

# *Clostridoides difficile* Profiling

Kah Yen Claire Yeak



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# Chapter 1

## Pathogen Overview

### 1.1 Characteristics

- Gram-positive, anaerobic, spore-forming bacterium, causing *Clostridioides difficile* infection (CDI)
- It is a major cause of healthcare-associated infections (HAI), particularly in hospital and long-term care settings

#### 1.1.1 Symptoms

- **Gastrointestinal symptoms** are most common, including **diarrhoea** (often watery and frequent), **abdominal pain**, **fever**, **nausea**, and **loss of appetite**.
- Severe cases may lead to **pseudomembranous colitis**, **toxic megacolon**, **septic shock**, and, in extreme cases, **colon perforation**.

#### 1.1.2 Disease

- CDI refers to the clinical disease caused by **toxin-producing** *C. difficile* strains colonizing the gut.
- CDI typically follows the **disruption of the gut microbiota** by antibiotics, which reduces microbial competition and allows *C. difficile* spores to germinate and grow.

#### 1.1.3 Duration of Sickness

- **Mild to moderate cases** usually last **1–2 weeks** with appropriate antibiotic treatment.
- **Severe or recurrent cases** can persist for weeks or longer, especially in cases where standard treatments are less effective or where reinfection occurs.

#### 1.1.4 Duration of onset

- Most infectious periods for potential donors to support transmission of *C. difficile* were 1 week (65%), with only 10% > 8 weeks.
- Most incubation periods in recipients were 4 weeks (61%), with few > 12 weeks (13%)
- 82% of CDIs occurred within 4 weeks of a potential donor infection
- For rare cases, onset of sickness to death takes only 30 days.

#### 1.1.5 Recurrence rate

- Recurrence occurs in approximately **20–30%** of patients after the initial infection, with some cases experiencing multiple recurrences, further increasing morbidity.

**Recurrence Factors:**

1. **Age:** Older adults (65+) are more likely to experience recurrent CDI due to reduced immune function
2. **Previous CDI:** A history of CDI increases the likelihood of future recurrences.
3. **Antibiotic Use:** Continued use of broad-spectrum antibiotics disrupts gut microbiota, increasing recurrence risk.
4. **Immune Response:** Insufficient immune response to *C. difficile* toxins predisposes patients to recurrence

**1.1.6 Severity of Sickness**

- **Mortality Rate:** CDI has an estimated 5-10% mortality with some cases **up to 20% mortality rate**, especially in older, hospitalized patients or those with weakened immune systems. Mortality rates may vary by country, healthcare setting, and patient age.

**1.1.7 Likelihood of Infection**

- CDI is a leading cause of healthcare-associated infections, with **incidence rates of 3–10 per 1,000 hospital admissions** in North America and Europe. Rates are on the rise in some regions, linked to high antibiotic usage and inadequate infection control in healthcare facilities.

**1.1.8 Risk Factors**

Basically similar to factors causing recurrence in patients as mentioned above.

**1.1.8.1 Age:**

- Older adults (65+) are at higher risk due to reduced immune function.

**1.1.8.2 Hospitalization and Long-Term Care:**

- Patients in these settings are more exposed to *C. difficile* spores, especially those undergoing lengthy treatments.

**1.1.8.3 Antibiotic Use:**

- Broad-spectrum antibiotics, such as fluoroquinolones, clindamycin, cephalosporins, and penicillin, disrupt normal gut microbiota, making patients more susceptible.

**1.1.8.4 Immunocompromised Status:**

- Previous CDI: Individuals who have had CDI before are at a higher risk for recurrence.
- Patients with weakened immune systems (e.g., cancer patients, organ transplant recipients) are more vulnerable.
- Underlying Health Conditions: Conditions such as inflammatory bowel disease (IBD) and kidney disease increase the risk of severe outcomes.

**1.1.9 Antibiotics Used for Treatment****1.1.9.1 Primary Antibiotics**

- **Vancomycin:** Often used as a first-line treatment due to its efficacy against *C. difficile*.
- **Fidaxomicin:** Another first-line antibiotic, favoured for its narrow-spectrum effects that minimize microbiota disruption.
- **Metronidazole:** Previously a first-line treatment, it is now often reserved for mild cases or used when other options are unavailable.

### 1.1.9.2 Adjunct Treatments

- In cases of recurrent CDI, **faecal microbiota transplantation (FMT)** is often considered to restore a healthy microbiome

## 1.1.10 Diagnostics for CDI

### 1.1.10.1 Glutamate Dehydrogenase (GDH) EIA

- Screens for the presence of *C. difficile* but cannot distinguish between toxigenic and non-toxigenic strains.
- **Use:** Typically combined with a toxin test for confirmation due to high sensitivity.

### 1.1.10.2 Enzyme Immunoassay (EIA)

- Detects toxins A and/or B directly from stool samples.
- **Advantages:** Rapid results, but sensitivity ranges widely (29–86%) depending on the setting and kit used.
- **Limitation:** Due to lower sensitivity, EIA is generally not recommended as a standalone diagnostic tool

### 1.1.10.3 Nucleic Acid Amplification Test (NAAT)

- Detects toxigenic genes directly from stool samples, offering high sensitivity for identifying *C. difficile*'s presence.
- **Limitation:** While highly sensitive, NAAT may detect asymptomatic colonization, potentially leading to overtreatment. Often used alongside toxin assays for accurate diagnosis.

### 1.1.10.4 Cell Cytotoxicity Assay (CTA)

- Identifies toxin-induced damage in a cell culture, serving as a reference standard with high accuracy.
- **Limitation:** The process is time-intensive and technically demanding, making it less practical for routine diagnostics

For more information on diagnostics for CDI, see [Diagnostics for CDI](#).

## 1.2 Epidemiology

- CDI rates have increased due to antibiotic usage and hypervirulent strains like ribotype 027
- It is prevalent in North America and Europe and has recently expanded to community-associated infections (CA-CDI), affecting a broader demographic, including younger, healthier individuals
- The rising rates of CDI have largely been attributed to the presence of BI/NAP1/027 but are not limited to the spread of this strain
- Depending on the country, other strains (including PCR ribotypes 001, 053 and 106) can be often associated with outbreaks and severe cases
- Ribotype 078 has increased recently from 3% to 13% in several countries in Europe. In the Netherlands, patients infected with ribotype 078 were younger (67.4 versus 73.5 years) and had community-associated disease more frequently (17.5% versus 6.7%; odds ratio = 2.98; 95% confidence interval = 2.11–8.02) than patients infected with ribotype 027.
- Ribotype 027, new hypervirulent strains (e.g., ribotype 078 and ribotype 181) have been identified, particularly in community-associated settings and Environmental and Zoonotic Reservoirs
- **Global Trends:** Different regions showed varying strain distributions, with North America and Europe still seeing hypervirulent strains like ribotype 027, while Asia and Australia documented distinct ribotypes, such as 017, 018, and 244.

### 1.2.1 Historical Context and Rise in Incidence

#### 1935 – Initial Identification

- *C. difficile* was first isolated and identified by researchers Hall and O'Toole, who discovered it as part of the normal gut flora in neonates. The bacterium was named *difficile* due to the difficulty in culturing it.

#### 1970s – Recognition as a Pathogen

- **1974:** The association between antibiotics and diarrhoea began to be studied more intensively, leading to the identification of certain bacterial causes.
- **1978:** Researchers discovered that *C. difficile* was the primary cause of antibiotic-associated diarrhoea and pseudomembranous colitis. The discovery highlighted that *C. difficile* produced toxins responsible for severe colitis in patients who had undergone antibiotic treatment, which disrupted their gut microbiota.

#### 1980s – Emergence as a Major Healthcare Concern

- CDI gained recognition as a significant nosocomial (hospital-acquired) infection. The understanding of *C. difficile* spores and their resistance to conventional disinfectants emphasized the importance of stringent infection control practices in hospitals.

#### 1990s – Advances in Diagnostic and Typing Methods

- Improvements in laboratory techniques allowed for better detection of *C. difficile* toxins in stool samples, enhancing the diagnosis of CDI.
- The development of PCR ribotyping and other genetic typing methods helped in tracking outbreaks and understanding strain variations.

#### 2000s – Hypervirulent Strains and Global Spread

- **2003:** The emergence of hypervirulent strains, particularly ribotype 027 (also known as BI/NAP1/027), was first reported in North America and soon observed in Europe. These strains were associated with increased severity, higher recurrence rates, and greater resistance to fluoroquinolones.
- **2005:** Major outbreaks of CDI linked to ribotype 027 were reported in Canada and the U.S., resulting in significant mortality and reinforcing CDI as a public health priority.
- **2011–2013:** The EUCLID study in Europe highlighted underreporting and gaps in the recognition of CDI, emphasizing the need for standardized diagnostics and increased awareness.

#### 2010s – Recognition of Community-Associated CDI (CA-CDI)

- Studies began to identify CDI cases outside of traditional healthcare settings, affecting younger, healthier individuals without recent antibiotic use or hospitalization. This indicated new transmission pathways and potential environmental or foodborne sources.
- **2011/12:** The European Centre for Disease Prevention and Control (ECDC) conducted a large survey, estimating CDI's prevalence at around 3.7% in healthcare-associated infections across Europe, revealing a significant annual burden.
- **2011:** Fidaxomicin was approved as a treatment option, offering a targeted approach to reduce recurrence by preserving more of the normal gut microbiota compared to other antibiotics.

#### 2020s – Continued Research and Novel Strains

- Ongoing studies focus on understanding the spread of CDI in community settings and further improving diagnostic methods. Research into alternative treatment methods, including bacteriophages and CRISPR-based strategies, continues to evolve.

### 1.2.2 Incidence in Healthcare-Associated Infections (HAIs)

- **CDI in North America:** CDI is a major contributor to HAIs in these regions. In the U.S., CDI accounts for approximately 14,000 deaths annually, positioning it as a leading cause of hospital-associated infections.
- **CDI in Europe:**
  - **Early Studies: In 2000,** CDI incidence in Europe was estimated at around 1.1 cases per 1,000 patient admissions. By 2005, it increased to 2.5 cases per 10,000 patient days, and by 2008, reached 4.1 cases per 10,000 patient days.
  - **EUCLID Study (2011-2013):** This large, multicenter European study reported an incidence of 7.0 cases per 10,000 patient days among hospitalized patients with diarrhoea. The study highlighted that CDI may be underreported due to limited awareness and testing by healthcare providers.
  - **ECDC Survey (2011/12):** The European Centre for Disease Prevention and Control (ECDC) survey of over 230,000 patients across 33 European countries estimated a CDI prevalence of 3.7% in HAIs, with an annual burden of around 123,997 cases in the EU and EEA countries.

### 1.2.3 Community-Associated CDI (CA-CDI)

- **Expansion Beyond Hospitals:** Initially, CDI primarily affected hospitalized and elderly patients with recent antibiotic exposure. Recently, however, there has been a significant increase in community-associated CDI cases affecting younger, healthier individuals with little or no recent antibiotic exposure.
- **Demographic Shift:** This shift suggests changes in the transmission patterns and epidemiology of CDI, potentially linked to environmental contamination, foodborne sources, or asymptomatic carriers

#### Definitions

- Community-Associated CDI (CA-CDI): Refers to CDI cases in patients with no recent hospitalization or healthcare exposure. Typically, CA-CDI is diagnosed in individuals who have not been admitted to a healthcare facility in the previous 12 weeks, making it truly community-acquired.
- Community-Onset CDI (CO-CDI): Refers to CDI that is diagnosed outside of a hospital setting but may involve recent healthcare exposure (such as recent outpatient procedures or short-term hospital stays within the past 12 weeks). CO-CDI cases often represent an overlap between traditional hospital-acquired CDI and community-acquired cases.

#### 1.2.3.1 Epidemiology and Rising Trends

- Both CA-CDI and CO-CDI cases have been increasing in recent years, making up a larger portion of all CDI cases. Studies estimate that between **20-40% of CDI** cases in some regions now occur outside healthcare settings.
- CA-CDI has been noted more frequently among younger, healthier individuals who traditionally would be at lower risk, such as children, pregnant women, and adults without recent antibiotic use or hospital exposure.

#### 1.2.3.2 Potential Sources and Transmission Pathways for non HAIs

- **Environmental Contamination:** *C. difficile* spores, which are highly resilient, are found in various environments, including soil, water, and public spaces. This environmental persistence contributes to community-based transmission, as spores can be ingested via contaminated surfaces, food, or water sources.
- **Foodborne Transmission:** Recent studies have detected *C. difficile* in retail meats (e.g., beef, pork, and chicken), suggesting a potential foodborne route, although direct evidence of foodborne infection remains limited.

- **Animal and Zoonotic Sources:** Certain *C. difficile* ribotypes found in livestock (like ribotype 078) are increasingly implicated in CA-CDI, especially in regions with intensive farming practices. Cross-species transmission from animals to humans could contribute to community transmission.

### 1.2.3.3 Risk Factors for CA-CDI

Unlike healthcare-associated CDI, where antibiotics are the main risk factor, CA-CDI cases often have more varied backgrounds. Risk factors include:

- **Antibiotic Use:** Although less common than in healthcare-associated CDI, prior outpatient antibiotic use (e.g., broad-spectrum antibiotics like cephalosporins) remains a significant risk factor.
- **Acid-Suppressing Medications:** Proton pump inhibitors (PPIs) and other acid-suppressing medications have been linked to increased CDI risk, possibly due to changes in gut pH that favour *C. difficile* growth.
- **Underlying Health Conditions:** Conditions like inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS) can increase susceptibility to CDI by altering gut microbiota.
- **Household Transmission:** Some studies suggest household transmission among family members or those in close contact, particularly when one individual has been recently colonized or infected.

### 1.2.4 Global Trends and Regional Variations

- **North America and Europe:** Hypervirulent strains such as ribotype 027 are especially prevalent in these regions, driving higher CDI rates and more severe disease cases.
- **Australia and Asia:** Distinct regional ribotypes are observed, such as ribotypes 017, 018, and 244, with different levels of virulence and transmission patterns. These strains indicate geographic variability in *C. difficile* distribution, potentially influenced by regional healthcare practices, antibiotic usage patterns, and genetic differences among strains.

In Europe, CDI incidence has doubled over the past few decades, with the prevalence reaching about 3.7% in HAIs. Major regional studies like the ECDC's report indicate an annual CDI burden of approximately 124,000 cases across the EU and EEA.

## 1.3 Transmission route

Other than HAI, community sources for CDI include soil, water, pets, animals used for food, meats and vegetables. There is no conclusive evidence that *C. difficile* contamination of food has led to clinical CDI in humans, and Community-associated CDI without previous direct or indirect contact with a hospital environment remains rare compared with hospital-acquired CDI. While direct contact with contaminated surfaces and person-to-person spread in healthcare settings are the primary transmission routes, environmental reservoirs, food, animals, and even aerosolization can contribute to *C. difficile* spread. Understanding these routes is essential for effective control and prevention.

### 1.3.1 Direct Contact Transmission

- **Person-to-Person Contact:** Transmission via hands or skin contact with an infected or colonized individual, particularly in healthcare settings. Hospital visitors who are not always under strict hygiene protocols might spread spores inadvertently, especially if they visit multiple rooms or interact closely with patients.
- **Healthcare Workers:** *C. difficile* spores can be transferred from healthcare workers' hands, clothing, or equipment if hygiene protocols are not followed.
- **Human Carriers:** Asymptomatic carriers can shed *C. difficile* spores into the environment without displaying symptoms. These carriers, particularly healthcare workers or patients with recent hospital visits, can unknowingly spread spores in both healthcare and community settings.

### 1.3.2 Hospital Infrastructure-Related Transmission

- **Open-Plan Wards or Shared Bathrooms:** The layout and use of shared bathrooms in health-care facilities can contribute to transmission by increasing the chance of contact with contaminated surfaces or aerosolized particles from toilets.
- **Shared Ventilation and Airflow:** Although less common, there is some evidence that spores may spread within hospitals through shared ventilation systems, especially in inadequately ventilated or overcrowded facilities.
- **Clothing and Personal Items:** Personal items like mobile phones, stethoscopes, and clothing can act as vectors, particularly in hospitals where cross-contamination is common.

### 1.3.3 Fomite Transmission

- **Environmental Surfaces:** *C. difficile* spores can survive on surfaces such as bed rails, medical equipment, door handles, and bathrooms for long periods, allowing indirect transmission.
- **Hospital and Healthcare Equipment:** Shared medical devices, bed linens, and even handrails can harbor spores if not properly disinfected.

### 1.3.4 Transmission via Aerosols

*C. difficile* spores can potentially spread via aerosols, although it is not the primary mode of transmission. The spores are highly resilient and can survive on surfaces for long periods. While direct contact and surface contamination are the most common ways *C. difficile* spreads, studies have shown that spores can be released into the air during certain activities, such as when removing bed linens or flushing toilets. This highlights the importance of strict infection control measures in healthcare settings to prevent aerosol spread.

