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**Clostridium difficile** infection: advances in epidemiology, diagnosis and understanding of transmission.

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Key points:

- *C. difficile* infection (CDI) is a continually evolving global healthcare problem.
- Community onset CDI is increasing and multiple potential reservoirs of infection exist including environmental sources, animals, asymptomatic patients and symptomatic patients.
- Highly discriminatory typing techniques such as whole-genome sequencing and multi-locus variable-number tandem-repeat analysis offer the potential for illuminating previously under-recognized routes of *C. difficile* transmission.
- The optimal approach to sampling and testing for CDI remains a contentious issue.
- Multistep algorithms are recommended to improve diagnostic sensitivity and specificity.

Abstract

*Clostridium difficile* infection (CDI) continues to affect many patients in hospitals and communities worldwide. Modern technologies, such as whole genome sequencing, are helping to track *C. difficile* transmission across healthcare facilities, countries and continents. However, comparison of CDI epidemiology between countries is challenging due to the varied approaches to sampling and diagnosis. In this Review, we describe recent advances in the understanding of *C. difficile* epidemiology, transmission and diagnosis and discuss the impact these developments have on clinical management of CDI.

Introduction

*Clostridium difficile* is an anaerobic spore-forming Gram-positive bacillus which is able to colonise and proliferate in the human gut, especially following changes in the indigenous colonic microbiota.
after antibiotic use. The period from spore ingestion to symptom onset is typically short,² and is
dependent on microbiological, biochemical and immune factors present in the patient’s bowel
(figure 1). Importantly, human ingestion of spores does not always result in symptomatic infection.
The clinical outcome after spore acquisition is variable and ranges from transient colonisation of the
gut and persistent asymptomatic carriage to fulminant disease.²,³ These asymptomatic carriers of *C. difficile* provide a potential reservoir for onward transmission, especially within a hospital
population.³

*C. difficile* is a genetically diverse species,⁴ including both pathogenic (toxin-producing) and non-
pathogenic strains (figure 1). Thus, diagnostic tests for CDI should ideally detect only strains (or their
toxins) that have the potential to cause disease. Clinical infection develops when successful
germination of *C. difficile* spores results in toxin production within the gut lumen. The actions of two
protein exotoxins, TcdA (toxin A) and TcdB (toxin B), disrupt colonic epithelial cells and stimulate the
release of pro-inflammatory cytokines and chemokines. This leads to an intense inflammatory
response causing acute inflammation of the large intestine. The spectrum of clinical disease ranges
from mild diarrhoea to toxic mega-colon (grossly dilated bowel), colonic perforation and death.
Recent research suggests that both strain characteristics and the host’s immune response influence
CDI severity, recurrence risk and mortality.⁵-⁷

Recently, several advances have contributed to a new understanding of CDI. Its epidemiology is now
recognised to be heterogeneous in terms of incidence and strain types reflecting different stages of
epidemic spread.⁸ Novel fingerprinting techniques, notably whole genome sequencing (WGS) and
multi-locus variable-number tandem-repeat analysis (MLVA), have indicated that *C. difficile* can be
acquired from a wide range of sources,⁹ possibly including undiagnosed symptomatic cases,
asymptomatic carriers (including infants), animals and food. Historically, CDI diagnosis has been
complicated by the wide range of commercial tests available, and the different bacterial targets
detected. In this review, we summarize the key advancements in CDI epidemiology, diagnosis and
understanding of transmission.

