the emergence of certain epidemic types of *C. difficile*^{7,8}. The first complete genome sequence for *C. difficile*⁹, together with the development of tools for the genetic manipulation of *C. difficile*^{10,11}, has greatly stimulated research on the bacterium. *C. difficile* is now recognized as the leading cause of health-care-associated infective diarrhoea and is increasingly being linked to community-acquired cases of colitis¹². *C. difficile* can be found in the intestinal tracts of both humans and animals, but its spores are also ubiquitous in the environment and can be isolated from food¹³. Importantly, people with an adequate immune response will either eliminate the infection and/or become asymptomatic carriers¹⁴. In 2013, it was proposed that *C. difficile* should be reclassified as *Peptoclostridium difficile* on the basis of a detailed phylogenetic analysis¹⁵, and this has been adopted by the US National Center for Biotechnology Information. However, considering the public awareness of the disease, the large body of scientific literature using *C. difficile* and the lack of formal acceptance of this proposal, we will refer to the organism as *C. difficile* throughout this Primer, and to the disease it causes as CDI. We describe key aspects of the epidemiology of *C. difficile* infections, the mechanisms underlying the disease, strategies for diagnosis, prevention and management, and summarize the impact that CDIs have on patients and society.

Epidemiology

Molecular typing (BOX 1) is the characterization of organisms beyond the species level, enabling clustering of individual bacterial isolates in a meaningful manner¹⁶. Typing is crucial for epidemiological studies and facilitates effective infection prevention and disease management. Different types of *C. difficile* are known; here, we will refer to PCR ribotypes when relevant, a typing system that is based on a banding pattern obtained from PCR amplifying ribosomal 16S–23S intergenic spacer sequences¹⁷. Certain PCR ribotypes of *C. difficile* (such as PCR ribotype 010) are non-pathogenic as they lack the toxin genes. Epidemic PCR ribotypes are distinguished from non-epidemic types by their frequent occurrence in multiple settings across several countries.

Box 1**Molecular typing of *Clostridium difficile***

Epidemiological studies are dependent on standardized typing methods. Many typing methods have been developed for *Clostridium difficile* that evaluate either phenotypic or genotypic traits¹⁷ (see the figure). Given their high reproducibility, typability (ability to type a strain unambiguously) and discriminatory power, genotyping methods have become standard for typing of *C. difficile*. Band-based typing methods, such as restriction enzyme analysis (REA), pulsed-field gel electrophoresis (PFGE) and PCR ribotyping, are common. Historically, REA and PFGE have been the methods of choice in North America, whereas PCR ribotyping has primarily been used in Europe. As a result, epidemic strains are often indicated with multiple typing designations²⁴⁷. For instance, PCR ribotype 027 strains that have caused outbreaks globally⁸ have been classified as REA group BI and PFGE type NAP1. Similarly, PCR ribotype 078 strains are known as REA group BK and PFGE type NAP7/NAP8. Global surveillance is becoming key for health-care management, and efforts have been made to harmonize the different typing schemes. Capillary gel-based electrophoresis ribotyping has been standardized and validated in a collaborative effort of the European Centre for Disease Control and Prevention, the US Centers for Disease Control and Prevention and the Public Health Agency of Canada, and is likely to become commonplace throughout the world²⁴⁸. In addition, sequence-based methods, such as multilocus sequence typing (MLST) and whole-genome sequencing (WGS; specifically single-nucleotide polymorphism (SNP) typing), have gained interest specifically to study evolutionary relationships between various *C. difficile* strains (phylogeny)¹⁷. Overall, different lineages can be discriminated and several different PCR ribotypes have been shown to be closely related to the epidemic types using these sequence-based methods^{98,230}. WGS is also used to study transmission and outbreaks^{25,231}, although this approach can be costly and is predominantly performed retrospectively. Relatedness of strains in an outbreak setting is more commonly performed using multilocus variable-number tandem repeat analysis¹⁷.



Owing to a lack of systematic surveillance, no comprehensive data are available on circulating *C. difficile* types before 2003. After 2003, large increases in incidence and

mortality rates in North America — and subsequently in several European countries — were observed, which were associated with PCR ribotype 027 (REF. 8) and, to a lesser extent, PCR ribotype 078 (REF. 7). Such increases have also been observed in Australia, Asia and Central America after 2008 (REF. 18). It should be noted, however, that CDI and CDI-related epidemics are not limited to these types. Ribotypes 001, 002 and 014/020 frequently cause CDI clusters in the United States and Europe^{19,20}. Furthermore, outbreaks have also been reported for strains of other PCR ribotypes, such as 017 (REF. 12), 018 (REF. 21), 106 (REF. 12), 176 (REF. 22) and 244 (REF. 23). Epidemiological data on *C. difficile* in Africa and the Middle East are sparse¹².

CDI is historically regarded as a nosocomial infection; antibiotic exposure (either prophylactic or as treatment) during hospitalization is the foremost risk factor for CDI. However, *C. difficile* is increasingly being recognized as a cause of community-associated diarrhoea. Indeed, PCR ribotypes of strains isolated from patients with community-associated CDI have a large overlap with strains cultured from patients with health-care-associated CDI in an endemic setting, suggesting a common source (or sources) of *C. difficile*^{24,25}. The incidence of community-associated CDI is estimated at 30 to 120 cases per 100,000 persons per year in the United States²⁶. The incidence of community-associated CDI in the Netherlands is estimated at 390 to 730 per 100,000 person years, similar to the incidence of *Campylobacter* spp. infection and higher than the incidence of *Salmonella* spp. infection²⁷. More than 30% of patients who develop community-associated CDI do not have typical risk factors for CDI, such as antibiotic treatment or recent hospitalization (see below)^{28,29}.

The US Centers for Disease Control and Prevention (CDC) estimated that almost 500,000 patients had CDI, with 29,000 attributable deaths in the United States in 2011 (REF. 26). However, there is uncertainty regarding the precision of these estimates given the accuracy of the testing methodology, although data have been adjusted based on the frequency of nucleic acid amplification test use. Between 2001 and 2010, the incidence of CDI among hospitalized adults in the United States approximately doubled according to International Classification of Diseases (ICD)-9 discharge diagnoses³⁰. Again, ascertainment bias secondary to awareness (frequency of requesting) and to diagnostic methods could be relevant here.

The first European Centre for Disease Prevention and Control (ECDC) point-prevalence survey in 2011 and 2012 estimated that ~124,000 patients developed health-care-associated CDI within the European Union each year³¹. However, this surveillance was performed with a variety of diagnostic tests, and CDI was often only tested upon physician request, resulting in considerable underestimation of the true CDI incidence; on average, ~80 cases of CDI are missed per hospital per annum in Europe³². A pilot study supported by the ECDC using standardized CDI surveillance performed in 37 hospitals in 14 European countries demonstrated large differences in the incidence of CDI per hospital and per country³³. The incidence of health-care-associated CDI by hospital was in the range of 4.2–131.8 per 10,000 discharges (median 16.4 per 10,000 discharges) and 0.6–18.5 per 10,000 patient days (median 3.7 per 10,000 patient days). In some countries, a high incidence of PCR ribotype 027 strains has been noted, but other countries demonstrated a decrease in the incidence of

these strains, most probably as a consequence of effective management strategies (see below)³².

The all-cause mortality associated with CDI due to non-epidemic PCR ribotypes has been reported to be ~15–20% within a month of diagnosis, and approximately half of the deaths are directly attributed to CDI³⁴. Given the multiple comorbidities (such as respiratory disease and renal failure) typically present in patients with CDI, mortality is often related to one or more of these conditions.

C. difficile is a human pathogen but can also infect and cause disease in animals that can enter the food chain; however, the relevance of foodborne transmission in human disease is unclear¹³. Since the beginning of 2000, *C. difficile* has been reported as a major cause of neonatal enteritis in piglets. The predominant strains found in piglets (belonging to PCR ribotype 078) are similar to isolates from some human patients with CDI and to isolates from asymptomatic farm workers, suggesting zoonotic transmission^{13,35}. As a consequence of increasing pressure from spores present in the environment and animals, humans might become colonized more frequently. One meta-analysis revealed an increased rate (>8% of admitted patients) of asymptomatic colonization by toxigenic isolates in patients at the time of admission to the hospital³⁶. It should be noted, however, that patients who have been admitted to hospital typically have a much higher colonization rate than people in the community, because of exposure to a hospital environment, comorbidities and/or antibiotic use.

Mechanisms/pathophysiology

C. difficile lifecycle

C. difficile is transmitted via the oral–faecal route (FIG. 2). Spores are dormant cells that are highly resistant to environmental conditions³⁷, including some disinfectants and many antimicrobials (BOX 2), which generally target metabolically active cells. Spores are thought to be the infectious vehicle given that vegetative (metabolically active) cells of obligate anaerobic bacteria are unlikely to survive the oxygenated environment outside the host or the acidic environment of the stomach. Indeed, an asporogenic strain of *C. difficile* is unable to persist in the environment or transmit between hosts³⁸.

Box 2

Antibiotic resistance of *Clostridium difficile*

Most antimicrobial compounds target metabolically active cells and have limited or no activity against dormant cells, such as spores. This intrinsic resistance of spores ensures that *Clostridium difficile* can persist in the presence of antibiotics or in the host immune system^{249,250}. *C. difficile* also demonstrates extensive acquired antimicrobial resistance^{9,251}. Interestingly, *C. difficile* vegetative cells are sensitive to teicoplanin and vancomycin despite harbouring a genomic region that resembles a *vanG* glycopeptide resistance cluster^{9,252}. This cluster can confer vancomycin resistance to a heterologous host, but why it is not functional in *C. difficile* is unclear²⁵³. The mobile genome of *C. difficile* (which consists of transposons, insertion sequences and (pro)phages) probably

contributes to antibiotic resistance because these elements commonly contain resistance determinants^{9,254}. For example, the Tn6218 element of *C. difficile* contains a *cfr*-like gene that can confer resistance to peptidyl transferase inhibitors such as linezolid²⁵⁵ and the Tn5397 element carries a tetracycline resistance determinant²⁵⁶. Reduced susceptibility or resistance to the commonly used antibiotics vancomycin, metronidazole and fidaxomicin has been noted^{257–260}. Although this is cause for concern, the clinical relevance of resistance to these antibiotics so far is limited. However, *C. difficile* antibiotic resistance is only part of the reason why *C. difficile* has been classified as an Urgent Antibiotic Resistance Threat by the US Centers for Disease Control and Prevention²⁶¹. Other major factors include the bacterium affecting people treated by antibiotics for other infections, the ageing of the general population and the emergence of epidemic types, such as PCR ribotype 027. At least in this ribotype, epidemic lineages are associated with resistance against fluoroquinolones⁸; although this class of antibiotics is not used for the treatment of *C. difficile* infections, the antibiotics can select for *C. difficile* when used to treat other infections. Fluoroquinolone resistance is also common in other PCR ribotypes²⁶².

Germination of spores is dependent on sensing primary bile acids from the liver, such as taurocholate, by the germinant receptor CspC and is inhibited by secondary bile acids in the colon^{37,39,40}. In addition, glycine can act as a germinant through an uncharacterized mechanism⁴¹. A proteolytic cascade then leads to the degradation of the spore peptidoglycan, release of calcium dipicolinic acid and rehydration of the spore, ultimately resulting in outgrowth of the cells^{37,40}.