- **Aerosolized Particles:** Spores may become airborne during activities such as bed linen removal, patient cleaning, or toilet flushing, although this is a less common transmission route than surface contamination.
- **Close Proximity Aerosols:** In some settings, spores could theoretically spread through short-range airborne particles, especially in rooms with poor ventilation.

### 1.3.5 Foodborne and Waterborne Transmission

- **Foodborne Transmission:** *C. difficile* spores have been found in food products, including meat and shellfish, potentially posing a foodborne transmission risk.
- **Contaminated Water:** Spores can survive in water sources, which may contribute to transmission in community settings if individuals come into contact with contaminated water.

### 1.3.6 Zoonotic Transmission (Animal-to-Human)

- **Livestock and Pets:** Certain ribotypes common in livestock, such as ribotype 078, suggest potential zoonotic transmission. Contact with animals (e.g., cattle, pigs, and pets) and their environments may increase infection risk.
- **Soil and Environmental Exposure:** Animal waste can introduce spores into soil, which may reach humans through direct contact or indirectly through food contamination.

### 1.3.7 Community-Associated Transmission

- **Household Contacts:** Transmission can occur between household members, especially if one person is colonized or infected.
- **Public Spaces:** *C. difficile* spores may be present in community spaces such as public restrooms, gyms, and transportation vehicles, though these routes are less common.

### 1.3.8 Transmission via Biofilm

- **Water Systems and Biofilms:** *C. difficile* spores can integrate into biofilms in water systems (e.g., sinks, drains) within healthcare facilities. Biofilms can harbor spores, allowing them to persist and spread over extended periods.

These additional routes highlight nuances in how *C. difficile* can spread, especially within healthcare settings, and underscore the importance of strict hygiene, infrastructure design, and environmental cleaning to minimize transmission risks.

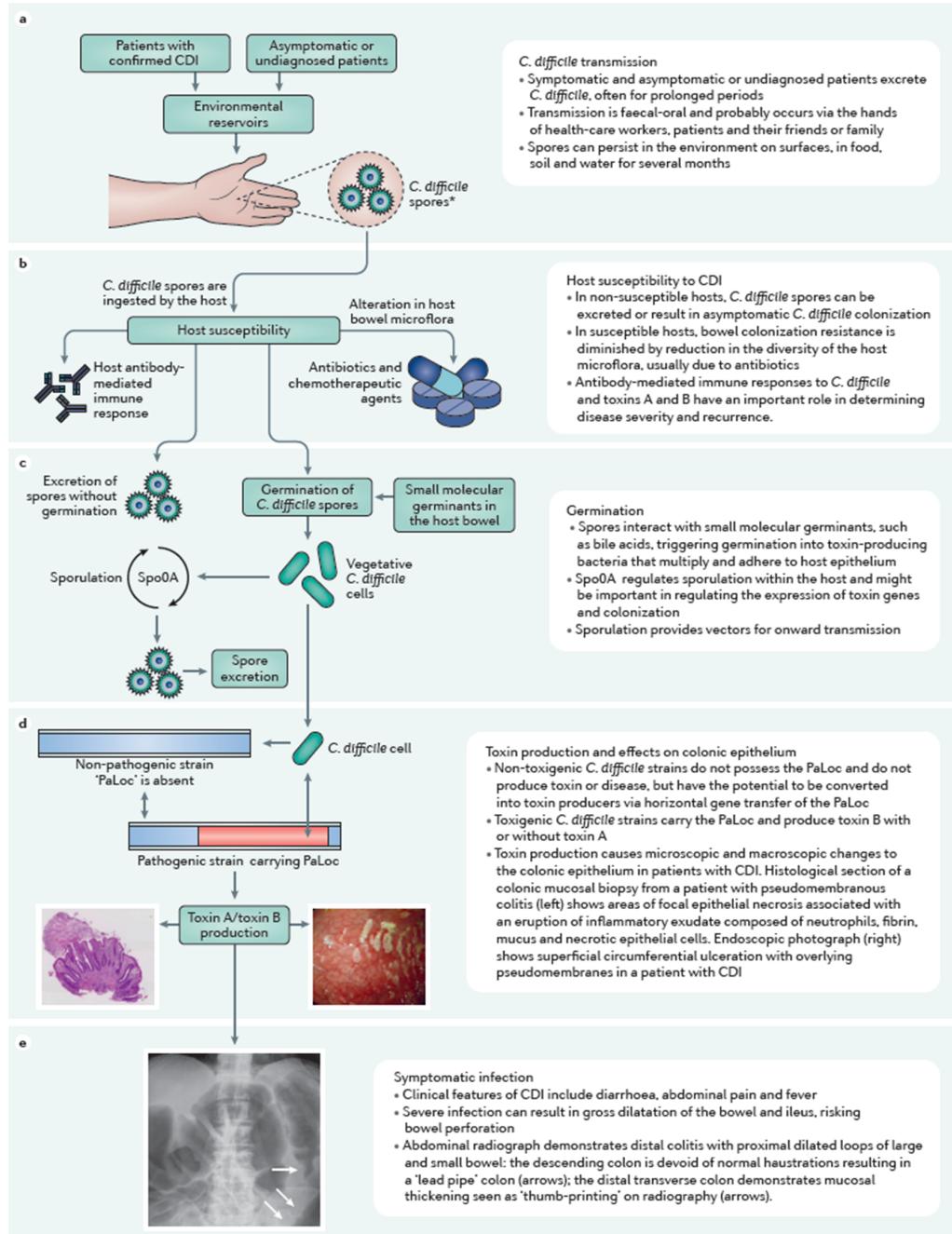


Figure 1.1: *C. difficile* transmission (Martin, Monaghan, and Wilcox 2016)

## 1.4 Reservoirs

*C. difficile* reservoirs are locations or hosts where the bacterium can survive, persist, and potentially spread to new hosts. These reservoirs are critical to understanding the transmission pathways of both

symptomatic and asymptomatic CDI. Here are the main types:

- **Primary Reservoirs:** CDI can spread through direct contact with symptomatic or asymptomatic individuals, healthcare workers, and contaminated environments. Spores survive on surfaces for extended periods, posing long-term risks.
- **Asymptomatic Carriers:** Asymptomatic individuals, especially in hospital and long-term care settings, can spread CDI. Roughly 4-10% of patients are colonized with *C. difficile* upon admission to healthcare facilities, increasing during their stay.
- **Environmental and Zoonotic Reservoirs:** CDI spores are found in soil, water, and foods (e.g., meats, shellfish). There is potential zoonotic transmission from animals, particularly in agricultural regions with high-density farming, although foodborne outbreaks are rare.

#### 1.4.1 Human Reservoirs

- **Asymptomatic Carriers**

Humans can carry *C. difficile* without symptoms, especially in high-risk groups (e.g., infants, the elderly, and immunocompromised individuals), and shed spores that can infect others. Asymptomatic carriers can shed the bacteria and contribute to transmission without showing symptoms of infection themselves. Several groups are recognized as common or significant carriers:

- **Infants and Young Children**

Infants, particularly under one year of age, frequently carry *C. difficile* asymptotically due to underdeveloped gut microbiota and potential immune tolerance to *C. difficile* toxins. This group is unique in that *C. difficile* is often present without causing disease, possibly due to reduced toxin receptor expression in their intestines.

- **Elderly Individuals**

Older adults, especially those in long-term care facilities, are more likely to carry *C. difficile* asymptotically. Factors include frequent antibiotic use, age-related gut microbiome changes, and increased exposure in healthcare settings.

- **Hospitalized Patients**

Patients admitted for reasons other than CDI often carry *C. difficile* asymptotically, especially after receiving antibiotics that disrupt their gut microbiota. These carriers pose a transmission risk in hospitals, as they can shed spores that survive on surfaces, leading to healthcare-associated infections.

- **Healthcare Workers**

While not commonly symptomatic carriers, healthcare workers can carry *C. difficile* spores transiently on their skin or clothing, making them potential vectors for hospital-based transmission, especially without rigorous hand hygiene.

- **Individuals Recently Treated with Antibiotics**

Antibiotic use disrupts the gut microbiota, increasing susceptibility to *C. difficile* colonization without necessarily leading to symptoms, especially in patients not on acid-suppressing therapy.

- **Close Contacts of CDI Patients**

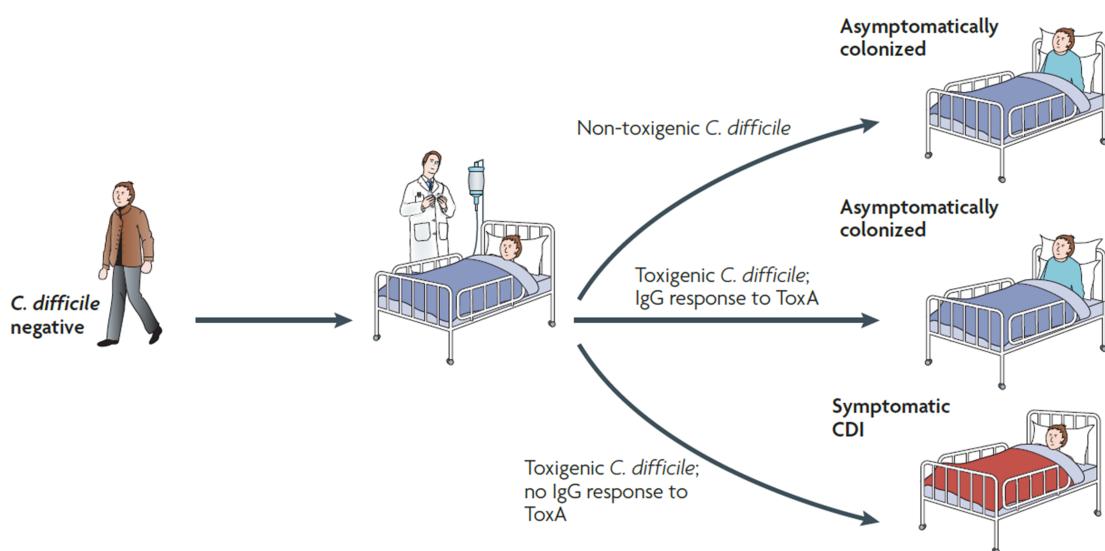
Family members or household contacts of individuals with symptomatic CDI can become asymptomatic carriers, likely through environmental exposure to spores.

- **Immunocompromised Individuals**

People with weakened immune systems (e.g., cancer patients, those on immunosuppressive therapy) are at a higher risk of asymptomatic colonization due to compromised defenses against microbial overgrowth. These groups represent asymptomatic carriers who may either pose a risk for community transmission or act as reservoirs within healthcare environments, thereby contributing indirectly to the spread of CDI.

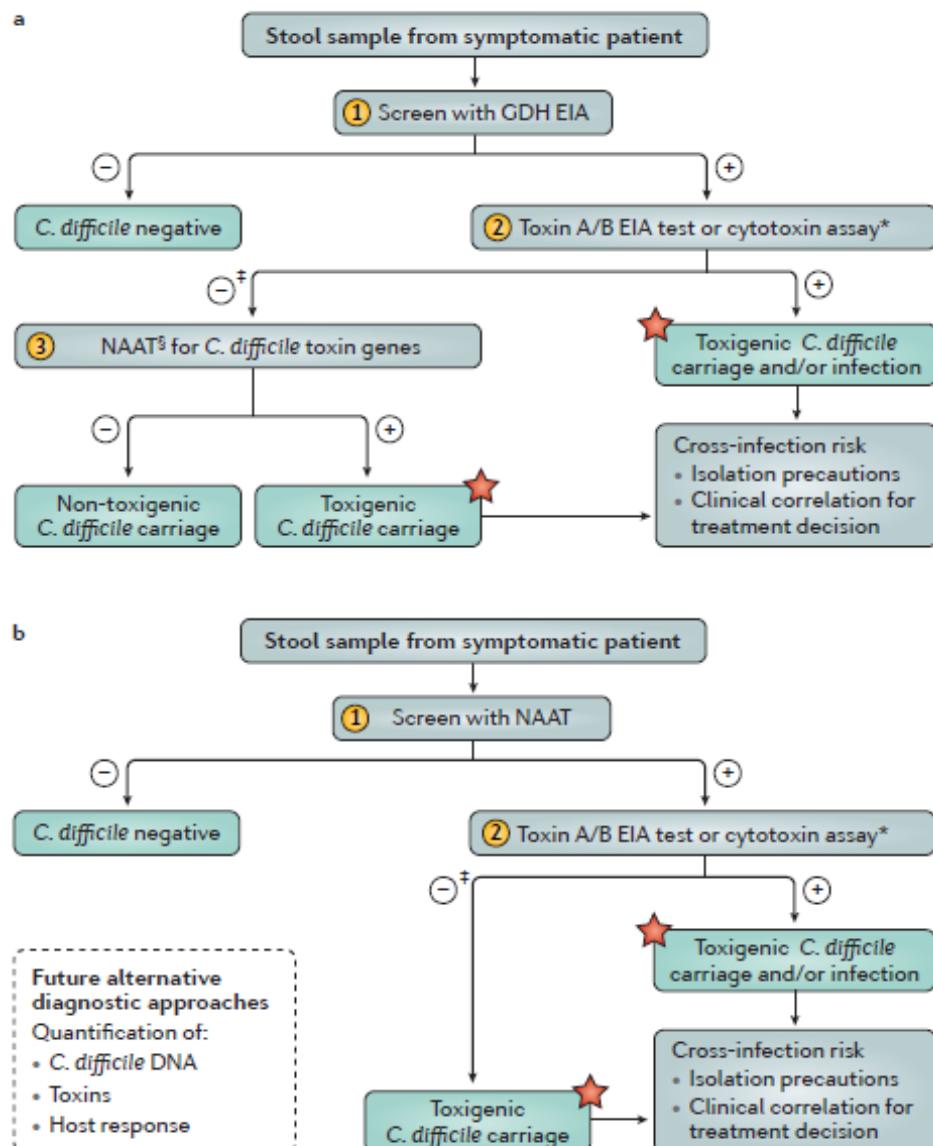
- **Symptomatic Individuals**

Patients with active CDI are also reservoirs, especially in healthcare settings where spores can spread easily.



**Figure 1 | Model for the acquisition of *Clostridium difficile* infection (CDI).** Patients are exposed to *C. difficile* spores through contact with the hospital environment or health care workers. After taking an antibiotic, they develop CDI if they acquire a toxigenic *C. difficile* strain and fail to mount an anamnestic serum immunoglobulin G (IgG) antibody response to toxin A (ToxA; also known as TcdA)<sup>64</sup>; if they can mount an antibody response they become asymptotically colonized with *C. difficile*. If they acquire a non-toxigenic *C. difficile* strain, they also become asymptotically colonized. Colonized patients have been shown to be protected from CDI<sup>126</sup>.

Figure 1.2: Model of accquisition of *C. difficile* infection (Rupnik, Wilcox, and Gerding 2009)



**Figure 3 | Examples of multistep algorithms for testing stool samples for rapid diagnosis of CDI.** **a | Diagnostic pathways using GDH as first-line test:** GDH+toxin EIA (1); GDH+cytotoxin assay (2); and GDH+toxin EIA+PCR (3). **b | Diagnostic pathways using NAAT as first-line test (1):** NAAT+toxin EIA (2); NAAT+cytotoxin assay. CDI, *Clostridium difficile* infection; EIA, enzyme immunoassay; GDH, glutamate dehydrogenase; NAAT, nucleic acid amplification test. \*Cytotoxin assay method takes longer than toxin EIA, but has greater sensitivity. <sup>†</sup>A negative toxin test result could be false-negative and so management of the patient should always include a clinical assessment. <sup>§</sup>NAAT or PCR does not distinguish between CDI and colonization.

Figure 1.3: Example of multistep algorithms for testing the presence of *C. difficile* (Martin, Monaghan, and Wilcox 2016)

#### 1.4.2 Animal Reservoirs

- **Livestock:** Animals, particularly pigs, cows, and chickens, can harbor *C. difficile*, especially certain ribotypes like ribotype 078, which has been linked to zoonotic transmission. These animals can spread spores through direct contact or indirectly through their waste.
- **Pets:** Dogs, cats, and other pets can carry *C. difficile* asymptotically, potentially shedding spores in household environments.

- **Wildlife:** Some wild animals have been identified as carriers of *C. difficile* in the environment, which can facilitate the spread to other animals and, indirectly, humans.

### 1.4.3 Environmental Reservoirs

- **Soil:** *C. difficile* spores are resilient and can survive in soil, where they may be transmitted through contact or ingestion, potentially reaching food sources or water supplies.
- **Water Sources:** *C. difficile* spores can persist in water systems, including rivers, lakes, and wastewater, providing another means for transmission in community settings.
- **Food:** *C. difficile* has been isolated from foods, especially meats, suggesting foodborne transmission as a potential route. Meat contamination may occur at processing stages, particularly with livestock-associated strains.

