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Understanding *Clostridium difficile* colonization

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Running title: *Clostridium difficile* colonization

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1 **SUMMARY**

2 *Clostridium difficile* is the main causative agent of antibiotic-associated and health care  
3 associated infective diarrhea. Recently, there has been growing interest in alternative  
4 sources of *C. difficile*, other than patients with *Clostridium difficile* infection (CDI) and the  
5 hospital environment. Notably, the role of *C. difficile* colonized patients as a possible source  
6 of transmission has received attention. In this review, we present a comprehensive  
7 overview of the current understanding of *C. difficile* colonization. Findings from gut  
8 microbiota studies yield more insights in determinants that are important for acquiring or  
9 resisting colonization and progression to CDI. When discussing the prevalence of *C. difficile*  
10 colonization among populations and its associated risk factors, colonized patients at  
11 admission to the hospital merit more attention as findings from the literature have pointed  
12 to their role both in health care associated transmission of *C. difficile* and a higher risk of  
13 progression to CDI once admitted. *C. difficile* colonization among patients at admission may  
14 have clinical implications, although further research is needed to identify if interventions are  
15 beneficial to prevent transmission or overcome progression to CDI.

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## 24 INTRODUCTION

25 *Clostridium difficile* is a spore-forming, gram-positive rod causing *Clostridium difficile*  
26 infection (CDI), which may range from mild diarrhea to life-threatening pseudomembranous  
27 colitis. *Clostridium difficile* infection has been considered as a healthcare associated  
28 infection transmitted primarily from other symptomatic CDI patients. Recent studies,  
29 notably based on highly discriminatory techniques like whole genome sequencing, have  
30 emphasized that assumptions about the sources and transmission of *C. difficile* may not be  
31 correct (1-3). The realization that a large proportion of CDI cases are not due to transmission  
32 from other CDI cases has underlined the need to re-examine the many diverse potential  
33 sources of *C. difficile*, and to determine their contribution to the epidemiology of this  
34 disease. Paramount to our understanding is the issue of colonization of *C. difficile*, which is  
35 the subject of this review.

36

## 37 DEFINITIONS

### 38 Definition of *C. difficile* colonization

39 The authors of this review define "*C. difficile* colonization" as the detection of the organism  
40 in the absence of CDI symptoms and "*C. difficile* infection" as the presence of *C. difficile*  
41 toxin (ideally), or a toxigenic strain type, and clinical manifestations of CDI (Figure 1). Clinical  
42 presentations compatible with CDI include diarrhea (defined as Bristol stool chart type 5-7,  
43 plus a stool frequency of three stools in 24 or fewer consecutive hours, or more frequently  
44 than is normal for the individual), ileus (defined as signs of severely disturbed bowel  
45 function such as vomiting and absence of stool with radiological signs of bowel distention)  
46 and toxic megacolon (defined as radiological signs of distention of the colon, usually  $\geq 10$  cm  
47 diameter, and signs of a severe systemic inflammatory response) (4).

48 However, as a previous review highlighted, definitions for CDI used in the Infectious Disease  
49 Societies of America (IDSA) and European Society of Clinical Microbiology and Infectious  
50 Diseases (ESCMID) guidelines differ (5-7). IDSA guidelines accept a CDI diagnosis if *C. difficile*  
51 symptoms are identified in combination with either the presence of a toxigenic strain, free  
52 toxin in the stool or histopathological evidence of pseudomembranous colitis, whereas  
53 recent ESCMID guidelines require the additional exclusion of alternative etiologies for  
54 diarrhea. Differences in definitions for CDI may affect the proportion of patients regarded as  
55 asymptotically or symptomatically colonized instead of having symptomatic CDI.  
56 Moreover, the criteria used to define asymptomatic carriage/colonization vary considerably  
57 among studies. Strict definitions of colonization have been described (8, 9), including  
58 classifying asymptomatic carriers as those testing positive for *C. difficile* toxins but no signs  
59 of CDI for 12 weeks pre- or post-specimen collection, based on a retrospective record  
60 review (2). Highly restrictive definitions are difficult to apply in practice, and therefore use  
61 of a simplified definition of multiple positive stools from multiple time points to determine  
62 colonization has been recommended (10). In contrast, other studies utilized the less strict  
63 definition of colonization as a single *C. difficile* positive stool and the absence of diarrhea  
64 (11-13). Clearly, this has implications for who is classified as *C. difficile* colonized and how  
65 asymptomatic cohorts are perceived as potential transmission sources. Donskey and  
66 colleagues demonstrated that a single *C. difficile* positive fecal sample could imply either  
67 colonization, transient carriage or even 'pass-through' (10). We thus indicate the  
68 importance of further delineation of asymptomatic carriage into transient and persistent  
69 colonization, as outlined in a transmission study by Curry et al. (2). Differentiating between  
70 repeat, persistent detection (carriage) and point detection (colonization) would enable a  
71 greater understanding of transmission events and the infection control practices necessary

72 to prevent CDI spread. However, at the moment longitudinal studies on this topic are  
73 lacking.

74

#### 75 **Assessing asymptomatic colonization**

76 The rates of asymptomatic colonization vary considerably due to the different definitions of  
77 diarrhea and laboratory methodological differences.

78

79 Standardization of the definition of diarrhea is essential, since McFarland et al. defined  
80 diarrhea as  $\geq 3$  unformed stools for at least two consecutive days (14), whilst others  
81 accepted the same number of loose stools, but over a single 24 hour period (12, 15).

82 Therefore, the absence of diarrhea is not synonymous with lack of loose stools, potentially  
83 resulting in inconsistent designations of asymptomatic patients.

84 Besides the disparate definitions for diarrhea, assays or methodologies to test for CDI or *C.*  
85 *difficile* colonization also vary and impact incidence rates of both conditions (13). (See Table  
86 1) Methods used for CDI diagnosis can sometimes also be used for diagnosing *C. difficile*  
87 colonization, but on the other hand, some methods used for routinely diagnosing CDI may  
88 falsely classify colonized patients with diarrhea (due to a non-*C. difficile* cause) as CDI  
89 patients.

90 Despite its labor intensive and time consuming characteristics and susceptibility to toxin  
91 degradation in stool samples with incorrect storage, cell cytotoxicity neutralization assay  
92 (CCNA) is frequently considered as the gold standard for CDI due to its high specificity and  
93 direct detection of the main virulence factor (toxin) (16, 17). However, as CCNA detects *C.*  
94 *difficile* toxins and not the presence of the organism itself, its utility is limited in detecting *C.*  
95 *difficile* colonization. Nonetheless, in infants, a positive CCNA without clinical symptoms has

96 been used to consider these infants as *C. difficile* colonized (18), indicating the aberrant  
97 association between toxin presence and clinical symptoms in this age group.

98 An alternative gold standard for CDI is toxigenic culture, which includes culture of the  
99 organism followed by detection of its in vitro toxin producing capacity by toxin enzyme  
100 immunoassay (Tox A/B EIA), CCNA or detection of the toxin genes by nucleic acid  
101 amplification test (NAAT). A major study by Planche et al. of greater than 12,000 fecal  
102 specimens highlighted no increase in mortality in patients harboring a toxigenic *C. difficile*  
103 strain without the presence of detectable toxin (19), suggesting that free toxin positivity  
104 reflects CDI, while toxigenic culture positivity encompasses some patients with colonization.  
105 Therefore, the use of toxigenic culture to diagnose CDI could lead to an over-diagnosis of  
106 CDI and hence an underestimation of *C. difficile* colonization. However, if the goal is  
107 detection of toxigenic *C. difficile* colonization in asymptomatic patients, toxigenic culture is a  
108 suitable option.

109 As both gold standard methods for diagnosing CDI are time-consuming and laborious, rapid  
110 assays are more appealing for CDI testing in daily practice. When rapid assays are used to  
111 test for CDI, it is recommended to use them in an algorithm in order to optimize positive  
112 and negative predictive values. Concerning the relationship between free toxins and true  
113 disease as described above, the algorithm should include a Tox A/B EIA to test for free  
114 toxins in stool. However, in clinical practice, rapid assays and especially NAATs, are often  
115 used as stand-alone test instead of as part of an algorithm, and this may again lead to *C.*  
116 *difficile* colonization being erroneously classified as CDI. A study by Polage et al.  
117 demonstrated that 39.9% of NAAT positive specimens tested negative for toxin by cell  
118 cytotoxicity assay (20), showing how reliance on stand-alone NAAT could lead to over-

119 diagnosis of CDI and consequently an underestimation of asymptomatic colonization, similar  
120 to the situation described above for TC.

121

122 There are some specific limitations that have to be taken into account when assessing *C.*  
123 *difficile* colonization. In *C. difficile* colonization, bacterial loads can be lower than in CDI.  
124 Direct culture of the organism is quite sensitive, although detection rates will differ as the  
125 sensitivity of the culture media varies. Nonetheless, culture-independent detection  
126 techniques, such as enzyme immunoassays, have lower sensitivity and specificity than  
127 culture methods. As stools with lower counts of *C. difficile* could be deemed falsely negative,  
128 these assays may lead to underestimation of the asymptomatic colonization rates, making  
129 them less suitable for detection of colonization. For example, glutamate dehydrogenase  
130 (GDH) screening is regarded as highly specific for detection of *C. difficile* in clinical  
131 specimens (7, 21); however, potential issues have been highlighted with the use of this  
132 methodology for reporting asymptomatic colonization (22). In a study by Miyajima et al.,  
133 only one out of five positives determined by an enrichment culture method was positive by  
134 GDH assay (22), probably due to low levels of GDH antigen in non-diarrheal stools, below  
135 the lower limits of detection for this assay.

136

137 As the above illustrates, the diagnosis of CDI should not be based on laboratory results  
138 alone, but should always be supported by clinical signs and symptoms suggestive of CDI (7,  
139 23). This is especially important when methodologies which cannot discern CDI from  
140 colonization (stand-alone NAAT, TC) are applied in routine CDI testing.

141 Likewise, we suggest that an optimal diagnostic method for the determination of  
142 asymptomatic colonization should include a confirmation of the absence of clinical

143 symptoms (i.e. absence of diarrhea, ileus and toxic megacolon per the criteria described  
144 above), or the presence of an alternative explanation for these clinical symptoms. The  
145 laboratory methods should include (enrichment) stool culture and either toxigenic culture  
146 or PCR confirmation. This combination of sensitive techniques, although expensive, will yield  
147 more reliable data and support inter-study comparisons.

148

#### 149 **MECHANISMS OF *C. DIFFICILE* COLONIZATION**

150 After having defined *C. difficile* colonization, a closer look at mechanisms that underlie *C.*  
151 *difficile* colonization is needed. Key factors in acquiring or resisting colonization (and  
152 subsequent infection) are the gut microbiota and the host immune response against *C.*  
153 *difficile*.