The propensity of spores to outgrow and colonize the intestine is greatly influenced by the host microbiota and its associated metabolome^{39,42}. For example, antibiotic-induced shifts in the microbiota can generate an environment conducive to *C. difficile* infection⁴³. Mucolytic enzymes, such as cell surface protein Cwp84 (REF. 44), are secreted by the bacterium and degrade the colonic mucosa. Bacterial cell surface-associated proteins have been shown to affect the adhesion of the bacterium to colon epithelial cells *in vitro*^{45–48}; mutations in genes encoding these proteins, or in the genes encoding proteins for their processing, generally attenuate virulence^{49,50}. The expression of at least a subset of colonization factors by the bacterium, such as cell surface protein Cwp84 and surface layer protein A (SlpA), is stimulated in the presence of the antibiotics ampicillin and clindamycin⁵¹.

Before germination into vegetative cells, spores are also capable of adhering to colon cells⁵². *C. difficile* is a motile bacterium, and the switch between the sessile and motile phase is regulated by the secondary messenger cyclic di-GMP^{53–55}. *C. difficile* is also capable — at least *in vitro* — of forming robust biofilms^{56–58}. Cell–cell signalling contributes to both colonization and virulence factor expression^{59,60}. Host recognition of *C. difficile* is mediated via pattern recognition receptors and myeloid differentiation primary response 88 (MYD88)- and nucleotide-binding oligomerization domain-containing protein 1 (NOD1)-dependent pathways^{61,62} (FIG. 3). Specifically, SlpA can activate a Toll-like receptor 4 (TLR4)-dependent response⁶³, flagellin can stimulate TLR5 (REF. 64) and NOD1 is probably

activated via peptidoglycan-derived compounds⁶⁵. The first line of host defence against *C. difficile* is the production of antimicrobial compounds such as lysozyme and cationic antimicrobial peptides^{66,67}. Interestingly, the bactericidal α -defensins, but not β -defensin 1 or cathelicidin antimicrobial peptide (also known as LL-37), can also inhibit the activity of the TcdB (see below) through a mechanism that involves direct binding to the toxin⁶⁶. Innate lymphoid cells, specifically class 1, are implicated in resistance against CDI⁶⁸.

Resistance of the bacterium to host-produced antimicrobial compounds is multifactorial⁶⁹ and mediated by, for example, the extracytoplasmic function (ECF) sigma factor CsfV⁷⁰, the site-1 protease PrsW⁷¹, the proteins encoded by the *cpr*-locus⁷² and modulation of cell wall charge⁷³.

The large clostridial toxins TcdA and TcdB

Regulation of expression—Although several virulence factors contribute to the retention of *C. difficile* within the gastrointestinal tract^{74,75}, the symptoms of CDI correlate with the presence of a toxin-encoding pathogenicity locus (PaLoc) in the bacterial genome^{76,77}. In most strains, the PaLoc is located at the same site in the chromosome^{77,78}. The PaLoc in most pathogenic *C. difficile* strains encodes two large homologous toxins (TcdA and TcdB) and three proteins that seem to regulate toxin production and secretion (TcdR, TcdE and TcdC)^{74,75,78} (FIG. 4a).

TcdR is a member of the ECF family of alternative sigma factors and is critical for the initiation of TcdA and TcdB production in *C. difficile*^{79,80}. TcdC is thought to encode an anti-sigma factor that negatively regulates toxin production⁸¹. Epidemic ribotype 027 strains carry a nonsense mutation within the *tcdC* gene, leading to the suggestion that derepression of the toxin genes by the inactivation of TcdC might contribute to the increased virulence of these strains⁸². However, despite many studies aimed at defining the role of TcdC in toxin production and virulence, conflicting findings have been reported and the functional role of this protein remains unclear⁷⁵.

TcdE has homology with bacteriophage holin proteins, which are involved in the release of progeny phages from infected bacterial cells⁸³. The role of TcdE is also poorly understood, although some evidence suggests that it facilitates toxin (TcdA and TcdB) secretion^{78,84,85}. TcdA and TcdB do not possess any recognizable signal or export sequences, suggesting that they might be exported from the bacterial cell by lysis or a non-classic secretion pathway, possibly involving TcdE^{84,85}.

The synthesis of TcdR and the subsequent activation of *tcdA* and *tcdB* expression is influenced by many environmental stimuli, including short-chain fatty acids such as butyric acid that are common in the gut and sub-inhibitory concentrations of certain antimicrobials that may be relevant in the context of disease^{79,86–89}. Amino acids such as proline, cysteine and certain branched chain amino acids in the local environment of the bacterium repress toxin production through the action of the global transcriptional regulator CodY (known as GTP-sensing transcriptional pleiotropic repressor CodY)⁹⁰. The presence of glucose or other rapidly metabolizable carbon sources in the local environment of the bacterium also inhibits the production of TcdA and TcdB via the carbon catabolite control protein A (CcpA)^{91,92}.

The sigma factor SigD, which is associated with the expression of motility genes, promotes toxin gene expression by binding to a SigD-dependent promoter sequence upstream of *tcdR*^{93,94}. The master regulator of sporulation in both *Bacillus* and *Clostridium* species, Spo0A, can also regulate toxin production in *C. difficile*, but only in some strains⁹⁵. Specifically, Spo0A negatively regulates TcdA and TcdB production in epidemic type PCR ribotype 027 strains but not in others that have been tested^{95–97}. These results highlight the heterogeneous nature of *C. difficile* isolates and the need to study strains belonging to distinct evolutionary lineages^{95,98}. Finally, growth signals and cell density play an important part in toxin regulation^{59,60}. Cell–cell signalling is partially dependent on an accessory gene regulator quorum signalling system, which is mediated by a novel thiolactone quorum signalling peptide^{59,60}. Overall, *C. difficile* toxin synthesis is closely connected to the metabolic state of the bacterium and its environment⁹⁹.

Mechanism of action—Structural and functional studies have provided insight into the mechanisms of action of all of the *C. difficile* toxins, particularly TcdA and TcdB. Once secreted, TcdA and TcdB bind and enter the colonic epithelium to cause inflammatory chemokine and cytokine production, an influx of neutrophils, disruption of tight junctions, fluid secretion and epithelial cell death¹⁰⁰ (FIGS 5,6). Given the homology between the two proteins, it is notable that TcdA and TcdB have markedly different functions in animal toxicity models. Historically, TcdA has been viewed as the more potent enterotoxin, as administration of purified TcdA into the intestines of rabbits and rodents causes tissue necrosis and an intense infiltration of immune cells^{101–103}. High levels of TcdB in identical experiments failed to induce these effects, although it should be noted that most of these studies were conducted in an ileal loop model and, accordingly, represent only the response of the small intestine. In studies involving human colonic tissue, TcdB seems to be a potent inflammatory toxin^{104,105}, whereas TcdA is weaker¹⁰⁴. These data suggest that the differential toxin responses might partially stem from differences in receptor tropism and highlight the importance of conducting mechanistic studies within the colon, which is the site of bacterial outgrowth in the host.

TcdA and TcdB have four functional domains^{100,106}: an amino-terminal glucosyltransferase domain (GTD), an autoprotease domain (APD), a pore-forming and delivery domain, and the combined repetitive oligopeptides (CROPS) domain, which extends from around residue 1,830 to the carboxyl terminus (FIG. 5a,b). A combination of electron microscopy and X-ray crystallography studies has revealed the structural organization of these domains in TcdA and suggests that the structure of TcdB is similar^{106,107}. TcdA and TcdB enter cells via receptor-mediated endocytosis¹⁰⁸. Historically, receptor binding has been associated with a CROPS domain located at the C termini of TcdA and TcdB¹⁰⁹. The CROPS domain is capable of binding carbohydrates, which is consistent with the model wherein TcdA engages glycosylated receptors¹¹⁰. Evidence supporting the idea that the TcdA CROPS domain contributes to receptor binding includes the observations that antibodies against the TcdA CROPS domain can block intoxication¹¹¹ and that excess TcdA CROPS domain can compete with TcdA holotoxin for cell binding¹¹². TcdA binds a variety of carbohydrates, and although multiple glycolipids and glycosylated proteins have been proposed as receptors¹⁰⁹, the specific receptors used to bind human epithelial cells remain unknown.

However, accumulating evidence suggests that domains other than CROPS participate in receptor binding. Indeed, TcdA and TcdB lacking CROPS domains are still capable of intoxicating cells^{113,114}, and the homologous toxin TpeL from *Clostridium perfringens* lacks a CROPS domain entirely¹¹⁵. Recently, two protein receptors have been reported for TcdB: poliovirus receptor-like protein 3 (PVRL3; also called nectin 3)¹¹⁶ and chondroitin sulfate proteoglycan 4 (CSPG4)¹¹⁷. PVRL3 is highly expressed on the surface of human colon epithelial cells and co-localizes with TcdB in tissue resected from a *C. difficile*-infected individual¹¹⁶, suggesting that PVRL3 could serve as the initial receptor that TcdB encounters in the context of infection. CSPG4 is highly expressed in the intestinal subepithelial myofibroblasts of mouse and human intestines¹¹⁸, suggesting that this receptor could be engaged after initial damage to the colonic epithelium¹¹⁷. Both CSPG4 and PVRL3 bind outside the CROPS domain^{116,117}. It is conceivable that additional alternative receptors for TcdB exist.

Following receptor binding and endocytosis, acidification of the endosome is thought to trigger a structural change in the delivery domain, facilitating pore formation and translocation of the GTD into the cytosol¹⁰⁹ (FIG. 5c). The APDs share sequence homology with the cysteine protease of the MARTX family of toxins^{119,120}, but one publication has shown that the catalytic dyad of TcdA and TcdB serves to coordinate a zinc ion that is essential for function¹⁰⁶. Activation of the APD by eukaryotic inositol hexakisphosphate (InsP₆) results in the release of the GTD into the cell, enabling access to cytosolic substrates^{119,120}. Host *S*-nitrosylation at the conserved cysteine of the APD can inactivate the protease activity in an InsP₆-dependent manner¹²¹. It has been hypothesized that the autoprotease and translocation efficiency of the epidemic PCR ribotype 027 strain contribute to its increased virulence¹²². The GTD transfers glucose from UDP glucose onto RHO family GTPases such as RHO, RAC1 and cell division control protein 42 (CDC42)^{123,124}. These modifications cause a cytopathic effect resulting from rearrangement of the actin cytoskeleton and can lead to apoptosis¹²⁵ (FIG. 5c). At higher concentrations, TcdB is also capable of coordinating the assembly of the NADPH oxidase complex on endosomes¹²⁶. The resulting production of reactive oxygen species leads to cell death by a necrotic mechanism¹²⁶ (FIG. 5d). Both mechanisms might be important in the context of disease; cytopathic effects promote inflammation and disruption of the tight junctions, whereas TcdB-induced necrosis contributes to the colonic tissue damage observed in severe cases of CDI.

Knowledge of the mechanisms of action of *C. difficile* toxins has served as a foundation for several preclinical studies aimed at identifying small-molecule inhibitors of intoxication, particularly those that are already approved for other uses¹²⁷. A recent screen for inhibitors of TcdB-induced cytopathic effects identified compounds that act by inhibiting toxin binding to cells, endosomal maturation or glucosyltransferase function¹²⁸. Another screen, conducted using an activity-based probe for inhibitors of the APD, identified ebselen, a FDA-approved compound, which reduced tissue pathology in a mouse infection model¹²⁹. Notably, ebselen has also been reported to block NADPH oxidase 1 activity¹³⁰, a function that could contribute to a decrease in toxin-induced production of reactive oxygen species. Similarly, *N*-acetylcysteine, an FDA-approved antioxidant, prevented TcdB-induced tissue damage in a colonic explant model¹²⁶.

