



UNIVERSITY OF LEEDS

This is a repository copy of *Clostridium difficile* infection: advances in epidemiology, diagnosis and transmission.

White Rose Research Online URL for this paper:  
<http://eprints.whiterose.ac.uk/93246/>

Version: Accepted Version

---

**Article:**

Martin, JS, Monaghan, TM and Wilcox, MH (2016) *Clostridium difficile* infection: advances in epidemiology, diagnosis and transmission. *Nature Reviews Gastroenterology and Hepatology*, 13 (4). pp. 206-216. ISSN 1759-5045

<https://doi.org/10.1038/nrgastro.2016.25>

---

(c) 2016, The Author(s). This is an author produced version of a paper published in *Nature Reviews Gastroenterology and Hepatology*. Uploaded in accordance with the publisher's self-archiving policy.

**Reuse**

Unless indicated otherwise, fulltext items are protected by copyright with all rights reserved. The copyright exception in section 29 of the Copyright, Designs and Patents Act 1988 allows the making of a single copy solely for the purpose of non-commercial research or private study within the limits of fair dealing. The publisher or other rights-holder may allow further reproduction and re-use of this version - refer to the White Rose Research Online record for this item. Where records identify the publisher as the copyright holder, users can verify any specific terms of use on the publisher's website.

**Takedown**

If you consider content in White Rose Research Online to be in breach of UK law, please notify us by emailing [eprints@whiterose.ac.uk](mailto:eprints@whiterose.ac.uk) including the URL of the record and the reason for the withdrawal request.



[eprints@whiterose.ac.uk](mailto:eprints@whiterose.ac.uk)  
<https://eprints.whiterose.ac.uk/>

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

3 Dr Jessica Martin - University of Leeds. Leeds Teaching Hospitals NHS Trust, Leeds, UK.

4 Dr Tanya Monaghan - NIHR Nottingham Digestive Diseases Biomedical Research Unit, Nottingham University  
5 Hospitals NHS Trust, Nottingham, UK.

6 Professor Mark Wilcox - University of Leeds. Leeds Teaching Hospitals NHS Trust, Leeds, UK.

7 Corresponding Author - Professor Mark Wilcox ([Mark.Wilcox@leedsth.nhs.uk](mailto:Mark.Wilcox@leedsth.nhs.uk))

8

9 Final manuscript

10 Display items

11 Figure 1 – *C. difficile* acquisition, germination and infection

12 Figure 2 – Global epidemiology of common *C. difficile* ribotypes

13 Box 1 – *C. difficile* typing methods and ribotype O27/NAP1/BI/ST1.

14 Box 2 – CDI diagnostic tests.

15 Figure 3 – Examples of multistep algorithms for rapid diagnosis of CDI

16 Key points:

- 17 • *C. difficile* infection (CDI) is a continually evolving global healthcare problem.
  - 18 • Community onset CDI is increasing and multiple potential reservoirs of infection exist  
19 including environmental sources, animals, asymptomatic patients and symptomatic patients.
  - 20 • Highly discriminatory typing techniques such as whole-genome sequencing and multi-locus  
21 variable-number tandem-repeat analysis offer the potential for illuminating previously  
22 under-recognized routes of *C. difficile* transmission.
  - 23 • The optimal approach to sampling and testing for CDI remains a contentious issue.
  - 24 • Multistep algorithms are recommended to improve diagnostic sensitivity and specificity.
- 25

26 **Abstract**

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

34

35 **Introduction**

36 *Clostridium difficile* is an anaerobic spore-forming Gram-positive bacillus which is able to colonise  
37 and proliferate in the human gut, especially following changes in the indigenous colonic microbiota

38 after antibiotic use. The period from spore ingestion to symptom onset is typically short,<sup>1</sup> and is  
39 dependent on microbiological, biochemical and immune factors present in the patient's bowel  
40 (figure 1). Importantly, human ingestion of spores does not always result in symptomatic infection.  
41 The clinical outcome after spore acquisition is variable and ranges from transient colonisation of the  
42 gut and persistent asymptomatic carriage to fulminant disease.<sup>2,3</sup> These asymptomatic carriers of *C.*  
43 *difficile* provide a potential reservoir for onward transmission, especially within a hospital  
44 population.<sup>3</sup>