**C. difficile epidemiology**

*C. difficile* was first identified as a pathogen related to antibiotic-associated diarrhoea in the late
1970s.¹⁰ In the last decade of the 20th Century, CDI incidence escalated,¹¹ becoming a well-publicised
cause of hospital-acquired infection in developed countries. From 2000 onwards, there was a further
rise in CDI dominated by epidemic strains with increased pathogenicity leading to high transmission rates, increased severity and greater mortality.12

Today, there are on average 7 CDI cases for every 10,000 overnight patient stays in European hospitals.13 The incidence is similar in the US where C. difficile is the leading cause of hospital associated infection, with an estimated 14,000 deaths each year.14,15 Approximately 4-10% of patients are colonised with toxigenic C. difficile on admission to a healthcare facility; this proportion rises during their stay but is extremely variable between institutions, regions and countries.16-18 The risk of acquisition is known to increase with proximity to a symptomatic case, increasing age and longer admissions.19-20

In recent years, the proportion of CDI occurring in patients outside the hospital setting has increased suggesting endemicity in the wider community, not just in hospital attendees.21 Studies have shown that community-associated CDI has been shown to affect younger, healthier patients who are less likely to have been exposed to antibiotics compared with hospital-acquired cases.21-25 A large US study (15,451 CDIs) recently demonstrated 48 community cases per 100,000 population, higher than previously reported.26 In this study, a third of CDIs were community-associated and only 24.2% of cases became symptomatic during hospitalisation. This study may have over-diagnosed cases by using a highly sensitive molecular assay that fails to differentiate between C. difficile colonisation and disease. However, these data support the view that the boundaries between hospital and community CDI are becoming less distinct.

**Global epidemiology**

First recognised in 2002, C. difficile BI/NAP1/027 clones (Box 1) have caused large epidemics across the developed world with significant morbidity and mortality.8,12 In addition to toxins A and B, this strain produces binary toxin (known as C. difficile transferase [CDT]), also produced by a number of other strains (e.g. ribotype 078, 023).27 Infection caused by these binary toxin-producing strains has been associated with increased disease severity and 30 day mortality (though it is not clear whether it is binary toxin which contributes to adverse outcome, or other, as yet unknown factors).28-30 The incidence of BI/NAP1/027 has recently fallen in some areas of Western Europe (figure 2), due to a reduction in the total incidence of CDI as well as a reduction in the proportion of cases attributed to this strain type.31-32 However, BI/NAP1/027 persists in North America and is increasing in other areas, especially Eastern Europe.13,33-35
In Australia, similar binary toxin-producing strains, such as ribotypes 244, have recently emerged, but interestingly, *C. difficile* BI/NAP1/027 has yet to become established. The reasons for this are unclear, but this may reflect the relative pressures of antimicrobial selection of particular strains. For example, *C. difficile* BI/NAP1/027 is relatively fluoroquinolone resistant and these antibiotics are infrequently prescribed in Australia. In Asia, non-binary toxin strains such as ribotypes 017, 018 and 014 remain dominant. Further understanding of global CDI epidemiology is hindered by a lack of surveillance, especially in the developing world.

In the UK, where CDI surveillance is mandatory, the prevalence of *C. difficile* BI/NAP1/027 has markedly decreased since 2007 (figure 2). It has not been superseded by an alternative dominant strain, but instead, a picture of increasing diversity of strains has emerged. Such heterogeneity is well documented elsewhere; a review of 6 US centres demonstrated 98 ribotypes in 720 toxigenic isolates and an Australian study showed 32 ribotypes in 70 samples. Such observations may simply reflect the epidemiology of *C. difficile* prior to the emergence of successful clones.

Using *C. difficile* typing for epidemiological investigations

*C. difficile* strains can be differentiated by a variety of typing techniques (Box 1) which can be used to scrutinise epidemiologically linked cases. However, challenges arise when attempting to standardise these techniques between laboratories, particularly when typing methods depend on gel banding patterns (e.g. PFGE). Multi-locus sequence typing (MLST), described by both Griffiths *et al* and Lemee *et al* in 2010, is a typing strategy which overcomes these challenges by sequencing multiple housekeeping gene fragments and using an internet-accessible database to interpret results. MLST has the additional benefit of grouping strains into ‘clades’ based on common molecular lineage. A study looking at CDI outcomes based on clades demonstrated interesting differences in disease severity and mortality (25% 14 day mortality rate in clade 5 [ST11, ribotype 078] vs. 7% in clade 3 [ST 22/5, ribotype 023]).