154

#### 155 **Disruptions in microbiota**

156 The gut microbiota has a prominent role in the whole life cycle of *C. difficile* from  
157 germination and colonization to establishing symptomatic disease. Results from studies on  
158 the differences in microbial composition in patients with CDI, asymptomatic carriers and  
159 non-infected patients can elucidate which alterations determine either the susceptibility to  
160 colonization and/or disease development or colonization resistance (defined as the  
161 resistance to colonization by ingested bacteria or inhibition of overgrowth of resident  
162 bacteria normally present at low levels within the intestinal tract) (24, 25). The optimal  
163 method to study the impact of the microbiota in spore germination, colonization and toxin  
164 production by *C. difficile* would be to take luminal samples and biopsies to study both  
165 microbiota attached to the intestinal wall and present in the lumen, as *C. difficile* was  
166 actually found in biofilm-like structures in the mucus layer of the murine gut and in a human

167 CDI gut model (26, 27). Also, ideally samples should be examined from different locations  
168 along the intestine, because it was demonstrated that in mice, *C. difficile* spores did  
169 germinate and grow in ileal contents, while this was not possible in cecal contents unless  
170 the mice had been treated with specific antibiotics (28). Obtaining these samples in human  
171 subjects is not feasible, though ingestible remotely controlled capsules that are capable of  
172 taking samples from the small intestinal tract are in development. However, most human  
173 studies use easy-to-obtain fecal samples for analyzing the intestinal microbiota, although  
174 these may actually not optimally reflect the microbial composition in the more proximal  
175 intestine where bile acid induced germination of the ingested spores occurs (see below).

176  
177 Alterations in gut microbial composition that have been described for CDI patients include a  
178 lower species richness and lower microbial diversity compared with healthy controls (29-  
179 31). Between samples from CDI patients, a greater heterogeneity was observed than  
180 between individual samples from healthy controls (31). At the phylum level, *Bacteroidetes*  
181 were less prevalent in CDI patients than in healthy controls, while there was an increase in  
182 *Proteobacteria*. Within the *Firmicutes* phylum, a decrease in the *Clostridia*, especially from  
183 the *Ruminococcaceae* and *Lachnospiraceae* families and butyrate-producing anaerobic  
184 bacteria from *Clostridium* clusters IV and XIVa was noted in CDI patients (31). In addition to  
185 these depletions, increases in the orders of the *Enterobacteriales* and *Pseudomonales*  
186 (*Proteobacteria*) and *Lactobacillales* (*Firmicutes*) were observed (30, 31). Also, in human  
187 fecal samples collected prior to onset of a first CDI episode, a decreased diversity, a  
188 decrease in the phylum *Bacteroidetes* and changes within the phylum *Firmicutes* (a decrease  
189 in *Clostridiales Incertae Sedis XI* and an increase in *Enterococacceae* from the order  
190 *Lactobacillales*) were observed in comparison to samples from hospitalized patients who did

191 not develop CDI (32). A reduction in the family *Clostridiales Incertae Sedis XI* in these  
192 samples was demonstrated to be independently associated with CDI development .  
193 Moreover, changes in microbial composition comparable to those found in CDI patients  
194 have been described for patients with nosocomial diarrhea who tested negative for  
195 *C.difficile* or its toxins. These changes included a comparable decrease in species richness  
196 and microbial diversity and again a decrease in butyrate producing bacteria from the  
197 *Ruminococcaceaea* and *Lachnospiraceae* families in comparison to healthy controls (30, 31,  
198 33). This may indicate that patients with nosocomial diarrhea not due to CDI are also  
199 susceptible to development of CDI once they are exposed to *C. difficile* spores. It also  
200 suggests that the CDI itself did not much alter the gut microbial composition (31). Among  
201 mice that were given clindamycin to render them susceptible to CDI development, luminal  
202 samples and biopsies generally confirm the findings in humans and demonstrate a  
203 decreased species richness (34). Mice without antibiotic pre-exposure, and therefore  
204 undisturbed microbiota, do not develop CDI symptoms after administration of *C. difficile*  
205 spores (34). Also, in mice with CDI a microbiota dominated by *Proteobacteria* was  
206 demonstrated, instead of a *Firmicutes* and *Bacteroidetes* dominated microbiota as found in  
207 healthy mice (34, 35).

208

209 Alterations in gut microbial composition in *C. difficile* carriers are less well described, but  
210 may give more insight in the mechanisms that allow for colonization whilst protecting  
211 against the development of overt disease. One of the few available studies reports a  
212 decreased species richness and decreased microbial diversity not only in samples from 8 CDI  
213 patients but also in samples from 8 asymptomatic carriers, compared to 9 healthy subjects  
214 (29). However, the structure of the microbial community was significantly different among

215 CDI patients and carriers and therefore it is suggested that the absence or presence of  
216 certain bacterial taxa is more important in determining the development of CDI or *C. difficile*  
217 colonization than the diversity of species richness alone. In carriers, fewer *Proteobacteria*  
218 and a higher proportion of *Firmicutes* and *Bacteroidetes* were found than in CDI patients  
219 and so this distribution resembled that of healthy individuals more (29). Another study  
220 among 98 hospitalized patients (including 4 CDI patients and 4 *C. difficile* colonized patients)  
221 showed that, compared with CDI patients, a higher level of *Clostridiales Family XI Incertae*  
222 *Sedis, Clostridium* or *Eubacterium* was found just before *C. difficile* colonization was  
223 detected, also supporting the notion that the presence of certain bacterial taxa is important  
224 to prevent overgrowth or progression from colonization to overt infection (36). Evidence  
225 from murine studies also indicates that colonization with certain bacterial taxa may prevent  
226 the progression from colonization to CDI; mice precolonized with a murine *Lachnospiraceae*  
227 isolate showed significantly reduced *C. difficile* colonization (37). Similarly, administration of  
228 *Clostridium scindens* in antibiotic-treated mice is associated with resistance to CDI (38).  
229 Moreover, in antibiotic-exposed mice who were challenged with *C. difficile* spores, different  
230 patterns in microbiota composition were seen in those that developed severe CDI  
231 symptoms versus animals who became only *C. difficile* colonized (35). In the first group, a  
232 shift towards *Proteoabacteria* was noted, while the latter group had a microbiota that was  
233 dominated by *Firmicutes* (including *Lachnospiraceae*) resembling that of mice who had not  
234 been exposed to antibiotics. The presence of a *Firmicutes* dominated microbiota seemed to  
235 be protective against the development of clinical symptoms in this experiment (35).  
236 Interestingly, a recent longitudinal study in a *C. difficile* colonized infant showed important  
237 changes in microbiota composition during weaning. An increase in the relative abundance of  
238 *Bacteroides, Blautia, Parabacteroides, Coprococcus, Ruminococcus, and Oscillospira* was

239 noted suggesting that these bacterial genera likely account for the expulsion of *C. difficile*  
240 (39).

241

242 In conclusion, there are only a few studies on the intestinal microbiota in patients with  
243 asymptomatic *C. difficile* colonization, which are also very limited in sample sizes. However,  
244 these studies and findings from mice studies support the idea that a decreased species  
245 richness and decreased microbial diversity appear to allow for colonization, although the  
246 presence of certain bacterial taxa seems to protect from progression to CDI. Mechanisms by  
247 which the microbiome and in particular the presence of certain bacterial taxa may offer  
248 colonization resistance and protection against infection will be described below.

249

#### 250 **The role of the microbiota: bile acid metabolism**

251 The first step in establishing *C. difficile* colonization is the germination of spores. Primary  
252 bile acids are known to stimulate this germination process (40). The physiological function  
253 of primary bile acids is to assist in digesting fat. To be able to do so, after being produced in  
254 the liver, primary bile acids are released into and reabsorbed from the small intestine.  
255 However, a small amount of the primary bile acids is not reabsorbed and is passed into the  
256 colon. In the colon, these primary bile acids are metabolized into secondary bile acids by  
257 certain members of the normal gut microbiota. Secondary bile acids inhibit *C. difficile*  
258 growth (40). The capacity to metabolize primary bile acids into secondary bile acids by the  
259 production of bile acid 7 $\alpha$ -dehydroxylating enzymes is shown in members of the  
260 *Lachnospiraceae*, *Ruminococcaceae* and *Blautia* families, all belonging to the phylum  
261 *Firmicutes* (28, 41). A disruption in the intestinal microbiota and depletion of *Firmicutes* may  
262 therefore cause an increase in primary bile acids and a decrease in secondary bile acids. This

263 was shown in antibiotic-treated mice, where loss of members of the *Lachnospiraceae* and  
264 *Ruminococcaceae* families was found to be correlated to a significant loss of secondary bile  
265 acids (28). More specifically, this was also shown for one of the members of the  
266 *Lachnospiraceae* family, *C. scindens*; the administration of this bacterium was shown to  
267 restore physiological levels of secondary bile acid synthesis (38). Loss of secondary bile acids  
268 and an increase in primary bile acids creates a favorable environment for *C. difficile*. Support  
269 for the role of bile acid metabolism in this susceptibility to *C. difficile* colonization is  
270 obtained from both in vitro and in vivo studies. In vitro, spores are able to germinate in the  
271 presence of bile acids concentrations found in feces of CDI patients; however, spore  
272 germination and vegetative growth was inhibited in the presence of bile acids at  
273 concentrations found in patients after fecal microbiota transplant (FMT) or in mice resistant  
274 to *C. difficile* (28, 42). In vivo significantly higher levels of primary bile acids and lower levels  
275 of secondary bile acids were found in feces from CDI patients compared with controls,  
276 especially in patients with a recurrent CDI episode (43). Notably, the amount of germination  
277 in response to bile acids seems to vary between strains, which may be related to mutations  
278 in the CspC germinant receptor (called CspC) that recognizes the primary bile acids (42). A *C.*  
279 *difficile* mutant completely deficient for the CspC receptor gene was demonstrated to cause  
280 less severe clinical symptoms in a hamster model (40).

281

## 282 **The role of the microbiota: other mechanisms**

283 Apart from the altered bile acid composition, other mechanisms also induced by disruptions  
284 of the microbiota are suggested to play a role in conferring susceptibility to *C. difficile*.  
285 First, disruptions in the microbiota that lead to a diminished production of short chain fatty  
286 acids (SCFAs) may be of importance. SCFAs are produced from dietary and host-derived

287 carbohydrates mainly by *Lachnospiraceae* and *Ruminococcaceae*, the families that were less  
288 abundant in CDI patients and carriers. They may have effect on colonization resistance  
289 through reducing the luminal pH (and thereby creating an unfavorable environment for *C.*  
290 *difficile*) (44) and stimulating the defense barrier as one of the SCFAs (butyrate) is the main  
291 energy source of the gut epithelium (45, 46). Amino acids may also play a role in the  
292 susceptibility to *C. difficile* colonization, as they can enhance germination in the presence of  
293 secondary bile acids and may influence the immune system. Moreover, the digestion of  
294 carbohydrates in the gut results may impact susceptibility for CDI development.  
295 *Bacteroidetes* are mainly responsible for this carbohydrate digestion which results in  
296 production of substrates essential for homeostasis of colonocytes (47). A reduction in  
297 *Bacteroidetes* may therefore negatively impact colonic health.  
298 Besides the indirect mechanisms described above, the microbiota may also have direct  
299 resistant mechanisms against *C. difficile*. These include competition for niches and nutrients  
300 and the production of antimicrobials (48, 49).