45

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

56

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

66

### 67 ***C. difficile* epidemiology**

68 *C. difficile* was first identified as a pathogen related to antibiotic-associated diarrhoea in the late  
69 1970s.<sup>10</sup> In the last decade of the 20<sup>th</sup> Century, CDI incidence escalated,<sup>11</sup> becoming a well-publicised  
70 cause of hospital-acquired infection in developed countries. From 2000 onwards, there was a further

71 rise in CDI dominated by epidemic strains with increased pathogenicity leading to high transmission  
72 rates, increased severity and greater mortality.<sup>12</sup>

73

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

81

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

92

### 93 *Global epidemiology*

94 First recognised in 2002, *C. difficile* B1/NAP1/027 clones (Box 1) have caused large epidemics across  
95 the developed world with significant morbidity and mortality.<sup>8,12</sup> In addition to toxins A and B, this  
96 strain produces binary toxin (known as *C. difficile* transferase [CDT]), also produced by a number of  
97 other strains (e.g. ribotype 078, 023).<sup>27</sup> Infection caused by these binary toxin-producing strains has  
98 been associated with increased disease severity and 30 day mortality (though it is not clear whether  
99 it is binary toxin which contributes to adverse outcome, or other, as yet unknown factors).<sup>28-30</sup> The  
100 incidence of B1/NAP1/027 has recently fallen in some areas of Western Europe (figure 2), due to a  
101 reduction in the total incidence of CDI as well as a reduction in the proportion of cases attributed to  
102 this strain type.<sup>31-32</sup> However, B1/NAP1/027 persists in North America and is increasing in other  
103 areas, especially Eastern Europe.<sup>13,33-35</sup>

104

105 In Australia, similar binary toxin-producing strains, such as ribotypes 244, have recently emerged,  
106 but interestingly, *C. difficile* BI/NAP1/027 has yet to become established.<sup>36-38</sup> The reasons for this are  
107 unclear, but this may reflect the relative pressures of antimicrobial selection of particular strains. For  
108 example, *C. difficile* BI/NAP1/027 is relatively fluoroquinolone resistant and these antibiotics are  
109 infrequently prescribed in Australia.<sup>39</sup> In Asia, non-binary toxin strains such as ribotypes 017, 018 and  
110 014 remain dominant.<sup>40</sup> Further understanding of global CDI epidemiology is hindered by a lack of  
111 surveillance, especially in the developing world.

112

113 In the UK, where CDI surveillance is mandatory, the prevalence of *C. difficile* BI/NAP1/027 has  
114 markedly decreased since 2007 (figure 2).<sup>31</sup> It has not been superseded by an alternative dominant  
115 strain, but instead, a picture of increasing diversity of strains has emerged. Such heterogeneity is  
116 well documented elsewhere; a review of 6 US centres demonstrated 98 ribotypes in 720 toxigenic  
117 isolates<sup>41</sup> and an Australian study showed 32 ribotypes in 70 samples.<sup>42</sup> Such observations may  
118 simply reflect the epidemiology of *C. difficile* prior to the emergence of successful clones.

119

120 *Using C. difficile typing for epidemiological investigations*

121 *C. difficile* strains can be differentiated by a variety of typing techniques (Box 1) which can be used to  
122 scrutinise epidemiologically linked cases. However, challenges arise when attempting to standardise  
123 these techniques between laboratories, particularly when typing methods depend on gel banding  
124 patterns (e.g. PFGE). Multi-locus sequence typing (MLST), described by both Griffiths *et al* and  
125 Lemee *et al* in 2010,<sup>43-44</sup> is a typing strategy which overcomes these challenges by sequencing  
126 multiple housekeeping gene fragments and using an internet-accessible database to interpret  
127 results. MLST has the additional benefit of grouping strains into 'clades' based on common molecular  
128 lineage. A study looking at CDI outcomes based on clades demonstrated interesting differences in  
129 disease severity and mortality (25% 14 day mortality rate in clade 5 [ST11, ribotype 078] vs. 7% in  
130 clade 3 [ST 22/5, ribotype 023]).<sup>5</sup>