It is important to note that, whilst there is some concordance between typing methods (as for strain BI/NAP1/027), there are also key differences that reflect the different aspects of the genome targeted by each technique. Ribotyping uses the 16S-23S intergenic spacer region in the ribosomal RNA gene complex; it is the preferred *C. difficile* typing method in Europe and Australia and its use has recently increased in North America. MLST and ribotyping have similar discriminatory powers but different ribotypes may be seen as a single strain by MLST, and vice versa. For instance, ribotype 014 falls into a number of sequence types (ST-2, ST-14, ST-50 and ST-132) and ST2 includes...
multiple ribotypes (ribotype 014, 020, 076, 220, 095, 006). Similar problems arise when comparing results from other typing strategies such as PFGE, REA and MLVA (Box 1), thus hindering investigations into global epidemiology.

New \textit{C. difficile} typing methods, such as Whole Genome Sequencing (WGS), provide a high level of discrimination between strains aiding epidemiological investigations.\textsuperscript{4,8,9} \textit{C. difficile} genomic evolution is estimated to occur at a rate of 0.74-1.\textsuperscript{9,46}9 single nucleotide variants (SNVs) per year, and therefore near identical strains (e.g. <2 SNVs apart) in two individuals strongly imply transmission or recent exposure to a common source, strains >10 SNVs are highly likely to be distinct.\textsuperscript{9} For interpretation of strains between 3-10 SNVs, the time between potential donor and recipient samples should be considered in the context of the \textit{C. difficile} evolutionary rate. WGS can also be used to assemble phylogenetic trees to investigate the common origins of clinically relevant \textit{C. difficile} strains. As an example, WGS has been used to demonstrate transcontinental spread of two distinct lineages of the BI/NAP1/027 strain.\textsuperscript{8} One lineage (FQR1) originated in the north eastern US and was transmitted to South Korea and Switzerland. A second lineage (FQR2) demonstrated wider spread with multiple trans-Atlantic transmission events between the US and Europe, some of which led to well publicised CDI outbreaks in the UK and cases in Austria, Poland and the Netherlands. The spread of both strains was preceded by the acquisition of fluoroquinolone resistance, likely due to the selection pressure of high level use of this antibiotic in North America.\textsuperscript{8} This study highlights the influence of global travel on the spread of emerging infectious organisms and suggests there is a growing need for an internationally uniform approach to \textit{C. difficile} typing.

\textbf{CDI transmission}

Both host susceptibility and strain characteristics are likely to contribute to the probability of effective \textit{C. difficile} transmission, and subsequent infection, when two individuals come into contact (figure 1).\textsuperscript{5,47} The host variables associated with increased CDI risk are well characterised, and include increasing age, antibiotic use and co-morbidities.\textsuperscript{47} The characteristics leading to the success of particular strains are less well understood.

Host susceptibility to CDI is known to be enhanced by changes in the host intestinal microbiota, often following hospital admission and/or antibiotic exposure.\textsuperscript{48} Recent analysis of the gut microbiome using 16S ribosomal RNA sequencing demonstrates a reduction in the diversity of host bacteria and altered predominant species in patients with CDI compared to those with either asymptomatic \textit{C. difficile} colonisation or healthy subjects.\textsuperscript{49} Antimicrobial-induced dysbiosis also results in loss of protective toll-like receptor (TLR) signalling, accumulation of pro-inflammatory T
helper 17 (Th17) T cells and increased epithelial permeability. Thus, subsequent infection with C. difficile leads to toxin-mediated epithelial injury and perpetuation of the pro-inflammatory response.

C. difficile sporulation and germination
Currently, we have a limited understanding of the reasons why some C. difficile strains have led to large transatlantic epidemics (i.e. BI/NAP1/ribotype 027), whilst 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 may influence the success of some strains.