301

### 302 **The role of the immune system: innate immunity**

303 The precise protective factors of the innate immunity that prevent colonization and  
304 progression to CDI are unknown, but are probably less important than the role of the  
305 microbiota and bile acid metabolism. Virulence factors of *C. difficile* induce a rapid innate  
306 immune response resulting in an inflammatory response which is necessary to induce  
307 adaptive immunity.

308 CDI is characterized by a severe intestinal inflammatory response in which neutrophils  
309 infiltrate the mucosa. TcdA and TcdB play an important role in eliciting this inflammatory

310 response (50). After epithelial barrier disruption, TcdA and TcdB trigger inflammatory  
311 signaling cascades through activation of NF- $\kappa$ B, AP-1 and inflammasome, and stimulate  
312 production of pro-inflammatory cytokines and chemokines in epithelial cells. This promotes  
313 the recruitment of immune cells including neutrophils and induces the production of  
314 defensins. Surface proteins also trigger an innate immune response. Challenge of  
315 macrophages with *C. difficile* surface proteins (surface layer proteins, SLPs) leads to pro-  
316 inflammatory cytokine production such as TNF- $\alpha$ , IL-1 $\beta$  and IL-8 (51).

317 Additionally, *C. difficile* SLPs interact *in vitro* with TLR4 leading to dendritic cell (DC)  
318 maturation, robust Th1 and Th17 responses with production of IFN $\gamma$  and IL-17, and a weak  
319 Th2 response leading to antibody production (52). Ryan *et al.* showed that TLR4 and myeloid  
320 differentiation primary-response protein 88 (MyD88) deficient mice were more prone to *C.*  
321 *difficile* infection (53). *C. difficile* flagellin FliC also activates an innate immune response via  
322 its interaction with TLR5 inducing predominantly activation of p38 MAPK and, to a lesser  
323 extent NF- $\kappa$ B, resulting in up-regulation of the expression of pro-inflammatory cytokine  
324 genes and the production of pro-inflammatory factors (54, 55). In vivo, Batah *et al.* showed  
325 a synergic effect of *C. difficile* flagellin and toxins in inducing mucosal inflammation (56).

326 In summary, the innate immune response induces an inflammatory response which  
327 promotes an adaptive immune response with memory and long-lasting immunity (see  
328 below), but its effects on *C. difficile* colonization are unknown.

329

330 **The role of the immune system: adaptive immunity**

331 The adaptive immunity against colonization or CDI has mainly been studied for its antibody-  
332 mediated response whereas the role of the cell-mediated immune response remains  
333 unknown.

334 Serum antibodies against somatic antigens and surface components have been found in  
335 asymptomatic carriers and patients recovered from CDI (57, 58), which suggests that surface  
336 proteins induce an immune response and modulate disease outcome. Vaccination assays  
337 with these proteins have been performed in animal models. Parenteral or mucosal  
338 vaccination with the S-layer proteins led to specific antibody production but only partial  
339 protection in the hamster model (59, 60). Immunization studies that were performed in  
340 animals with Cwp84 and the flagellar proteins FLiC and FliD by mucosal route resulted in a  
341 significant decrease in intestinal *C. difficile* colonization in the mouse model and partial  
342 protection in the hamster model (61, 62). Likewise, Ghose *et al.* immunized mice and  
343 hamsters intra-peritoneally with FliC adjuvanted with alum, inducing a high circulating anti-  
344 FliC IgG response in animal sera, full protection in mice against a clinical 072/NAP1 strain,  
345 but only partial protection in hamsters against 630 $\Delta$ erm strain (63). All these results suggest  
346 that antibodies against *C. difficile* surface proteins have a protective role against  
347 colonization. At the moment, studies with surface protein-based vaccines to prevent  
348 colonization in humans are lacking.

349 Antibodies to TcdA and TcdB do not protect from colonization, but influence disease  
350 susceptibility and subsequently the progression from colonization into CDI. Kyne *et al.*  
351 studied anti-TcdA IgG antibody levels in patients who became colonized after *C. difficile*  
352 exposure. They found that patients who remained asymptotically colonized had greater

353 increases in anti-TcdA IgG antibodies than patients who progressed from colonization to CDI  
354 (64).

355 Monoclonal antibody (Mab)-based passive immunotherapy directed to toxins was able to  
356 protect hamsters from CDI. In humans, two Mabs, one targeting TcdA (actoxumab) and  
357 another targeting TcdB (bezlotuxumab) were tested in human clinical trials aimed at the  
358 prevention of recurrent disease (65). Bezlotuxumab prevented approximately 40% of the  
359 recurrences. A recently published hypothesis suggested that this reduction in recurrences is  
360 presumably due to limiting epithelial damage and facilitating rapid microbiome recovery  
361 (66), suggesting that reduced (re)colonization may be an important factor, although this  
362 should be explored further. Currently, two pharmaceutical firms (Pfizer and Valneva) have  
363 vaccine clinical trial development programmes with the two toxins (toxoids or toxin  
364 fragments) but no colonization factors as antigens (67); Sanofi Pasteur has recently  
365 announced the cessation of its vaccine development programme, which was also based on  
366 toxin antigens alone. Therefore these vaccines protect against the toxic effects of *C. difficile*  
367 on the intestinal mucosa, and can thereby hinder the progression from colonization to CDI.

368

369 In conclusion, a rapid innate immune response induces adaptive immunity to CDI, of which  
370 the antibody-mediated response is best understood. Antibodies against *C. difficile* surface  
371 proteins are thought to protect against colonization, while antibodies against *C. difficile*  
372 toxins protect against disease, directly by its toxin neutralizing effect and possibly also  
373 indirectly by limiting epithelial damage and restoring colonization resistance.

374

375 **SOURCES OF *C. DIFFICILE* - HUMAN**

376 Patients with CDI can shed *C. difficile* not only during the diarrheal episode, but also after  
377 completion of therapy. In a study of 52 patients receiving CDI treatment, samples from  
378 stool, skin and environmental sites were cultured for *C. difficile* before treatment, every 2-3  
379 days during treatment and weekly after therapy was completed (68). Prior to treatment,  
380 100% of stool samples and approximately 90% of skin and environmental samples were  
381 culture positive for *C. difficile*. Stool cultures became *C. difficile* negative in most patients by  
382 the time diarrhea resolved at a mean 4.2 days. However, at the same time, skin and  
383 environmental contamination with *C. difficile* remained high at 60% and 37% respectively. In  
384 addition, stool detection of *C. difficile* was 56% at 1-4 weeks post treatment among  
385 asymptomatic patients recovering from CDI. Moreover, 58% had skin contamination with *C.*  
386 *difficile* 1-4 weeks after completion of treatment and 50% had sustained environmental  
387 shedding. Persistent skin and environmental contamination was associated with receipt of  
388 additional antibiotic therapy. Prior to treatment, the mean density of *C. difficile* in stool  
389 samples was significantly higher than at the time that the diarrhea resolved, at end of  
390 treatment and at 1-6 weeks post treatment. This study highlights that patients with CDI can  
391 be a source of *C. difficile* spores and that they can potentially transmit *C. difficile* to other  
392 patients even after diarrhea has resolved. In addition, similar to animal models, continued  
393 antibiotic treatment can trigger a “supershedder” state in patients, in which there is *C.*  
394 *difficile* overgrowth and excretion of high concentrations of spores (69).

395

396 CDI was historically regarded as a healthcare associated infection transmitted primarily  
397 (directly or indirectly) by symptomatic patients, but a growing body of evidence  
398 demonstrates that asymptomatic carriers can also transmit the disease.

399 One study, using MLST (Multi Locus Sequence Typing) could link only 25% of patients with  
400 symptomatic CDI to a previously identified CDI patient (1). A follow-up study of the same  
401 large patient cohort (>1200 cases) used whole genome sequencing and was able to link at  
402 most only 55% (and more likely only 35%) of new cases to previous patients with CDI (3). A  
403 much smaller study (~50 cases) using MLVA (Multiple-Locus Variable number tandem repeat  
404 Analysis) found that only 30% of new cases could be linked to previously identified cases (2).  
405 One could argue that the inability to link new cases to previous ones might be caused by  
406 patients with CDI who are clinically undetected. However, strict criteria were used to  
407 determine which samples should be tested for CDI in the large UK study (1, 3); although a  
408 toxin EIA was used, which is not as sensitive as a reference test, repeat sampling was carried  
409 out according to clinical suspicion of CDI. Depending on the reference test used, the  
410 sensitivity of toxin EIA is approximately 60-85%, which means that 15-40% of patients with  
411 CDI may go undetected. Nonetheless, this does not account completely for the 45 to 75% of  
412 cases that were not closely linked to symptomatic patients (1, 3). This raises the question of  
413 what is/are the source(s) accounting for approximately half of new CDI cases? Curry et al.  
414 examined patients for *C. difficile* carriage who were selected to undergo screening for  
415 vancomycin-resistant enterococci. They found that 29% of CDIs could be linked to  
416 asymptomatic *C. difficile* carriers (2).

417

418 As asymptomatic carriers and the associated shedding of spores usually goes undetected  
419 because of lack of routine screening, they can play a role in spread of *C. difficile* to the  
420 environment and other patients. Although transmission events from one individual  
421 asymptomatic carrier may be rare, as was shown in a relatively small study (15),  
422 asymptomatic carriers may still importantly contribute to the transmission of the disease as

423 they likely outnumber symptomatic CDI patients. A recent study showed that 2.6% of  
424 patients who were not exposed to *C. difficile* colonized patients developed CDI, while this  
425 percentage increased to 4.6% in patients who were exposed (70). Unfortunately, however,  
426 the case definition of CDI in this study was based on detection of toxin gene rather than  
427 toxin, and so over-diagnosis of true cases likely occurred. Asymptomatic carriers who are  
428 colonized at admission appear to contribute to sustaining transmission in the ward. Already  
429 in 1992, it was recognized that *C. difficile* strains introduced to the ward by asymptomatic  
430 carriers were important sources of onwards health care associated transmission (71),  
431 although definitive proof of linkage was hampered by use a non-specific typing technique.  
432 More recently, using an epidemiological model of *C. difficile* transmission in healthcare  
433 settings, Lanzas et al. confirmed that patients colonized on admission likely play a significant  
434 role in sustaining ward based transmission (72).

435

## 436 **ANIMAL AND ENVIRONMENTAL SOURCES OF *C. DIFFICILE***

### 437 **Animals**

438 Similar to humans, CDI or asymptomatic carriage can occur among domestic, farm and wild  
439 animals (73-80). Carriage rates in these studies range from 0-100%. These varied observed  
440 rates may be related to different culture methodologies and different study settings. Much  
441 of this subject has been reviewed in this journal but new information has emerged on  
442 possible transmission from domestic and farm animals (81, 82).