Pathology—Although the roles of TcdA and TcdB in the context of CDI have been difficult to assess, recent progress has come through advances in the genetic manipulation of *C. difficile*. Four studies have been conducted in both hamster and mouse models of infection. All studies indicate that TcdB is capable of inducing the phenotypes of disease in the absence of TcdA, but differ on the interpretation of the role of TcdA in the absence of TcdB on the survival of animals^{2,3,131,132}. Histological examination of colonic and caecal tissue from mice infected with TcdB-positive *C. difficile* strains (either wild-type TcdA⁺ TcdB⁺ or TcdA⁻ TcdB⁺ mutants) showed severe gut damage associated with eroded and often absent crypts, mucosal ulceration and goblet cell loss¹³². Polymorphonuclear cell influx into the lamina propria, enterocyte hyperplasia and severe submucosal oedema associated with haemorrhage were also observed in these tissues. TcdB-negative strains (TcdA⁺ TcdB⁻) caused less tissue damage that was confined to mild oedema and polymorphonuclear cell influx¹³². Tissue damage was strictly dependent on TcdB or TcdA given that tissues from mice infected with a strain that did not produce TcdA or TcdB (TcdA⁻ TcdB⁻) resembled those of mock-infected control animals¹³² (FIG. 6).

Consistent with the finding that TcdB is independently capable of causing disease, a considerable number of clinical *C. difficile* isolates express only TcdB¹³³. The prevalence of these strains, which include PCR ribotype 017, has been increasing, sometimes to epidemic proportions¹³⁴. Recently, the first strain with an intact *tcdA* gene, but no *tcdB* gene, in a different genomic context than the PaLoc has been characterized⁷⁸. This work has raised the hypothesis that the single toxin-encoding loci might have fused to form the typical two-toxin locus (PaLoc), which is the most common form currently detected in clinical isolates. The study also suggests a conserved relationship between the presence of toxin genes and holin genes, and demonstrates that the PaLoc does not always encode a *tcdC* homologue⁷⁸. However, it should be noted that confirmed clinical cases of CDI caused by strains that only produce TcdA are extremely limited⁷⁸.

Binary toxin CDT

Regulation of expression—*C. difficile* transferase (CDT; or binary toxin) is a third toxin produced by some *C. difficile* strains, including the epidemic PCR ribotypes 027 and 078. CDT has received increased attention in recent years because of its increasing prevalence in isolates of both human and animal origin¹³⁵. CDT is encoded by two genes, *cdtA* and *cdtB*, that are located in an operon on the binary toxin locus (CdtLoc)^{136,137} (FIG. 3b). In CDT-negative strains, this locus contains an ~2 kb deletion¹³⁸. The CdtLoc also harbours a response regulator gene, *cdtR*, upstream of the *cdtAB* operon¹³⁹. CdtR is an orphan LytTR-like positive transcriptional regulator of the *cdt* operon and CDT production. The cognate sensor histidine kinase that interacts with the orphan histidine kinase CdtR has yet to be identified¹³⁹. A truncation in the *C. difficile* PCR ribotype 078 CdtR does not abrogate CDT expression, suggesting that full-length CdtR is not essential for expression¹⁴⁰. In contrast to the PaLoc, the environmental signals that regulate the expression of the CdtLoc genes are not known.

Mechanism of action—Recent studies have provided insight into the mechanisms of action of CDT, although the role of this toxin in disease pathogenesis remains unclear. CDT

belongs to the binary ADP-ribosylating toxin family and consists of two components: an enzymatic component (CDTa) and a binding/translocation component (CDTb). CDTa has ADP ribosyltransferase activity, whereas CDTb facilitates the passage of the enzymatic component to the cell cytosol¹³⁵ (FIG. 7). CDTa leads to the complete destruction of the actin cytoskeleton and, ultimately, cell death^{135,141}.

Pathology—Despite a thorough understanding of the mechanism of action of CDT on intoxicated cells, the role of this toxin in disease pathogenesis is unclear. Experimental data have suggested that CDT results in the formation of microtubule-based protrusions on epithelial cells that might increase the adherence and colonization of *C. difficile*¹⁴². Importantly, the increasing presence of CDT in clinically relevant strain types commonly associated with severe CDI, such as PCR ribotypes 027 and 078, and the isolation of TcdA[−] TcdB[−] CDT⁺ strains suggest that this toxin is likely to play an important but as yet undefined part in CDI^{143,144}.

Experimental models

Multiple experimental models have been developed as a proxy for CDI and its treatment in humans^{145–147}. The most common models are the female golden (Syrian) hamster model, which is exquisitely sensitive to toxin and is primarily suited to study acute disease, and the mouse model. The mouse model mimics certain aspects of human disease that are difficult to assess in hamsters; for instance, mice can be colonized asymptomatically, they exhibit differential sensitivity towards different PCR ribotypes and they can experience relapsing disease. For these reasons, the model is suitable to study colonization, transmission and persistence phenotypes^{38,148,149}. A piglet model is of particular interest to study *C. difficile* strains that are problematic in both animal and human populations^{13,35,150}. *In vitro* gut models have been developed to study interactions of *C. difficile* with therapeutics in the context of a complex microbiome^{13,151,152}. Each model is greatly influenced by variables such as qualitative and quantitative differences in inoculum and the choice of *C. difficile* strain¹⁴⁷. Furthermore, non-animal models (for example, the *in vitro* gut models) may not fully reflect the interaction with the host, but some have been shown to be more reflective of human CDI than animal models¹⁴⁷.

Diagnosis, screening and prevention

Symptoms and risk factors

The clinical symptoms associated with CDI range from mild, self-limiting diarrhoea to fulminant colitis, and can include pseudomembranous colitis (FIG. 1d), toxic megacolon (severe dilatation of the colon), bowel perforation and sepsis, and/or multiple organ dysfunction syndrome^{14,132,153}. Given that the characteristics (for example, age, use of medication or underlying comorbidities) of patients who can acquire CDI are highly variable, there is considerable variation in the possible severity assessments for this disease, which is reflected in the differing criteria used in guidelines^{154,155}. Severe diarrhoea associated with *C. difficile* is often accompanied by a typical endoscopic picture of pseudomembranous colitis with haemorrhage and deep ulcerations. Toxic megacolon is considered the most serious disease entity and is characterized by systemic toxicity and high

mortality. Extra-intestinal manifestations of CDI (including bacteraemia) are extremely rare, and this emphasizes that it is the localized effects of toxins, associated with depleted intestinal microbiota, that cause the range of signs and symptoms of CDI. *C. difficile* toxins in sera from patients with CDI can be detected with an ultrasensitive cell-based assay¹⁵⁶, but studies are required to assess the relationship between severe CDI and levels of toxemia.

Known risk factors for CDI are previous hospitalization, underlying disease, advanced age (>65 years) and, most importantly, the use of antibiotics. All antibiotic classes can be associated with CDI, but clindamycin, cephalosporins and fluoroquinolones are the most frequently cited¹⁵⁷. Antibiotic-induced dysbiosis of the protective intestinal microbiota often underlies *C. difficile* outgrowth and toxin production^{39,43}. Thus, even low-risk antibiotics (such as trimethoprim and piperacillin-tazobactam) can predispose the patient to CDI, especially when two or more courses of (different) antibiotics are prescribed; the cumulative damage to the intestinal microbiota could be sufficient to enable *C. difficile* to proliferate.

In addition to the antibiotic class, the number of administered antibiotics, dose and duration of therapy have been identified as risk factors for CDI. Given that disruption of the intestinal flora persists for >3 months after antibiotic therapy, patients can remain susceptible to CDI development long after ending the treatment²⁸. Acid suppression by proton pump inhibitors (commonly used for dyspepsia, peptic ulcer disease and gastroesophageal reflux disease) has frequently been associated with CDI^{158,159} but the precise role of proton pump inhibitors in CDI (and a causal relationship) remains unclear^{160,161}. Of all patients with antibiotic-associated diarrhoea, 20–30% is caused by *C. difficile*¹⁶². A differential diagnosis could consider a role for *Staphylococcus aureus*, *C. perfringens*, *Clostridium sordellii* or *Klebsiella oxytoca* as causative agents of antibiotic-associated diarrhoea¹⁶³.

Diagnosis

The mainstay for diagnosing CDI is the presence of clinical symptoms plus a well-chosen laboratory assay. The diagnostic tests for *C. difficile* can be divided into tests for *C. difficile* products (glutamate dehydrogenase (GDH), aromatic fatty acids, TcdA and/or TcdB); culture methods for the detection of toxin-producing *C. difficile* (toxigenic culture); and nucleic acid amplification tests for *C. difficile* genes (detecting 16S rRNA, toxin genes or the gene encoding GDH)¹⁶². The test selection is important to differentiate between patients with CDI and asymptomatic carriers^{34,164}. Tests that detect toxins are specific to CDI, whereas those that detect (a component of) the bacterium might indicate colonization rather than disease^{34,164}.

Exclusive reliance on molecular tests for CDI diagnosis without tests for toxins is likely to result in overdiagnosis and overtreatment^{34,165}. Owing to large variations in sensitivity and specificity of various diagnostic tests, the European Society of Clinical Microbiology and Infectious Diseases recommends using a two-step algorithm, including a test for the presence of *C. difficile* and one to detect free toxins in the faeces¹⁶⁶. As tests remain positive for toxins during treatment and toxins can even be found after successful treatment¹⁶⁷, regular monitoring using toxin tests as follow up for treatment is not advised. If free toxins are absent, CDI is highly unlikely (FIG. 8). Importantly, *C. difficile* toxin assays vary markedly in their sensitivity^{34,164,168}. If *C. difficile* is present but the toxin test result is

negative, CDI cannot be definitively excluded. Patients could be (asymptotically) colonized by *C. difficile* with diarrhoea due to an alternative cause or could be experiencing CDI with toxin levels below the lower limit of detection of the assay used. For these patients, clinical evaluation is required to decide whether treatment for CDI is warranted.

An alternative diagnostic algorithm uses a coupled enzyme immune assay that simultaneously detects both GDH and TcdA or TcdB¹⁶⁹. As the sensitivity of the toxin component is unclear, samples that are GDH-positive but toxin-negative could undergo reflex testing using a nucleic acid amplification test to determine whether a toxigenic *C. difficile* strain is present. Ideally, every laboratory should also have the opportunity to isolate *C. difficile* from faecal samples. Isolation enables toxigenic culture and susceptibility testing, and offers the ability to perform molecular typing that is required for surveillance (BOX 1). Many countries have implemented reference laboratories for this purpose³³. The most recent US guidelines on testing were published in 2010 (by the Society for Healthcare Epidemiology of America and the Infectious Diseases Society of America); the guidelines did not make a firm recommendation regarding the routine diagnosis of CDI¹⁵⁴.