C. difficile spores are a key feature in transmission. Once ingested, spores interact with small molecular germinants, such as bile acids, triggering a series of events committing the spore to germinate into toxin-producing bacteria. Recently, a germination-specific protease, CspC, has been shown to play an active and essential role in germination by functioning as the C. difficile bile acid germinant receptor. Recent data has also begun to elucidate the activity of Spo0A, the master regulator of sporulation, which controls the transition of the bacterium into the spore form. Spo0A has also been implicated in controlling toxin gene expression, intestinal colonization and disease in mice. There is early evidence that SpoOA may vary between ribotypes but further research is required to confirm the influence this may have on transmission and clinical disease.

Toxins, immunity and C. difficile disease
Investigations seeking to link quantitative toxin production with C. difficile virulence have not been conclusive. The clinical impact of a particular C. difficile strain is related not only to its pathogenicity, but also to the host immune response to toxin. Toxins A (TcdA) and B (TcdB) stimulate the release of multiple pro-inflammatory cytokines and chemokines (e.g. interleukin (IL)-1β, tumour necrosis factor alpha (TNF-α), IL-8, IL-12, IL-18, IL-23, macrophage inflammatory protein 1α (MIP-1α), MIP-2, leptin) from epithelial cells and mucosal immune cells. This inflammatory response is a major determinant of disease severity and has recently been shown to correlate with persistent diarrhoea and poor clinical outcome. Importantly, toxins A and B are themselves targets of immune recognition. Antibody-mediated responses to toxins have an important role in determining asymptomatic carriage and predisposition to recurrent infection. Symptomless carriers of toxigenic C. difficile and those with a single episode of CDI show more robust antitoxin immune responses than those with symptomatic and recurrent disease. Recently, circulating TcdA and TcdB-specific
memory B cells have been detected following CDI, strengthening the evidence for the importance of
the humoral immune response against both toxins.\textsuperscript{74}

\textit{Hospital acquired infection}

Control of CDI has conventionally centred on symptomatic cases, especially during hospital
outbreaks, since these are the most recognizable sources of transmission. Whilst the exact temporal
relationship between antibiotic exposure, spore exposure and symptom onset is unknown in most
instances, a recent study found that most infectious periods for potential donors to support
transmission of \textit{C. difficile} were $\leq$1 week (65%), with only 10% >8 weeks.\textsuperscript{1} Most incubation periods in
recipients were $\leq$4 weeks (61%), with few >12 weeks (13%).\textsuperscript{1}

CDI transmission between symptomatic patients has recently been studied in Oxford, UK, using
detailed epidemiological data and WGS of consecutive isolates.\textsuperscript{9} The analysis revealed that 45% of
new cases were genetically distinct from all previous cases and only 13% had recent ward contact
with a previous sequence matched case ($\leq$ 2 SNVs).\textsuperscript{9} Many genetically–matched cases showed no
epidemiological relationship (either hospital or community-associated) which suggests alternative
sources of infection.\textsuperscript{9} This study used a suboptimal diagnostic test (a toxin enzyme-immunoassay),
which lacks sensitivity, and it is likely that a proportion of true CDI cases will have remained
undiagnosed. However, these findings have been confirmed by a confirmatory UK study using a
reference standard diagnostic test\textsuperscript{75} and alternative sources of \textit{C. difficile} in the healthcare setting
are likely, potentially including untested symptomatic patients, asymptomatic patients,
environmental contamination and healthcare workers (HCWs).

The contribution of asymptomatic carriers to transmission has been confirmed in a recent study
from Pittsburgh, US, in which 10.4% of inpatients carried \textit{C. difficile} regardless of their symptom
profile.\textsuperscript{17} MLVA typing (Box 1) showed that a third of diagnosed CDI cases had been in recent contact
with a symptomatic patient, whilst another third had had contact only with an asymptomatic \textit{C.
\textit{difficile} carrier}.\textsuperscript{17} This confirms earlier work suggesting a significant role for asymptomatic patients.\textsuperscript{3}