### 1.4.4 Healthcare Environment Reservoirs

- **Surfaces and Equipment:** Hospital rooms, shared medical devices, and other surfaces can become reservoirs of *C. difficile* spores, especially in healthcare facilities where disinfecting practices may vary.
- **Healthcare Workers' Clothing:** Healthcare workers can inadvertently carry spores on their clothing, hands, or personal items, contributing to environmental contamination and patient-to-patient transmission.

These reservoirs represent critical sources of *C. difficile* spores in healthcare, community, and natural environments, and understanding them helps guide effective infection control and prevention strategies.

## 1.5 Global geographical distribution

Globally, regions most affected by CDI include North America, Europe, and parts of East Asia. In the United States and Canada, the incidence and severity of CDI are notably high, particularly in healthcare settings. Europe also reports high rates of infection, with increasing antibiotic resistance complicating treatment. Other regions, like Latin America and parts of Asia, are experiencing rising incidence, likely due to increased antibiotic usage and healthcare-associated infections. High-risk populations are often the elderly and hospitalized patients worldwide.

### 1.5.1 Regional Patterns

#### 1.5.1.1 North America and Europe

- Dominated by **ribotypes 027 and 106**, associated with HAIs. High fluoroquinolone use in these regions contributes to the prevalence of resistant strains.

#### 1.5.1.2 Asia

- **Ribotype 017** is widespread, with increased cases linked to both healthcare and community sources. The region has seen distinct strains due to regional antibiotic practices and infection control policies

#### 1.5.1.3 Australia

- **Ribotype 244** is emerging as a prevalent strain, causing severe cases across both hospital and community settings.

### 1.5.2 Regional Factors Influencing Spread

#### 1.5.2.1 Antibiotic Usage Patterns

- Regions with higher fluoroquinolone usage have seen a greater prevalence of fluoroquinolone-resistant ribotypes like 027. This is likely due to selective pressure that allows resistant strains to thrive, especially in healthcare settings where these antibiotics are frequently used.

### 1.5.2.2 Healthcare Practices

- Variations in infection control practices and CDI surveillance can impact ribotype prevalence. For example, regions with strict infection control policies may report fewer cases of certain hypervirulent strains within healthcare facilities but may still see their spread in community settings.

### 1.5.2.3 Agricultural Practices

- Ribotypes associated with animals, such as ribotype 078, are more prevalent in regions with high-density livestock farming (e.g., the Netherlands). The presence of *C. difficile* in soil, water, and meat products suggests that agricultural practices, including the use of antibiotics in livestock, contribute to regional ribotype prevalence.

### 1.5.2.4 International Travel and Global Spread

- Ribotypes like 027 have spread globally in part due to increased international travel and patient movement across healthcare systems. WGS studies have tracked strains across continents, showing how certain ribotypes spread through healthcare tourism and hospital transfers.

### 1.5.2.5 Zoonotic Transmission and Regional Expansion

- Ribotype 078's association with livestock has led to increased community-acquired infections in agricultural areas and food supply chains. This has led to its spread outside traditional agricultural regions, affecting urban areas with less direct contact with livestock but potential exposure through food.

## 1.6 Ribotypes

### 1.6.1 Ribotypes Shift

#### 1.6.1.1 Changing Dominance of Ribotypes:

- The predominant ribotypes in Europe shifted over time, from RT001 (13%) in 2005 to RT014/020 (16%) in 2008.
- By 2012/13, RT027 (19%) emerged as the predominant strain due to its higher outbreak potential and association with severe outcomes, influencing global CDI surveillance policies.

### 1.6.2 Key Ribotypes

#### 1.6.2.1 Ribotype 027 (BI/NAP1/027)

- This hypervirulent strain has been associated with severe CDI outbreaks and is particularly prevalent in North America and Europe, where it's linked to HAIs. Its spread has been facilitated by its high resistance to fluoroquinolones.

#### 1.6.2.2 Ribotype 078

- Commonly found in Europe and increasingly in North America, ribotype 078 is associated with community-associated CDI and zoonotic transmission, especially from livestock. This ribotype has been isolated from pigs, cows, and other animals, leading to concerns about foodborne transmission and its persistence in the community.

#### 1.6.2.3 Ribotype 017

- Found widely in Asia, particularly in East Asia (e.g., China, Japan, and South Korea), ribotype 017 is known for its unique toxin profile (**TcdA-negative, TcdB-positive**). This ribotype is also common in Australia and has been involved in both healthcare- and community-associated outbreaks.

#### 1.6.2.4 Ribotype 106

- Predominantly seen in the United Kingdom and Ireland, ribotype 106 has caused outbreaks in healthcare facilities and is increasingly linked to severe infections, similar to ribotype 027.

#### 1.6.2.5 Ribotype 244

- First detected in Australia, ribotype 244 is an emerging hypervirulent strain associated with severe infections and higher toxin production. It's becoming more common in both hospital and community settings within Australia.

### 1.6.3 Typing Techniques

#### 1.6.3.1 PCR Ribotyping

- Principle:** This method uses PCR to amplify the intergenic spacer region between the 16S and 23S rRNA genes. Differences in the length of these spacer regions result in unique banding patterns after gel electrophoresis, which are used to classify strains into specific ribotypes.
- Applications:** Effective for tracking strain distribution in epidemiological studies and identifying specific strains in healthcare settings.

#### 1.6.3.2 Multilocus Sequence Typing (MLST)

- Principle:** MLST sequences several conserved housekeeping genes (typically seven) to create an allelic profile for each strain. Each unique allelic combination is assigned a sequence type (ST), allowing for the characterization of genetic relationships between strains.
- Applications:** MLST is useful for understanding the genetic diversity, evolutionary relationships, and global distribution of *C. difficile* strains.

#### 1.6.3.3 Whole genome sequencing (WGS)

- Principle:** WGS involves sequencing the entire genome of the *C. difficile* strain. This provides comprehensive genetic information, including details on virulence factors, resistance genes, and evolutionary mutations.
- Applications:** WGS is highly precise and useful for tracking transmission routes, identifying outbreak sources, and conducting high-resolution epidemiological studies.

#### 1.6.3.4 Restriction Endonuclease Analysis (REA)

- Principle:** REA uses restriction enzymes to cut *C. difficile* DNA at specific nucleotide sequences, producing a unique pattern of DNA fragments. These fragments are then separated by gel electrophoresis, creating a strain-specific restriction pattern.
- Applications:** Traditionally used in research and clinical labs to differentiate strains, though less common now due to its labour-intensive nature.

#### 1.6.3.5 Toxinotyping

Toxinotyping helps researchers understand *C. difficile* genetic diversity and link certain toxinotypes with outbreaks and disease trends, even though toxinotype alone is not a definitive predictor of clinical disease expression. The rise of variant strains in human populations, especially those associated with severe outbreaks, highlights the need for ongoing monitoring and research.

- Principle:** Toxinotyping examines variations within the pathogenicity locus (PaLoc), where the toxin genes *tcdA* and *tcdB* are located. This method involves PCR amplification and sequencing of PaLoc to identify differences in the toxin gene regions.
- Applications:** Toxinotyping helps distinguish between strains based on toxin gene variations, useful in identifying hypervirulent strains and understanding virulence differences among *C. difficile* strains.

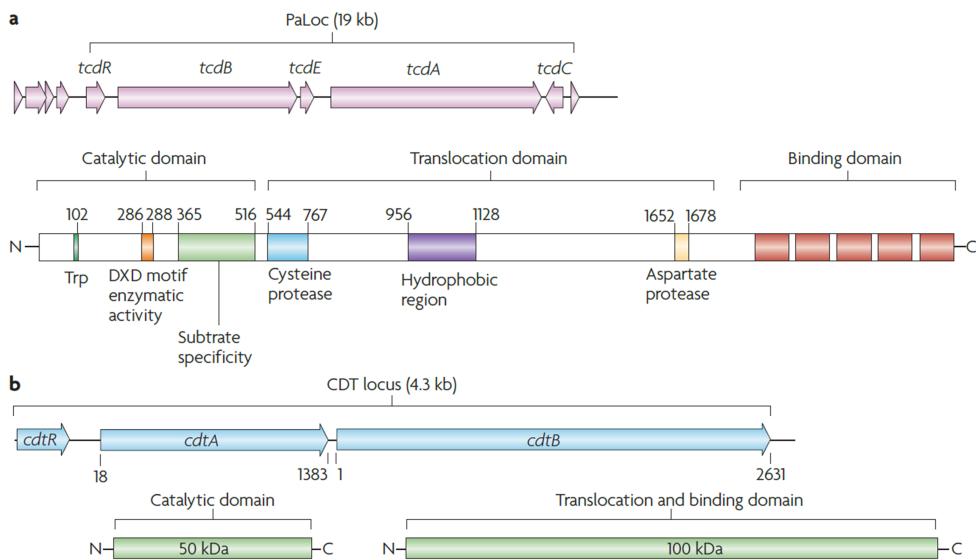


Figure 3 | Toxins produced by *Clostridium difficile*. **a** | Two large toxins, toxin A and toxin B (TcdA and TcdB), are encoded on the pathogenicity locus (PaLoc), which comprises five genes. In non-toxigenic strains, this region is replaced by a short 115 bp sequence. Both toxins are single-chain proteins, and several functional domains and motifs have been identified. TcdB is shown in detail below the PaLoc. **b** | A third toxin, the binary toxin or CDT, is encoded on a separate region of the chromosome (CdtLoc) and comprises three genes. The binary toxin is composed of two unlinked proteins, CdtB and CdtA. CdtB has a binding function and CdtA is the enzymatic component.

Figure 1.4: *C. difficile* toxin production operon (Rupnik, Wilcox, and Gerding 2009)

#### 1.6.3.6 Multilocus Variable-Number Tandem Repeat Analysis (MLVA)

- **Principle:** MLVA analyzes variations in the number of tandem repeat sequences across multiple loci in the *C. difficile* genome. By counting these repeats, a unique fingerprint for each strain is generated.
- **Applications:** MLVA is particularly useful in outbreak investigations to track the spread of specific *C. difficile* strains within healthcare facilities.

#### 1.6.3.7 Amplified Fragment Length Polymorphism (AFLP)

- **Principle:** AFLP typing involves digesting *C. difficile* DNA with restriction enzymes, followed by selective PCR amplification of the DNA fragments. The resulting amplified fragments produce a unique pattern when separated on a gel, forming a fingerprint for each strain.
- **Applications:** AFLP is used for assessing genetic diversity among strains, though it is less commonly applied due to its complexity and the need for specialized equipment.

#### 1.6.3.8 Pulsed-Field Gel Electrophoresis (PFGE)

- **Method:** PFGE uses restriction enzymes to generate large DNA fragments that are separated by pulsed-field gel electrophoresis to produce a strain-specific banding pattern.
- **Applications:** Historically popular for outbreak investigation, but now largely replaced by WGS due to PFGE's complexity and lower resolution.

#### 1.6.4 Problems with Ribotyping

- The lack of a universally accepted typing strategy has limited the comparison of strain patterns between countries and continents
- Each technique is reported with its nomenclature, thus ribotype 027 is also known as NAP1 (PFGE), BI (REA) and ST1 (MLST)

- The various typing methods have different relative discriminatory powers; REA and MLVA show greater discrimination than ribotyping or MLST, which in turn provide greater power to separate strains than PFGE
- WGS provides the best resolution to date

## 1.7 Virulence factor

### 1.7.1 Toxins

- **TcdA and TcdB:** Primary virulence factors encoded in the pathogenicity locus (PaLoc), disrupting intestinal cells and causing inflammation. TcdB is a key factor in colitis severity, while TcdA aids in gut colonization.
- **Binary Toxin (CDT):** Present in certain hypervirulent strains like ribotype 027, CDT enhances adhesion and invasion, contributing to increased virulence and mortality rates

### 1.7.2 Non-Toxin Virulence Factors

- **Adhesion and Biofilm Formation:** Proteins like fibronectin-binding proteins aid in adherence to host cells, supporting biofilm formation that provides resistance against host immune responses and antibiotics.
- **Regulatory Systems (c-di-GMP):** Modulates virulence by switching between motile/toxin-producing and biofilm-forming states, aiding in colonization and persistence within the gut environment

### 1.7.3 Genetic Variability

- Different toxin types, defined by the PaLoc variations, are linked to unique toxin profiles and clinical outcomes. For example, toxin type III (ribotype 027) and toxin type VIII (ribotype 017) show increased virulence, with significant outbreak associations

## 1.8 Infection cycles

The *C. difficile* infection cycle involves several stages, from spore ingestion to active infection, followed by spore shedding that can lead to further transmission. Here's a breakdown of the infection cycle:

### 1.8.1 Spore Ingestion

- **Entry Point:** The infection cycle begins when a host ingests *C. difficile* spores, typically through contaminated hands, surfaces, food, or water.
- **Resistant to Stomach Acidity:** Spores are highly resilient and can survive the acidic environment of the stomach, allowing them to reach the intestines intact.

### 1.8.2 Germination in the Gut

Germination is a critical step for *C. difficile* to establish infection but only occurs in the lower GI tract (O<sub>2</sub> concentration is negligible at this site), as it allows the spores to transition into their vegetative form, which can produce toxins and cause disease. Therefore, preventing germination could be an effective strategy to reduce the risk of infection. Targeting germination processes may help decrease the infectious dose and limit the ability of *C. difficile* to colonize the gut.

For *C. difficile*, germination starts when specific molecules, called germinants, interact with receptors on the spore's surface. Unlike other bacteria like *Bacillus* spp., *C. difficile* lacks the typical GerA, GerB, and GerK germinant receptors. Instead, it has a unique receptor called CspC, which binds to bile acids.

**Bile Acid Trigger:** In the small intestine, exposure to primary bile acids triggers spore germination, transforming the spores into vegetative, toxin-producing cells that are controlled by the *cspBAC* gene locus.

- **Primary Bile Acids:**

- Taurocholate and glycocholate (both primary bile acids) trigger spore germination when they interact with the CspC receptor, encoded by the *cspBAC* gene locus.
- These bile acids stimulate the transformation from spores to vegetative cells without promoting further vegetative growth.

- **Secondary Bile Acids:**

- Deoxycholate, a secondary bile acid, also promotes germination but inhibits vegetative growth of *C. difficile* cells, limiting their ability to thrive post-germination.
- Chenodeoxycholate has a unique effect: it not only inhibits germination by competing with taurocholate but also suppresses vegetative cell growth

**The CspC Receptor:**

- CspC is the primary bile acid receptor for *C. difficile* spores. Studies showed that CspC specifically binds to taurocholate, enabling the spore to sense the gut environment and begin germination. Mutations in CspC, such as a substitution at residue 457 (glycine to arginine), can change how *C. difficile* responds to inhibitory bile acids, like chenodeoxycholate.
- CspC works alongside other proteins encoded by the *cspBAC* locus, including CspB and SleC. CspB activates the enzyme SleC, which is crucial for breaking down the spore cortex during germination.

**Activation Process of SleC:**

- During germination, taurocholate binding to CspC initiates a chain reaction
- SleC, activated by CspB, degrades the protective cortex layer of the spore, releasing nutrients and preparing the spore for vegetative growth.
- The lipoprotein GerS further enhances SleC's activity, ensuring that germination proceeds efficiently

**Gene Regulation During Germination**

Germination is a complex process that triggers the regulation of over 500 genes, as identified through transcriptional profiling. This regulation varies among clinical strains of *C. difficile*, and researchers are studying how these differences might affect the virulence of each strain

**Disruption of Gut Microbiota**

In a healthy gut, the normal microbiota prevents *C. difficile* from proliferating. However, when gut microbiota is disrupted (e.g., by antibiotics), *C. difficile* has less competition and can colonize the gut more effectively.

### 1.8.3 Colonization and Toxin Production

- **Adherence and Growth:** The vegetative cells adhere to the gut lining, where they multiply and produce two main toxins, TcdA and TcdB. These toxins disrupt the intestinal epithelial cells, leading to inflammation and damage.
- **Inflammatory Response:** Toxins trigger an inflammatory immune response that causes tissue damage, resulting in the symptoms associated with CDI, such as diarrhea, abdominal pain, and colitis.

### 1.8.4 Symptomatic or Asymptomatic Infection

- **Symptomatic CDI:** If toxin levels and inflammation are high, patients experience symptoms like diarrhea, fever, and abdominal pain. In severe cases, it can lead to pseudomembranous colitis or toxic megacolon.
- **Asymptomatic Colonization:** Some people become asymptomatic carriers without developing symptoms. They can still shed spores and serve as a reservoir for transmission to others.

### 1.8.5 Spore Formation and Shedding

- **Sporulation:** When environmental conditions within the gut become unfavorable for vegetative cells, *C. difficile* forms spores. These spores are highly resistant and can survive for long periods outside the host.
- **Fecal Shedding:** Infected individuals, both symptomatic and asymptomatic, shed spores in their feces. These spores can contaminate the surrounding environment, surfaces, and even hands, perpetuating the transmission cycle.