443

444 *C. difficile* can cause diarrhea in domestic companion animals such as dogs and cats, but  
445 asymptomatic transient carriage of *C. difficile* by household pets is common (11-40%) (73,  
446 78, 83, 84). However, many of these studies did not analyze isolates from humans and pets

447 within the same household. A recent study examined the potential for transmission to pets  
448 from 8 patients with recurrent CDI (85), but in this study *C. difficile* was not found in any of  
449 the pets. In contrast, Loo et al. studied 51 families with 15 domestic pets that included 9  
450 cats, 5 dogs and 1 bird (86). During follow-up visits, toxigenic *C. difficile* was found in  
451 cultures of 2 cats and 2 dogs. Probable transmission occurred in 3 of the 15 domestic pet  
452 contacts. None of the domestic pets had diarrhea. Typing by pulsed-field gel electrophoresis  
453 showed that the profiles of all 4 domestic pet isolates were indistinguishable or closely  
454 related to those of their respective index patients. It is conceivable that household pets can  
455 serve as a potential source of *C. difficile* for humans.

456

457 Transmission from farm animals to humans has been examined using whole genome  
458 sequencing using 40 Australian ribotype 014/NAP4 isolates of human and porcine origin  
459 (87). A clonal relationship with one or more porcine strains was demonstrated among 42%  
460 of human strains underscoring potential interspecies transmission. Similar findings were  
461 obtained in a study on 65 *C. difficile* 078/NAP7 isolates collected between 2002 and 2011  
462 that included 12 pairs of human and pig isolates from 12 different pig farms (88). Five  
463 (41.7%) of the 12 farmer-pig pairs were colonized with identical and nearly identical *C.*  
464 *difficile* clones (88); the remaining 7 (58.3%) farmer-pig pairs were not clonal suggesting  
465 exposure to different sources such as the environment.

466

#### 467 **Food**

468 With reports that *C. difficile* can be detected among farm animals, studies of *C. difficile*  
469 detection in retail food products appeared.

470

471 Studies from Canada and the United States report that *C. difficile* can be recovered from  
472 retail meat including ground beef, ready to eat beef, ground pork, ground turkey, pork  
473 sausage, summer sausage, pork chorizo and pork braunschweiger, with prevalences ranging  
474 from 20-63% (89-92).

475 However, the prevalence of *C. difficile* in retail meat products was lower in European  
476 countries, ranging from 0-6.3% (93-95). The observed differences in prevalence of *C. difficile*  
477 culture positivity in retail meats in North American and Europe is striking. This may be  
478 related to seasonal and temporal changes, or may be true observed geographical  
479 differences.

480

481 Using both quantitative and enrichment culture, Weese et al. sought to provide a measure  
482 of the degree of contamination from 230 samples of retail ground beef and pork (96). *C.*  
483 *difficile* was isolated from 28 (12%) and notably, approximately 70% of samples were  
484 positive by enrichment culture only. Among the samples that were positive on direct  
485 culture, the concentration of spores ranged from 20 to 240 spores/gram. Although the  
486 infectious dose of *C. difficile* is not known, these findings suggest that although *C. difficile*  
487 can readily be recovered from retail meat products, the concentration of *C. difficile* spores is  
488 low.

489

490 Stabler et al. investigated the MLST profiles of 385 *C. difficile* isolates from human, animal  
491 and food sources and from geographically diverse regions (97). Animal and food strains  
492 were associated with the ST-1 and ST-11 profiles and these strains have been associated  
493 with CDI outbreaks in humans. Although the majority of *C. difficile* isolates recovered from  
494 retail food products are toxigenic and are of the same ribotypes or MLST to those of human

495 isolates, there have not been any human CDI cases that have been confirmed to be  
496 foodborne in origin.

497

#### 498 **Environment**

499 *C. difficile* spores can survive in the environment for months or years due to their resistance  
500 to heat, drying, and certain disinfectants. Within hospitals, the surface environment is  
501 frequently contaminated with *C. difficile*. *C. difficile* has been cultured from many surfaces  
502 including floors, commodes, toilets, bed pans and high-touch surfaces such as call bells and  
503 overbed tables (14, 98). The frequency of environmental contamination depends on the *C.*  
504 *difficile* status of the patient: fewer than 8% of rooms of culture-negative patients, 8-30% of  
505 rooms of patients with asymptomatic colonization and 9-50% of rooms of CDI patients were  
506 found to be contaminated with *C. difficile*, respectively (14, 99, 100).

507

508 To examine environmental sources outside of the healthcare milieu, Al Saif and Brazier  
509 undertook a large study of 2580 samples in Cardiff, South Wales from various sources  
510 including water, domestic and farm animals, soil, raw vegetables, surface samples from  
511 healthcare facilities, veterinary clinics and private residents (101). One hundred and eighty-  
512 four (7.1%) samples were positive. Water samples gave the highest yield of culture positivity  
513 at 36%, followed by soil at 21% and healthcare environments at 20%. *C. difficile* was found  
514 in 59% of lawn samples collected in public spaces in Perth, Australia and toxigenic ribotypes  
515 014/NAP4 and 020/NAP4 were predominant (102). A Canadian study demonstrated that *C.*  
516 *difficile* was found in 39% of sediments sampled from rivers connected to the discharge  
517 effluent pipe of waste water treatment plants (103). The most common PCR ribotype was  
518 078/NAP7.

519

520 In summary, *C. difficile* has been isolated from animals, retail food and the environment.

521 Using ribotyping and whole genome sequencing techniques, there appears to be

522 interspecies and environmental transmission but the directionality of the transmission

523 remains to be elucidated.

524

## 525 **EPIDEMIOLOGY OF ASYMPTOMATIC COLONIZATION**

526 After having discussed possible sources of *C. difficile* and underlying mechanisms of

527 colonization, a description of the epidemiology of colonization, including the prevalence of

528 colonization rates among different populations, is essential.

529

### 530 **Infants (0-24 months)**

531 Asymptomatic colonization rates in neonates and infants (<2 years) are widely reported as

532 high, but range between 4-71% (18, 104-108). Although the clinical relevance of *C. difficile*

533 colonization in infants is considered as less significant, due to low rates of disease in this

534 population (109), its potential as a transmission reservoir for adult populations remains.

535

536 An early study researching the prevalence of *C. difficile* in the neonate population found

537 that approximately 30% of all newborns were asymptotically colonized within their first

538 month of life (18). However, these data included four specimens deemed positive with no

539 identifiable organism, only toxin. Nonetheless, the transient nature of colonization at this

540 early stage was highlighted with only 4 of 10 babies who were culture positive in the first

541 week of life remaining positive at 14 and 28 days. A more recent review corroborated these

542 early figures, pooling data from 5887 subjects to determine a colonization rate of

543 approximately 35% of infants under one year of age (105). This large-scale analysis suggests  
544 that colonization peaks between 6-12 months, before substantially decreasing towards  
545 adult rates. Although this major review provides a valuable assemblage of data, the  
546 variability across methodologies used by the included studies should be taken into  
547 consideration.

548

549 Geographical differences in infant colonization rates have been identified, with one study  
550 indicating a variance of 4-35% across Estonian and Swedish infant populations respectively  
551 (108). The colonization rate was inversely associated with an elevated presence of inhibitory  
552 Lactobacilli in Estonian subjects, which may be determined by variation in diet and  
553 environmental exposure. A US study of hospitalized infants demonstrated a 20%  
554 colonization rate (110) whereas Furuichi et al. found no evidence of *C. difficile* colonization  
555 amongst Japanese newborns (111). However, the Japanese data were based on culture only,  
556 with no attempt to utilize EIA or NAAT to detect low levels of organism. These studies  
557 emphasize the variable epidemiology amongst diverse geographical populations.

558

559 The source of infant colonization is uncertain, with suggestions that the presence of *C.*  
560 *difficile* in the urogenital tract implicated vaginal delivery as a potential route of  
561 transmission to neonates (112). However, later work contradicted this suggestion, failing to  
562 detect any *C. difficile* positive vaginal swabs from post-partum mothers (18, 104). Molecular  
563 analysis of both infant and environmental isolates demonstrate likely acquisition from  
564 environmental sources and patient to patient transmission (113).

565

566 Infants are rarely diagnosed with CDI. Bolton and colleagues found that almost 50% carried  
567 toxin positive strains, but showed no sign of diarrhea, suggesting that although the relevant  
568 toxin genes may be present, they may be minimally (or not) expressed and so fail to cause  
569 disease; alternatively, absent or immature toxin receptors may explain the infrequency of  
570 CDI despite high colonization rates (18). However, understanding toxigenic strain  
571 colonization rates may provide a greater insight into the relevance of this population as a  
572 reservoir for transmission to adults. Isolates from infants have shown predominance of  
573 ribotypes associated with CDI (106). Adlerberth et al. found that 71% of colonized infants  
574 had toxigenic strains with more than half identified as ribotypes 001/NAP2 and 014/NAP4  
575 that can cause endemic CDI (114). A comparison of *C. difficile* strains in children (<30  
576 months) with those circulating in the adult ( $\geq 18$  years) CDI population within the same  
577 institution, determined nine shared sequence types among the 20% asymptomatic pediatric  
578 subjects (115). This may further implicate infants as a potential reservoir for *C. difficile*  
579 dissemination; nonetheless, no direct transmission events were documented in this limited  
580 pilot study. Potential community-based transmission from infant carriers to the adult  
581 population was alluded to in a longitudinal study demonstrating colonization in all 10 infants  
582 at some point in the first year of life, with 3 infants colonized for 4-9 months (116).

583

#### 584 **Children (2-16 years)**

585 Meta-analysis of studies examining pediatric *C. difficile* epidemiology reported  
586 asymptomatic colonization in children older than 1 year at 15%, with prevalence reducing to  
587 5% in those greater than 2 years of age (117). One explanation for the reduction in  
588 colonization rates after infancy is that by 12 months the distribution of gut flora begins to  
589 closely resemble that of a healthy adult, providing a colonization resistance effect.

590 Nonetheless, contemporaneous studies have reported higher rates of up to 30%  
591 asymptomatic colonization amongst non-infant pediatric populations (111, 118, 119).  
592 Similarly, Merino and colleagues found that around a quarter of US children aged 1-5 years  
593 were colonized by *C. difficile* asymptotically (120). By using a molecular identification  
594 method, classifying groups by the presence of the Toxin A gene (*tcdA*), the Toxin B gene  
595 (*tcdB*) and binary toxin genes (*cdtA/B*), they found that although 3/37 asymptotically  
596 colonized children harbored a strain with toxigenic genes *tcdA* & *tcdB*, none carried the  
597 binary toxin genes *cdtA/cdtB*. Ferreira et al. (121) found low levels of toxigenic *C. difficile* in  
598 Brazilian children, arguing that the majority of acute diarrhea in this cohort is likely to be  
599 associated with entirely different enteropathogens. These epidemiological variations should  
600 be considered in the context of widely differing enteric pathogen populations between  
601 developing and developed countries.

602

### 603 **Healthy adults**

604 Previous studies indicate that the asymptomatic colonization rates amongst healthy  
605 individuals range from 4-15% (Figure 2). However, these studies have often been based on  
606 point prevalence detection of *C. difficile*, making a true carriage rate difficult to ascertain.  
607 Nevertheless, such a prevalence of even transient colonization by *C. difficile* suggests  
608 significant potential for exposure to the bacterium in the community setting among healthy  
609 populations.