Larger multi-centre studies are needed to fully appreciate the impact of asymptomatic
colonisation/carriage. A recent meta-analysis of North American studies suggests that toxigenic \textit{C.
\textit{difficile} carriage on admission increases the risk of subsequent CDI six-fold (21.8% vs. 3.4%, p=0.03),
contrary to previous understanding that carriage reduced CDI risk.\textsuperscript{22} Notably, at present we do not
have a proven intervention to address such individuals, and the resource implications for global
sampling and source isolating asymptomatic patients are daunting.
Another possible source of CDI transmission is health care workers (HCWs). Although asymptomatic intestinal *C. difficile* colonisation in HCWs is generally uncommon, other factors, such as poor hand hygiene, have been shown to play a significant role in CDI transmission.\(^7\)\(^6\) Taken together, these data suggest that source isolation of symptomatic CDI patients and control of epidemic strains, although essential interventions, may no longer be sufficient to further reduce the burden of disease in settings where outbreaks are uncommon. Further investigation of alternative sources of CDI in the hospital setting is necessary, with a focus on asymptomatic and untested patients. Alternative approaches, such as controlling exposure to antibiotics and reducing patient’s susceptibility to CDI (e.g. by vaccination) may also be required.

**Community-associated and community-onset CDI**

Community-associated infections (CA-CDI) are typically defined as those with symptom onset in the community (or within 3 days of hospital admission) without a history of hospitalisation within the previous 12 weeks. Testing for CDI in the community often lacks consistency and low diagnostic suspicion can lead to missed cases; one recent Dutch study demonstrated that only 40% of community CDI cases are successfully detected.\(^7\)\(^7\) CA-CDI should not be confused with ‘community-onset’ CDI, a term encompassing both CA-CDI and community-onset healthcare facility associated CDI. A recent large US surveillance study demonstrated that over a half of their CDI cases were community-onset.\(^2\)\(^6\) This study estimated that two-thirds of hospital-associated cases had symptom onset in the community, split equally between nursing home residents and patients in their own homes. The majority of community-onset hospital acquired cases occur soon after discharge suggesting either hospital acquisition or possible the use of inciting antibiotics during hospital admissions.\(^7\)\(^8\)\(^-\)\(^7\)\(^9\) Approximately 25% of true CA-CDIs are hospitalised for treatment;\(^2\)\(^1\) thus strains are likely to circulate frequently between hospital and community settings.

It has been suggested that more than half CA-CDIs have outpatient (OPD) healthcare exposure in the weeks prior to infection.\(^2\)\(^4\) CA-CDI is also well recognised in residents of long term care (LTC) facilities (LTC cases are classified as healthcare associated in some countries including the US),\(^7\)\(^7\) with hospital strains being regularly introduced by both asymptomatic and symptomatic hospital attendees.\(^8\)\(^0\)\(^-\)\(^8\)\(^1\) A recent meta-analysis including 9 studies, mainly from North America, demonstrated a 14.8% rate of asymptomatic carriage of toxigenic strains in LTC residents.\(^8\)\(^1\) Colonisation was associated with previous CDI, prior hospitalisation and antimicrobial use. Previously, a survey in Germany demonstrated an approximate five-fold increase in *C. difficile* colonisation of LTC residents...
compared to elderly people living in their own homes.\textsuperscript{82} Further data are needed on the significance of both OPD visits and LTC residence in CA-CDI transmission; in both instances, CDI incidence is likely to be dependent on the susceptibility of other attendees.

Asymptomatic \textit{C. difficile} carriage in infants is another potential reservoir for CDI. Contact with children <2 years old has previously been shown to be a risk factor for CA-CDI.\textsuperscript{83} More recently, two small studies have shown 22-45\% of healthy infants to be colonised with \textit{C. difficile} at a single point in time, with the majority of these infants being affected during the first year of life.\textsuperscript{84-85} Pathogenic strains were retrieved in both studies, but ribotypes seen most frequently in adult CDI (e.g. ribotypes 027/078) were not seen in healthy children. Not all studies have demonstrated the association between infants and CDI.\textsuperscript{77}

Finally, environmental sources may be relevant to CA-CDI. Toxigenic and non-toxigenic \textit{C. difficile} strains can be recovered from the faeces of piglets, cattle, horses and poultry risking transmission to humans via direct contact, food and the environment.\textsuperscript{25,86} Recent studies in the Netherlands, where high density pig farming is present in some parts of the country, have demonstrated potential spread of \textit{C. difficile} between farm animals and humans.\textsuperscript{87-88} The reported prevalence of \textit{C. difficile} in ‘off the shelf’ foods is generally low but extremely variable (0-42\%), with ground meat, shellfish, vegetables and pre-packed salads most commonly contaminated.\textsuperscript{86-89} However, no food-related outbreaks have been reported.