### 1.8.6 Environmental Persistence and Transmission

- **Environmental Contamination:** Spores shed in faeces contaminate surfaces, hospital equipment, and personal items, where they remain viable for months.
- **Infection of New Hosts:** Spores can be transferred to new hosts through direct contact with contaminated surfaces or hands, reinitiating the infection cycle when they are ingested by another individual.

This infection cycle underscores the resilience of *C. difficile* and the challenges in controlling CDI, particularly within healthcare environments where high transmission risk persists due to environmental spore contamination.

## 1.9 Infectious Dose

### 1.9.1 Evidence for Dose-Response in CDI

- Studies suggest a **dose-response relationship** for CDI, where the risk of infection increases with the number of spores ingested. In animal models, even a relatively low dose of **100 to 1,000 spores** has been shown to induce infection, especially when normal gut microbiota has been disrupted, typically by antibiotics.
- In humans, this susceptibility to infection after antibiotic treatment reflects a lowered microbial competition, allowing *C. difficile* to colonize and cause infection even at low spore counts.

### 1.9.2 Factors Influencing Infectious Dose

- **Antibiotic Use:** The effective infectious dose of *C. difficile* is thought to decrease significantly in individuals whose microbiota is compromised by antibiotics, which eliminates protective colonization resistance from the gut flora.
- **Immune Status:** Individuals with weakened immune defences, such as the elderly or immunocompromised, may have increased susceptibility to infection at lower spore doses.

### 1.9.3 Implications for Infection Control and Prevention

- **Lowering Environmental Contamination:** Understanding that low doses can trigger infection in susceptible individuals highlights the importance of rigorous sanitation practices, particularly in healthcare settings where individuals may be predisposed due to recent antibiotic use.
- **Protective Role of Microbiota Restoration:** Strategies such as FMT or probiotics, which aim to re-establish colonization resistance in the gut, help increase the threshold infectious dose for *C. difficile* by reintroducing beneficial microbial competition.

While the exact infectious dose in humans remains undetermined, existing studies underscore that susceptibility is highly context-dependent, with antibiotic use and compromised immune status significantly lowering the threshold for infection by *C. difficile* spores.

### 1.9.4 Mathematical models

Mathematical models to determine the infectious dose of *C. difficile* spores may be derived based on dose-response models used for other pathogens. These models may be used to relate the probability of

infection to the number of spores ingested, but precise quantification for *C. difficile* is challenging due to individual host factors, including microbiota status and antibiotic exposure. However, here are some of the primary approaches and types of models that could be adapted or suggested for *C. difficile*:

Some useful models to look at:

#### 1.9.4.1 Exponential and Beta-Poisson Models

- **Exponential Model:** This model assumes that each spore has an equal and independent probability of causing infection. It calculates the probability of infection ( $P_{\text{inf}}$ ) based on the dose ( $N$ ) and a pathogen-specific rate parameter ( $k$ ) as follows:  $P_{\text{inf}} = 1 - e^{-kN}$

In this model, a lower  $k$  value suggests a lower probability of infection per spore, which might represent a higher threshold for infection.

- **Beta-Poisson Model:** This model is commonly used when the host's response to pathogens varies significantly between individuals. It introduces variability by using parameters ( $\alpha$  and  $\beta$ ) to capture a range of susceptibilities:  $P_{\text{inf}} = 1 - (1 + N)^{-\beta}$

The Beta-Poisson model is often more accurate in capturing variations in pathogen infection risk, especially considering that antibiotic use or weakened immune systems can drastically reduce the effective infectious dose.

#### 1.9.4.2 Logistic Regression Models for Dose-Response

- **Logistic Regression Models:** These models apply a logistic function to dose-response data to predict infection probability based on dose. Logistic regression can help analyze specific patient data (e.g., recent antibiotic use, age, co-morbidities) and predict individual risk.
- **Threshold Models:** In cases where a threshold dose is hypothesized (e.g., a minimum number of spores required to achieve colonization), logistic models can estimate the dose at which infection probability begins to rise significantly.

#### 1.9.4.3 Quantitative Microbial Risk Assessment (QMRA) Models

- **Simulation-Based Approaches:** QMRA uses probabilistic simulation (e.g., Monte Carlo simulations) to model the variability in host susceptibility, environmental contamination levels, and dose-response relationships.
- **Risk Assessment in Healthcare:** QMRA has been adapted to assess infection risk in healthcare settings. This model accounts for variable patient susceptibility based on prior antibiotic use and environmental spore concentration to estimate likely infection outcomes.

#### 1.9.4.4 Agent-Based and Computational Models

- **Agent-Based Models (ABMs):** These models simulate individual hosts (agents) and interactions within healthcare settings or communities. ABMs can incorporate individual characteristics, such as microbiota diversity, immune status, and antibiotic history, making them highly adaptable to pathogen's unique infection dynamics.
- **Environmental Contamination Dynamics:** In healthcare settings, ABMs can simulate how spore concentrations change over time with environmental cleaning, predicting infection probabilities based on spore exposure levels.

#### Current Research Gaps and Limitations

- **Data Availability:** Precise infectious dose estimates are difficult to determine due to the complexity of *C. difficile* infection dynamics and the lack of consistent human dose-response data.
- **Impact of Microbiota:** Traditional models may not fully capture the variability in susceptibility caused by microbiota disruptions, which are known to lower the infectious dose threshold for *C. difficile*.



## Chapter 2

### Preface/Disclaimer



This book on *Clostridioides difficile* (*C. difficile*) has been compiled by the author for self-education purposes only, using information originally published in peer-reviewed articles and other credible sources. This book intends to gather all essential and valuable information on *C. difficile* into a single comprehensive resource. Some sections may include direct excerpts or closely paraphrased content from the original articles, supplemented with the author's interpretations for clarity and synthesis. Some information interpreted by the author may be subjective and may contain errors. If in doubt, please do further check

using other resources in literature.

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# Chapter 3

## Virulence mechanisms, environmental persistence and survival

*C. difficile*'s pathogenicity is primarily driven by its ability to produce toxins that damage host tissues, initiate inflammatory responses, and disrupt the intestinal epithelium. The major toxins involved include TcdA and TcdB, and in certain hypervirulent strains, the binary toxin CDT.

Other than virulence factors, *C. difficile* employs various non-toxic factors for colonization, adherence, and survival within the gut. These mechanisms enhance its pathogenicity and serve dual purposes, promoting both host infection (increase colonization resistance) and survival in external environments. This overlap is crucial to *C. difficile*'s ability to thrive in healthcare settings and facilitate its transmission through community and hospital surfaces, air particles, and medical devices.

### 3.1 Toxin production

Toxin production in *C. difficile* plays a critical role in its pathogenicity and infection dynamics. While these toxins facilitate nutrient availability and reduce competition by disrupting gut cells, excessive production leads to severe gut damage, inflammation, and potential loss of the bacterial niche. This disruption can hinder *C. difficile* persistence in the gut, initiating cycles of infection and clearance as the host immune response becomes activated. Toxin expression is influenced by genetic and environmental factors that modulate the bacteria's virulence, with therapeutic strategies targeting these toxins to mitigate symptoms and reduce recurrence risk in CDI.

#### 3.1.1 Major Toxins: TcdA and TcdB

##### 3.1.1.1 Genetic Basis (Pathogenicity Locus, PaLoc)

- The pathogenicity locus (PaLoc) encodes toxins A (TcdA) and B (TcdB), which are the primary virulence factors for *C. difficile*. This locus typically contains five genes: *tcdA* (encodes TcdA), *tcdB* (encodes TcdB), *tcdR* (a positive regulator), *tcdE* (possibly involved in toxin export), and *tcdC* (a negative regulator).
- Variations in the PaLoc influence toxin levels among strains, affecting infection severity. Mutations in *tcdC* in hypervirulent strains disrupt normal regulation, leading to increased toxin expression and virulence.

##### 3.1.1.2 Structure and Function

- TcdA and TcdB have four main domains: glucosyltransferase domain (GTD), autoprocessing domain, translocation domain, and receptor-binding domain.

- Their glucosyltransferase activity targets RHO GTPases in host cells, causing cytoskeletal disruption, cell rounding, and cell death. This activity compromises gut barrier integrity increases permeability, and induces inflammation.

### 3.1.1.3 Mechanism of Action

- **TcdA:** Known as an enterotoxin, TcdA binds to carbohydrates on colon epithelial cells, leading to fluid accumulation, inflammatory responses, and cell death. It initiates tissue damage and provides nutrient release, aiding bacterial colonization.
- **TcdB:** Acts as a potent cytotoxin and binds to host receptors like chondroitin sulfate proteoglycan 4, causing severe cellular disruption. TcdB is essential for colitis and severe disease, as **strains without TcdB are non-pathogenic**. Conversely, strains with TcdB but lacking TcdA can still cause colitis.

### 3.1.1.4 Regulation of Toxin Expression

- **TcdR** functions as an alternative sigma factor, activating toxin gene transcription, particularly in stationary growth phases.
- **TcdC** serves as a negative regulator during exponential growth. Hypervirulent strains, such as BI/NAP1/027, often harbour mutations in **tcdC** that dysregulate toxin expression, contributing to enhanced pathogenicity.

## 3.1.2 Binary Toxin (CDT)

### 3.1.2.1 Presence in Hypervirulent Strains:

- CDT, an additional toxin produced by certain strains like ribotype 027, consists of two subunits, **CdtA** and **CdtB**. CdtA has ADP-ribosyl transferase activity targeting actin, weakening host cell structure, while CdtB forms pores facilitating CdtA's entry.

### 3.1.2.2 Impact of CDT:

- Although CDT alone is not sufficient to cause disease, it enhances ***C. difficile*'s adhesive capacity**, potentially aiding in immune evasion and increasing virulence in severe or recurrent infections.

## 3.1.3 Toxin-Triggered Inflammatory Responses

### Host Immune Response

- **TcdA and TcdB** induce cellular damage, prompting the release of pro-inflammatory cytokines and chemokines. These signals recruit immune cells, exacerbating inflammation in the colon and causing CDI symptoms, such as diarrhoea and colitis.
- **RHO GTPase Glycosylation** by TcdB activates inflammasomes within host cells, leading to the release of interleukin-1 (IL-1) and promoting a strong inflammatory response that worsens tissue damage.

## 3.1.4 Regulation of Toxin Production

### 3.1.4.1 Environmental Cues

Nutrient availability, bile acids, and gut pH can influence toxin expression. Stress conditions, like nutrient limitation, prompt *C. difficile* to upregulate toxin production, disrupting host cells to release nutrients for bacterial sustenance.

### 3.1.4.2 Genetic Diversity and Strain Variability

Variability within the PaLoc, particularly in regulatory genes like **tcdC**, results in differing toxin production levels among strains. Hypervirulent strains often have dysregulated toxin expression, contributing to more severe disease outcomes. Different ribotypes (e.g., RT027, RT078) produce variable combinations of TcdA, TcdB, and CDT, while non-toxigenic strains lack these genes entirely.

### 3.1.4.3 Detection of Toxins

Protocols such as **multiplex PCR** and **whole genome sequencing (WGS)** identify the presence of virulence genes (*tcdA*, *tcdB*, *cdtA*, *cdtB*), aiding in differentiating between toxigenic and non-toxigenic strains and tailoring infection control strategies.

### 3.1.4.4 PaLoc and Toxin Gene Fragments

- The PaLoc includes ten fragments, but toxinotyping primarily analyzes the B1 and A3 fragments. These fragments contain regions of the *tcdA* and *tcdB* toxin genes.
- Comparison with a Reference Strain: Strains are compared to the reference strain *C. difficile* VPI 10463, revealing variations that define 27 toxin types (I to XXVII).

### 3.1.4.5 Types of PaLoc Variations

- Minor Changes: Some toxin types have small deletions or alterations in repetitive sequences, particularly in the A3 region of the *tcdA* gene.
- Major Variations: In other toxin types, changes are distributed across the entire PaLoc, resulting in major toxin types. These often correspond well with other typing methods like ribotyping.

### 3.1.4.6 Variant Toxin Production

- Variant toxin genes may produce toxins with altered properties or result in the absence of one or both toxins. For example, TcdA–TcdB+ strains produce only TcdB, the first discovered variant type.

### 3.1.4.7 Clinical Relevance

- Association with Disease Patterns:
  - Specific toxin types can sometimes correlate with disease characteristics or specific patient populations during outbreaks. However, a toxin type does not generally predict disease severity.
- Increased Human Variant Strains:
  - Historically, many variant toxinotypes were found in animal isolates, while human isolates were mostly non-variant. Recently, the proportion of variant strains in humans has risen, and some variant toxin types (e.g., toxin type III or ribotype 027, and toxin type VIII or ribotype 017) have been linked to major outbreaks worldwide.

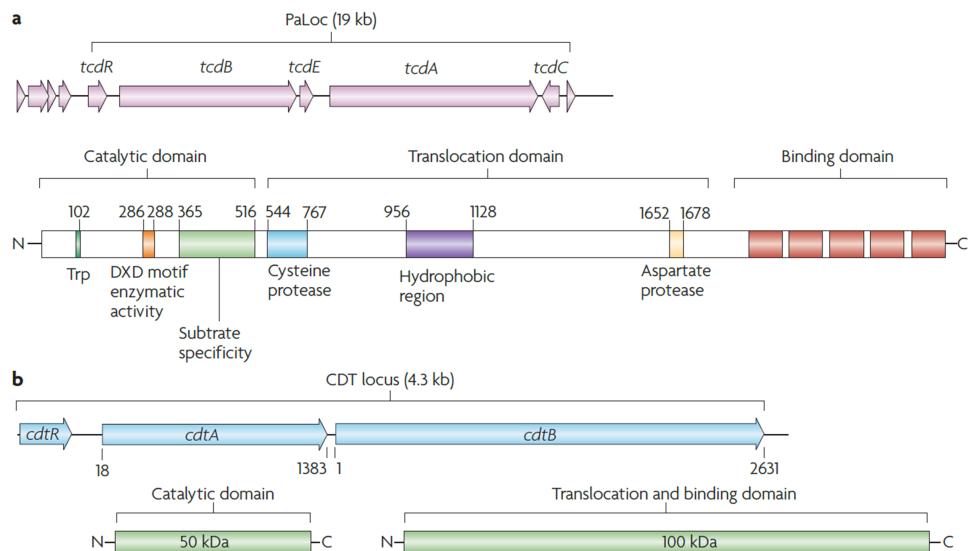


Figure 3 | **Toxins produced by *Clostridium difficile*.** **a** | Two large toxins, toxin A and toxin B (TcdA and TcdB), are encoded on the pathogenicity locus (PaLoc), which comprises five genes. In non-toxigenic strains, this region is replaced by a short 115 bp sequence. Both toxins are single-chain proteins, and several functional domains and motifs have been identified. TcdB is shown in detail below the PaLoc. **b** | A third toxin, the binary toxin or CDT, is encoded on a separate region of the chromosome (CdtLoc) and comprises three genes. The binary toxin is composed of two unlinked proteins, CdtB and CdtA. CdtB has a binding function and CdtA is the enzymatic component.