610

611 It is important to note the proportions of toxigenic strains because of their importance for  
612 transmission and potential for CDI. Work carried out amongst healthy Japanese adults  
613 reported a high colonization rate (15.4%), with around 70% harboring toxigenic strains

614 (122). However, a more recent US study discovered that all strains contributing to a 6.6%  
615 asymptomatic colonization rate were toxigenic (13). This rate is higher than seen in large  
616 patient transmission studies (2, 12, 71) suggesting that the healthy adult data may be  
617 skewed by relatively small study cohorts (n=149 (122); and n=139 (123)).

618

619 Ozaki et al. identified matching PCR ribotypes amongst a cohort of healthy company  
620 employees, as a potential indication of a shared work place as a common source or  
621 representing human cross-transmission within this cohort (123). As well, they highlighted  
622 the transient nature of colonization, with only 37.5% demonstrating carriage with the same  
623 strain within a follow-up period of 1 year. Galdys et al. also found that approximately 33% of  
624 participants remained positive with the same strain, in samples submitted one month apart  
625 (13). Another study used cluster analysis to highlight that although colonization amongst  
626 healthy groups acts as a reservoir for community acquired CDI, it may only occur  
627 infrequently between families (124). Although a previous study has implicated the family  
628 environment as a source of transmission of *C. difficile* (125), Kato et al. found only one  
629 instance of a shared strain type amongst family members, across 22 families with 1 *C.*  
630 *difficile* colonized index patient.

631

### 632 **Patients at admission to a hospital**

633 Patients at admission to a hospital are a considerable reservoir for *C. difficile* and,  
634 importantly, a potential source of nosocomial transmission. Asymptomatic colonization  
635 rates among patients at admission to a hospital range from 3-21% (11, 12, 98, 126-132).  
636 (Figure 2) A large study by Clabots and colleagues reported that 9.6% of admissions to the  
637 study ward were colonized; admissions from home had the lowest colonization rate (6%),

638 but nonetheless accounted for the second most prevalent method of *C. difficile*  
639 introduction, due to their greater numbers (71). A major Canadian study of over 5000  
640 admissions demonstrated a lower *C. difficile* prevalence rate, with 4.05% asymptotically  
641 colonized (133); this rate was very similar in a more recent large-scale study (4.8%) (134).  
642 Kong et al. suggested that these low rates may be due to regional distribution, as the  
643 majority of *C. difficile* colonized patients in this multi-institution study were based in  
644 hospitals with higher proportions of NAP1-associated CDI (133).  
645  
646 A recent meta-analysis of studies reporting toxigenic *C. difficile* colonization rates upon  
647 hospital admissions, reported a rate of 8.1% among almost 9000 patients (135). Although  
648 this overall rate provides a strong insight into the prevalence of toxigenic *C. difficile*  
649 colonization, the meta-analysis excluded certain large studies due to methodology  
650 differences, in order to attain maximum compatibility of data sets. Such exclusions may well  
651 have impacted on the reported colonization rates.  
652  
653 Two considerably smaller studies have reported higher *C. difficile* colonization rates,  
654 highlighting the potential for sampling bias. Hung et al. found that 20% of 441 patients  
655 admitted to a Taiwanese hospital were *C. difficile* positive, with two thirds carrying toxigenic  
656 *C. difficile* (11), whilst Alasmari and colleagues reported a rate of 21.2% (n=259), with almost  
657 75% harboring toxigenic strains (127). Prior healthcare exposure was very common and not  
658 statistically different between patients colonized with a toxigenic strain and non-colonized  
659 patients (prevalence of prior healthcare exposure 90% and 85%, respectively). However,  
660 Leekha and colleagues demonstrated recent health care exposure as a significant risk factor,  
when reporting a 9.7% toxigenic *C. difficile* colonization rate on admission (129).

661

662 **Hospitalized patients**

663 Determination of hospital *C. difficile* colonization rates is helpful to understanding the  
664 potential for nosocomial transmission. Asymptomatic acquisition during hospital admission  
665 has generally been demonstrated to range between 3-21% (11, 12, 14, 71, 98, 131, 136,  
666 137). McFarland et al. were able to separate their study cohort into early (<2 weeks) and  
667 late (>2 weeks) acquisition relative to hospital admission (14). The majority of patients had  
668 early colonization, with a significant increase in disease severity associated with those  
669 subjects progressing to CDI after late acquisition. However, this understandably correlates  
670 with significant increases in other recognized CDI risk factors, including exposure to  
671 antibiotics and multiple comorbidities.

672

673 Nevertheless, a study that involved mainly HIV positive (and younger) participants,  
674 demonstrated that all 44 *C. difficile* negative patients remained non-colonized throughout  
675 the period of hospitalization (138). This study population was largely accommodated in  
676 single rooms, which could have diminished the impact of positive carriers on transmission.  
677 In addition, Guerrero demonstrated that rectal and skin swabs from hospitalized, colonized  
678 patients yielded much lower counts than those from subjects with diarrhea, suggesting a  
679 reduced transmission potential associated with colonized individuals (8). Furthermore,  
680 Longtin and colleagues were able to show a significant decreasing trend in healthcare-  
681 associated CDI cases after the implementation of contact isolation precautions for colonized  
682 patients identified upon admission (134).

683

684 Length of hospital stay not surprisingly is related to the risk of *C. difficile* colonization; a  
685 large study reported a 50% acquisition rate for those patients with a length of stay greater  
686 than 4 weeks. For those patients screened negative on admission, the average duration of  
687 hospital stay before a positive *C. difficile* culture, ranges between 12-71 days (11, 14, 137).

688

### 689 **Patients in long-term care facilities**

690 Previous reports of *C. difficile* colonization rates amongst residents of long-term healthcare  
691 facilities (LTHF) have ranged widely (4-51%) (139-142). A major caveat in the study reporting  
692 the highest colonization rate was that it was conducted during a CDI outbreak (143).

693 Furthermore, two studies that found high rates examined relatively small cohorts (n=68  
694 (143) and n=32 (141)). Interestingly, the data from Riggs and colleagues showed 37% of  
695 colonized residents harbored the outbreak strain (RT027/NAP1) asymptotically, whilst  
696 Rea and O'Sullivan also isolated a range of outbreak-associated strains from the  
697 asymptomatic group, including RT027/NAP1, 078/NAP7, 018, 014/NAP4 and 026 (142).

698 These rates must be considered with caution, as the presence of an epidemic strain in a  
699 given community is likely to inflate asymptomatic colonization rates. For example, the  
700 asymptomatic colonization rate before and post a CDI outbreak was reported to be 6.5%  
701 and 30.1%, respectively ( $p=0.01$ ) (144).

702

703 Arvand et al. identified colonization rates that ranged from 0-10% across 11 nursing homes  
704 in Germany and concluded that additional factors influenced the asymptomatic colonization  
705 prevalence, including antibiotic exposure rates, comorbidities of residents and the individual  
706 facility's infection control procedures (140). Ryan et al. found similar distributions, likely  
707 reflecting differing resident morbidities and regional strain prevalence (139). Arvand and

708 colleagues found that nursing home residents were ten times more likely to be colonized  
709 with toxigenic strains than non-toxigenic types (140), similar to other reports (122, 139)  
710 demonstrating the presence of the toxin genes, *tcdA* and *tcdB*, in 70% of strains from the  
711 asymptomatic cohorts. Conversely, Rogers et al. found only toxigenic *C. difficile* in those  
712 with asymptomatic colonization (141). In one study where follow up samples from colonized  
713 residents (1-3 months after initial screening) were tested, 10/12 displayed persistent  
714 carriage by the same *C. difficile* PFGE type, possibly indicating a less transient nature  
715 amongst individuals in LTHFs (143). These data demonstrate the variability across studies,  
716 which likely reflect multiple confounders including stringency of infection control  
717 procedures, strain type, antibiotic use and comorbidities, and issues such as single room  
718 versus shared accommodation.

719

#### 720 **Healthcare workers**

721 Asymptomatic gut colonization of healthcare workers (HCW) is a potential, but unproven  
722 source for *C. difficile* transmission. HCWs may well have a role in transmission, due to their  
723 frequent patient contact, but this could simply be due to transient hand contamination.  
724 Kato et al. carried out a large-scale study of Japanese groups including two cohorts of HCWs,  
725 and identified 4.2% of hospital employees as colonized by *C. difficile* (124). Van Nood et al.  
726 attempted to clarify whether intestinal colonization was related to the presence of spores  
727 on HCW's hands. Of 50 Dutch hospital workers, 0% and 13% were *C. difficile* culture positive  
728 on hand print agar plates and fecal samples, respectively (145). Also, in demonstrating that  
729 colonization rates were similar across staff working on wards with and without CDI patients,  
730 they highlighted the potential for acquisition and/or transmission by means other than

731 HCW's hands. Unfortunately, no strain typing was carried out in this study and therefore  
732 definitive transmission relationships could not be determined.

733 Several studies demonstrated low to non-existent intestinal colonization levels with 0-1% of  
734 healthcare workers being *C. difficile* positive (146-149). Friedman et al. did, however, point  
735 out the voluntary nature of study recruitment, and thus HCWs with poorer hand hygiene  
736 may have opted out, leading to a nonrepresentative cohort (147). Furthermore, these  
737 studies only sampled subjects once.

738 Landelle et al. detected *C. difficile* spores on the hands of 24% of HCWs who were directly  
739 caring for CDI patients (150). Other studies have also shown that after caring for patients  
740 with CDI, the proportion of healthcare workers with hand contamination when gloves are  
741 not worn ranged from 8 to 59% (14, 151). This highlights the challenge in determining the  
742 relative importance of patients' fecal *C. difficile* burden, versus HCW hand or environmental  
743 contamination as potential sources of transmission.

744

#### 745 **Duration of carriage**

746 There is a paucity of research reporting duration of asymptomatic *C. difficile* carriage. Large-  
747 scale, longitudinal studies are required to investigate length of carriage and the associated  
748 determinants. Nonetheless, some research does provide follow up data on asymptomatic  
749 hosts.

750

751 Several studies have assessed duration of short term carriage (98, 152, 153). During weekly  
752 follow up of 32 asymptomatic subjects, Samore et al. found that 84% remained positive until  
753 discharge, although the mean duration of sampling was only 8.5 days (range 7-29 days) (98).

754 Johnson et al. continued surveillance on 51 asymptomatic long-term hospital stay patients

755 for up to nine weeks, with no development of CDI during this time (152). Later, when  
756 investigating treatment efficacies for asymptomatic carriage, the same investigators found  
757 that 60, 80 and 100% lost *C. difficile* colonization after 40, 70 and >90 days, respectively (in  
758 the absence of a targeted intervention) (153). Contemporaneous research demonstrated  
759 that only two of six healthy, colonized volunteers retained the same strain one month later  
760 (13). Although the data are limited, they indicate the short term, transient nature of  
761 symptomless *C. difficile* colonization, at least in the absence of repeated exposure to *C.*  
762 *difficile* risk factors such as antibiotics. Nonetheless, variation among patient cohorts and  
763 environments must be considered.