\textit{C. difficile} has also been recovered from water, soil\textsuperscript{90} and household environs; one small US study demonstrated toxigenic \textit{C. difficile} on 25/63 (39.7\%) of shoe swabs.\textsuperscript{91} However, the relative importance of exposure to infants, animals and the environment has remained elusive, perhaps reflecting the fact that the majority of the population lacks vulnerability to CDI due to colonisation resistance provided by healthy bowel microbiota.

In summary, it is likely that community and hospital exposure to \textit{C. difficile} is frequent and the consequences following spore acquisition relate to the host microbiome, host immune function and strain virulence. Further research into CDI acquisition and transmission will be challenging due to the ubiquitous nature of this bacterium, but whole genome sequencing has the potential to accurately link environmental reservoirs to human infection in the near future.

\textbf{CDI diagnosis}
Targets for C. difficile detection

CDI research has been complicated by the wide range of diagnostic tests available (Box 2), inconsistent use of reference standards and a lack of agreement in the clinical criteria used for sampling. C. difficile produces two closely related proteins to elicit its toxic effect: TcdA (toxin A) and TcdB (toxin B). Their corresponding genes (tcdA and tcdB) are encoded by the Pathogenicity Locus (PaLoc), a chromosomally integrated DNA sequence which is variably present among strains.

Strains lacking the PaLoc are not associated with disease. However, horizontal gene transfer of the PaLoc has demonstrated the potential to convert non-toxigenic strains into toxin-producers. Thus, tests for C. difficile infection can detect the bacterium itself, the presence of toxin or the capability of a particular strain to produce toxin. There are two reference standard tests in current use (Box 2), one for detection of toxin producing potential (cytotoxicity culture, CC), and the other for the detection of toxin (cell cytotoxicity assay, CTA). It is vital that new tests for CDI use the appropriate reference standard in order to create accurate sensitivity/specificity data.

Different C. difficile tests have been shown to correlate variably with clinical outcome. A recent prospective observational study by Polage et al demonstrated that detection of toxin genes alone (NAAT testing) over-diagnosed clinically relevant CDI. Toxin was detected in only half of the NAAT positive patients; toxin negative patients had a similar symptom duration as patients without toxigenic C. difficile, whilst toxin positive patients had a significantly increased risk of CDI complications and death. This study is particularly insightful since NAAT has been adopted by many centres within the US, increasing CDI rates by 50-100% in some laboratories. Notably, the study also confirmed the results of a recent, large, multi-centre, prospective study, which compared 12 test-reference standard combinations, including assays for all three bacteriological targets (cell wall antigen, toxin genes and toxin). Planche et al found that cell cytotoxicity assays (CTA) were associated with increased all-cause mortality (16.6% at 30 days). Mortality was similar in patients with NAAT positive/CTA negative (9.7%) samples and those for whom both tests were negative (8.6%) suggesting that the presence of toxin itself is more indicative of mortality risk than carriage of a strain with toxin-producing potential. Thus, tests detecting C. difficile toxigenic potential, rather than toxin, will be likely to over-daignose CDI with possible wide-ranging consequences. Ironically, one such consequence is overtreatment with antibiotics leading to perpetuation of gut dysbiosis, and so risking subsequent CDI.