Screening

There is considerable variation between and within countries in the diagnostic algorithm applied but also the frequency of testing (when to test). A study involving ~500 hospitals in 20 countries across Europe revealed that 23% of diarrhoeal samples with a positive CDI test result (at a reference laboratory) were initially missed owing to a lack of clinical suspicion³². Hence, restricting the testing of samples to those for which a physician has requested CDI testing will lead to underdiagnosis. All stool samples from hospitalized patients with diarrhoea should be tested unless a plausible alternative explanation (such as laxative use or diagnosis of inflammatory bowel disease) is available^{155,164,170} or if the patient is <2 years of age. Indeed, *C. difficile* is commonly found in the faeces of healthy infants, and there is no general agreement on how to define CDI in children or when to test for CDI in children, especially with respect to age, underlying disease and use of antibiotics^{1,171}. However, CDI can occasionally occur in infants and young children, and can cause microscopic intestinal lesions with symptoms other than diarrhoea. The American Academy of Pediatrics has recently recommended testing for CDI only if age-specific clinical criteria are met¹⁷².

As *C. difficile* is increasingly recognized as a causative agent of community-associated diarrhoea, one can consider testing all diarrhoeal samples from community patients, although it can be cost-prohibitive to test a large number of samples. Application of specific algorithms, such as testing those with diarrhoea who have previously been hospitalized or used antibiotics, results in recognition of only 61% of patients with CDI in the community²⁸. The introduction of multiplex molecular tests for enteric pathogens makes it easier to implement routine testing (screening) for CDI, although positive tests of *C. difficile* should be followed by a stool toxin detection test (two-step algorithm). In a European, multicentre, quarterly point-prevalence study of community-acquired diarrhoea using molecular tests, *C. difficile* was found among 709 samples to be the third most frequently occurring pathogen, after enteropathogenic *Escherichia coli* and *Campylobacter* spp.¹⁷³. In that study, children were also included (potentially identifying colonized rather than infected individuals) and a

large variation per country was observed¹⁷³. *C. difficile* occurred more frequently in the >60-year age group than in other age groups. Application of molecular diagnostics in a case-control study of patients with diarrhoea attending a general practitioner in the Netherlands revealed that *C. difficile* was present in 4.2% of 1,515 cases and 0.8% of 1,195 healthy controls¹⁷⁴. This number is considerably higher than the prevalence of *Salmonella* spp. and *Shigella* spp., and confirmed that *C. difficile* is frequently found in general practice²⁴.

Asymptomatic carriage

For both health-care- and community-associated infection, it is necessary to differentiate between colonization and disease^{34,164,165}. Carriage and colonization are often deemed to be interchangeable terms^{175,176}. Indeed, the criteria used to determine asymptomatic carriage and/or colonization vary markedly between studies. A clarification of terminology is required, as a single *C. difficile*-positive faecal sample could indicate colonization, transient carriage or 'pass through' (REF. 177). We prefer to use the term 'carriage' to refer to persistent colonization.

Most studies use just one single culture and, therefore, evaluate passage or transient colonization, defined here as asymptomatic colonization. Asymptomatic *C. difficile* colonization is common in health-care facilities and in the community, and can be attributable to toxin-positive or toxin-negative strains^{36,178–180}. In hospitals, the prevalence of asymptomatic colonization varies from 7% to 18%, depending on the length of stay, exposure to antibiotics and to *C. difficile* (infection pressure), underlying disease and possibly use of acid-suppressive medication. Indeed, the environment is most contaminated in rooms of patients with CDI, less so in rooms of patients asymptomatically colonized with *C. difficile* and least in patients who are not colonized¹⁸¹. During outbreaks, colonization rates may further increase to >50%¹⁷⁹. Asymptomatic colonization in the community is lower than in the health-care setting and is in the range of 2–4%. Several studies report high carriage rates (25–35%) in children in the first year; the rate drops (to 15%) for ages 1–8 years^{182–184}. This variation is probably associated with the unstable intestinal microbiota in the first 2–3 years of life, which enables *C. difficile* to remain established in the intestinal tract¹⁸⁵. Interestingly, high levels of free toxin can also be detected in faecal samples of young children without diarrhoeal symptoms, therefore, further reinforcing the need for information on the effects of toxins on intestinal epithelial cells in children.

Asymptomatic colonization has been considered a protective factor against the acquisition of new *C. difficile* isolates. A frequently cited meta-analysis of four studies from 1994 showed that patients colonized with *C. difficile* actually had a lower risk of subsequently developing CDI, but no distinction was made between colonization by toxigenic versus non-toxigenic strains¹⁸⁶. A recent meta-analysis included studies in which patients were colonized with toxigenic strains at hospital admission only³⁶. It should be noted that in this study, unlike the previous study¹⁸⁶, patients with previous CDI were not excluded and this could have confounded the results. Overall, the patients who were colonized had an increased risk of developing CDI (relative risk 5.86; 95% confidence interval 4.21–8.16). By contrast, patients asymptomatically colonized by non-toxigenic strains do not seem to have an

increased risk or are even protected from progressing to CDI. This concept was recently tested in humans; patients who could be colonized by a non-toxigenic *C. difficile* strain after receiving standard of care treatment for CDI had significantly reduced CDI recurrence rates¹⁸⁷. However, further explorations of this approach should consider that non-toxinogenic strains can become toxinogenic by horizontal gene transfer¹⁸⁸, although it is unknown whether this process also occurs in a human host.

Asymptomatically colonized patients can shed spores into their environment and consequently to other patients. As early as 1992, it was recognized that nosocomial acquisition of a *C. difficile* strain was preceded by introduction of that strain to the ward by an asymptomatically colonized patient¹⁸⁹. On the basis of an epidemiological model of *C. difficile* transmission in health-care settings, admission of colonized patients was shown to play an important part in sustaining ward-based transmission¹⁹⁰. This observation needs confirmation as it could have major implications for infection control and prevention measures.

Management

Infection control

Several reviews and guidelines for controlling CDI have been published^{154,155,157,191–195}. Although some differences exist, most of these guidelines have similar recommendations (BOX 3). If a patient is suspected of having CDI, rapid diagnostic testing should be performed to enable treatment initiation as soon as possible. Spores are highly infectious and problematic in health-care settings as they are able to persist on surfaces and are resistant to many disinfectants and alcohol-based hand washes³⁷. Up to 1×10^7 spores per gram of faeces are present in patients with CDI, representing considerable potential for environmental contamination. Consequently, treatment should always be combined with patient isolation to prevent spread of *C. difficile* or other enteropathogenic microorganisms.

Box 3

Infection control and prevention of *Clostridium difficile* infection

- Ensure rapid diagnostic testing of patients with diarrhoeal illness acquired in the hospital or associated with antimicrobial therapy
- A hospital-based infection control programme combined with active surveillance can help to decrease the incidence of *Clostridium difficile* infection (CDI); locally defined thresholds or triggers for the addition of enhanced control measures are needed
- Antibiotic stewardship, including restriction of specific high-risk antimicrobials (such as clindamycin, cephalosporins and fluoroquinolones), is recommended to reduce the risk of CDI
- Patient isolation and contact precautions (including hand hygiene with soap and water) should be maintained until at least the diarrhoea has resolved

- If isolation in a single room is not possible, alternatives are segregation within wards and/or cohorting of cases
- Disinfection of environmental surfaces is recommended using chlorine-releasing agents as a minimum in clinical areas with CDI cases
- Educate health-care personnel, cleaning staff and visitors on contact precautions to minimize the transmission of spores

In a health-care setting, transmission of *C. difficile* spores occurs primarily via the contaminated hands of health-care workers, but contact with a contaminated environment, contaminated utensils or medical devices has also been implicated; *C. difficile* spores have been identified in rooms of patients who have tested negative. Environmental decontamination of clinical areas, ideally using chlorine-releasing agents or a sporicidal product, is recommended; however, in practice, compliance with cleaning protocols is often suboptimal¹⁵⁴. Newer alternatives for environmental decontamination have been introduced, notably gaseous hydrogen peroxide and, more recently, UV decontamination¹⁹⁶. The former is particularly effective at killing *C. difficile* spores, but the cost-effectiveness of these approaches is unclear.

Antimicrobial therapy and surgery

The currently available antibiotics that are recommended for treatment of CDI are metronidazole, vancomycin and fidaxomicin (FIG. 8). Stopping the inciting antibiotics and clinical observation can treat very mild CDI that has been induced by antibiotics¹⁵⁵. Patients with mild-to-moderate CDI can be treated with oral metronidazole whereas oral vancomycin is recommended for severe or complicated infections^{154,155,193}. Two multinational randomized controlled trials included patients managed with either vancomycin or metronidazole¹⁹⁷. Metronidazole was inferior to vancomycin on an intent-to-treat basis (clinical cure rates 72.7% versus 81.1%). Also, in a post-hoc multivariate analysis, vancomycin, treatment-naïve status and mild-to-moderate CDI severity predicted treatment success.

Concomitant antibiotics are associated with reduced clinical cure, increased recurrence rates and longer time to cessation of diarrhoea¹⁹⁸. Consequently, additional measures to curb CDI include discontinuation of unnecessary antimicrobial therapy (that is, for the presenting infection), as well as avoidance of anti-motility medications and reviewing proton pump inhibitor use¹⁵⁵. Patients with complicated CDI with ileus (paralysed bowel) or toxic megacolon, in whom oral antibiotics cannot reach the disease site, can be treated with vancomycin delivered per rectum plus intravenous metronidazole.

Fulminant CDI is a highly lethal disease with mortality rates of up to 80%. These patients often require a total abdominal colectomy, but there is no established management protocol. Alternatively, a diverting loop ileostomy and colonic lavage might be associated with reduced morbidity and mortality¹⁹⁹. Surgical therapy should be considered in patients with toxic megacolon, clinical signs of sepsis and severe organ dysfunction, acute abdomen and

severe ileus. A white blood cell count of 15,000–20,000 per μl and elevated serum lactate ($>5.0\text{ mM}$) might serve as markers of disease severity^{154,155,200}.

Recurrent infection

After treatment of an initial episode of CDI, the chance of a recurrence within 8 weeks is 15–25%; for a patient with 1–2 previous recurrences, the risk of further recurrences is 40–65%²⁰¹. Recurrences are associated with an impaired immune response to *C. difficile* toxins and/or alteration of the colonic microbiota. Fewer recurrences occur after treatment with oral fidaxomicin (13%) than with vancomycin (25%)^{202,203}. However, after a first recurrence, optimal treatment options are less clear, but fidaxomicin might be effective^{198,202,203}. Despite these shortcomings, fidaxomicin is generally used for treating a first recurrence of CDI, unless disease has progressed from non-severe to severe (FIG. 8). Given that the main strength of fidaxomicin is prevention of recurrent infections (except for those due to PCR ribotype 027 that generally respond less well to antibiotics), clinical prediction markers for recognizing patients at risk for recurrent CDI could be helpful. Multiple risk factors for recurrent CDI have been suggested in the literature, but as not all of these are evident at the time of treatment initiation, it is difficult to select appropriate parameters. Age >65 years, concomitant antibiotics, renal failure, history of previous CDI, possibly continued use of antacid medications and initial disease severity^{155,204} are associated with increased risk of recurrence.