131

132 It is important to note that, whilst there is some concordance between typing methods (as for strain  
133 BI/NAP1/027), there are also key differences that reflect the different aspects of the genome  
134 targeted by each technique. Ribotyping uses the 16S-23S intergenic spacer region in the ribosomal  
135 RNA gene complex;<sup>45</sup> it is the preferred *C. difficile* typing method in Europe and Australia and its use  
136 has recently increased in North America. MLST and ribotyping have similar discriminatory powers  
137 but different ribotypes may be seen as a single strain by MLST, and *vice versa*.<sup>43</sup> For instance,  
138 ribotype 014 falls into a number of sequence types (ST-2, ST-14, ST-50 and ST-132) and ST2 includes

139 multiple ribotypes (ribotype 014, 020, 076, 220, 095, 006). Similar problems arise when comparing  
140 results from other typing strategies such as PFGE, REA and MLVA (Box 1), thus hindering  
141 investigations into global epidemiology.

142

143 New *C. difficile* typing methods, such as Whole Genome Sequencing (WGS), provide a high level of  
144 discrimination between strains aiding epidemiological investigations.<sup>4,8,9</sup> *C. difficile* genomic  
145 evolution is estimated to occur at a rate of 0.74-1.4<sup>9,46</sup> single nucleotide variants (SNVs) per year,  
146 and therefore near identical strains (e.g. <2 SNVs apart) in two individuals strongly imply  
147 transmission or recent exposure to a common source, strains >10 SNVs are highly likely to be  
148 distinct.<sup>9</sup> For interpretation of strains between 3-10 SNVs, the time between potential donor and  
149 recipient samples should be considered in the context of the *C. difficile* evolutionary rate. WGS can  
150 also be used to assemble phylogenetic trees to investigate the common origins of clinically relevant  
151 *C. difficile* strains. As an example, WGS has been used to demonstrate transcontinental spread of  
152 two distinct lineages of the BI/NAP1/027 strain.<sup>8</sup> One lineage (FQR1) originated in the north eastern  
153 US and was transmitted to South Korea and Switzerland. A second lineage (FQR2) demonstrated  
154 wider spread with multiple trans-Atlantic transmission events between the US and Europe, some of  
155 which led to well publicised CDI outbreaks in the UK and cases in Austria, Poland and the  
156 Netherlands. The spread of both strains was preceded by the acquisition of fluoroquinolone  
157 resistance, likely due to the selection pressure of high level use of this antibiotic in North America.<sup>8</sup>  
158 This study highlights the influence of global travel on the spread of emerging infectious organisms  
159 and suggests there is a growing need for an internationally uniform approach to *C. difficile* typing.

160

### 161 **CDI transmission**

162 Both host susceptibility and strain characteristics are likely to contribute to the probability of  
163 effective *C. difficile* transmission, and subsequent infection, when two individuals come into contact  
164 (figure 1).<sup>5,47</sup> The host variables associated with increased CDI risk are well characterised, and include  
165 increasing age, antibiotic use and co-morbidities.<sup>47</sup> The characteristics leading to the success of  
166 particular strains are less well understood.

167 Host susceptibility to CDI is known to be enhanced by changes in the host intestinal microbiota,  
168 often following hospital admission and/or antibiotic exposure.<sup>48</sup> Recent analysis of the gut  
169 microbiome using 16S ribosomal RNA sequencing demonstrates a reduction in the diversity of host  
170 bacteria and altered predominant species in patients with CDI compared to those with either  
171 asymptomatic *C. difficile* colonisation or healthy subjects.<sup>49</sup> Antimicrobial-induced dysbiosis also  
172 results in loss of protective toll-like receptor (TLR) signalling, accumulation of pro-inflammatory T

173 helper 17 (Th17) T cells and increased epithelial permeability.<sup>50</sup> Thus, subsequent infection with *C.*  
174 *difficile* leads to toxin-mediated epithelial injury and perpetuation of the pro-inflammatory  
175 response.<sup>51</sup>

#### 176 *C. difficile* sporulation and germination

177 Currently, we have a limited understanding of the reasons why some *C. difficile* strains have led to  
178 large transatlantic epidemics (*i.e.* BI/NAP1/ribotype 027), whilst others remain at a local or sporadic  
179 level. Several explanations for this ‘hypervirulence’ have been proposed,<sup>52-55</sup> and it seems likely that  
180 pathogenic factors such as germination, sporulation, epithelial adherence and toxin production may  
181 influence the success of some strains.