Figure 3.1: *C. difficile* toxin production operon (Rupnik, Wilcox, and Gerding 2009)

## 3.2 Sporulation

**Stages of Sporulation:** Sporulation is a four-step process in *C. difficile*:

- **Initiation:** Triggered by environmental stress, leading *C. difficile* to decide to form spores.
- **Development:** The bacterium begins forming the spore structure, sequestering essential DNA and proteins.
- **Maturation:** The spore develops a tough, resistant coat, enabling survival under adverse conditions.
- **Release:** The mature spore exits the mother cell and becomes capable of enduring extreme environments and eventually infecting new hosts

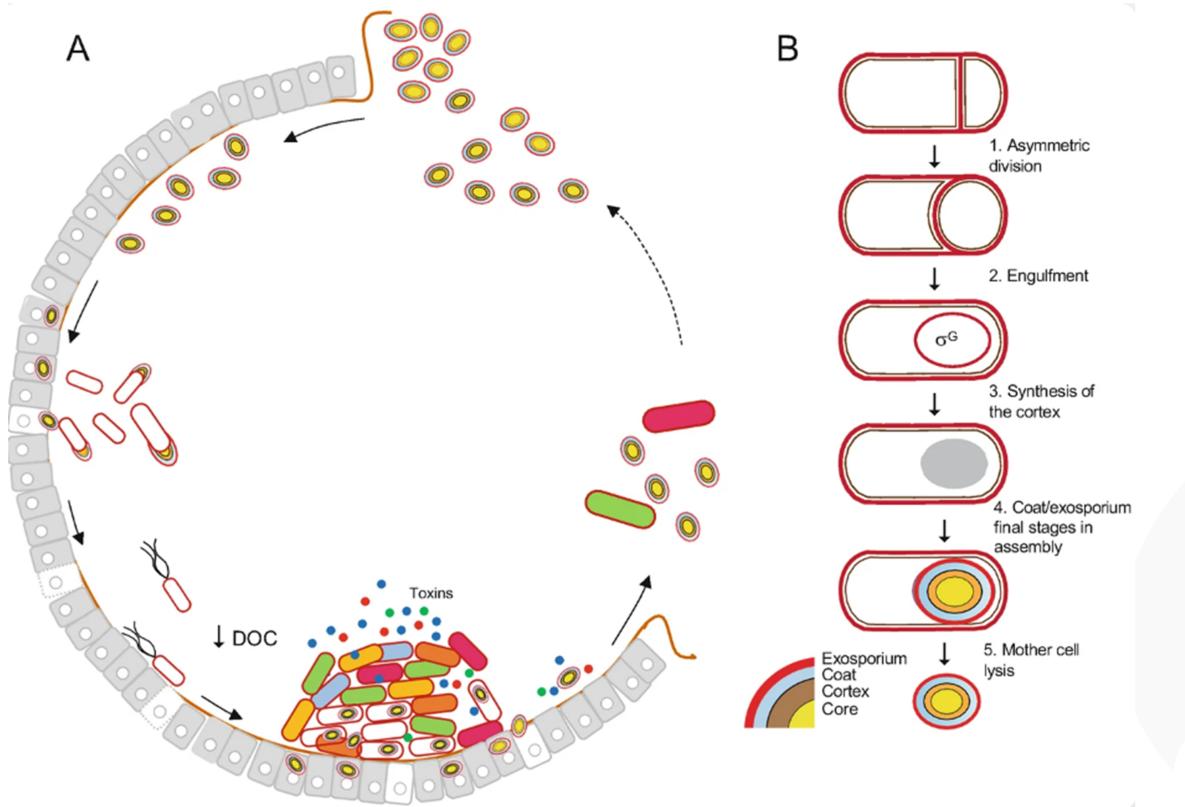


Figure 3.2: *C. difficile* life cycle reported by (Serrano, Martins, and Henriques 2024). (a) An obligate anaerobe, *C. difficile* is usually found outside the host in the form of dormant spores. Infection starts with the ingestion of spores. Once in the small intestine, spores are exposed to bile salts and germinate. While primary bile salts, such as cholate (CA), induce spore germination and promote vegetative growth, secondary bile salts, such as deoxycholate (DOC), more abundant in the large intestine, inhibit vegetative growth. Bile salts levels are influenced by the commensal gut microbiota. *C. scindens*, for instance, encodes a 7 -dehydroxylating activity which converts CA into DOC. After antibiotic treatment the commensal gut microbiota is disturbed and the representation of species capable of 7 -dehydroxylation is reduced. Thus, growth is enhanced in the large intestine, leading to host colonization. The vegetative cells are motile and formation of flagellum was shown to be important for infection. Once the cell finds a surface, it can divide and form cell clusters or microcolonies. Evidence suggests that toxins and spores are produced within the biofilm. Toxins contribute for spore adherence and internalization by the intestinal epithelial cells. Spore may be released from the cell during the normal renewal of the intestinal epithelium. Shedding of the spores to the environment will allow the infection of new hosts, while spores that remain inside the host can be the cause of disease recurrence. (b) Sequence of the morphological events leading to spore differentiation: (1) asymmetric division of the sporangium; (2) intermediate stage in the process of engulfment of the forespore (the future spore) by the larger mother cell; (3) engulfment completion, isolating the forespore from the surrounding medium; (4) synthesis of the spore surface layers, the spore cortex and the coat and exosporium. (5) Finally, upon lysis of the mother cell the spore is released to the environment. The various layers detected in mature spores are indicated.

**Benefits:** Sporulation allows *C. difficile* to persist outside the host and resist environmental factors like heat, desiccation, and antimicrobials, making it central to transmission and survival.

**In-Host Function:** Sporulation allows *C. difficile* to survive in the host when conditions become hostile (e.g., low nutrients or immune attack).

**Environmental Survival:** Sporulated *C. difficile* spores are highly resistant to environmental factors like desiccation, heat, UV light, and chemical disinfectants. This resilience allows spores to survive on surfaces in healthcare settings and other environments for months, contributing to transmission.

Currently, there is limited understanding of why some *C. difficile* strains have led to large transatlantic epidemics (such as BI/NAP1/ribotype 027), whereas others remain at a local or sporadic level. Several explanations for this ‘hypervirulence’ have been proposed, and it seems likely that pathogenic factors such as germination, sporulation, epithelial adherence and toxin production could influence the success of some strains.

Early evidence indicates that Spo0A might vary between ribotypes, but further research is required to confirm the influence this factor might have on transmission and clinical disease

### 3.3 Biofilm Formation

*C. difficile* has limited but significant biofilm-forming capabilities, which are characterized by the production of different biofilm structures that exhibit unique metabolic types. Biofilm formation is induced by factors such as deoxycholate and is associated with reduced sporulation and toxin production, allowing *C. difficile* to adapt to nutrient availability during its persistent lifestyle in vivo. This ability to form biofilms may enhance its survival in the gut environment and contribute to recurrent infections, highlighting the need for further research on the clinical implications of *C. difficile* biofilm formation.

- **Structure and Composition:** In biofilm form, *C. difficile* secretes an extracellular matrix made of proteins, polysaccharides, and extracellular DNA. This matrix helps the bacteria survive against antibiotics, oxidative stress, and immune responses.
- **Acid trigger:** Biofilm formation in *C. difficile* is triggered by bile acids like deoxycholate, especially under nutrient limitations. This adaptation allows *C. difficile* to transition between active infection and persistence in the gut.
- **Limited Biofilm Formation:** Although *C. difficile* does not form particularly robust biofilms, the biofilm state is significant for recurrent infections, as it provides a secure environment for spores to survive and persist within the host.

**In-Host Role:** Biofilm formation enables *C. difficile* to resist host defences and antibiotics by creating a protective matrix around itself.

**Environmental Benefit:** Biofilms enhance environmental persistence by embedding spores within a matrix, protecting them from physical and chemical disruptions. This trait is particularly useful in water systems, surfaces, and medical equipment, where spores can survive within biofilms for extended periods.

### 3.4 Stress Adaption

- **Sensitivity to Oxygen and pH:** As an anaerobic bacterium, *C. difficile* struggles in oxygen-rich environments, which limits its growth near the gut lining. Low pH conditions also restrict its ability to thrive, giving other microbes an advantage over *C. difficile* in oxygenated areas.
- **Nutrient depletion/environment stress:** *C. difficile* can activate sporulation or biofilm formation to survive hostile environments within the gut.
- **Heat and Solvent Stress:** Stress response proteins (e.g., heat shock proteins) and solvent resistance mechanisms support *C. difficile*'s survival and enable it to endure fluctuating environmental conditions within the gut.

**In-Host Adaptation:** Within the host, *C. difficile* uses stress adaptation proteins (e.g., heat shock proteins) to withstand immune responses, oxidative stress, and other gut environmental pressures.

**Environmental Resilience:** These adaptations also enable survival under environmental stresses, such as temperature fluctuations, limited nutrients, and exposure to disinfectants. This mechanism aids in survival in soil, water, and healthcare environments where conditions vary widely.

### 3.5 Flagella and Motility

- **Role in Virulence:** Flagella enhance *C. difficile* motility, allowing it to migrate and adhere to the gut lining. Additionally, flagellar expression appears to be linked with toxin regulation, meaning flagella plays a dual role in both adherence and virulence.

- Flagellar expression varies widely between strains, and strains without flagella show a reduced ability to adhere to intestinal cells, e.g., mutants lacking flagellar components show disrupted toxin regulation, indicating that flagella not only helps with movement and adherence but also influence toxin production.
- **Regulation by Cyclic dimeric guanosine monophosphate c-di-GMP:** High cyclic di-GMP (c-di-GMP) levels suppress flagellar and toxin production, shifting *C. difficile* to a biofilm-forming, adherent state. Lower c-di-GMP levels activate flagellar expression, allowing *C. difficile* to transition to a motile, toxin-producing form.

**In-Host Function:** Flagella facilitate movement toward the gut lining, aiding in colonization and evasion of gut defences.

**Environmental Impact:** While motility is primarily a factor in host colonization, flagella help *C. difficile* attach to surfaces, assisting in the initial stages of biofilm formation. The flagella-driven movement also plays a role in reaching favourable niches in environments outside the host, such as nutrient-rich spots on the surface.

## 3.6 Adhesion Proteins

- *Adhesins for C. difficile* uses a variety of adhesion proteins to attach to the gut lining:
  - **Fibronectin-binding protein A** and cell wall proteins like **Cwp66, S-layer protein A, and Cwp84** play critical roles in colonization by anchoring the bacterium to host cells.
- **Spo0A:** Interestingly, the sporulation regulator Spo0A not only regulates sporulation but also aids in adhesion, linking survival mechanisms with virulence.

**Host Colonization:** Adhesion proteins (e.g., fibronectin-binding proteins and S-layer proteins) are crucial for binding to intestinal cells, enabling stable colonization.

**Environmental Adhesion:** These proteins likely facilitate attachment to surfaces outside the host, such as medical equipment and healthcare surfaces, where *C. difficile* spores can anchor and persist. This attachment helps spores remain viable and increases their likelihood of infecting a new host.

## 3.7 Other Virulence Mechanisms

- **Nutrient Competition and Colonization in Mucus:** *C. difficile* colonizes the nutrient-rich mucus layer lining the gut, with a preference for mucus associated with CDI. By-products from other gut microbes can stimulate *C. difficile* colonization, indicating how changes in the gut environment help the pathogen establish infection.
- **Immune Modulation:** *C. difficile* can adjust its toxin production to avoid excessive host immune response, reducing the likelihood of displacing itself from the gut niche. By managing toxin levels, it maintains a balance that favours long-term survival within the host.

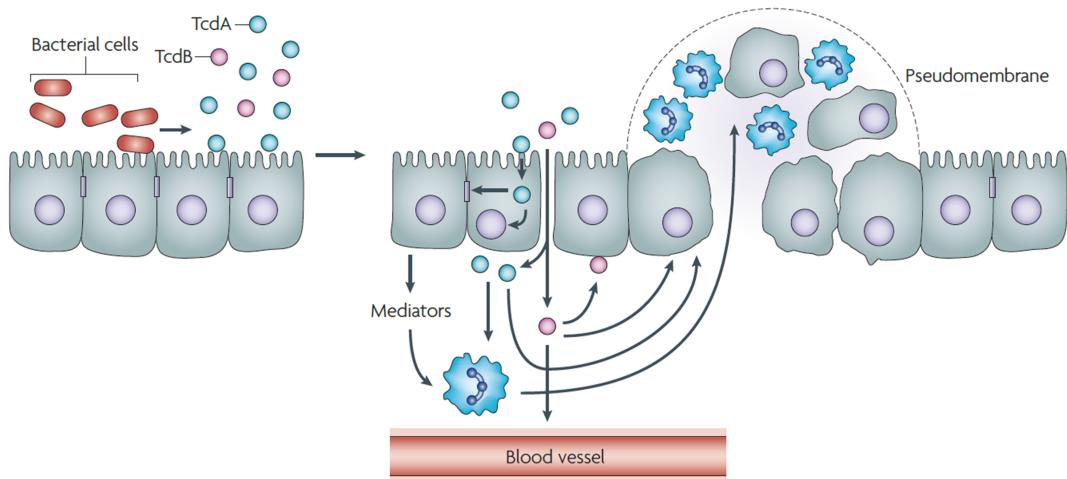
**In-Host Utility:** Nutrient uptake mechanisms allow *C. difficile* to utilize resources in the gut, especially during antibiotic-induced dysbiosis. It can also modulate toxin production to avoid triggering a strong immune response.

**Environmental Adaptation:** The ability to persist in nutrient-scarce environments and to use diverse substrates may aid in survival outside the host, particularly in nutrient-limited environments like soil and water.

## 3.8 Gut Interactions and Colonization Resistance

Gut microbiota plays a critical role in preventing *C. difficile* colonization, a process known as colonization resistance. Antibiotics disrupt this balance, reducing competitive pressures and enabling *C. difficile* spores to germinate and colonize. Bile acid modulation is another important factor: primary bile acids (e.g., taurocholate) promote germination, whereas secondary bile acids (e.g., deoxycholate) inhibit it. Understanding these dynamics offers potential targets for interventions. For example, modulating bile

acid composition in the gut or targeting specific receptors like CspC could inhibit spore germination and reduce infection rates.



**Figure 5 | *Clostridium difficile* pathogenesis.** *C. difficile* colonizes the intestine (colon) after disruption of the normal intestinal flora. To what extent adhesion and biofilm production are involved in the pathogenesis of *C. difficile* is unknown; in the schematic, bacterial cells are shown as free cells and attached to host cells. Toxigenic strains produce toxin A and toxin B (TcdA and TcdB). TcdA binds to the apical side of the cell and, after internalization, causes cytoskeletal changes that result in disruption of tight junctions and loosening of the epithelial barrier, in cell death or in the production of inflammatory mediators that attract neutrophils. Disruption of tight junctions enables both TcdA and TcdB to cross the epithelium. TcdB binds preferentially to the basolateral cell membrane. Both toxins are cytotoxic and induce the release of various immunomodulatory mediators from epithelial cells, phagocytes and mast cells, resulting in inflammation and the accumulation of neutrophils. In an animal model, TcdB was shown to have a tropism for cardiac tissue, which would require that TcdB enter the bloodstream.

Figure 3.3: *C. difficile* pathogenesis (Rupnik, Wilcox, and Gerding 2009)

### 3.8.1 Metabolic Adaptability

- *C. difficile* is highly adaptable in using various gut nutrients, which aids in its survival and spread. It can break down mucus components like sialic acid and N-acetylglucosamine and uses amino acids through a process called Stickland fermentation. One nutrient, trehalose, has been linked to the spread of a specific, common strain (Ribotype 027), though not necessarily to increased severity. Understanding how different nutrients and additives impact *C. difficile* could improve treatments, possibly offering alternatives to FMT by targeting these metabolic pathways.

### 3.8.2 Role of Bile Acids

- Bile acids are key regulators of *C. difficile* spore germination. Primary bile acids like taurocholate induce spore germination, while secondary bile acids, such as chenodeoxycholate, inhibit both germination and vegetative growth. This effect depends on the type and concentration of bile acids present, with a balanced microbiota generally favouring secondary bile acid production, thereby maintaining colonization resistance against *C. difficile*.
- Antibiotic-treated animals have higher concentrations of primary bile acids in their stool, which promotes germination.
- In untreated animals, secondary bile acids dominate, aiding in colonization resistance (the gut's ability to resist pathogen growth).
- Certain bacteria, like *Clostridium scindens*, metabolize primary bile acids into secondary bile acids, creating an environment that resists *C. difficile* colonization. Studies suggest that multiple types of gut bacteria might contribute to this resistance.
- The balance of bile acids regulated by the microbiota along different areas of the gut could be key in both regulating spore germination and maintaining resistance to *C. difficile* colonization. This relationship indicates that a healthy microbiota composition could help prevent *C. difficile* infection by reducing conditions favourable for its germination and growth.

### 3.8.3 Iron and Zinc Scavenging

- To support growth, *C. difficile* scavenges essential nutrients, including iron and zinc, using specialized transporters and mechanisms that help reduce redox stress enabling it to survive and colonize more effectively. Elevated zinc levels have been shown to reduce the antibiotic dose needed for *C. difficile* colonization, indicating that limiting access to these nutrients in the gut is a natural defence mechanism against its colonization.

### 3.8.4 Sensitivity to Oxygen and pH

- As an obligate anaerobe, *C. difficile* thrives in low-oxygen areas of the gut, with higher oxygen levels limiting its colonization ability near the epithelial lining. Additionally, *C. difficile* grows slower in acidic (low-pH) environments, so gut regions with higher pH and low oxygen favour its establishment, especially when other bacteria are absent due to antibiotics.

### 3.8.5 Colonization of the Mucus Layer

- *C. difficile* specifically colonizes the mucus layer of the large intestine, where it is attracted by nutrient-rich by-products from other microbes breaking down the mucus. This layer contains polysaccharides, proteins, and other nutrients that support *C. difficile* growth. *C. difficile* is drawn to areas where microbial activity is high and binds preferentially to certain mucin types commonly found in individuals susceptible to CDI. This targeted attachment allows *C. difficile* to integrate into multispecies microbial communities in the mucus, enhancing its stability, persistence, and potential for recurrence within the host. Changes in the gut environment, such as alterations in mucin types during infection, appear to facilitate *C. difficile* colonization.



# Chapter 4

## Therapeutic Strategies Targeting Colonization Resistance

- **Microbiota Restoration:** Probiotics and FMT reintroduce beneficial bacteria that inhibit *C. difficile* through competition, secondary bile acid production, and other inhibitory metabolites.
- **Harnessing Beneficial Bacteria:** Key microbiota members—*Bacteroides*, *Firmicutes*, *Akkermansia muciniphila*, and *Faecalibacterium prausnitzii*—are central to colonization resistance and gut health.
- **Nutrient and Resource Modulation:** Limiting access to essential nutrients like trehalose, iron, and zinc can suppress *C. difficile* colonization.
- **Antibiotics and Alternative Treatments:** Standard antibiotics, bile acid modulation, antimicrobial peptides, and bacteriophages offer targeted approaches for reducing *C. difficile* without extensive disruption of the microbiota.