Bacteriotherapy and faecal transplantation

Limited evidence supports the use of probiotics to decrease recurrence of CDI, and no effective immunotherapy is currently available²⁰⁵. Faecal microbiota transplantation (FMT) is a highly effective rescue treatment and should be considered in patients who have had >2 recurrences, as the efficacy of antibiotics in these patients is $\sim 30\%$. A randomized controlled trial of FMT revealed that it is effective ($\sim 81\%$) in treating multiple recurrent CDI²⁰⁶ (FIG. 9). FMT is best reserved for patients with multiple recurrences of CDI who have failed to respond to other treatment options. Importantly, FMT is a non-standardized procedure, and the long-term consequences of altering a patient's gut microbiota are unknown. Several national guidelines have, therefore, been developed to standardize FMT, including donor screening and selection^{207–209}. Results from a preliminary study among patients with relapsing CDI revealed that administration of FMT using frozen encapsulated inoculum from unrelated donors also resulted in a good outcome²¹⁰. It is likely that future research will define mixtures of selected microorganisms, designed according to the microorganisms' roles in the microbiota against CDI, as 'pharmacological' alternatives to FMT. For example, a mixture of 33 bacteria has been shown to be effective in two patients with CDI²¹¹, although the selection of the isolates was not based on microbiota studies. Rectal bacteriotherapy with a mixture of 12 bacteria from healthy donors resolved CDI and prevented recurrence within 30 days in 64% of the patients²¹². A combination of four bacterial species selected on the basis of strongest association with resistance to CDI, protected mice from infection, most probably indirectly through an effect on bile acid metabolism⁴². As noted for FMT, long-term safety data on rectal bacteriotherapy will be needed, given the far-reaching effects of gut microbiota in health and disease.

Novel therapeutics

Several therapeutics targeting different stages of CDI are currently in clinical development (TABLE 1). In brief, these consist of treatments to restore a complex microbiota (SER-109)²¹³, to prevent off-target effects of antibiotic treatment on the intestinal microbiota (SYN-004), to neutralize *C. difficile* toxins (including monoclonal antibodies)²¹⁴ or to inhibit *C. difficile* proliferation (SMT19969, LFF571, surotomycin, cadazolid, PolyCAb and CRS3123).

Quality of life

Economic burden of CDI

The burden of health-care-associated CDI can be expressed in terms of mortality, recurrence, (additional) length of hospital stay or economic cost^{215–217}. Economic analyses of health-care-associated CDI have shown that direct health-care cost, and costs due to increased length of stay, were the main cost drivers. An integrative review showed a wide variation in the difference in length of stay between people with and those without CDI (2.8–16.1 days), which was attributed to differences in design and data collection²¹⁸. Overall, people with CDI stay longer in hospital than people without CDI despite this variation.

A systematic review of the effects of CDI in Europe showed that the median length of stay for patients with CDI was in the range of 8–27 days, with an additional length of stay (due to CDI) of between 2.8 days and 18 days²¹⁷. The incremental per-case cost of CDI in this study was UK£4,577 in Ireland and £8,843 in Germany, after standardization to 2010 prices²¹⁷. Others have estimated the incremental per-case cost of CDI, after standardization to 2008 prices, to be US\$2,871–90,644 (REFS 216,219) (FIG. 10). A recent meta-analysis identified a total of 45 studies (mostly from North America) that measured the economic impact of CDI. For hospitalized patients, attributable mean CDI costs ranged from \$8,911 to \$30,049. However, the authors noted that costing methods were heterogeneous, making inter-study and setting comparisons difficult. Standardization of such measurements would be helpful, although differences between health-care systems remain a barrier when comparing financial costs²²⁰.

The total direct cost of CDI to the European Union in 2006 was estimated at €3 billion per year⁶. Assuming a 3% annual inflation rate, this approximates to >€4 billion in 2015. Estimates for the economic burden of CDI in the United States and Canada are in excess of US\$1 billion²²¹ and CAN\$280 million²²², respectively. These figures do not include the indirect socioeconomic costs (see below). Only for Canada does the estimate include a parameter for community-associated CDI in addition to health-care-associated CDI²²²; so, the total burden in the United States and European Union probably exceeds the numbers given above.

Patient-reported quality of life

The stark mortality rates associated with CDI emphasize the serious consequences of this disease. Furthermore, given that CDI is characterized by diarrhoea, relatively frequent symptomatic recurrences and often altered bowel habit for possibly weeks or months

following cessation of acute symptoms, it is perhaps not surprising that patients regularly report that this disease is one of the worst that they have experienced²²³. As patients are typically older and have comorbidities, the additional burden of CDI can affect both their dignity and ability to cope. Despite these well-recognized effects of CDI, very few data are available to formally measure how the disease affects an individual's health status, functionality and quality of life. Two recent studies have begun to explore these under-reported issues.

A prospective study of 66 outpatients with CDI used the RAND Short-Form 36 (SF-36) Health Survey and concluded that CDI significantly decreased overall quality of life but that a more specific health-related questionnaire is needed. The Patient-Reported Outcome Measurement Information System (PROMIS; <http://www.nihpromis.org>) is a large, US National Institutes of Health-funded database of questions to measure patient-reported health status for physical, mental and social well-being. PROMIS has recently been explored in a prospective, observational, multicentre study as a potential way to evaluate self-reported health status in patients with CDI²²⁴. Patients ($n = 95$) with active CDI (58%) or who were hospitalized (42%) had worse scores with regard to bowel function, nausea and abdominal pain compared with controls ($P < 0.001$). Those with recurrent CDI had worse anxiety scores than any other group (patients with first occurrence of CDI and controls; $P < 0.001$). The authors concluded that the 18 patient-reported health status questions were discriminatory for active CDI and primary versus recurrent CDI. These questions might be suitable for measuring short-term and long-term differences in patient-reported health status in people with CDI. CDI can also have long-lasting effects on families, but there is no systematic evaluation of these effects.

More work is needed to optimize these measurements and to determine which interventions are associated with the best improvements in outcomes for both patients and relatives.

Outlook

Outstanding research questions

Substantial progress has been made in our understanding of *C. difficile* physiology and pathogenesis. Studies have provided insight into the workings of the pathogen and highlighted aspects of its biology that differ from the situation in other studied bacteria. For instance, the order, activation and function of sporulation sigma factors of *C. difficile* deviate from what is known for the best-studied Gram-positive spore former, *Bacillus subtilis*²²⁵. Perhaps this difference should not come as a surprise, as the last common ancestor of bacilli and clostridia dates back ~2.7 billion years, only shortly after the divergence of Gram-positive and Gram-negative bacteria (3.2 billion years ago)²²⁶. It is conceivable that more detailed investigations of the molecular biology of clostridia in general, and *C. difficile* in particular, will reveal unexpected features.

Laboratory investigations under controlled conditions have clearly demonstrated that the production of enterotoxins is regulated in a complex manner and integrates signals from both bacterial metabolism and the culture conditions⁹⁹. It is uncertain, however, whether this process reflects infection within the host. Mutants that are used to assess these effects in the

laboratory might display reduced virulence as a result of reduced fitness. Although the role of enterotoxins in disease is established, it remains unknown how toxin production is triggered *in vivo*, and how or when the toxins are secreted into the gastrointestinal tract. Similarly, it is unknown what triggers sporulation during infection. Both toxins (production and activity) and spores have been implicated in epidemicity^{82,122,227}. However, strong evidence supporting this link is lacking and the ability of *C. difficile* to cause epidemics is likely to be a multifactorial process that involves a delicate balance of factors that affect virulence and transmissibility^{74,75}. It should be noted that increased virulence might not correlate with transmissibility, which might be favoured when hosts remain relatively healthy⁷⁵.

Epidemic types of *C. difficile* have received considerable attention as a result of their higher mortality and morbidity in comparison to other, non-epidemic, types^{7,8,12,18,82}. Often, enhanced infection control measures are taken when transmission of epidemic *C. difficile* types is detected, which has probably contributed to the decline of PCR ribotype 027 in different countries in 2014 (REF. 32). But is the increased vigilance towards these strains warranted? First, epidemiological analyses ignore the fact that not all strains of the same PCR ribotype exhibit the same characteristics, as has been demonstrated for sporulation²²⁸. Second, other PCR ribotypes can also cause outbreaks of CDI^{20,21,229}. Third, with the advent of sequence-based typing methods, it is becoming clear that strains with different PCR ribotypes can be highly related^{98,230}. For instance, strains of PCR ribotypes 244 and 176, which are related to PCR ribotype 027, can cause outbreaks of severe CDI^{22,23}. PCR ribotype 244 seems to be primarily implicated in community-associated CDI, indicating that highly related strains might emerge as epidemic types in different settings²³¹. Finally, new types could emerge. Thus, care should be taken to not generate an unjustified bias towards certain strains in epidemiological vigilance.

Colonization and pathogenesis

What determines whether *C. difficile* successfully establishes an infection is an important question. The host microbiota and its associated metabolites greatly influence the ability of *C. difficile* spores to germinate and colonize the gut^{39,43}. However, niche-specific competition²³² or collaboration (for instance, in a multispecies biofilm^{56,58}) might also contribute. Most of the metagenomic studies have focused on bacterial species, whereas the contribution of fungal species and viruses (bacteriophages) is poorly explored. Notably, population groups with relatively unstable microbiomes (that is, infants¹⁸⁵ and the elderly²³³) are most commonly colonized by *C. difficile*.

Other host factors might also play a part. Failure to mount a protective immune response is associated with disease progression; patients with an adequate response could either eliminate the infection or become asymptomatic carriers¹⁴. Host-dependent expression levels of toxin or colonization factor receptors could also be an important determinant for disease development. It has been postulated that infants remain asymptomatic in the presence of high levels of toxins due to the absence of receptors in their still-developing gastrointestinal tract; however, there is no evidence yet to support this hypothesis. Detailed

studies of the interaction of *C. difficile* with the host are necessary to delineate the contribution of host factors to colonization and pathogenesis.

Clinical needs

Although antimicrobial resistance of *C. difficile* is not a major issue in the clinic, novel narrow-spectrum therapeutics are needed. The primary reason is that current (broad-spectrum) antibiotics with activity against *C. difficile*, such as metronidazole and vancomycin, can concurrently predispose patients to CDI (and possibly recurrent CDI) owing to their effects on other members of the microbiota²³⁴. Furthermore, some antibiotics are not cost-effective as first-line treatment options. It is possible that specific prophylactic elimination of *C. difficile* in high-risk groups could reduce the risk of CDI without altering the host microbiota. It is of interest that bacteriocins and viruses (bacteriophages) seem to be able to target *C. difficile* specifically^{232,235,236}.

FMT is an excellent potential alternative to antibiotic therapy²⁰⁶, but long-term safety, public acceptance and relative lack of standardized donor material are limiting broad application. Steps have been taken to generate standardized formulations of FMT or bacteria^{42,149,211,212}. However, it remains to be established whether a single species of bacteria or mixtures of different bacteria are effective for all (or most) patients. It is likely that bacterial or metabolic signatures need to be identified that confer broad protection or activity against CDI (FIG. 9).

Antivirulence strategies²³⁷ might become valuable additions or alternatives to the current therapeutic spectrum. Neutralizing antitoxin antibodies have shown clinical promise^{214,238} (TABLE 1), and toxin activity has also been targeted using small molecules^{128,129}. Interference with quorum sensing or colonization factors has been underexplored so far, although it is clear that these factors can also be targeted by antibodies^{239,240}. Small-molecule inhibition of extracellular protein processing or function could prove a viable strategy to reduce colonization or transmission of CDI.

A final issue is the clinical need for accurate prediction models¹⁵⁵. Accurate risk assessment tools that can be applied in real time would be beneficial to target diagnostic methods and for optimized treatment of those at risk of severe or complicated CDI, or recurrence^{241–244}. Although several tools have been developed, there is considerable room for simplification and improvements in predictive values to make these tools applicable at the bedside.