182 *C. difficile* spores are a key feature in transmission (figure 1). Once ingested, spores interact with  
183 small molecular germinants, such as bile acids,<sup>56-57</sup> triggering a series of events committing the spore  
184 to germinate into toxin-producing bacteria. Recently, a germination-specific protease, CspC, has  
185 been shown to play an active and essential role in germination by functioning as the *C. difficile* bile  
186 acid germinant receptor.<sup>58</sup> Recent data has also begun to elucidate the activity of Spo0A, the master  
187 regulator of sporulation, which controls the transition of the bacterium into the spore form.<sup>59-60</sup>  
188 Spo0A has also been implicated in controlling toxin gene expression,<sup>61</sup> intestinal colonization and  
189 disease in mice.<sup>62-63</sup> There is early evidence that Spo0A may vary between ribotypes<sup>61</sup> but further  
190 research is required to confirm the influence this may have on transmission and clinical disease.

191

#### 192 *Toxins, immunity and C. difficile* disease

193 Investigations seeking to link quantitative toxin production with *C. difficile* virulence have not been  
194 conclusive.<sup>64</sup> The clinical impact of a particular *C. difficile* strain is related not only to its  
195 pathogenicity, but also to the host immune response to toxin. Toxins A (TcdA) and B (TcdB) stimulate  
196 the release of multiple pro-inflammatory cytokines and chemokines (e.g. interleukin (IL)-1 $\beta$ , tumour  
197 necrosis factor alpha (TNF- $\alpha$ ), IL-8, IL-12, IL-18, IL-23, macrophage inflammatory protein 1 $\alpha$  (MIP-1 $\alpha$ ),  
198 MIP-2, leptin) from epithelial cells and mucosal immune cells.<sup>65-68</sup> This inflammatory response is a  
199 major determinant of disease severity<sup>69-70</sup> and has recently been shown to correlate with persistent  
200 diarrhoea and poor clinical outcome.<sup>5,71</sup> Importantly, toxins A and B are themselves targets of  
201 immune recognition. Antibody-mediated responses to toxins have an important role in determining  
202 asymptomatic carriage and predisposition to recurrent infection.<sup>70</sup> Symptomless carriers of toxigenic  
203 *C. difficile* and those with a single episode of CDI show more robust antitoxin immune responses  
204 than those with symptomatic and recurrent disease.<sup>72-73</sup> Recently, circulating TcdA and TcdB-specific

205 memory B cells have been detected following CDI, strengthening the evidence for the importance of  
206 the humoral immune response against both toxins.<sup>74</sup>

207

### 208 *Hospital acquired infection*

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

215

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

227

228 The contribution of asymptomatic carriers to transmission has been confirmed in a recent study  
229 from Pittsburgh, US, in which 10.4% of inpatients carried *C. difficile* regardless of their symptom  
230 profile.<sup>17</sup> MLVA typing (Box 1) showed that a third of diagnosed CDI cases had been in recent contact  
231 with a symptomatic patient, whilst another third had had contact only with an asymptomatic *C.*  
232 *difficile* carrier.<sup>17</sup> This confirms earlier work suggesting a significant role for asymptomatic patients.<sup>3</sup>  
233 Larger multi-centre studies are needed to fully appreciate the impact of asymptomatic  
234 colonisation/carriage. A recent meta-analysis of North American studies suggests that toxigenic *C.*  
235 *difficile* carriage on admission increases the risk of subsequent CDI six-fold (21.8% vs. 3.4%,  $p=0.03$ ),  
236 contrary to previous understanding that carriage reduced CDI risk.<sup>22</sup> Notably, at present we do not  
237 have a proven intervention to address such individuals, and the resource implications for global  
238 sampling and source isolating asymptomatic patients are daunting.