764 Longitudinal studies of Japanese healthy populations have followed asymptomatic carriers  
765 among students, employees and hospital workers. Kato et al. performed a longitudinal  
766 surveillance on 38 asymptomatic carriers for 5-7 months and determined 12 (31.6%)  
767 remained *C. difficile* positive during this time (124). Half of these remained with the same  
768 PFGE type, whilst five had acquired a new strain. The remaining participant retained the  
769 original strain and acquired a new type. Therefore, only 18.4% of participants retained the  
770 same strain after six months, again implying a high rate of transient colonization.

771 Nonetheless, analysis of a single, six-month follow up sample does not permit in-depth  
772 analysis of the dynamics of carriage and it remains unclear if carriage was lost after a few  
773 days, weeks or months. Testing of 18 asymptomatic subjects in three-month intervals, over  
774 one year period found that ten participants (55.6%) only tested positive for *C. difficile* on a  
775 single sampling occasion, indicating loss of carriage within three months; only three (16.7%)  
776 were persistently colonized throughout (123). This further supports the suggestion that  
777 intestinal colonization in healthy adults is largely a transient phenomenon. Of those testing  
778 positive on three or four instances, five harbored the same strain on consecutive sampling

779 occasions (3 students, 2 employees), potentially indicating an element of cross-transmission  
780 within cohorts sharing common physical areas, and even a possibility of a subject  
781 contaminating their own environment and reacquiring the strain later.

782

783 A recent study of healthy subjects from Pittsburgh, USA provided analysis of participant  
784 demographics and dietary data in relation to the duration of *C. difficile* carriage (13). No  
785 correlations were found between previous CDI, prior antibiotics, healthcare exposure, race,  
786 ethnicity, consumption of uncooked meat or seafood and duration of carriage.

787

#### 788 **Ribotype specific differences**

789 Determining the prevalence of ribotypes among asymptomatically colonized individuals may  
790 help to improve the understanding of potential sources of *C. difficile*, and specifically which  
791 toxigenic and common strain types originate from such individuals. Studies of colonizing  
792 strains have shown a broad distribution of PCR ribotypes, with reports of 37 ribotypes  
793 among 94 isolates (124) and 29 diverse sequence types from 112 carriers (115). Whilst it  
794 might be expected that there is a diverse strain distribution among asymptomatically  
795 colonized individuals, as with CDI patients, the prevalence of individual strain types is likely  
796 to vary depending on the virulence potential of a specific ribotype. Nonetheless, the  
797 relationship between ribotype prevalence in CDI patients and strain distribution among  
798 asymptomatic carriers remains unclear.

799

800 In the context of outbreaks, colonization rates by hyper-virulent strains appear to be  
801 markedly increased. Loo et al. and Riggs et al. found very similar (asymptomatic)  
802 colonization rates for PCR ribotype 027/NAP1 strain (36.1% and 37%, respectively) (12, 143).

803 Contemporaneous research highlighted the persistence of PCR ribotype 027/NAP1 in a New  
804 York, long-term care facility, where half of the asymptomatic population (19.3% of all  
805 residents) carried this strain (154). This is likely to be due to increased prevalence in the  
806 patient populations and consequent spore shedding in to the environment (155).  
807 Interestingly, three of the five asymptotically colonized patients that developed  
808 subsequent CDI harbored the epidemic 027/NAP1 strain, hinting at its potential superiority  
809 in progression from colonization to symptomatic disease.

810

811 Other ribotypes have also been implicated as dominant colonizing strains; earlier work  
812 reported that 51.7% of asymptotically colonized, elderly patients were positive for  
813 ribotype 001/NAP2 on admission, with the remaining 48.3% consisting of 12 other ribotypes  
814 (156). As ribotype 001/NAP2 was deemed to predominate in Welsh hospitals at the time,  
815 this may be as expected. Other prevalent European ribotypes (157), including 012/NAP<sub>cr1</sub>,  
816 014/NAP4 and 020/NAP4 have also been reported as predominant strains among  
817 asymptomatic populations (127, 140).

818

819 Conversely, in recent studies covering a period of marked reduction in PCR ribotype  
820 027/NAP1-associated CDI (157), asymptomatic colonization rates of this strain were  
821 considerably lower (140, 142). These data were supported by a large scale, UK transmission  
822 study (15), which also found no evidence of PCR ribotype 027/NAP1 colonization in UK  
823 hospitalized patients; no single strain predominated in this study.

824

825 **RISK FACTORS FOR *C. DIFFICILE* COLONIZATION**

826 Clinical and epidemiological risk factors for CDI are well known, but risk factors for  
827 colonization with *C. difficile* have only come to attention recently. An important distinction  
828 has to be made between risk factors *to be colonized* in the community or *at admission to a*  
829 *hospital*, as opposed to risk factors for acquiring *colonization during hospital admission*.

830

### 831 **Risk factors for colonization in a community-setting**

832 Risk factors for being or becoming colonized in the community are not extensively studied.  
833 Clusters of colonized patients with identical *C. difficile* types have been identified within  
834 community settings (e.g. employees, students) and families, indicating cross-transmission  
835 from colonized individuals or acquisition from a common source (124). A study among 106  
836 healthy adults in Pennsylvania found no statistically significant differences in patient's  
837 characteristics or exposures between 7 colonized and 99 non-colonized subjects, but this  
838 may be due to the small sample size (13). Living in the proximity of livestock farms was not  
839 found to be a risk factor in a recent study among 2494 adults in the Netherlands (158).  
840 Antibiotic exposure in the 3 preceding months was however associated with a 3.7-fold  
841 increased risk of *C. difficile* colonization in the same study (158). A recent study among 338  
842 predominantly healthy infants ( $\leq 2$  years of age) showed that *C. difficile* colonization  
843 increased with pet dogs (159).

844

### 845 **Risk factors for colonization at admission**

846 Recognition of risk factors for being colonized at admission is important, as patients with  
847 these risk factors may introduce and spread *C. difficile* into the hospital. Epidemiological and  
848 clinical risk factors for (overall or toxigenic) colonization at the time of admission include  
849 recent hospitalization (15, 129, 133), chronic dialysis (129), corticosteroid/

850 immunosuppressant use (15, 129, 133), gastric acid suppressant medication (15), and  
851 antibodies against Toxin B (133). (Table 2) The consistent association between previous  
852 healthcare contact and colonization by *C. difficile* likely means that hospitals remain  
853 important sources of *C. difficile*, related to host factors at time of admission (e.g. altered  
854 microbiota composition due to antibiotic use) and increased exposure to strains. However,  
855 patients colonized at admission may have acquired *C. difficile* from diverse sources. Notably,  
856 the healthcare associated *C. difficile* ribotype 027/NAP1 is less frequently found in carriers  
857 at admission, than in those who become colonized during admission (128, 133).

858

#### 859 **Risk factors for acquiring *C. difficile* during hospital admission**

860 Previous hospitalization in the last 2 months, use of proton-pump inhibitors H2-blockers or  
861 chemotherapy (within the 8 weeks preceding the hospitalization or during hospitalization  
862 but before colonization was acquired) and cephalosporin use during admission were  
863 significant risk factors for becoming colonized (with toxigenic or non-toxigenic strains)  
864 during admission (12, 128). (Table 2) In one study, cefepime use and a toll-like receptor 4  
865 polymorphism were risk factors for acquiring toxigenic *C. difficile* colonization during  
866 admission (11). The presence of Toxin B antibodies was associated with asymptomatic  
867 colonization during admission (12). Interestingly, antibodies against Toxin B may have  
868 protective effect against the development of CDI. Likewise, compared to patients who  
869 acquired *C. difficile* and subsequently developed CDI, patients who acquired *C. difficile*  
870 colonization but remained asymptomatic had higher levels of IgG antibody against Toxin A  
871 at time of colonization (160). These observations may indicate that antibodies and/or  
872 acquired immunity (e.g. due to previous hospitalizations) might confer resistance to the  
873 development of symptomatic CDI (see before). Patients who acquired *C. difficile* and

874 developed asymptomatic colonization were less frequently colonized with the hypervirulent  
875 ribotype 027/NAP1 strain compared to those who developed CDI (12, 128, 160). This  
876 suggests that the virulence of the acquired strain can influence the development of  
877 colonization or infection.

878

#### 879 **Risk factors for colonization by toxigenic versus non-toxigenic strains**

880 A recent study showed that hospitalized patients colonized by toxigenic strains and non-  
881 toxigenic strains do not share risk factors. Risk factors for colonization by a toxigenic strain  
882 included a higher number of admissions in the previous year, antimicrobial exposure during  
883 the current admission and the presence of gastro-esophageal reflux disease. Risk factors for  
884 colonization by a non-toxigenic strain were chronic kidney failure and chronic obstructive  
885 pulmonary disease. Unfortunately, the design of this study was cross-sectional and  
886 therefore the time period of *C. difficile* acquisition (i.e. before at admission or during  
887 admission) could not be established in these patients (161). Another study tried to  
888 determine if the type of antibiotics used during admission impacts the risk for acquisition of  
889 either toxigenic or non-toxigenic *C. difficile*. They found that the use of cephalosporins was a  
890 risk factor for both conditions: acquisition of a toxigenic strain was associated with the use  
891 of cefepime, while the acquisition of a non-toxigenic strain was associated with the use of  
892 cefuroxime. Moreover, the use of glycopeptides was a risk factor for acquiring a non-  
893 toxigenic strain during admission (11). For patients colonized on admission, associations  
894 between classes of antibiotics used and the colonization of either toxigenic or non-toxigenic  
895 *C. difficile* have also been reported, but multivariate analyses to identify independent risk  
896 factors have not yet been performed (127).

897

898 **C. DIFFICILE COLONIZATION AND SUBSEQUENT CDI**

899 One of the major questions is, do *C. difficile* colonized individuals have an increased risk of  
900 developing subsequent CDI, or are they protected against disease? A lower risk for *C.*  
901 *difficile* colonized patients of subsequently developing CDI was found in a frequently cited  
902 but older meta-analysis of four studies (162). The major drawback of this review, however,  
903 is that patients colonized by toxigenic or non-toxigenic strains were not analyzed separately;  
904 this difference may be of importance as 44% of colonized patients in this meta-analysis  
905 harbored a non-toxigenic strain. Also, all four studies were performed pre-1994, before the  
906 emergence of hypervirulent strains and recognition of community-associated CDI.  
907 Furthermore, colonization was determined at different time points: at admission (71, 98), at  
908 start of tube feeding with patients colonized at admission excluded (163) or after a hospital  
909 stay of at least 7 days (152). Colonized patients therefore included some patients that  
910 acquired colonization during admission. The risk that these latter patients go on to develop  
911 CDI during the hospital stay may be different from that for individuals already colonized at  
912 admission. A recent meta-analysis aimed to include studies in which patients were colonized  
913 *at admission* with *toxigenic* strains only (11, 15, 98, 127, 131, 135, 164-166). However, not  
914 all included studies succeeded in obtaining samples within 48hrs or 72hrs of admission (15,  
915 98). Also, a study that included patients at admission to a rehabilitation unit (after an  
916 average stay of 30 days in acute care) was included (166). In one study, the distinction  
917 between colonization of a toxigenic strain and CDI was difficult to establish, as all patients  
918 received a hematopoietic stem cell transplantation and donor lymphocyte infusion; almost  
919 all such patients subsequently develop diarrhea. In patients known to carry a toxigenic *C.*  
920 *difficile* strain, diarrhea may have been falsely attributed to CDI (164). Notwithstanding  
921 these limitations, all studies pointed to an increased risk for patients colonized with

922 toxigenic *C. difficile* at admission to progress to CDI: overall, the relative risk was 5.86 (95%  
923 CI 4.21-8.16). (Table 3) Some recent studies were not included in this meta-analysis. A  
924 recent large study, which screened n=3605 of 4508 hospital admissions, found that patients  
925 carrying toxigenic strains on admission were at a much increased risk of developing CDI (CDI  
926 rates 9.4% vs 2.3% for non-toxigenic *C. difficile* carriers) (70). The risk of CDI in non-  
927 colonized patients who were exposed to subjects colonized by a toxigenic strain was also  
928 significantly increased (4.6% vs 2.6% for non-exposed patients; odds ratio for CDI if exposed  
929 to carrier, 1.79; 95% CI, 1.16–2.76). However, this study appeared to diagnose CDI based on  
930 the presence of toxigenic *C. difficile* strains rather than toxin, and so the case incidence is  
931 likely to have been overestimated. In turn, the association between colonization by, or  
932 exposure to, toxigenic strains and subsequent CDI may have been exaggerated (70). A much  
933 smaller study did not report any CDI cases among 37 patients colonized on admission (128)  
934 (Table 3).