The burden of CDI

Current conservative estimates of the economic burden of CDI are based on estimates of incidence and an incremental — per (hospitalized) patient — cost. These estimates fail to take into account the changes in demographics that are projected over the coming years. Age is a risk factor for CDI, although it is unclear whether this is an independent factor or caused by underlying confounders (such as immune senescence, comorbidities, need to stay at long-term care facilities and additional required health care). The European Union has projected that the demographic old-age dependency ratio (the ratio of those aged >65 years old to those aged 15–64 years) will increase from 27.8 to 50.1% between 2013 and 2060 (REF. 245). Similarly, US-based population projections foresee an increase in the percentage of

people aged >65 years of 13.7–20.9% between 2012 and 2050 (REF. 246). On the basis of these projections, the impact of CDI is expected to become considerable in coming years.

Epidemiological evidence suggests that an increasing number of CDI cases is linked to populations that are generally considered to be at low risk for CDI. These community-associated infections generally affect a different demographic (younger patients) who have frequently not been exposed to antibiotics. Further studies are required to determine risk factors for community-associated CDI. As many cases of community-acquired CDI go undetected^{24,27,29}, more studies are required to determine its contribution to the total burden of CDI at national and international levels.

Finally, *C. difficile* is increasingly recognized in veterinary medicine, with highly variable disease entities in different species of animals. No information is available to date on the economic burden of CDI in the food (animal) production industry.

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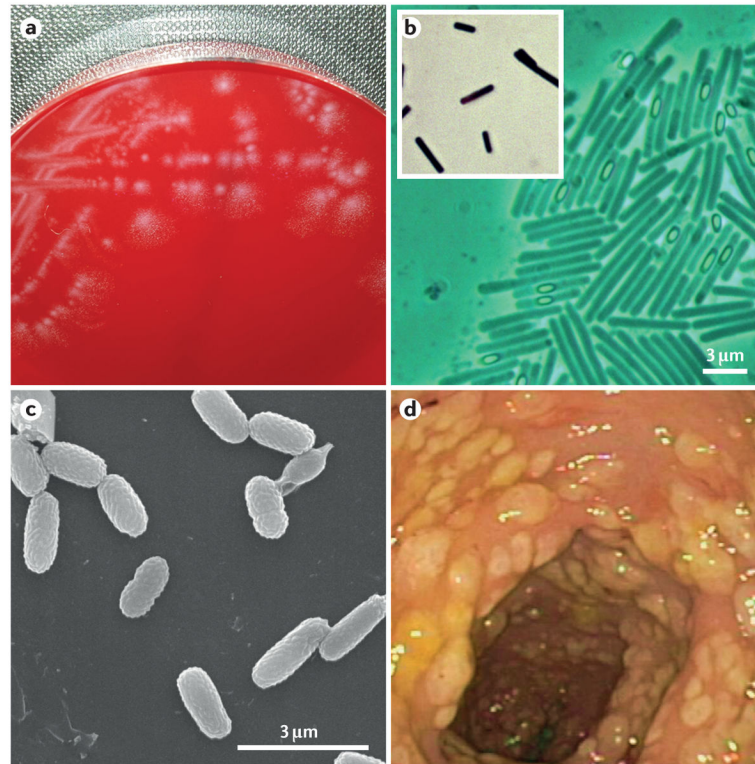


Figure 1. *Clostridium difficile*

a | Typical image of *Clostridium difficile* colonies on a blood agar plate. **b** | Phase-contrast microscopy image of a *C. difficile* culture with vegetative cells (elongated rods), phase-dark spores (subterminal dark spots) and phase-bright spores (bright ellipsoids). Inset, Gram stain of culture. **c** | Scanning electron micrograph of *C. difficile* spores. **d** | Endoscopic picture of pseudomembranous colitis caused by *C. difficile*. Healthy colon tissue is pink, pseudomembranes resulting from *C. difficile* infection are yellow.

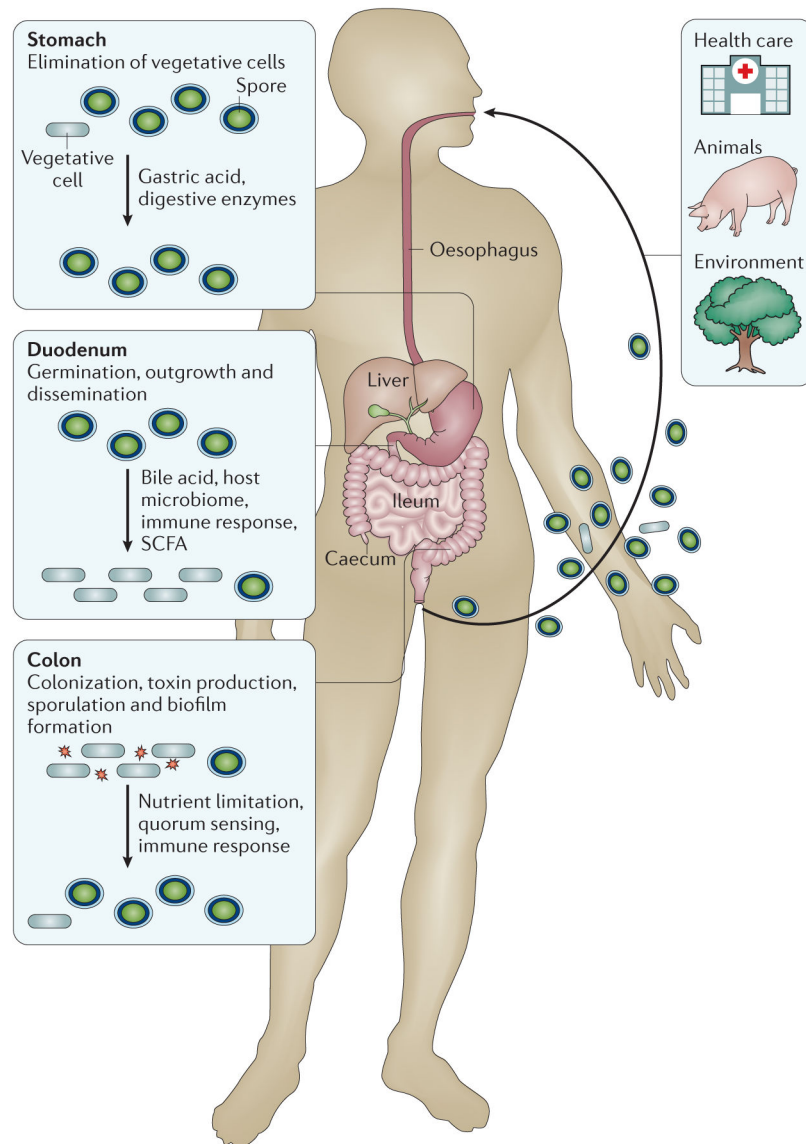


Figure 2. Stages of the *Clostridium difficile* life cycle in the human gastrointestinal tract

Three sources of infection (health care, animal and environmental) are indicated. A range of host factors influence the *Clostridium difficile* life cycle, as well as the relative numbers of spores and vegetative (metabolically active) cells in the gut. Note that passage through the stomach eliminates most vegetative cells (but spores survive), and spores germinate and grow out in the duodenum. In the caecum and colon, *C. difficile* starts producing spores again, and vegetative cells are excreted by the patient during infection. Toxin is produced in the colon. As *C. difficile* is an obligate anaerobic bacterium, transmission occurs primarily via spores. SCFA, short-chain fatty acid (such as butyrate).

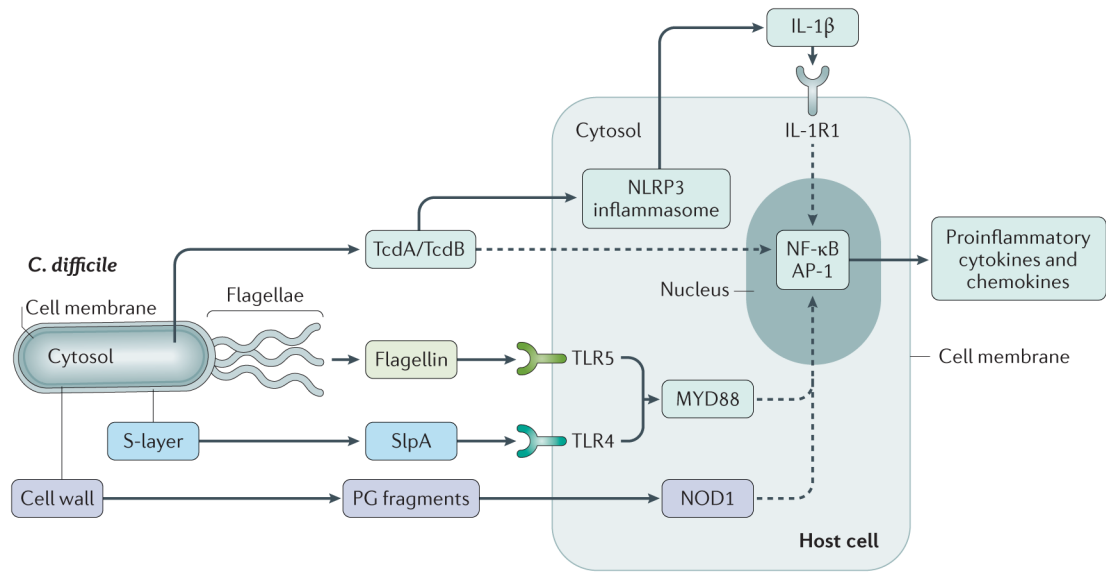


Figure 3. Innate immune response of host cells to *Clostridium difficile*

Clostridium difficile elicits the innate immune response via at least four different effectors, leading to the induction of proinflammatory cytokines and chemokines via nuclear factor- κ B (NF- κ B) and transcription factor AP-1. The large clostridial toxins toxin A (TcdA) and TcdB act via NLRP3 (NOD-, LRR- and pyrin domain-containing 3) inflammasome-dependent and -independent pathways. Surface layer protein A (SlpA) and flagellin act via myeloid differentiation primary response 88 (MYD88)-dependent pathways through Toll-like receptor 4 (TLR4) and TLR5, respectively. The nucleotide-binding oligomerization domain-containing protein 1 (NOD1)-dependent pathway of induction most probably detects fragments of peptidoglycan (PG) derived from the cell wall of *C. difficile*. Dashed lines indicate indirect effects. IL-1R1, IL-1 receptor type 1.