239

240 Another possible source of CDI transmission is health care workers (HCWs). Although asymptomatic  
241 intestinal *C. difficile* colonisation in HCWs is generally uncommon, other factors, such as poor hand  
242 hygiene, have been shown to play a significant role in CDI transmission.<sup>76</sup> Taken together, these data  
243 suggest that source isolation of symptomatic CDI patients and control of epidemic strains, although  
244 essential interventions, may no longer be sufficient to further reduce the burden of disease in  
245 settings where outbreaks are uncommon. Further investigation of alternative sources of CDI in the  
246 hospital setting is necessary, with a focus on asymptomatic and untested patients. Alternative  
247 approaches, such as controlling exposure to antibiotics and reducing patient's susceptibility to CDI  
248 (e.g. by vaccination) may also be required.

249

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

251 Community-associated infections (CA-CDI) are typically defined as those with symptom onset in the  
252 community (or within 3 days of hospital admission) without a history of hospitalisation within the  
253 previous 12 weeks. Testing for CDI in the community often lacks consistency and low diagnostic  
254 suspicion can lead to missed cases; one recent Dutch study demonstrated that only 40% of  
255 community CDI cases are successfully detected.<sup>77</sup> CA-CDI should not be confused with 'community-  
256 onset' CDI, a term encompassing both CA-CDI and community-onset healthcare facility associated  
257 CDI. A recent large US surveillance study demonstrated that over a half of their CDI cases were  
258 community-onset.<sup>26</sup> This study estimated that two-thirds of hospital-associated cases had symptom  
259 onset in the community, split equally between nursing home residents and patients in their own  
260 homes. The majority of community-onset hospital acquired cases occur soon after discharge  
261 suggesting either hospital acquisition or possible the use of inciting antibiotics during hospital  
262 admissions.<sup>78-79</sup> Approximately 25% of true CA-CDIs are hospitalised for treatment;<sup>21</sup> thus strains are  
263 likely to circulate frequently between hospital and community settings.

264

265 It has been suggested that more than half CA-CDIs have outpatient (OPD) healthcare exposure in the  
266 weeks prior to infection.<sup>24</sup> CA-CDI is also well recognised in residents of long term care (LTC)  
267 facilities (LTC cases are classified as healthcare associated in some countries including the US),<sup>77</sup> with  
268 hospital strains being regularly introduced by both asymptomatic and symptomatic hospital  
269 attendees.<sup>80-81</sup> A recent meta-analysis including 9 studies, mainly from North America, demonstrated  
270 a 14.8% rate of asymptomatic carriage of toxigenic strains in LTC residents.<sup>81</sup> Colonisation was  
271 associated with previous CDI, prior hospitalisation and antimicrobial use. Previously, a survey in  
272 Germany demonstrated an approximate five-fold increase in *C. difficile* colonisation of LTC residents

273 compared to elderly people living in their own homes.<sup>82</sup> Further data are needed on the significance  
274 of both OPD visits and LTC residence in CA-CDI transmission; in both instances, CDI incidence is likely  
275 to be dependent on the susceptibility of other attendees.

276

277 Asymptomatic *C. difficile* carriage in infants is another potential reservoir for CDI. Contact with  
278 children <2 years old has previously been shown to be a risk factor for CA-CDI.<sup>83</sup> More recently, two  
279 small studies have shown 22-45% of healthy infants to be colonised with *C. difficile* at a single point  
280 in time, with the majority of these infants being affected during the first year of life.<sup>84-85</sup> Pathogenic  
281 strains were retrieved in both studies, but ribotypes seen most frequently in adult CDI (e.g. ribotypes  
282 027/078) were not seen in healthy children. Not all studies have demonstrated the association  
283 between infants and CDI.<sup>77</sup>