Overall, these approaches aim to restore the gut microbiota balance, inhibit *C. difficile* colonization, and prevent CDI recurrence by leveraging natural mechanisms of colonization resistance and gut health maintenance.

The gut microbiota competes with *C. difficile* for resources and produces inhibitory metabolites, which creates “colonization resistance” that prevents *C. difficile* colonization. A deeper understanding of these interactions is crucial for developing effective CDI treatments.

### 4.1 Probiotics and Fecal Microbiota Transplantation (FMT)

- **Probiotics:** Probiotics are supplements that introduce beneficial bacterial strains, aiming to restore gut microbial diversity and compete against *C. difficile* for resources. Common probiotic strains include *Bacteroides* and *Lactobacillus*, which can produce secondary bile acids and other metabolites that inhibit *C. difficile* growth and spore germination.
- **Fecal Microbiota Transplantation (FMT):** FMT involves transferring fecal material from a healthy donor into a CDI patient’s gut, to restore a balanced microbiota. By reintroducing a diverse range of beneficial microbes, FMT helps re-establish colonization resistance, reduces CDI recurrence, and promotes the production of secondary bile acids that suppress *C. difficile*.

### 4.2 Nutrient Competition

- **Limiting Access to Specific Nutrients:** Certain nutrients are essential for *C. difficile* growth, and restricting access to these can help prevent colonization. For instance:
  - **Trehalose:** Some hypervirulent strains of *C. difficile* (e.g., ribotype 027) can metabolize trehalose, a sugar linked to increased colonization and virulence. Limiting trehalose in the diet could reduce infection risk from these strains.

- **Iron and Zinc Modulation:** *C. difficile* relies on iron and zinc for growth and toxin production. Limiting these nutrients in the gut, or reducing their bioavailability, creates an unfavorable environment for *C. difficile*. High zinc levels, for example, are associated with increased colonization risk, so modulating these levels in CDI patients may be beneficial.

## 4.3 Primary Treatment Options

### 4.3.1 Antibiotics

- Standard antibiotic treatments, such as fidaxomicin and vancomycin, target *C. difficile* directly but often disrupt the broader gut microbiota, which can lead to recurrence if colonization resistance is not restored. Fidaxomicin has a narrower spectrum than other antibiotics, which may help preserve some beneficial gut bacteria.

### 4.3.2 FMT

- FMT is also used as a primary treatment, especially in recurrent CDI cases where antibiotics alone are ineffective. FMT aims to re-establish colonization resistance by repopulating the gut with a healthy microbiota.

### 4.3.3 Emerging Microbiota-Based Therapies

- New therapies focus on restoring microbial balance without disrupting the gut environment. These include microbiota-derived therapies that mimic the effects of FMT, using synthetic microbial communities or cultured strains known to suppress *C. difficile*.

## 4.4 Alternative Treatment Mechanisms

### 4.4.1 Bile Acid Modulation

- Since bile acids play a crucial role in *C. difficile* germination, therapies that promote secondary bile acid production may inhibit spore germination and vegetative cell growth. Modifying bile acid composition could be a key approach in CDI treatment and prevention.

### 4.4.2 Antimicrobial Peptides

- **Antimicrobial Peptides (AMPs):** AMPs are small, host-produced peptides that can kill or inhibit *C. difficile* without disrupting other gut bacteria.

### 4.4.3 Bacteriophages

- **Bacteriophage Therapy:** Bacteriophages are viruses that infect specific bacterial species. Phage therapy for *C. difficile* targets the pathogen without impacting the surrounding microbiota.

### 4.4.4 Short-Chain Fatty Acids (SCFAs)

- Produced by beneficial gut bacteria, SCFAs help lower gut pH and inhibit *C. difficile* growth. Therapy with SCFAs or SCFA-promoting bacteria is being studied as a way to maintain an environment hostile to *C. difficile*.

### 4.4.5 Biofilm Inhibition and Nutrient Deprivation

- Strategies that disrupt biofilm formation prevent *C. difficile* from establishing long-term infections. Reducing nutrients essential for *C. difficile* growth, such as iron and zinc, is also being explored as a therapeutic strategy.

## 4.5 Role of Beneficial Gut Microbiota in Preventing CDI

Certain beneficial bacterial groups play a vital role in maintaining a healthy gut microbiome and preventing *C. difficile* overgrowth. FMT and probiotic therapies often aim to restore these beneficial bacteria, enhancing colonization resistance against *C. difficile*.

### 4.5.1 Bacteroides

- **Function:** Bacteroides species help break down complex carbohydrates, releasing short-chain fatty acids (SCFAs) and other by-products that create an inhospitable environment for pathogens like *C. difficile*. SCFAs lower gut pH, indirectly inhibiting *C. difficile* growth.

### 4.5.2 Firmicutes (e.g., *Lactobacillus* and *Clostridium scindens*)

- **Function:** Firmicutes are key producers of secondary bile acids, which inhibit *C. difficile* spore germination and growth. *Clostridium scindens*, in particular, converts primary bile acids into secondary bile acids, maintaining colonization resistance. *Lactobacillus* species also help stabilize gut pH and produce antimicrobial compounds that suppress *C. difficile*.

### 4.5.3 *Akkermansia muciniphila*

- **Function:** This bacterium helps strengthen the gut lining and enhances mucosal barrier integrity, making it harder for pathogens like *C. difficile* to establish infection. It also supports immune functions, providing additional defences against colonization.

### 4.5.4 *Faecalibacterium prausnitzii*

- **Function:** Known for its anti-inflammatory properties, *F. prausnitzii* produces butyrate, an SCFA that supports gut health and inhibits *C. difficile*. Its presence is often associated with reduced inflammation, helping maintain a balanced gut environment resistant to CDI.



# Chapter 5

## Diagnostic Techniques and Challenges

### 5.1 Diagnostic Complexity

CDI diagnosis is complex due to the need to distinguish between non-toxigenic and toxigenic strains.

**Two primary reference tests exist:**

- Cytotoxigenic Culture: Detects toxigenic *C. difficile*, indicating infection risk but not always symptomatic disease.
- Cell Cytotoxicity Assay (CTA): Detects toxins A and B in stool samples, closely associated with clinical symptoms.

Only toxigenic strains of *C. difficile*, which produce toxin A and/or toxin B, are considered pathogenic and capable of causing clinical infection. According to the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) guidelines, a diagnosis of CDI is established if:

1. The patient presents a clinical picture that aligns with CDI symptoms, with laboratory evidence of toxin A and/or toxin B in stool samples and no other identified cause of diarrhoea.
2. Alternatively, the presence of pseudomembranous colitis (PMC) alone, a severe form of inflammation in the colon, is also a definitive indicator of CDI

Similarly, the Society for Healthcare Epidemiology of America and the Infectious Diseases Society of America (SHEA/IDSA) define a CDI case based on:

- Symptoms consistent with CDI, often diarrhoea, alongside a positive stool test for *C. difficile* toxins or toxigenic strains.
- Confirmatory findings of PMC through colonoscopy or histopathology can also establish a CDI diagnosis

Both definitions emphasize the critical role of toxin presence and clinical symptoms in diagnosing CDI, as only toxigenic strains can cause infection.

There are several diagnostic tests for CDI, each targeting different aspects of the bacterium or its toxins

Table 1 | Diagnosing *Clostridium difficile* infection

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

\*There are recent contradictory data regarding assay sensitivity.

Figure 5.1: CDI diagnosing questions (Rupnik, Wilcox, and Gerding 2009)

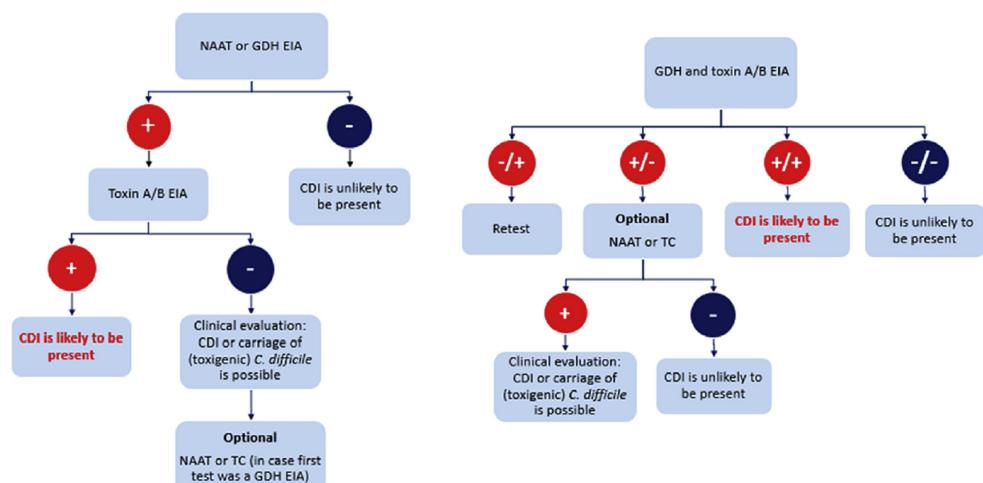
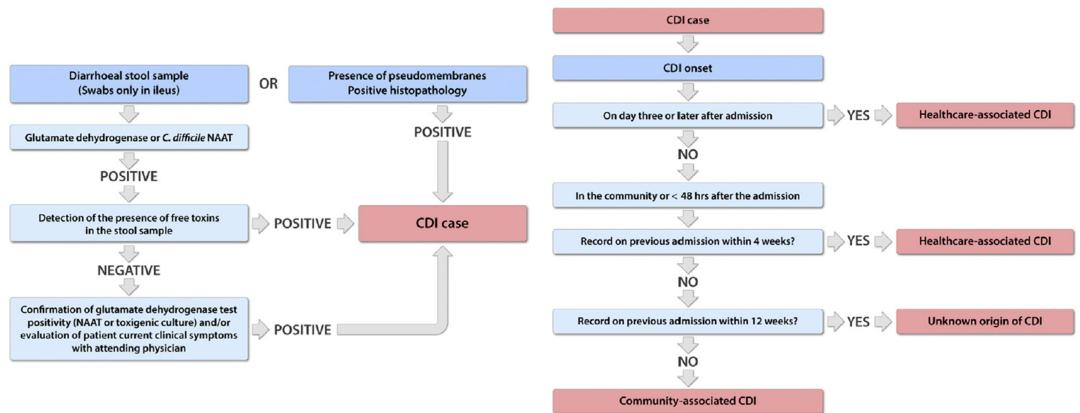


Fig. 2. Algorithms for the diagnosis of *Clostridium difficile* infection (CDI) (adapted from the ESCMID guidelines [28]). Abbreviations: EIA, enzyme immunoassay; GDH, glutamate dehydrogenase; NAAT, nucleic acid amplification test; TC, toxigenic culture.

Figure 5.2: ESCMID CDI decision algorithms guidelines (Gateau et al. 2018)



**Fig. 1.** Decision algorithms for (a) application of *Clostridium difficile* infection (CDI) case definition using ESCMID-recommended diagnostic algorithm [8] and (b) designation of the origin of CDI cases.

Figure 5.3: CDI decision algorithms (Krutova et al. 2018)

## 5.2 Screening Techniques

1. **Cytotoxicity Cell Assay (CTAs):** This test identifies toxin B activity by observing the cytotoxic effects on cultured cells, which is then neutralized with antitoxin. This test is considered a gold standard in terms of specificity but is time-consuming and requires specialized laboratory facilities.

**Gold Standard:** CTA is considered the reference method for detecting free toxins, mainly toxin B, in stool samples.

- **Procedure:**

- A filtrate of stool is applied to a cell culture, where the presence of toxins causes a specific cytopathic effect, notably cell rounding.
- This effect is observed after 1 to 2 days of incubation at  $36 \pm 1^\circ\text{C}$ .
- Specificity is verified by neutralizing the effect with antisera against *C. difficile* toxin B or *Clostridium sordellii* toxins.

- **Advantages:**

- Proven sensitivity and specificity, as CTA results correlate better with clinical outcomes than simply detecting toxigenic *C. difficile* strains.
- Lower cost compared to some other tests.

- **Limitations:**

- CTA is used by only a few laboratories due to a lack of standardization (e.g., choice of cell line, sample dilution, incubation time).
- Has a long turnaround time, limiting its practicality for routine diagnosis.

2. **Enzyme Immunoassays (EIAs):** Commonly used but can lack sensitivity and specificity, especially as single tests. These detect toxins A and/or B directly in stool samples. While fast and cost-effective, EIAs have variable sensitivity, which can lead to false negatives.

**Mechanism:** EIA detects both toxins A and B using monoclonal or polyclonal antibodies. This test can be formatted in either micro-well (ELISA) or lateral flow membrane devices.

**Commercial Availability:** Numerous commercial EIA kits are available, designed to provide rapid, easy-to-interpret results.

- **Advantages:**

- Rapid results and ease of use make EIA a convenient option for initial testing.

- **Limitations:**

- Many studies have noted EIA's lower sensitivity (ranging from 29% to 86%) compared to CTA, making it unsuitable as a stand-alone diagnostic for CDI.

**3. Glutamate Dehydrogenase (GDH):** GDH is an enzyme produced by *C. difficile*, and its presence in stool suggests colonization. While the GDH test is sensitive, it cannot differentiate between toxigenic and non-toxigenic strains, it may overdiagnose CDI so is often used as a preliminary screen.

***GDH as a Marker:***

- Glutamate dehydrogenase (GDH) is a metabolic enzyme present in all *C. difficile* strains.
- GDH can be detected using immuno-enzymatic (ELISA) or immuno-chromatographic assays.
- GDH tests are widely recommended as a screening method for CDI due to their high negative predictive value (NPV).

***Negative Predictive Value (NPV):***

The high NPV of the GDH test (80-100%) means a negative result generally rules out CDI, making it a useful initial screening tool. However, NPV can be influenced by the prevalence of CDI in the population: For example, with an NPV of 99% and a CDI prevalence of 10%, there is a risk that one out of every ten positive stool samples could be missed if only GDH screening is used.

***Confirmation of Positive Results:***

A positive GDH result requires confirmation with a second, more specific test, such as a toxin test, to verify the presence of toxigenic *C. difficile*. This second step ensures diagnostic accuracy by detecting the actual toxin production.

**4. Nucleic Acid Amplification Tests (NAATs):** These include PCR-based tests that detect genes responsible for toxin production (e.g., tcdA or tcdB genes). NAATs are highly sensitive and provide rapid results but can sometimes detect colonization without active infection, potentially leading to overtreatment.

**5. Emerging and Experimental Tests**

- **Loop-Mediated Isothermal Amplification (LAMP):** A rapid molecular test with high sensitivity and specificity, similar to PCR, but does not require complex thermal cycling.
- **Mass Spectrometry:** Techniques like MALDI-TOF (Matrix-Assisted Laser Desorption/Ionization-Time of Flight) are being explored for rapid pathogen identification directly from stool samples.
- **CRISPR-based Detection:** Experimental CRISPR-based tests are being developed for highly specific and rapid detection of toxin genes in *C. difficile*.

**6. Culture-Based Tests:** TC involves first isolating *C. difficile* strains on selective media and then testing them in vitro for toxin production.

- **Anaerobic Stool Culture:** This method involves culturing *C. difficile* under anaerobic conditions, followed by testing for toxin production. While highly sensitive, culture tests are labour-intensive and slow, making them more useful for research and epidemiology than routine diagnosis.
- **Cytotoxigenic Culture (TC):** This combines stool culture with subsequent toxin testing to confirm toxigenic strains specifically. It is highly sensitive but time-consuming and complex.

***Selective Media for Isolation:***

- Commonly used selective media are based on cycloserine cefoxitin fructose agar (CCFA), initially described by George et al.
- Additives like sodium taurocholate or lysozyme may be added to encourage spore germination and improve recovery rates.

### Chromogenic Media:

- Newer chromogenic media offer sensitivity comparable to other selective media, allowing for identification within 24 hours of incubation.

### Incubation Conditions:

- Plates are incubated in an anaerobic environment for 48 hours at 36°C ( $\pm 1^\circ\text{C}$ ).