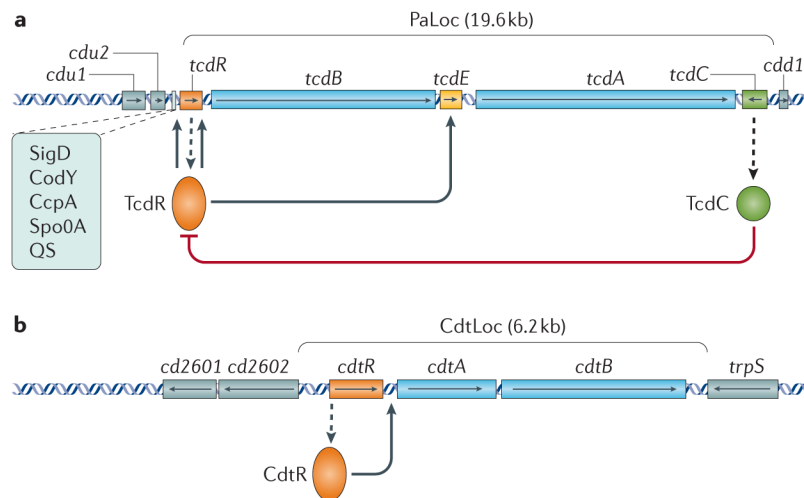


Figure 4. Regulation of the *Clostridium difficile* toxins

a | Schematic representation of the pathogenicity locus (PaLoc) and the flanking regions with regulatory interactions of *Clostridium difficile*. Boxes with arrows indicate open reading frames with the direction of the arrows showing the direction of transcription. Toxin genes *tcdA* and *tcdB* are in blue, regulatory genes are in orange (positive) and green (negative); *tcdE* is in yellow and genes located outside the PaLoc are in grey. Dashed arrows indicate the production of protein from a gene transcript. Other regulators (Sigma D (SigD), the nutritional repressor CodY (known as GTP-sensing transcriptional pleiotropic repressor CodY), catabolite control protein A (CcpA), Stage 0 sporulation protein A (Spo0A) and quorum sensing (QS)) that affect toxin gene transcription (boxed) mostly act via expression of the *tcdR* gene. The TcdR protein is involved in the initiation of the production of toxin A (TcdA) and TcdB. **b** | Schematic representation of the binary toxin locus (CdtLoc) and flanking regions with regulatory interactions. Boxes with arrows indicate open reading frames with arrows showing the direction of transcription. The *cdtA* and *cdtB* toxin genes are in blue, the regulatory gene *cdtR* is in orange and genes located outside the CdtLoc are in grey.

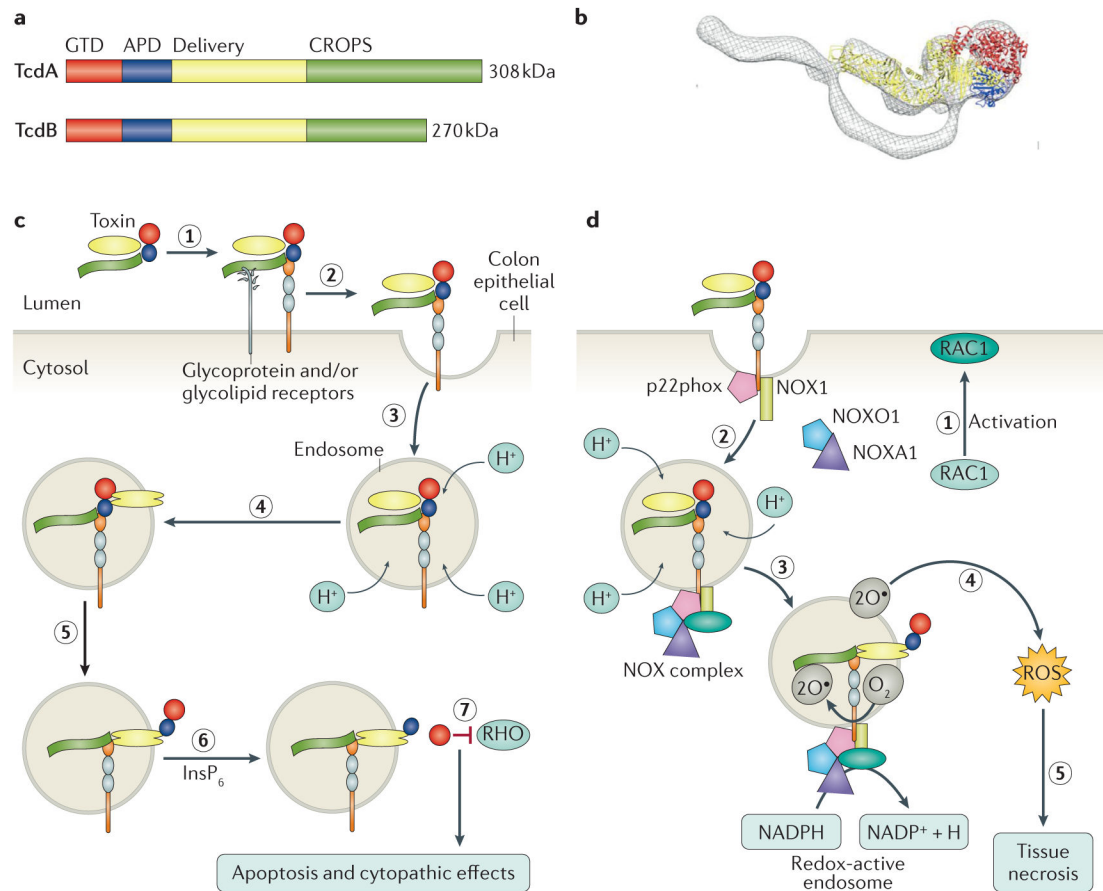


Figure 5. Structure and function of the large clostridial toxins

a | Schematic of the toxin A (TcdA) and TcdB primary structures, highlighting the four functional domains: the glucosyltransferase domain (GTD; red), the autoprotease domain (APD; blue), the delivery domain (yellow) and the combined repetitive oligopeptides (CROPS) domain (green) that binds carbohydrates on the host cell surface to facilitate bacterial entry. **b** | Overlay of an electron microscopy reconstruction of the structure of TcdA with the X-ray crystal structure of TcdA lacking the CROPS domain (Protein Data Bank code: 4R04). Colour-coding reflects the domain structure in part **a**. **c** | The discrete structural and functional domains of the toxins contribute to a multi-step mechanism of intoxication. Toxins bind to one or more receptors (carbohydrate and/or protein) on the cell surface (step 1) and are internalized by receptor-mediated endocytosis (step 2). As the endosome matures, the V-ATPase contributes to a decrease in pH (step 3). The acidic pH causes a conformational change in the toxin delivery domain, resulting in pore formation (step 4) and the translocation of the APD and GTD into the cytosol (step 5). Inositol hexakisphosphate ($InsP_6$) binds and activates the APD, resulting in the release of the GTD (step 6), which can inactivate RHO family proteins (step 7) to cause apoptosis and cytopathic ‘rounding’ effects. **d** | At concentrations >0.1 nM, TcdB can promote RAS-related C3 botulinum toxin substrate 1 (RAC1) activation (step 1) and complex formation between p22phox (also known as cytochrome b-245 light chain), NADPH oxidase 1 (NOX1), NADPH oxidase activator 1 (NOXA1), NADPH oxidase organizer 1 (NOXO1) and

RAC1 on the endosomal membrane to form the NOX complex (step 2). The fully assembled NOX complex generates superoxide by transferring an electron from NADPH to molecular oxygen (step 3). Superoxide generation leads to the production of reactive oxygen species (ROS), which — at high levels — promote necrosis by causing mitochondrial damage, lipid peroxidation and protein oxidation (step 5).

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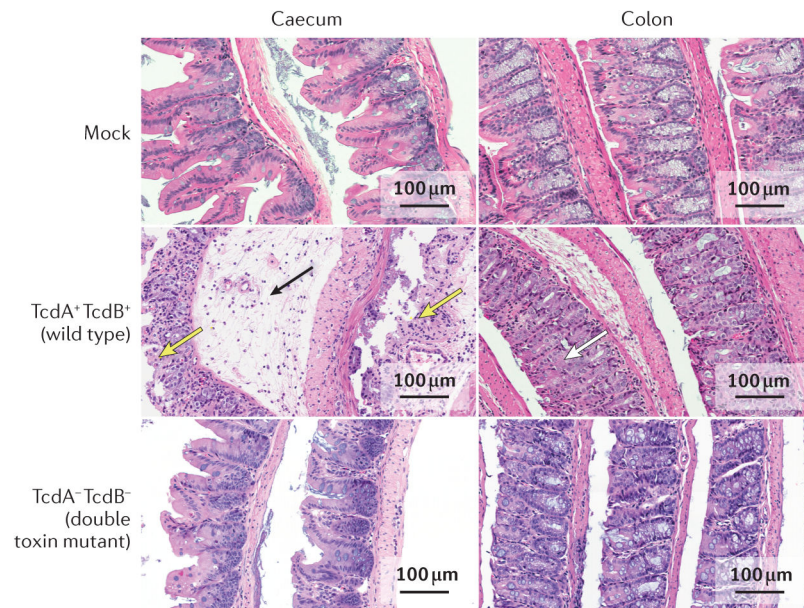


Figure 6. Histopathology of *Clostridium difficile* infection in a mouse model

Histopathological analysis of haematoxylin and eosin-stained caecal and colonic tissues collected from mice infected with a wild-type PCR ribotype 027 strain (TcdA⁺ TcdB⁺), infected with an isogenic double *tcdA* and *tcdB* mutant (TcdA⁻ TcdB⁻) or mock-infected with phosphate buffered saline (Mock). Note that both wild-type and double-mutant strains contain an intact binary toxin locus. Arrows indicate major histological differences; oedema and polymorphonuclear cell influx into the lamina propria (black arrows), erosion of crypts and goblet cell loss (yellow arrows) and hyperplasia (white arrows).

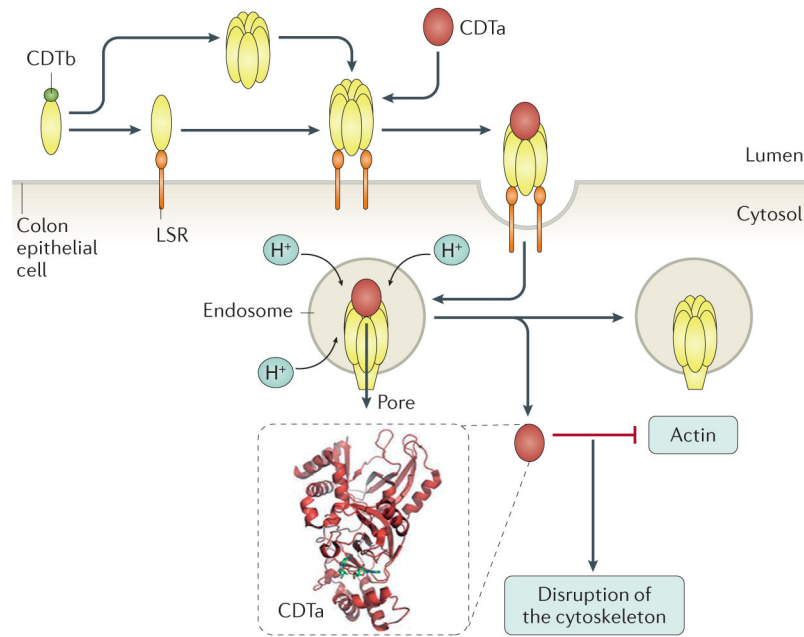


Figure 7. Mechanism of action of *Clostridium difficile* transferase (binary toxin)

Clostridium difficile transferase (CDT) is a binary toxin consisting of the CDTa ADP-ribosyltransferase (red) and the CDTb protein (yellow and green). The monomeric form of CDTb binds to the lipolysis-stimulated lipoprotein receptor (LSR)²⁶³, which is found in many tissues, including the gut. CDTb undergoes proteolytic activation and oligomerizes to form a heptameric prepore, which facilitates the binding of CDTa to the prepore–receptor complex. This complex enters cells by endocytosis and as the endosome matures, the V-ATPase contributes to a decrease in pH. The low pH of the endosome triggers pore formation and the translocation of CDTa into the cell. Once in the cytosol CDTa, ribosylates actin at arginine 177, resulting in a dual effect whereby G-actin polymerization is inhibited and F-actin depolymerization is favoured, which leads to the complete destruction of the actin cytoskeleton and, ultimately, cell death^{135,141}. Inset, structure of CDTa (Protein Data Bank code: 2WN7).