Despite the association of CDI diagnostic test results and mortality in study populations, no such assay can be guaranteed to correlate with clinical outcome in an individual; patients with a positive toxin EIA can be asymptomatic and severe infection/death can occur in patients with negative toxin
Thus, clinical judgement is always required in conjunction with test results in order to diagnose CDI accurately. Clinical strategies to minimise inappropriate testing are also needed to avoid the risks associated with CDI treatment in asymptomatic carriers.

**Two-step diagnostic algorithms.**

Due to the failure of single commercial tests to accurately diagnose CDI, two-step algorithms for CDI diagnosis have been recommended for several years (figure 3). Forthcoming revised European guidelines on CDI diagnosis recommend two stage testing in line with UK practice. Algorithm design requires consideration of cost, speed, sensitivity and specificity. Although the optimal diagnostic strategy is still under debate, recent data suggest that using a high sensitivity test (GDH or NAAT) to screen for *C. difficile* can yield a rapid result with very high negative predictive value (NPV). This approach allows swift communication of negative results to clinical teams. If the screening test is positive, a second *C. difficile* toxin test (Toxin EIA or CTA) provides specificity for CDI. If a two-step approach gives discordant results (e.g. a positive GDH followed by a negative toxin test) then a third test can be used to increase sensitivity, e.g. NAAT. Alternatively, it is reasonable to manage the patient as if infection is present if there is a strong clinical suspicion of CDI. Despite recent guidelines that recommend GDH or NAAT testing followed by toxin EIA, only 29% of European laboratories are using such a combination of tests, with 45% still using a single test.

**Sampling patients for CDI**

It is important that the correct clinical criteria for sampling are used to identify true cases. A US study found that over a third of clinician requested tests did not meet the criteria for ‘significant diarrhoea’ meaning that the detection of *C. difficile* in these patients would be unlikely to indicate disease. Equally, there are also examples of CDI patients being missed due to a failure to test them, such as a Spanish point prevalence study which tested all unformed stools, regardless of whether the clinician had requested *C. difficile* testing, and found two-thirds of positive samples would have been missed. Furthermore, a pan-European study in 482 hospitals across 20 countries found that a quarter of all patients with toxigenic *C. difficile* were missed due to inadequate sampling/testing strategies. Together, these data confirm that despite the prominence of CDI as a clinical threat, considerable challenges remain when trying to correctly recognise this infection.

As mentioned previously, 4-10% of asymptomatic patients are colonised with toxigenic *C. difficile* on arrival to hospital. Non-infectious diarrhoea is frequent in hospital patients and clinical differentiation between infectious and non-infectious symptoms is unreliable, making it difficult to
know who to test. In order to fully understand and manage CDI, it is important to examine the
interrelationship between diagnostic tests and the wide-ranging spectrum of C. difficile carriage and
disease. It is then possible to identify patients who require no intervention (i.e. carrying non-
toxigenic strains), those who pose an infection risk to others (i.e. carrying toxigenic strains) and
symptomatic patients requiring treatment (i.e. positive for C. difficile toxin). A clear and unified
global approach, separately defining infectious individuals and clinical cases, would allow improved
understanding of epidemiology as well as empowering clinicians to make informed decisions on who
to treat and how best to utilise infection control resources.

Conclusions

Novel C. difficile strains have been shown to rapidly emerge and spread across countries and
continents with global health impact. The reasons for the increased transmissibility of certain strains
may relate to a number of factors including antibiotic resistance, sporulation and toxin production.
Improved surveillance of C. difficile, in particular use of a unifying typing strategy such as WGS, is
likely to revolutionize our understanding of the epidemiology of CDI. In particular, future research
will help determine the main transmission routes and sources of C. difficile in non-outbreak settings
and help to identify methods to reduce CDI incidence in populations at risk. However, further
reduction in CDI will be challenging as evidence suggests that asymptomatic carriage may play a
significant role in transmission and, unlike in other hospital associated infections such as MRSA,
effective screening and decolonization strategies are not in current clinical practice.