935 Two other recent studies describe the risk of colonized ICU patients to develop CDI. The  
936 study by Tschudin-Sutter et al. in a cohort of 542 ICU patients described a relative risk to  
937 develop CDI of 8.6 for patients colonized on admission and a relative risk of 10.9 for patients  
938 who became colonized during hospitalization (132). Zhang and colleagues however,  
939 identified 6 patients who were colonized on admission to the ICU, but none of them  
940 developed CDI. During their study period 4 patients developed CDI, but all were not  
941 colonized on admission to the ICU (167). These conflicting results are probably caused by  
942 small samples sizes, a relatively rare outcome event (3 vs 0 colonized patients progressed to  
943 CDI) and different predominant strains.

944

945 From the above we can conclude that patients asymptotically colonized by toxigenic  
946 strains may progress to CDI during admission. However, for patients asymptotically  
947 colonized by non-toxigenic strains there seems to be no increased risk of progressing to CDI  
948 and these patients may even be protected from developing CDI.

949

## 950 **INFECTION CONTROL AND ANTIMICROBIAL STEWARDSHIP IMPLICATIONS FOR**

### 951 **ASYMPTOMATIC CARRIERS**

952 Symptomatic CDI patients are believed to be the main source of nosocomial transmission,  
953 and current guidelines recommend their systematic detection and isolation (5). Due to a  
954 paucity of data at the time of writing of this review, the isolation of asymptomatic carriers is  
955 not recommended. Whether these carriers should be isolated remains an important clinical  
956 question stemming from the growing body of literature on the subject. Mathematical  
957 modeling of *C. difficile* transmission and simulation of screening and isolation of carriers has  
958 shown the intervention to be effective at reducing CDI rates (168, 169). However, a clinically  
959 based study to directly answer this question has not been conducted until recently (134).

960

961 Longtin et al. explored the effect of isolating asymptomatic *C. difficile* carriers on the  
962 incidence of hospital acquired CDI in an acute care hospital in Quebec, with high baseline  
963 rates of CDI (134). A quasi-experimental design was employed, using change in CDI  
964 incidence in other Quebec hospitals as controls. The effect of the intervention (isolation of  
965 carriers) was evaluated through a time series analysis. Compared with the pre-intervention  
966 period, the incidence of CDI decreased significantly after the intervention. In addition, the  
967 effect was confirmed using two methods of analysis, segmented regression analysis and  
968 autoregressive integrated moving average (ARIMA) modeling, indicative of the robustness of

969 the results. Incidence rates of CDI in the study hospital remained low a year after the study  
970 terminated, demonstrating the sustained effect of this intervention.

971 This study provides the most convincing evidence to date for the significant effect of  
972 isolating carriers. The authors assessed confounding elements; such as intensity of CDI  
973 testing, total antimicrobial use and proton pump inhibitor use, which remained stable  
974 during the study period. Concurrently, a significant decrease in the use of metronidazole  
975 and oral vancomycin suggested true clinical impact from the observed decrease in incidence  
976 and trend. Compliance with hand hygiene increased, but utilized alcohol-based solution not  
977 effective against *C. difficile* spores. Some potential confounders that were not assessed  
978 include compliance with isolation precautions, environmental cleaning, improvement in  
979 appropriate antibiotic use, and knowledge of *C. difficile* carrier status on the management of  
980 a patient (170).

981

982 Ultimately, these promising findings need to be reproduced in a multicenter, cluster  
983 randomized trial, prior to being considered for widespread implementation. If these results  
984 are confirmed in various different hospital settings, adoption of screening and isolation of  
985 asymptomatic carriers may be an important strategy to decrease CDI rates. However, this  
986 will raise several practical questions, such as whether universal versus targeted screening  
987 should be adopted and what the optimal screening method is. Given known risk factors for  
988 colonization on admission, a reasonable approach may be to selectively target high-risk  
989 patients and isolate them on admission to hospital (133). Other issues that would need to  
990 be addressed include frequency of screening during hospitalization, the optimal isolation  
991 protocol, the impact on patient perception of care and the additional workload burden on  
992 frontline healthcare workers and the microbiology laboratory.

993 Reducing inappropriate antimicrobial use through antimicrobial stewardship programs  
994 (ASPs) has been shown to decrease rates of CDI (171-173), but given the lack of widespread  
995 screening for asymptomatic carriers, ASPs targeted at this population have not been  
996 studied. It does not necessarily follow that targeting colonized patients, as a whole group,  
997 would decrease CDI rates, as some of these patients may be long-time colonized patients  
998 with immunity and decreased risk of developing symptomatic CDI. These patients are likely  
999 different from patients who may still be colonized with *C. difficile* after an episode of  
1000 symptomatic CDI (10, 68). One study showed a three-fold increase in recurrence of CDI in  
1001 patients exposed to antimicrobials after resolved CDI, compared with those who were not  
1002 exposed (174). Therefore, patients with prior CDI, an easily identifiable subset of  
1003 asymptomatic carriers, probably represent colonized patients at highest risk of developing  
1004 infection, and may represent suitable targets for focused stewardship efforts.

1005

#### 1006 **CONCLUDING REMARKS AND FUTURE DIRECTIONS**

1007 The intriguing concept of *C. difficile* colonization has garnered much attention during the  
1008 last decade. Gut microbiota studies and immunologic studies have provided some insight in  
1009 the conditions that allow for colonization and protect against disease progression. However,  
1010 more studies are needed to assess the precise role of changes in microbiota and the precise  
1011 triggers of spore germination and colonization, as well as changes and initiators that lead to  
1012 toxin production. It also needs to be explored why some individuals' transition to *C. difficile*  
1013 carrier status and what interventions could terminate colonization or could block the  
1014 progression to CDI.

1015 The realization that *C. difficile* colonized patients may be the most important unexplained  
1016 reservoir for *C. difficile* transmission has led to epidemiological studies investigating

1017 colonization rates among different populations and risk factors for this condition. Colonized  
1018 patients on admission appear to play an important role in introducing and maintaining  
1019 transmission in the ward and hence, risk factors for colonization on admission are of specific  
1020 interest. To further study the acquisition and transmission of *C. difficile*, all patients  
1021 admitted to the hospital should be screened for colonization by (and preferably sustained  
1022 carriage of) *C. difficile*. *C. difficile* positive individuals should be questioned about risk factors  
1023 for acquisition and should be followed during admission for the development of  
1024 symptomatic CDI. Epidemiological investigations and molecular typing methods should be  
1025 applied to examine possible linkage of *C. difficile* colonized individuals to CDI cases. In this  
1026 way, risk factors for *C. difficile* colonization can be identified and the role of *C. difficile*  
1027 positive individuals in transmission of the disease can be elucidated. It would be interesting  
1028 to determine if there are host and pathogen factors that affect transmissibility of *C. difficile*.  
1029 More evidence from different settings is needed to determine whether specific control  
1030 measures targeting colonized patients may be justified to prevent spread. In addition, the  
1031 protective effects of *C. difficile* vaccines are being examined, but information on the  
1032 consequences of colonization and spread to non-vaccinated individuals would be relevant.

1033 **Conflicts of interest**

- 1034 MHW has received: consulting fees from Actelion, Astellas, bioMerieux, MedImmune, Merck, Pfizer,  
1035 Qiagen, Sanofi-Pasteur, Seres, Summit, Synthetic Biologics and Valneva; lecture fees from Alere,  
1036 Astellas, Merck & Pfizer; and grant support from Actelion, Astellas, bioMerieux, Da Volterra, Merck,  
1037 Sanofi-Pasteur, Seres and Summit.
- 1038 VGL has received consulting fees from Merck.
- 1039 MJC, JJV, LYK, SP, EJK: no conflicts of interest

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1599 gut modelling of *C. difficile* infections for investigation of treatment regimen and recurrence, efficacy  
1600 testing of novel compounds for antimicrobial activity, and work as part of the *Clostridium difficile*  
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1622 studied *Clostridium difficile* and especially the surface proteins and their interest in the development  
1623 of vaccine strategies at the Paris-Sud University. She has developed an expertise on *Clostridium*  
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1632 Director of Infection Prevention (4 years), Infection Control Doctor (8 years), Clinical Director of  
1633 Pathology (6 years) at LTHT and Head of Microbiology (15 years). Professor Wilcox heads a

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1637 **Ed J. Kuijper** is professor and head of Experimental Bacteriology at the Department of Medical  
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1640 "*Aeromonas*-associated diarrhoea in the Netherlands" . In 1987 he also completed his training to  
1641 medical microbiologist and worked as researcher on the topics of meningococcal infections, fungal  
1642 infections and mycobacterial infections until 2000. In 2001 he was appointed at Leiden University  
1643 and started a research group on *Clostridium difficile* infections (CDI), in close collaboration with the  
1644 National Center for Infectious Diseases at the RIVM. The research group focusses on the  
1645 pathogenesis, epidemiology and treatment of CDI.

**Figure 1. *C. difficile* colonization versus *C. difficile* infection.** CDI - *Clostridium difficile* infection.

**Figure 2. Prevalence of colonization among community-dwelling adults, patients at hospital admission to the hospital and LTCF residents.** Hollow circles represent CDC prevalences, solid circles represent tCDC prevalences. Size of the circles represents samples size. The different colors represent the different studies (see legend). CDC - *C. difficile* colonization (including non-toxigenic and toxigenic strains), tCDC - toxigenic *C. difficile* colonization, LTCF - long term care facility.

**Table 1: Diagnostic methodologies detecting *C. difficile* or its toxins.** tCD – toxigenic *C. difficile*, ntCD – nontoxigenic *C. difficile*, GDH – glutamate dehydrogenase, EIA – enzyme immunoassay, CCNA – cell cytotoxicity neutralization assay, CDI – *Clostridium difficile* Infection, PCR – polymerase chain reaction.