284

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

293

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

299

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

305

306 **CDI diagnosis**

307 *Targets for C. difficile detection*

308 CDI research has been complicated by the wide range of diagnostic tests available (Box 2),  
309 inconsistent use of reference standards and a lack of agreement in the clinical criteria used for  
310 sampling.<sup>92-93</sup> *C. difficile* produces two closely related proteins to elicit its toxic effect: TcdA (toxin A)  
311 and TcdB (toxin B). Their corresponding genes (*tcdA* and *tcdB*) are encoded by the Pathogenicity  
312 Locus (PaLoc), a chromosomally integrated DNA sequence which is variably present among strains.<sup>94-</sup>  
313 <sup>95</sup> Strains lacking the PaLoc are not associated with disease. However, horizontal gene transfer of the  
314 PaLoc has demonstrated the potential to convert non-toxigenic strains into toxin-producers.<sup>95</sup> Thus,  
315 tests for *C. difficile* infection can detect the bacterium itself, the presence of toxin or the capability of  
316 a particular strain to produce toxin. There are two reference standard tests in current use (Box 2),  
317 one for detection of toxin producing potential (cytotoxigenic culture, CC), and the other for the  
318 detection of toxin (cell cytotoxicity assay, CTA). It is vital that new tests for CDI use the appropriate  
319 reference standard in order to create accurate sensitivity/specificity data.

320

321 Different *C. difficile* tests have been shown to correlate variably with clinical outcome.<sup>96-98</sup> A recent  
322 prospective observational study by *Polage et al* demonstrated that detection of toxin genes alone  
323 (NAAT testing) over-diagnosed clinically relevant CDI.<sup>99</sup> Toxin was detected in only half of the NAAT  
324 positive patients; toxin negative patients had a similar symptom duration as patients without  
325 toxigenic *C. difficile*, whilst toxin positive patients had a significantly increased risk of CDI  
326 complications and death. This study is particularly insightful since NAAT has been adopted by many  
327 centres within the US, increasing CDI rates by 50-100% in some laboratories.<sup>99</sup> Notably, the study  
328 also confirmed the results of a recent, large, multi-centre, prospective study, which compared 12  
329 test-reference standard combinations, including assays for all three bacteriological targets (cell wall  
330 antigen, toxin genes and toxin).<sup>100</sup> *Planche et al* found that cell cytotoxicity assays (CTA) were  
331 associated with increased all-cause mortality (16.6% at 30 days). Mortality was similar in patients  
332 with NAAT positive/CTA negative (9.7%) samples and those for whom both tests were negative  
333 (8.6%) suggesting that the presence of toxin itself is more indicative of mortality risk than carriage of  
334 a strain with toxin-producing potential.<sup>100</sup> Thus, tests detecting *C. difficile* toxigenic potential, rather  
335 than toxin, will be likely to over-diagnose CDI with possible wide-ranging consequences. Ironically,  
336 one such consequence is overtreatment with antibiotics leading to perpetuation of gut dysbiosis,  
337 and so risking subsequent CDI.

338 Despite the association of CDI diagnostic test results and mortality in study populations, no such  
339 assay can be guaranteed to correlate with clinical outcome in an individual; patients with a positive  
340 toxin EIA can be asymptomatic and severe infection /death can occur in patients with negative toxin

341 EIA.<sup>101-104</sup> Thus; clinical judgement is always required in conjunction with test results in order to  
342 diagnose CDI accurately. Clinical strategies to minimise inappropriate testing are also needed to  
343 avoid the risks associated with CDI treatment in asymptomatic carriers.

344

#### 345 *Two-step diagnostic algorithms.*

346 Due to the failure of single commercial tests to accurately diagnose CDI, two-step algorithms for CDI  
347 diagnosis have been recommended for several years (figure 3).<sup>105-106</sup> Forthcoming revised European  
348 guidelines on CDI diagnosis recommend two stage testing in line with UK practice. Algorithm design  
349 requires consideration of cost, speed, sensitivity and specificity. Although the optimal diagnostic  
350 strategy is still under debate, recent data suggest that using a high sensitivity test (GDH or NAAT) to  
351 screen for *C. difficile* can yield a rapid result with very high negative predictive value (NPV).<sup>107-108</sup> This  
352 approach allows swift communication of negative results to clinical teams. If the screening test is  
353 positive, a second *C. difficile* toxin test (Toxin EIA or CTA) provides specificity for CDI. If a two-step  
354 approach gives discordant results (e.g. a positive GDH followed by a negative toxin test) then a third  
355 test can be used to increase sensitivity, e.g. NAAT. Alternatively, it is reasonable to manage the  
356 patient as if infection is present if there is a strong clinical suspicion of CDI. Despite recent guidelines  
357 that recommend GDH or NAAT testing followed by toxin EIA,<sup>105,107-108</sup> only 29% of European  
358 laboratories are using such a combination of tests, with 45% still using a single test.<sup>13</sup>