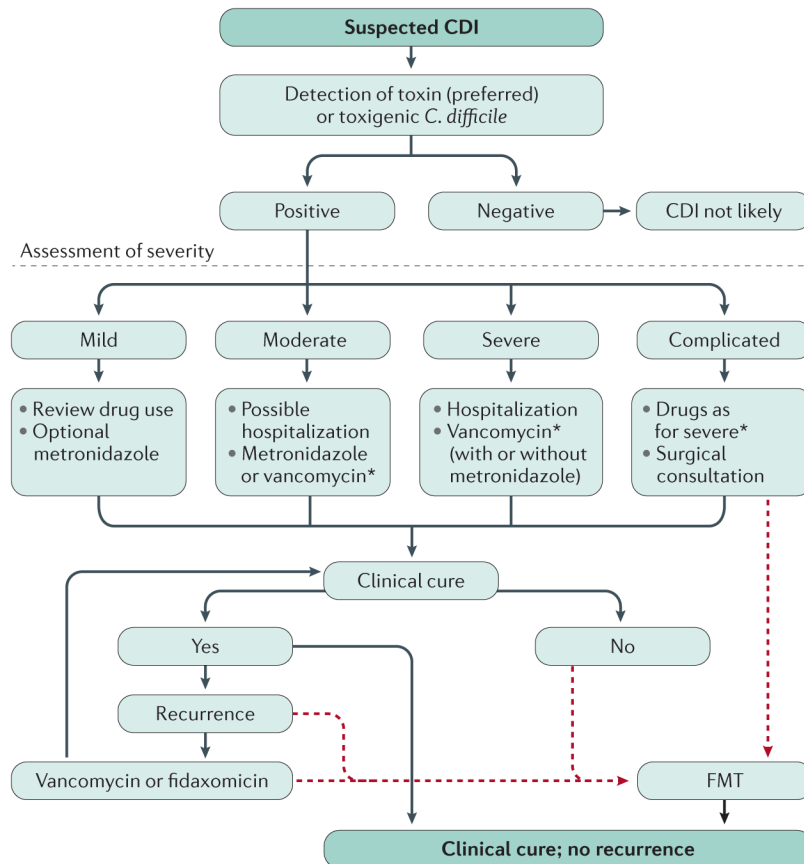


Figure 8. Diagnosis and treatment options for *Clostridium difficile* infections

When a patient is suspected of having *Clostridium difficile* infection (CDI), the recommended option is to detect toxins of *C. difficile* in the stool. Several diagnostic algorithms have been condensed into this figure²⁶⁴. Treatment options indicated here are based on reports by Leffler and Lamont¹⁴, and Debast *et al.*¹⁵⁵. For moderate CDI, metronidazole is given orally, in severe cases intravenously. Hospitalization refers to admission as a result of CDI (not as a result of comorbidities; patients might already be hospitalized). Note that recurrence after clinical cure (resolution of symptoms) can be observed. Faecal microbiota transplant is an effective but non-standard form of treatment (shown by a dashed line). *Fidaxomicin is a treatment option if the risk of recurrence is high, but not for complicated CDI.

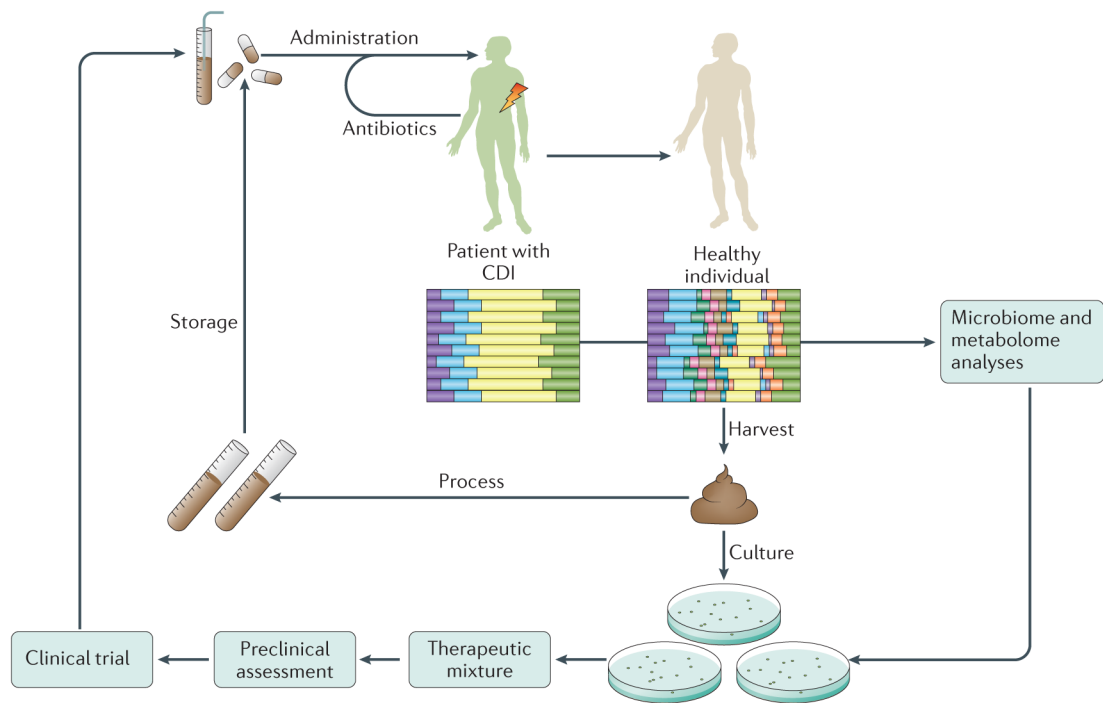


Figure 9. Faecal microbiota transplant

In faecal microbiota transplant (FMT), faecal material from a healthy donor is collected. The material is processed (blending, filtration) into pills or a solution. As part of this process, a check for the presence of pathogenic and multidrug-resistant organisms is performed. The processed material can be stored before (one-off) administration by nasoduodenal infusion, colonoscopic infusion or rectal enema for solution formulations or orally for pill formulations. Antibiotic treatment generally precedes the administration of the FMT to reduce *Clostridium difficile* levels. Alongside FMT, efforts are ongoing to standardize bacteriotherapy. On the basis of microbiome and metabolome analyses, signatures of resistance to colonization by *C. difficile* are identified. After collecting faecal material from healthy donors, species identified in these microbiome signatures or believed to be responsible for the metabolomic signature are cultured. Defined mixtures of these strains are tested for safety and ability to confer colonization resistance in preclinical trials and subsequently validated in clinical studies. Coloured bars indicate diversity of the microbiota, which is severely reduced in the patient with *C. difficile* infection (CDI) compared with the healthy subject.

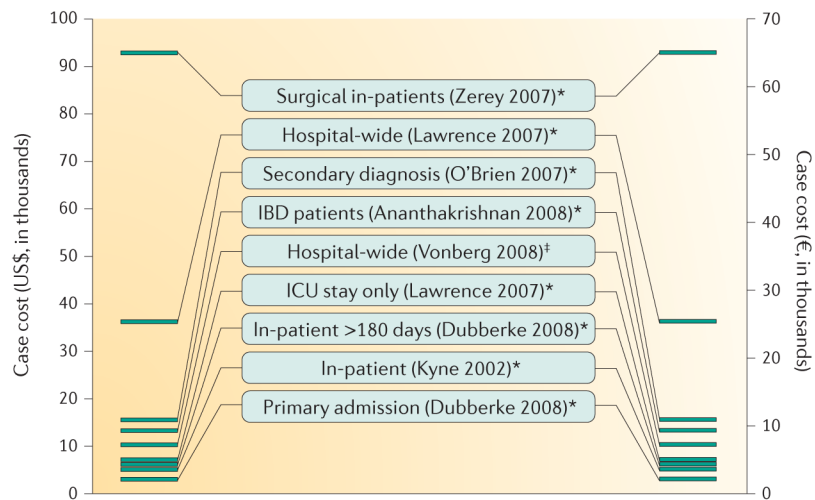


Figure 10. Cost per case of *Clostridium difficile* infection

Data are from Ghantoji *et al.*²¹⁹ (indicated with *) and Vonberg *et al.*²¹⁶ (indicated with †); surnames of first authors and years indicate the original studies described in these publications. Conversion between US\$ and € is based on 2008 exchange rates. Note that several studies have estimated the cost of *Clostridium difficile* infections more recently^{220,265,266}. IBD, inflammatory bowel disease; ICU, intensive care unit.

Table 1

Selected agents for the treatment and prevention of CDI in clinical trials

Agent (manufacturer)	Indication	Notes	Clinical trial identifier
<i>Phase III</i>			
Actoxumab and bezlotoxumab alone or in combination (Merck)	Prevention of recurrent CDI	Antitoxin A (MK-3415) and antitoxin B (MK-6072) monoclonal antibodies given intravenously as adjuncts to standard treatment	NCT01241552, NCT01513239
Surotomycin (Merck)	Treatment	Cyclic lipopeptide antibiotic related to daptomycin but administered orally	NCT01598311, NCT01597505
Cadazolid (Actelion)	Treatment	Hybrid antibiotic molecule, consisting of fluoroquinolone and oxazolidinone moieties, for oral administration	NCT01987895, NCT01983683
Cdiffense (Sanofi Pasteur)	Prevention	Vaccine containing toxoids of TcdA and TcdB from <i>C. difficile</i>	NCT01887912
<i>Phase II</i>			
IC84 vaccine (Valneva)	Prevention	Vaccine consisting of recombinant protein of the two truncated toxins TcdA and TcdB from <i>C. difficile</i>	NCT02316470
LFF571 (Novartis)	Treatment of moderate CDI	Semi-synthetic thiopeptide	NCT01232595
SER-109 (Seres Therapeutics)	Treatment of recurrent CDI	Oral microbiome therapeutic (mixture of bacterial spores) granted orphan drug designation by the FDA	NCT02437500
SMT19969 (Summit Pharmaceuticals)	Treatment	Oral non-absorbable antibiotic with a narrow spectrum of activity and high selectivity for <i>C. difficile</i>	NCT02092935
<i>C. difficile</i> vaccine (Pfizer)	Prevention	Bivalent toxin vaccine	NCT02561195, NCT02117570
SYN-004 (Synthetic Biologics)	Prevention	Class A β -lactamase designed to protect gut microbiota from the action of systemically administered β -lactam antibiotics that might otherwise predispose for CDI	NCT02563106
VP20621 (Shire)	Prevention of recurrent CDI	Orally administered non-toxicogenic <i>C. difficile</i>	NCT01259726
<i>Phase I</i>			
PolyCAb (Micropharm)	Treatment of severe CDI	Polyclonal antibodies against <i>C. difficile</i> given intravenously	Not available
CRS3123/REP3123 (National Institute of Allergy and Infectious Diseases)	Treatment	Methionyl-tRNA synthetase inhibitor oral antibiotic	NCT02106338, NCT01551004

CDI, *Clostridium difficile* infection; Tcd, *C. difficile* toxin.