Over recent years, choosing ‘who to sample’ and ‘how to test’ for CDI have been contentious issues
for clinicians. The answers to these important questions are likely to significantly influence local CDI
epidemiology, diagnosis and treatment. Unlike the ease of modern rapid identification strategies for
other organisms (such as mass spectrometry), C. difficile diagnosis is complicated by the need to
differentiate toxin-producing from non-toxigenic strains. Evidence now suggests that using any
single test as a blunt tool for both diagnosis and assessment of transmission risk is too simplistic.
Improved diagnostics for CDI are needed to identify who is truly infected with, as opposed to
colonized by, C. difficile. Better (more sensitive and specific) toxin detection methods would help.
Improved real time CDI case ascertainment will mean that the most appropriate treatment option(s)
can be targeted at the right patients whilst onward transmission can be interrupted by identification
of those carrying toxigenic strains.
Box 1. *C. difficile* typing methods and ribotype 027/NAP1/BI/ST1.

The lack of a universally accepted typing strategy has limited the comparison of strain patterns between countries and continents delaying a comprehensive global understanding of *C. difficile* epidemiology. Commonly used methods for *C. difficile* typing include ribotyping, pulsed field gel electrophoresis (PFGE), restriction endonuclease analysis (REA), multi-locus sequence typing (MLST) and multi-locus variable-number tandem-repeat analysis (MLVA). Each technique is reported with its own 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.

More recently, Whole Genome Sequencing (WGS) has been used as a highly discriminatory technique allowing phylogenetic analysis of *C. difficile* strains to establish genetic relatedness and historical lineages. WGS is similar in power to MLVA, but can also demonstrate the presence of specific genes and mutations. High throughput technologies will soon allow WGS to be applied to clinical situations within realistic time and cost constraints. Current limitations of WGS include the high acquisition cost of sequencing platforms, the complexity of sequence analysis and the need to improve nomenclature for use in routine practice; thus, a consistent global approach is required. Other technologies, such as mass spectrometry (e.g. Maldi-TOF), provide the potential for rapid *C. difficile* typing but currently lack discriminatory power compared to routine methods.
Box 2 CDI diagnostic tests.

Reference standards

The two reference tests for *C. difficile* have different bacterial targets. Cytotoxigenic culture (CC) demonstrates the presence of *C. difficile* isolates with the ability to produce toxin when cultured, whereas cell cytotoxicity assay (CTA) detects the presence of toxin in stool (toxin B). Thus, CC demonstrates the presence of a toxigenic strain of *C. difficile*, which is important for infection control purposes but does not necessarily imply infection, whilst CTA is more closely related to disease but may miss individuals with the potential to transmit toxigenic strains to others. CTA yields fewer positive tests than CC by ignoring toxigenic strains not currently producing toxin in the patient. CTA positivity has been shown to correlate more closely with clinical outcome and mortality.\(^{100}\)

Toxin enzyme immunoassays (EIAs)

EIAs directly detect *C. difficile* toxin (A/B) in stool samples. EIAs are rapid, simple to use and inexpensive, which has led to their widespread use. However, they have poor diagnostic accuracy for CDI as single tests (sensitivity 60-92%).\(^{100}\) Thus, the use of toxin EIAs as stand-alone tests is no longer recommended.\(^{107}\)

Glutamate dehydrogenase (GDH) and nucleic acid amplification tests (NAAT)

GDH testing detects a cell wall antigen (the GDH enzyme) which is produced by toxigenic and non-toxigenic strains of *C. difficile*.\(^{120}\) GDH tests have high sensitivity but low specificity.\(^{100,120}\) A growing number of NAATs for the detection of toxin genes are now available.\(^{121}\) The two methods employed are real-time polymerase chain reaction (PCR) targeting the toxin genes (tcdA/B) and loop mediated isothermal amplification (LAMP) of DNA.\(^{122-123}\) NAATs have high sensitivity compared with CC, but not CTA (sensitivity 87%).\(^{105,124}\) GDH tests and NAATs are widely used but neither detects *C. difficile* toxins so they cannot differentiate between asymptomatic carriage and true infection. Clinical correlation of positive results or further testing is required.