359

#### 360 *Sampling patients for CDI*

361 It is important that the correct clinical criteria for sampling are used to identify true cases. A US  
362 study found that over a third of clinician requested tests did not meet the criteria for 'significant  
363 diarrhoea' meaning that the detection of *C. difficile* in these patients would be unlikely to indicate  
364 disease.<sup>93</sup> Equally, there are also examples of CDI patients being missed due to a failure to test them,  
365 such as a Spanish point prevalence study which tested all unformed stools, regardless of whether  
366 the clinician had requested *C. difficile* testing, and found two-thirds of positive samples would have  
367 been missed.<sup>109</sup> Furthermore, a pan-European study in 482 hospitals across 20 countries found that  
368 a quarter of all patients with toxigenic *C. difficile* were missed due to inadequate sampling/testing  
369 strategies.<sup>13</sup> Together, these data confirm that despite the prominence of CDI as a clinical threat,  
370 considerable challenges remain when trying to correctly recognise this infection.

371

372 As mentioned previously, 4-10% of asymptomatic patients are colonised with toxigenic *C. difficile* on  
373 arrival to hospital. Non-infectious diarrhoea is frequent in hospital patients and clinical  
374 differentiation between infectious and non-infectious symptoms is unreliable<sup>110</sup> making it difficult to

375 know who to test. In order to fully understand and manage CDI, it is important to examine the  
376 interrelationship between diagnostic tests and the wide-ranging spectrum of *C. difficile* carriage and  
377 disease. It is then possible to identify patients who require no intervention (i.e. carrying non-  
378 toxigenic strains), those who pose an infection risk to others (i.e. carrying toxigenic strains) and  
379 symptomatic patients requiring treatment (i.e. positive for *C. difficile* toxin). A clear and unified  
380 global approach, separately defining infectious individuals and clinical cases, would allow improved  
381 understanding of epidemiology as well as empowering clinicians to make informed decisions on who  
382 to treat and how best to utilise infection control resources.

383

### 384 **Conclusions**

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

395

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

407

408 **Box 1.** *C. difficile* typing methods and ribotype 027/NAP1/BI/ST1.

409

410 The lack of a universally accepted typing strategy has limited the comparison of strain patterns  
411 between countries and continents delaying a comprehensive global understanding of *C. difficile*  
412 epidemiology. Commonly used methods for *C. difficile* typing include ribotyping, pulsed field gel  
413 electrophoresis (PFGE), restriction endonuclease analysis (REA), multi-locus sequence typing (MLST)  
414 and multi-locus variable-number tandem-repeat analysis (MLVA).<sup>111</sup> Each technique is reported with  
415 its own nomenclature, thus ribotype 027 is also known as NAP1 (PFGE), BI (REA) and ST1 (MLST). The  
416 various typing methods have different relative discriminatory powers; REA and MLVA show greater  
417 discrimination than ribotyping or MLST, which in turn provide greater power to separate strains than  
418 PFGE.

419

420 More recently, Whole Genome Sequencing (WGS) has been used as a highly discriminatory  
421 technique allowing phylogenetic analysis of *C. difficile* strains to establish genetic relatedness and  
422 historical lineages.<sup>4,8-9</sup> WGS is similar in power to MLVA, but can also demonstrate the presence of  
423 specific genes and mutations.<sup>112</sup> High throughput technologies will soon allow WGS to be applied to  
424 clinical situations within realistic time and cost constraints.<sup>113</sup> Current limitations of WGS include the  
425 high acquisition cost of sequencing platforms, the complexity of sequence analysis and the need to  
426 improve nomenclature for use in routine practice; thus, a consistent global approach is required.<sup>8,114</sup>  
427 Other technologies, such as mass spectrometry (e.g. Maldi-TOF), provide the potential for rapid  
428 *C. difficile* typing but currently lack discriminatory power compared to routine methods.<sup>115</sup>

429

430 Box 2 CDI diagnostic tests.

431

432 *Reference standards*

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

441

442 *Toxin enzyme immunoassays (EIAs)*

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

447

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

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

457