### Strain Identification:

- Various methods are available for identifying isolated strains:
  - Gallery strips for biochemical profiling
  - Gas-liquid chromatography
  - Latex agglutination to detect GDH enzyme
  - MALDI-TOF mass spectrometry for rapid identification

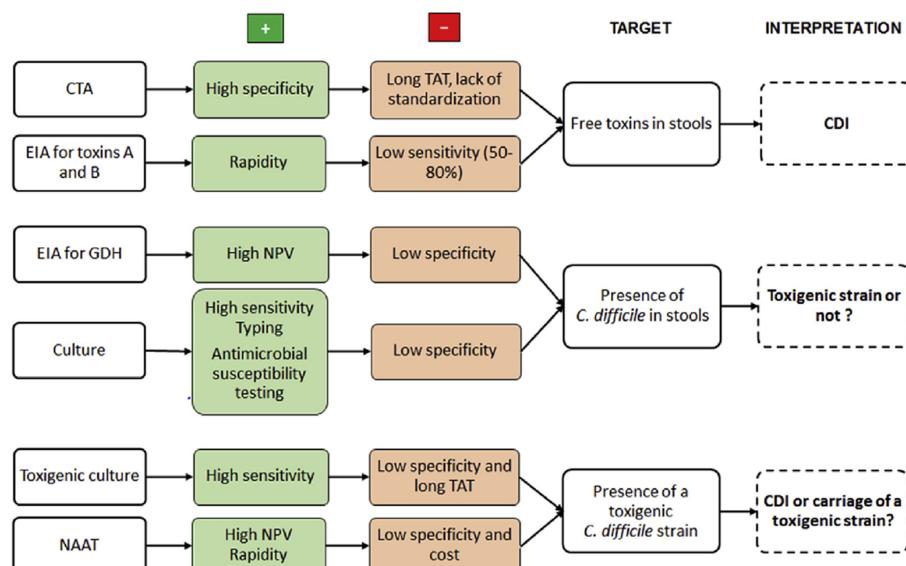
### Toxin Production Testing:

- Once isolated, the strain's pathogenic potential is assessed by testing for toxin production. This can be done either from a suspension of colonies or the supernatant from bacterial growth.

## 5.3

### 7. Emerging and Experimental Tests

- **Loop-Mediated Isothermal Amplification (LAMP):** A rapid molecular test with high sensitivity and specificity, similar to PCR, but does not require complex thermal cycling.
- **Mass Spectrometry:** Techniques like MALDI-TOF (Matrix-Assisted Laser Desorption/Ionization-Time of Flight) are being explored for rapid pathogen identification directly from stool samples.
- **CRISPR-based Detection:** Experimental CRISPR-based tests are being developed for highly specific and rapid detection of toxin genes in *C. difficile*.



**Fig. 1.** Advantages, disadvantages and targets of the different methods used for the diagnosis of *Clostridium difficile* infection (CDI). Abbreviations: CTA, cytotoxicity assay; EIA, enzyme immunoassay; GDH, glutamate dehydrogenase; NAAT, nucleic acid amplification test; NPV, negative predictive value; TAT, turnaround time.

Figure 5.4: CDI diagnosis method (Gateau et al. 2018)

## 5.4 Two-Step and Multi-Step Algorithms

**Two-Step Algorithms:** Combining initial sensitive screening (e.g., GDH or NAAT) with confirmatory toxin testing is recommended to improve diagnostic accuracy, reduce false positives, and ensure symptomatic cases receive treatment.

**Three-Step Algorithm:** Some laboratories use a three-step algorithm where GDH-positive, toxin-negative samples are confirmed with NAAT to reduce false positives from colonization.

## 5.5 Reference Standards:

- CTA and TC are considered the gold standards for detecting free toxins and toxigenic strains, respectively. However, both tests are rarely used in routine practice due to technical challenges and long processing times (TC due to a 2-5 day turnaround), making faster but less sensitive methods like EIA and NAAT more commonly implemented. TC is essential for subsequent strain typing, molecular analysis, and antimicrobial susceptibility testing to inform treatment and epidemiological studies.
- The ESCMID guidelines do not recommend using NAAT as a stand-alone test for *C. difficile* diagnosis but rather use NAAT as a screening test given its high NPV for CDI.
- Most international guidelines agree that Enzyme Immunoassay (EIA) for toxins alone is insufficient for diagnosing CDI and should not be used as a standalone test. The presence of a toxigenic strain without detectable free toxins (toxin-negative, NAAT-positive) in stool raises challenges in clinical interpretation, as not all toxin-negative cases reflect active disease

### Study Findings on Diagnostic Correlation with Clinical Outcomes:

- In a large UK observational study of over 12,000 patients with diarrhoea, those with a positive Cytotoxicity Assay (CTA), indicating free toxin presence, had higher mortality rates and blood leukocyte counts compared to those who were negative for *C. difficile*.
- Toxigenic strain presence without free toxins (TC-positive, CTA-negative) showed no association with increased mortality or clinical complications, indicating that toxin presence in stool correlates more directly with severe CDI outcomes.
- A similar US study found that patients positive for both NAAT and toxin experienced more complications, higher faecal lactoferrin levels, and higher blood leukocyte counts than NAAT-positive, toxin-negative patients. This reinforces that toxin presence, rather than just the toxigenic strain, best indicates active CDI.

### Implications for Asymptomatic Carriers:

- Patients who test positive for a toxigenic strain (TC-positive) but negative for free toxin (CTA-negative) are often classified as potential carriers. While some guidelines recommend isolating these patients to prevent transmission, decisions should be case-specific.
- Although a negative stool toxin test generally suggests no CDI, some studies show that around 11% of patients with toxigenic *C. difficile* but no detectable stool toxin still develop pseudomembranes, a hallmark of CDI, during endoscopic exams. This suggests that toxin tests alone may miss certain CDI cases.

## 5.6 Available Commercial tests

- *C. difficile Quik Chek Complete* (TechLab, Alere), *CERTEST Clostridium difficile GDH + toxin A + B* (Theradiag), and *C. difficile GDH-toxins A-B* (MonlabTest, Orgentec) can simultaneously detect both GDH and toxins A and B.

### Diagnosis Based on Results:

- Negative for Both GDH and Toxins: CDI can be reliably excluded if results are negative for both GDH and toxins.

- Positive for Both GDH and Toxins: Patients with both positive GDH and toxin results are classified as having CDI.
- Inconclusive Results:
  - \* GDH-Negative and Toxin-Positive: This combination is rare, and samples should be retested to confirm results.
  - \* GDH-Positive and Toxin-Negative: For these samples, the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) suggests an optional Nucleic Acid Amplification Test (NAAT) to confirm the presence of a toxigenic *C. difficile* strain.



# Chapter 6

## Treatment and Control Strategies

Current treatment methods include antibiotics (e.g., vancomycin, fidaxomicin), faecal microbiota transplantation (FMT), and emerging therapies that modulate the gut microbiota to restore balance.

Biocide applications targeting environmental control are also crucial. Opportunities lie in developing formulations that interfere with spore germination, biofilm formation, and nutrient acquisition, all of which are key survival mechanisms for *C. difficile*.

**Relevant treatment** (Sholeh et al. 2020)

Antibiotic	Use	Resistance	Reference
Vancomycin	First-line treatment for CDI, administered orally	Resistance is rare	ARIC Journal
Fidaxomicin	First-line treatment, especially if vancomycin is ineffective	Resistance is uncommon	ARIC Journal
Metronidazole	Reserved for mild CDI cases or when other treatments are unsuitable	Rare resistance cases reported	ARIC Journal
Rifaximin	Follow-up treatment after vancomycin to prevent recurrence	Resistance observed	ARIC Journal
Tigecycline	Alternative treatment, especially in severe cases	Resistance is rare	ARIC Journal
Bezlotoxumab*	Monoclonal antibody used with antibiotics to reduce recurrence	Not applicable (not an antibiotic; no resistance issues)	(Johnson et al. 2021)
Clindamycin	Not used for CDI; associated with high CDI risk due to resistance	High resistance in <i>C. difficile</i> strains	ARIC Journal
Fluoroquinolones	Not used for CDI; linked to hypervirulent strains with resistance	High resistance; associated with CDI outbreak strains	ARIC Journal
Cephalosporins	Not used for CDI treatment; often associated with CDI development	High risk of CDI due to gut microbiota disruption. <i>C. difficile</i> strains can show resistance.	ARIC Journal
Penicillins	Not used for CDI treatment; certain types associated with CDI risk	Broad-spectrum penicillins (e.g., ampicillin) may increase CDI risk by altering gut microbiota. Resistance patterns vary.	ARIC Journal

## 6.1 Antibiotic Therapies

### 6.1.1 Vancomycin

- An effective oral antibiotic for CDI, often used for moderate to severe cases. It works by inhibiting bacterial cell wall synthesis, helping to reduce *C. difficile* numbers in the gut.

### 6.1.2 Fidaxomicin

- A narrow-spectrum antibiotic that targets *C. difficile* specifically, preserving other gut microbiota and reducing recurrence rates. Often used for initial treatment and recurrent cases.

### 6.1.3 Metronidazole

- Traditionally used for mild to moderate CDI but now less favoured due to lower efficacy compared to other antibiotics.

### 6.1.4 Rifaximin

- An oral antibiotic that is sometimes used as a follow-up after vancomycin therapy to help prevent recurrence. Rifaximin has limited systemic absorption, primarily acting in the gut, where it targets residual *C. difficile* cells. It is often reserved for patients who have experienced recurrent CDI.

### 6.1.5 Tigecycline

- An intravenous antibiotic for severe or refractory CDI cases, especially when other treatment options have been ineffective. Tigecycline works by inhibiting protein synthesis in bacteria, but its use is generally limited to cases where first-line therapies like vancomycin and fidaxomicin are inadequate.

### 6.1.6 Bezlotoxumab (Monoclonal Antibody)

- While not an antibiotic, this monoclonal antibody targets toxin B produced by *C. difficile*, reducing recurrence risk when used alongside antibiotics.

### 6.1.7 Clindamycin

- While not used for treating CDI, clindamycin is noteworthy because of its association with an increased risk of developing CDI due to its significant impact on gut microbiota. The widespread use of clindamycin has been linked to the selection of *C. difficile* strains resistant to standard antibiotics.

### 6.1.8 Fluoroquinolones (e.g., Ciprofloxacin, Levofloxacin)

- Similar to clindamycin, fluoroquinolones are not used to treat CDI but are known to increase CDI risk. Their use is linked to the emergence of hypervirulent *C. difficile* strains, particularly those resistant to fluoroquinolones. These antibiotics disrupt the gut microbiota significantly, favouring *C. difficile* overgrowth.

### 6.1.9 Cephalosporins

- Not used for CDI treatment but are associated with a high risk of CDI development due to their broad-spectrum activity, which disrupts gut flora. Cephalosporin use is often linked to CDI outbreaks, especially in hospital settings.

### 6.1.10 Penicillins (e.g., Ampicillin)

Broad-spectrum penicillins like ampicillin are associated with increased CDI risk by disrupting gut microbiota balance. Narrow-spectrum penicillins pose a lower risk but are still considered cautiously in patients with a history of CDI.

## 6.2 Microbiota Restoration Therapies

### 6.2.1 Fecal Microbiota Transplantation (FMT)

- FMT involves transplanting stool from a healthy donor into the patient's colon to restore gut microbiota diversity, which helps reestablish colonization resistance against *C. difficile*. This is particularly effective for recurrent CDI.

### 6.2.2 Probiotics

- Certain probiotics containing beneficial bacterial strains like *Lactobacillus* and *Saccharomyces boulardii* may aid in rebalancing gut flora and preventing recurrence, though evidence is mixed on their efficacy in CDI treatment.

### 6.2.3 Microbiota-Based Therapeutic

- Emerging therapies include standardized bacterial consortia or synthetic microbiome capsules (e.g., SER-109), which provide select beneficial gut bacteria to restore a balanced microbiota without the need for donor material.

## 6.3 Toxin-Targeting Treatments

### 6.3.1 Monoclonal Antibodies (e.g., Bezlotoxumab)

- Administered alongside antibiotics, bezlotoxumab targets toxin B, a major virulence factor, to prevent recurrent infections.

### 6.3.2 Vaccines

- Several vaccines targeting *C. difficile* toxins (TcdA and TcdB) are under development, aiming to induce immunity that neutralizes the toxins and prevents infection.

### 6.3.3 Antitoxins

- Although experimental, antitoxin approaches aim to neutralize the effects of TcdA and TcdB in active infections, potentially reducing symptoms and severity.

## 6.4 Antimicrobial Peptides and Small Molecule Inhibitors

### 6.4.1 Antimicrobial Peptides

- Synthetic or host-derived peptides can disrupt *C. difficile* cell membranes, providing a targeted approach that minimizes impacts on the gut microbiota.

### 6.4.2 Small Molecule Inhibitors

- Some research focuses on developing small molecules that inhibit *C. difficile* toxin production or biofilm formation, targeting bacterial processes without killing other gut bacteria.

## 6.5 Bile Acid Modulation and Nutrient Limitation

### 6.5.1 Bile Acid Modifiers

- Treatments that increase secondary bile acid production or inhibit primary bile acids are being studied to reduce spore germination and vegetative cell growth in the gut.

### 6.5.2 Nutrient Competition

- Limiting access to specific nutrients that *C. difficile* relies on, such as trehalose, iron, and zinc, may create an unfavourable environment for bacterial colonization and persistence.

## 6.6 Emerging and Experimental Therapies

### 6.6.1 Bacteriophage Therapy

- Phages that specifically target *C. difficile* are being investigated as a means to reduce bacterial load without affecting other gut bacteria.

### 6.6.2 CRISPR-Cas Systems

- Gene-editing approaches using CRISPR-Cas systems are under exploration to target and deactivate *C. difficile* genes responsible for virulence or survival in the gut.

### 6.6.3 Short-Chain Fatty Acids (SCFAs)

- SCFAs, produced by beneficial gut bacteria, lower gut pH and inhibit *C. difficile* growth. Supplementing SCFAs or promoting SCFA-producing bacteria may help maintain colonization resistance.

## 6.7 Infection Control Measures in Healthcare Settings

### 6.7.1 Rigorous Hygiene and Cleaning

- Use of sporicidal agents (e.g., bleach-based cleaners) on surfaces in healthcare facilities to eliminate *C. difficile* spores and prevent transmission.

### 6.7.2 Hand Hygiene

- Handwashing with soap and water is recommended over alcohol-based sanitizers, as alcohol is less effective at killing *C. difficile* spores.

### 6.7.3 Isolation Precautions

- Infected patients are often isolated, and healthcare workers use protective equipment (e.g., gloves, PPE) to prevent cross-contamination.

### 6.7.4 Antibiotic Stewardship

- Reducing unnecessary antibiotic use to prevent disruption of gut microbiota and lower the risk of CDI, particularly in hospitals and long-term care facilities.

## 6.8 Infection Control Measures in Community Settings

- Strategies include monitoring *C. difficile* in livestock and regulating antibiotic use in agriculture
- Enhanced Surveillance in Travel and Healthcare Settings: Cross-border ribotype tracking, especially in regions with high patient mobility, has become a priority to prevent international spread of hypervirulent ribotypes.

## 6.9 Modelling control

Stochastic models, specifically MCMC and Bayesian inference, enable accurate CDI tracking by accommodating random infection spread, handling data gaps, and analyzing population-wide immunity. These methods provide insight into how infection risks change and how

interventions might affect overall outcomes, essential for devising targeted infection control strategies in hospital settings

- Stochastic Models handle randomness, making them ideal for unpredictable events like concert arrivals or sporadic CDI cases.
- Monte Carlo Simulations explore numerous possible scenarios by running repeated trials, providing a range of outcomes and highlighting patterns.
- Bayesian Statistics incorporate both observed and missing data to continually refine predictions, helping address uncertainties in real-life and medical contexts.

By combining these approaches, epidemiologists and event planners alike can make better-informed decisions, accounting for randomness, refining their models with each new data point, and exploring a wide range of possible scenarios.

### 6.9.1 Spatio-temporal Time Series Models:

Spatio-temporal models are mathematical tools used to understand how CDI infections spread over time and across different locations, which is crucial for developing effective infection control strategies.

- These methods, like STARIMA (Spatiotemporal Autoregressive Integrated Moving Average), rely on autocorrelation, meaning they look at how sequential data points (e.g., infection cases over time) are related. They create rules to describe trends over time without assuming specific biological mechanisms of disease spread.
- Advantages: These models are good for examining long-term trends, especially in large datasets where values change smoothly, like annual counts of *C. difficile* cases across a country.
- Limitations: These models smooth out random fluctuations and struggle with “spiky” data (e.g., sudden, sporadic outbreaks). They are also sensitive to missing data, which can weaken their predictions.

### 6.9.2 Deterministic Models:

- These models work by defining transition rates between disease states (e.g., susceptible to infected, infected to resistant). This approach is based on the biological understanding of infection dynamics and can be applied widely in epidemiology.
- Advantages: Deterministic models allow researchers to include assumptions about how infections progress. They work well with complete data and allow for testing different scenarios, helping evaluate the impact of risk factors like patient proximity.
- Limitations: Similar to time series models, deterministic approaches smooth out data. This is fine for analyzing large datasets (e.g., national case numbers), but not useful for small datasets where slight fluctuations can impact the results (e.g., cases in a single hospital ward).

### 6.9.3 Stochastic Models:

- Newer mathematical methods can handle stochastic events, or random chance variations. These are particularly useful in cases with small numbers where minor fluctuations (like one or two extra cases) are significant, such as tracking real-time cases in a single hospital unit.