Diagnostic Test	Target of detection	Able to detect colonization?	Remarks
<b>Direct culture</b>	<i>C. difficile</i>	Yes	Does not differentiate between colonization or infection by CD, does not differentiate between tCD and ntCD
<b>Enrichment culture</b>	<i>C. difficile</i>	Yes	Does not differentiate between colonization or infection by CD, does not differentiate between tCD and ntCD, thought to be more sensitive than direct culture when low numbers of vegetative cells or spores are present
<b>GDH EIA</b>	GDH	Yes	Does not differentiate between colonization or infection by CD, does not differentiate between tCD and ntCD
<b>Toxigenic culture</b>	Toxigenic <i>C. difficile</i>	Yes	Does not differentiate between infection and colonization by tCD
<b>PCR of toxin genes</b>	<i>tcdA</i> , <i>tcdB</i> , binary toxin genes	Yes	Does not differentiate between infection and colonization by tCD
<b>Toxin A/B EIA</b>	Toxins A and B	No	Detects Toxins A and B and not the presence of the organism, therefore cannot be utilized to identify asymptomatic colonization
<b>CCNA</b>	Toxin B	No	Detects Toxin B and not the presence of the organism, therefore cannot be utilized to identify asymptomatic colonization

**Table 2. Studies investigating risk factors for *C.difficile* colonization on admission or acquisition of *C. difficile* acquisition during admission.** Studies were included if: publication since 1994, investigating either risk factors for colonization at admission or risk factors for colonization acquisition during admission (studies investigating risk factors for being colonized at a certain time point during hospitalization were excluded), sample size > 100, risk factors assessed by multivariate regression. CDC -*Clostridium difficile* colonization, tCDC-toxigenic *Clostridium difficile* colonization.

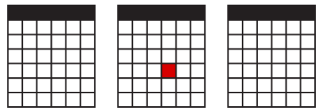
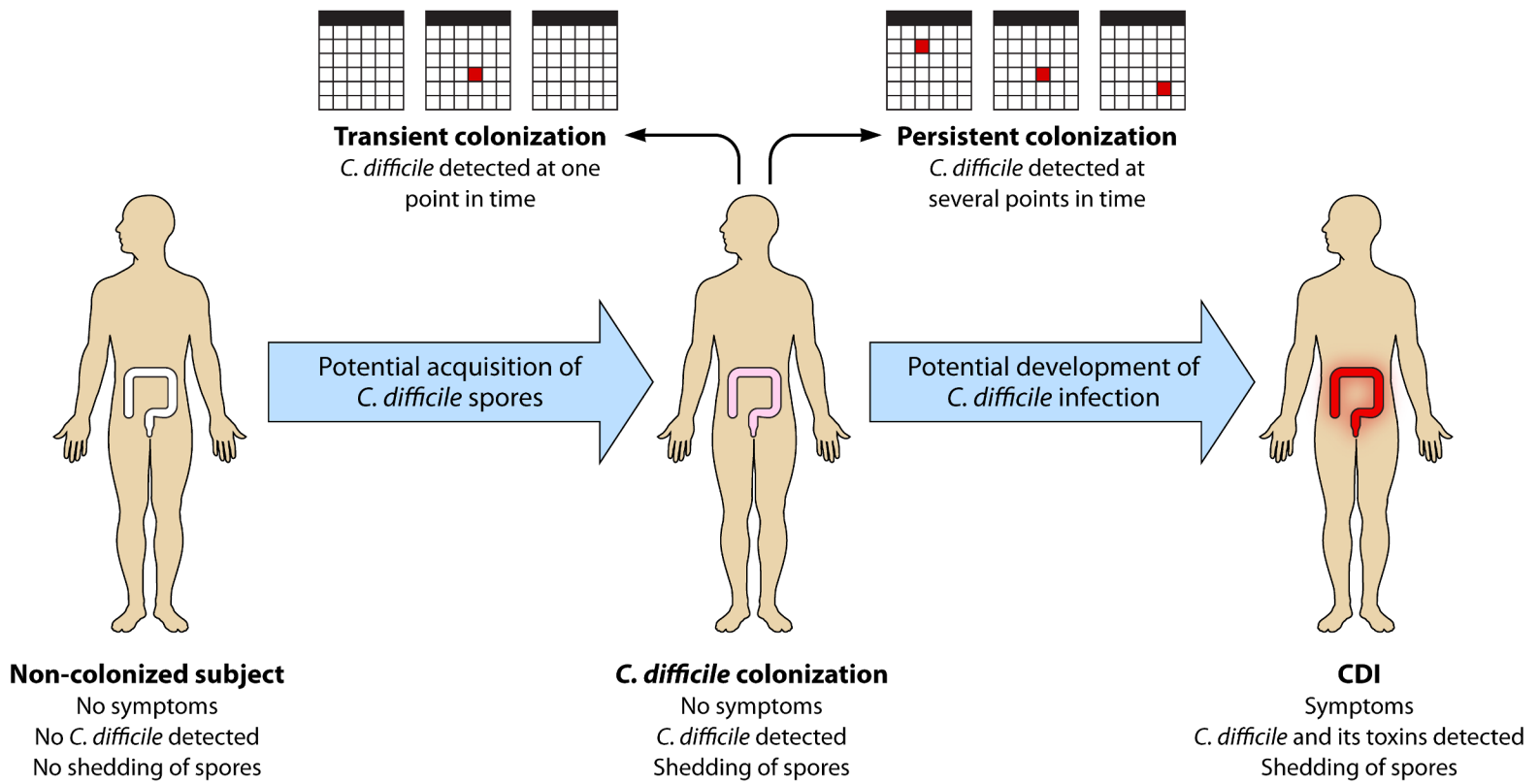
Condition	Identified risk factor	Reference
<i>Risk factors for colonization at admission</i>		
CDC	previous hospitalization	133, 15
	previous CDI episode	133
	previous use of corticosteroids or other immunosuppressant medication	133, 15
	presence of antibodies against Toxin B	133
	current loose stools/diarrhea but not meeting CDI criteria	15
tCDC	previous hospitalization	129
	chronic dialysis	129
	use of corticosteroids	129
<i>Risk factors for acquiring colonization during admission</i>		
CDC	previous hospitalization	12
	use of chemotherapy	12
	use of PPI or H2-blockers	12
	presence of antibodies against Toxin B	12
tCDC	TLR4 polymorphism	11
	cefepime use during admission	11

**Table 3. Studies investigating the risk of development of CDI among patients with toxigenic *C. difficile* colonization on admission.** Studies were included if: published since 1994, sample size > 100 patients, comparison of patients with toxigenic *C. difficile* colonization on admission with controls (patients with non-toxigenic *C. difficile* colonization and non-colonized patients together). Relative risks were calculated as the risk for tCDC patients compared to the risk for non-colonized and ntCDC patients together and were unadjusted. RR - relative risk, HSCT - hematopoietic stem cell transplantation, tCDC - toxigenic *Clostridium difficile* colonization, ntCDC - non-toxigenic *Clostridium difficile* colonization, CDI - *Clostridium difficile* infection, LOS - length of stay, ICU - intensive care unit, na - not available.

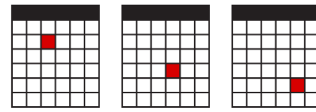
<b>Study</b>	<b>Country and period</b>	<b>Setting and patients</b>	<b>Follow up period</b>	<b>Included patients (N)</b>	<b>Prevalence tCDC (%)</b>	<b>CDI among tCDC (%)</b>	<b>CDI among controls (%)</b>	<b>RR for CDI (95% CI)</b>	<b>Remarks</b>
Samore (ref 98)	US 1991	patients with an anticipated LOS of at least 5 days admitted or transferred to general medical and surgical wards and ICUs	until discharge	496	24/496 (4.8)	1/24 (4.2)	8/472 (1.7)	2.46 (0.32-18.87)	90 of 496 samples (18.1%) were not obtained within 72hrs of admission
Soyletir (ref 131)	Turkey published 1996	patients admitted to a general medical ward with a LOS of at least 48hrs	until discharge	202	0/202 (0)	0/0 (0)	0/202 (0)	na	none of the patients was colonized at admission
Gupta (ref 165)	US and Canada 2009-2011	patients >60yrs admitted to general medical and surgical units, on antibiotics	until 30 days after discharge or 60 days in hospital (whichever came first)	1099	91/1099 (8.3)	9/91 (9.9)	11/1008 (1.1)	9.06 (3.86-21.30)	asymptomatic carriage was diagnosed by culture and REA typing but could have included both tCDC and ntDCD
Alasmari (ref 127)	US 2010-2011	adult patients with an anticipated LOS >48hrs admitted	until 60 days after discharge	259	40/259 (15.4)	1/40 (2.5)	2/219 (0.9)	2.74 (0.25-29.48)	

		to general medical and surgical wards							
Dubberke (ref 128)	US 2010-2012	adult patients admitted to medical or surgical wards with an anticipated LOS >48hrs	until 60 days after discharge	235	37/235 (15.7)	0/37 (0)	2/198 (1.0)	na	partly same patient cohort as Alasmari
Bruminhent (ref 164)	US 2011-2012	patients admitted to a bone marrow transplant unit for an HSCT	until 100 days after HSCT	150	16/150 (10.7)	14/16 (87.5)	23/134 (17.2)	5.10 (3.36-7.72)	distinction between CDI and colonization by toxigenic strains difficult to establish as almost all patients develop diarrhea after HSCT and CDI testing did not include free toxin detection in all cases
Hung (ref 11)	Taiwan 2011-2012	adult patients with an anticipated LOS of at least 5 days admitted to a general medical ward	until discharge from last hospitalization	441	58/441 (13.2)	8/58 (13.8)	6/383 (1.6)	8.80 (3.17-24.46)	
Blixt (ref 70)	Denmark 2012-2013	patients admitted to medical	one month (in and outside hospitals)	3464	213/346 (6.1)	20/213 (9.4)	76/3251 (2.3)	4.02 (2.50-6.44)	

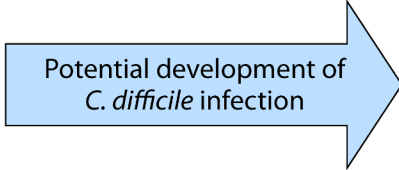
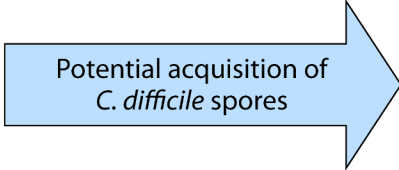
		departments at 2 university hospitals						
Tschudin- Sutter (ref 132)	US 2013	patients admitted to an ICU within 48hrs of hospital admission	until discharge	542	17/542 (3.1)	2/17 (11.8)	6/525 (1.1)	10.29 (2.24- 47.3)



**Transient colonization**  
*C. difficile* detected at one point in time



**Persistent colonization**  
*C. difficile* detected at several points in time



**Non-colonized subject**  
 No symptoms  
 No *C. difficile* detected  
 No shedding of spores

***C. difficile* colonization**  
 No symptoms  
*C. difficile* detected  
 Shedding of spores

**CDI**  
 Symptoms  
*C. difficile* and its toxins detected  
 Shedding of spores