Stochastic models, especially Markov chain Monte Carlo (MCMC) methods, are valuable for understanding the spread of *Clostridioides difficile* (*C. difficile*) infections in hospital settings because they incorporate randomness, essential for accurately modelling outbreaks that are irregular in time and space. Here's how these concepts relate and can be organized logically:

#### 6.9.3.1 1. Why Stochastic Models for CDI?

- CDI outbreaks often occur in clusters (short bursts in specific locations), with varying outbreak sizes. For example, outbreaks can range widely, from a few cases to hundreds.

- Unlike deterministic models, which smooth out data and are suitable for larger datasets (like annual national cases), stochastic models are better at capturing the random fluctuations of sporadic outbreaks. These models incorporate chance events, making them useful when patient cases are limited or unpredictable.

#### **6.9.3.2 2. Using Markov Chains to Model CDI**

- Markov Chains are mathematical sequences that model how an event (like infection spread) moves from one state to another (e.g., susceptible to infected). Originally used in atomic physics, Markov chains also apply to infections, as they can simulate random “chain reactions” similar to how infectious particles spread among susceptible individuals.
- By implementing continuous-time data (where events are tracked even if observation intervals vary), Markov models track infections realistically, modeling the disease’s rapid spread and clustering over time.

#### **6.9.3.3 3. Monte Carlo Simulations and Bayesian Inference**

- Monte Carlo Simulations allow multiple simulations of a Markov chain. This approach enables modelers to examine numerous potential outcomes based on the same infection spread model.
- Bayesian inference further supports these models by estimating missing information and hidden states, which is essential in infection tracking. Bayesian methods in CDI modeling can:
  1. Infer events between observation times, filling in the gaps.
  2. Estimate the effects of missing data, accounting for unobserved cases.
  3. Identify “hidden” infection states (e.g., whether certain individuals are resistant or susceptible).

#### **6.9.3.4 4. Application to Population Immunity**

- Stochastic models consider the “herd immunity” effect, assessing infection risk across the whole population rather than isolated individuals. The population’s overall susceptibility or resistance influences infection dynamics, which is essential for evaluating preventative measures.
- When the population is close to a critical susceptibility threshold (where slight changes drastically impact infection rates), having complete data is crucial. Bayesian methods help manage this by reducing missing data, allowing for precise model parameters.

## **6.10 Novel simulator for tracking HAI**

A realistic approach that includes patient interactions, hospital layout, and infection control policies. Traditional models for studying infection spread often use compartmental epidemiological models or contact networks, which are useful but lack essential hospital-specific details, such as patient location tracking, shift patterns, and spatial configurations. By integrating these overlooked aspects, this simulator provides a more practical and tailored model for hospital settings.

#### **6.10.1 Key Features of the Simulator:**

- Agent-Based Patient Modeling: Each patient is represented as an agent with individual characteristics, creating a more personalized and dynamic simulation of disease spread.
- Spatial-Temporal Hospital Constraints: The model incorporates physical hospital layout and movement constraints, making it possible to simulate infection spread realistically across different wards or rooms.
- Microorganism Behavior Models: The spread and behaviour of pathogens are modelled based on epidemiological principles, enabling realistic infection dynamics.

### 6.10.2 Model Validation and Utility:

The model's accuracy was assessed through several methods:

- Micro and Macro-Face Validation: Ensuring that the model aligns with both fine-grained (micro) and broader-scale (macro) expectations.
- Parameter Calibration: Adjusting model parameters based on literature to improve alignment with known infection dynamics.
- Sensitivity Analysis with Expert Input: Working with experts to refine the model and assess its reliability across various scenarios.

This simulation approach enables hospitals to monitor infection spread, detect patterns over time and space, and use predictive data to make more informed decisions about infection control policies. By addressing data gaps with simulated data, this model compensates for the limited high-volume clinical datasets currently available for AI-driven infection control solutions.



# Chapter 7

## Host immune response

The host response to CDI is driven predominantly by innate immune cells, especially innate lymphoid cells (ILCs) and neutrophils, which are essential for early containment of the infection and tissue repair. Adaptive immunity, including antibody production, is more critical for long-term protection and reducing recurrence rather than for resolving acute infection. As a result, the immune response to *C. difficile* often controls the bacterium's damage without completely clearing it, which can lead to persistent colonization.

The immune response to CDI involves a well-coordinated effort by both innate and adaptive immune systems. Innate immune cells, especially ILCs and neutrophils, are crucial for the initial control of infection and tissue repair, while adaptive immunity, primarily through antibody production, plays a key role in reducing recurrence. The inflammatory response, although essential for managing the infection, needs to be carefully balanced to avoid excessive tissue damage and further complications. Persistent colonization remains a challenge, highlighting the immune system's role in managing rather than fully eradicating *C. difficile* in the gut.

### 7.0.1 Early Cellular Response

- **Barrier Disruption:** *C. difficile* toxins TcdA and TcdB disrupt the epithelial barrier of the colon, increasing gut permeability and allowing bacterial and toxin translocation into deeper tissues.
- **Immune Cell Recruitment:** Damaged epithelial and resident immune cells release pro-inflammatory cytokines and chemokines (e.g., IL-8, CXCL1, CXCL2), which recruit innate and adaptive immune cells to the infection site.

### 7.0.2 Innate Immunity

#### 7.0.2.1 Innate Lymphoid Cells (ILCs)

- ILCs play a critical role in defending against CDI by responding to early inflammatory signals like IL-1, IL-12, and IL-23. They produce cytokines such as IL-22, IL-17a, IFN-, and TNF, which activate neutrophils and macrophages. These cytokines also promote antimicrobial peptide production, release of reactive oxygen (ROS) and nitrogen species (RNS), and help in epithelial repair.
- **Significance of ILCs:** Studies with Rag1<sup>-/-</sup> mice (which lack T and B cells) indicate that recovery from CDI can occur without adaptive immunity. However, in mice lacking both ILCs and adaptive immune cells (Rag1<sup>-/-</sup>Il2rg<sup>-/-</sup>), high mortality rates suggest that ILCs are crucial for early defense against *C. difficile*.

#### 7.0.2.2 Key ILC Subtypes

- **ILC1s:** Produce IFN-, enhancing macrophage activity and promoting phagocytosis.
- **ILC3s:** Secrete IL-22, which stimulates antimicrobial peptides and activates the complement system to clear bacteria that translocate to organs like the liver and lungs.

### 7.0.2.3 Role of Neutrophils

- Neutrophils are rapidly recruited to the infection site in response to cytokines (e.g., IL-23 and GM-CSF) and play a crucial role in CDI defence. They release ROS in response to *C. difficile* toxins, particularly TcdB, and produce IFN-, which boosts macrophage bactericidal activity.

### 7.0.3 Adaptive Immunity

#### 7.0.3.1 Antibody Production

- Although T and B cells are not essential for resolving acute CDI, toxin-specific antibodies (IgA and IgG) contribute to reducing disease severity and recurrence. Higher antibody levels are associated with reduced risk of recurrence.
- While T cells are not central to acute recovery, they support long-term immunity. Mice deficient in MHC II, which affects CD4+ T cell function, show reduced toxin-specific antibody responses and weaker secondary infection protection, indicating that CD4+ T cells support adaptive immune memory.
- **Monoclonal Antibody Therapy:** Clinical trials have shown that monoclonal antibodies targeting TcdA and TcdB reduce recurrence rates in CDI patients, highlighting the protective role of antibodies in long-term infection control.

### 7.0.4 Defense Mechanisms

- **Antimicrobial Peptides and ROS/RNS Production:** Immune cells produce antimicrobial peptides, ROS, and RNS to combat *C. difficile* and prevent further translocation. RNS can modify the cysteine protease domains of TcdA and TcdB through S-nitrosylation, inhibiting toxin activation by preventing the release of glucosyl transferase domains (GTDs) in the cytosol.
- **Toxin Neutralization:** This modification limits the toxins' potency, decreasing cellular damage and mitigating disease severity.

### 7.0.5 Inflammatory Cascade and Cytokine Production

- **NF- B and AP-1 Pathways:** Toxins trigger NF- B and AP-1 pathways in epithelial cells, leading to the production of various pro-inflammatory chemokines that attract more immune cells to the infected area.
- **Inflammasome Activation:** TcdB's glycosylation activity inactivates RHO GTPases, which are detected by the pyrin receptor in epithelial cells. This detection activates the inflammasome, causing the release of IL-1, a cytokine that amplifies the inflammatory response and recruits additional immune cells.

### 7.0.6 Balancing Inflammation

- **Protective vs. Harmful Inflammation:** While inflammation is essential for controlling CDI, excessive ROS production can exacerbate epithelial damage. Studies show that mice lacking the pro-inflammatory cytokine IL-23 have better survival outcomes, suggesting that moderate inflammation is protective, while excessive inflammation can worsen tissue damage.
- **Influence of Disease Variables:** Variables such as the type of antibiotic pre-treatment, *C. difficile* inoculum composition, and specific experimental protocols influence disease severity. These insights can help develop therapeutic strategies that aim to balance the immune response, minimizing tissue damage while effectively controlling infection.

### 7.0.7 Persistence of Colonization

- **Incomplete Clearance:** Even in hosts with competent immune systems, *C. difficile* may persist in the gut post-infection. The immune response generally limits the infection and aids in tissue repair, rather than completely clearing *C. difficile*, which may lead to asymptomatic colonization or recurrence if conditions favor bacterial growth again.

#### 7.0.8 Host Factors and Personalized Risk Profiles

- Genetic Susceptibility: Advances in genetic studies have identified specific host genetic markers, such as variations in immune response genes (e.g., TLR4, IL-8), that are associated with increased CDI susceptibility. This research has implications for personalized risk assessment and targeted prevention strategies.
- Immune Response Modulation: Studies reveal that certain immune-modulating treatments, such as checkpoint inhibitors used in cancer, can increase CDI susceptibility. Understanding immune responses to *C. difficile* in these populations is leading to new preemptive treatment strategies, such as prophylactic probiotics or FMT in at-risk patients.



# Chapter 8

# Future Research Directions and Opportunities

Ongoing research focuses on advancing diagnostics, including rapid toxin detection and identifying biomarkers for asymptomatic carriers. Other areas include developing vaccines, toxin-neutralizing monoclonal antibodies, and predictive infection tracking models, which can support both preventative and reactive measures against CDI outbreaks in healthcare settings.

- Improving Diagnostic Accuracy: Advanced methods to distinguish between true infections and asymptomatic carriage are needed to refine CDI management, prevent over-treatment, and accurately assess infection prevalence.
- Understanding Transmission Pathways: Whole-genome sequencing offers promise in tracing CDI sources, especially in community-onset cases, to mitigate hospital and environmental sources.
- Global Surveillance and Standardization: Uniform typing and reporting standards would help track and manage the spread of hypervirulent strains worldwide.

## 8.1 Modelling

We can build a robust infection prediction model for *C. difficile* that not only forecasts future trends but adapts dynamically to real-world changes, making it an effective tool for infection prevention and control.

### Step 1: Gather Data from Past Outbreaks

- Collect historical data on *C. difficile* cases, including infection rates, timing, affected areas, and patient demographics. Data should ideally span multiple years to capture seasonal and environmental variations.
- Identify key factors that influence outbreaks, like antibiotic use rates, hospital occupancy, and infection control measures.

### Step 2: Develop a Stochastic Model

- Model randomness by setting up a basic framework that accounts for chance events, such as sudden spikes in cases due to high patient turnover or lapses in infection control.
- Define infection stages for patients (e.g., susceptible, infected, resistant) to track transitions over time, helping you simulate patient status changes in a hospital environment.
- Include cluster effects, assuming that cases can spread more quickly in specific wards or during particular times.

### Step 3: Set Up Monte Carlo Simulations

- Run thousands of simulations, where each simulation represents a possible future infection scenario.

- Vary parameters like transmission rates, seasonal changes, patient flow, and random chance events in each simulation. For instance, one simulation might assume an increase in antibiotic resistance, while another might model stricter infection control practices.
- Analyze results across simulations to identify patterns and ranges, like peak infection times, high-risk areas, and potential yearly trends.

#### **Step 4: Apply Bayesian Statistics for Uncertain Data**

- Use prior knowledge about infection dynamics, like average infection rates or patient susceptibility, to establish a baseline prediction.
- As new data arrives in 2025, update predictions continuously. For example, if infections spike unexpectedly, Bayesian methods will adjust your model to reflect the new risk level.
- Bayesian methods allow you to handle missing or incomplete data, estimating infection risks even when some patient details (like exact susceptibility) are unknown.

#### **Step 5: Incorporate Real-Time Data for Dynamic Tracking**

- Collect real-time data from hospital records, including newly confirmed infections, antibiotic prescriptions, and patient movements. This data feeds directly into your model.
- Run daily or weekly simulations based on this updated information, adjusting risk predictions and identifying outbreak clusters as they develop.

#### **Step 6: Analyze and Interpret Predictions**

- Review Monte Carlo outcomes to understand the range of possible infection trends and likelihoods for each scenario (e.g., if a severe outbreak could happen in winter vs. summer).
- Identify high-risk periods and areas within the hospital or community based on the model's output, allowing for targeted preventative measures.

#### **Step 7: Create Actionable Interventions**

- Based on predictions, develop response plans for high-risk times, which might include increasing infection control measures or adjusting staff protocols.
- Use Bayesian-influenced predictions to adapt plans in real-time as new data on cases and treatment effectiveness becomes available.

### **8.1.1 Feasible software**

#### **8.1.1.1 R (with packages like EpiModel, spdep, INLA, and rstan):**

- EpiModel: Designed for infectious disease modelling, with support for stochastic simulations and compartmental models.
- spdep: Useful for spatial data analysis, allowing you to incorporate geographic data and visualize the spread of infections.
- rstan: Integrates with Bayesian models, ideal for applying Bayesian inference to real-time data.
- INLA: Great for handling spatio-temporal data, allowing you to incorporate both time and location-based data.

#### **8.1.1.2 Python (with libraries like PyMC3, EpiPy, and Geopandas):**

- PyMC3: Offers Bayesian modelling tools, allowing you to set up complex Bayesian inference models.
- EpiPy: Specialized for epidemiology, letting you model infectious disease dynamics, including stochastic and deterministic frameworks.
- Geopandas: Useful for managing spatial data to understand geographical patterns and create spatio-temporal models.

### 8.1.1.3 AnyLogic:

- A versatile software for developing agent-based models, system dynamics models, and discrete-event simulations. Useful for detailed simulations involving patient flow and disease transmission in healthcare settings.

### 8.1.1.4 Matlab (with the Simulink and SimEvents toolboxes):

- Matlab is powerful for mathematical modelling and can support stochastic and deterministic simulations. Simulink can be used to visualize the model flow, while SimEvents is great for time-based simulations.

### 8.1.1.5 STATA:

- While more commonly used for statistical analysis, STATA's support for time-series analysis and spatial modelling can assist with simpler, deterministic models.

## 8.1.2 Modified SEIR Model with a Carrier State (SECIR Model)

To start predicting *C. difficile* infections, the SEIR (Susceptible-Exposed-Infected-Recovered) model can be a strong base, especially for capturing infection dynamics with latent periods. Given the characteristics of *C. difficile*, we can modify the SEIR model with an additional “Carrier” state to represent asymptomatic carriers who might still spread the infection.

The modified model includes compartments for:

- S (Susceptible): Individuals at risk of infection.
- E (Exposed): Individuals who have been exposed but are not yet infectious.
- C (Carrier): Asymptomatic carriers who can transmit the infection without showing symptoms.
- I (Infected): Symptomatic individuals who can transmit the infection.
- R (Recovered): Individuals who have recovered and are assumed to have temporary immunity.

The equations for this SECIR model are as follows:

1. Susceptible:  $\frac{dS}{dt} = -\beta(S \cdot I + S \cdot C)$
2. Exposed:  $\frac{dE}{dt} = \beta(S \cdot I + S \cdot C) - \sigma E$
3. Carrier:  $\frac{dC}{dt} = \alpha \sigma E - \gamma C$
4. Infected:  $\frac{dI}{dt} = (1 - \alpha) \sigma E - \delta I$
5. Recovered:  $\frac{dR}{dt} = \gamma C + \delta I$

Where:

- $\beta$  = Transmission rate from infected or carriers to susceptible individuals.
- $\sigma$  = Rate at which exposed individuals become either carriers or symptomatic.
- $\alpha$  = Proportion of exposed individuals who become carriers.
- $\gamma$  = Recovery rate for carriers.
- $\delta$  = Recovery rate for symptomatic infected individuals.

Figure 8.1: Simulation for hospital infection spread and outbreak (Kim et al. 2023)

### Why This Model Works for *C. difficile*

- Carrier State: Many *C. difficile* cases are asymptomatic but can still spread the bacteria, so a carrier compartment reflects this dynamic.
- Exposed and Incubation: Including an “Exposed” state captures the latent period between exposure and either becoming symptomatic or a carrier.
- Spatio-temporal Adaptations: Adding spatial data can help predict where infections may spread in hospital settings, using software like R or Python with spatial libraries.



# Chapter 9

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#### Text

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