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Understanding *Clostridium difficile* colonization

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SUMMARY

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

INTRODUCTION

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

DEFINITIONS

Definition of *C. difficile* colonization

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

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

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

Assessing asymptomatic colonization

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

Standardization of the definition of diarrhea is essential, since McFarland et al. defined diarrhea as ≥3 unformed stools for at least two consecutive days (14), whilst others accepted the same number of loose stools, but over a single 24 hour period (12, 15). Therefore, the absence of diarrhea is not synonymous with lack of loose stools, potentially resulting in inconsistent designations of asymptomatic patients.

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

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

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

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

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

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

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

As the above illustrates, the diagnosis of CDI should not be based on laboratory results alone, but should always be supported by clinical signs and symptoms suggestive of CDI (7, 23). This is especially important when methodologies which cannot discern CDI from colonization (stand-alone NAAT, TC) are applied in routine CDI testing.
Likewise, we suggest that an optimal diagnostic method for the determination of asymptomatic colonization should include a confirmation of the absence of clinical

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

MECHANISMS OF *C. DIFFICILE* COLONIZATION

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

Disruptions in microbiota

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

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

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

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

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

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

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

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

The role of the microbiota: bile acid metabolism

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

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

The role of the microbiota: other mechanisms

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

carbohydrates mainly by *Lachnospiraceae* and *Ruminococcaceae*, the families that were less abundant in CDI patients and carriers. They may have effect on colonization resistance through reducing the luminal pH (and thereby creating an unfavorable environment for *C. difficile*) (44) and stimulating the defense barrier as one of the SCFAs (butyrate) is the main energy source of the gut epithelium (45, 46). Amino acids may also play a role in the susceptibility to *C. difficile* colonization, as they can enhance germination in the presence of secondary bile acids and may influence the immune system. Moreover, the digestion of carbohydrates in the gut results may impact susceptibility for CDI development. *Bacteroidetes* are mainly responsible for this carbohydrate digestion which results in production of substrates essential for homeostasis of colonocytes (47). A reduction in *Bacteroidetes* may therefore negatively impact colonic health.

Besides the indirect mechanisms described above, the microbiota may also have direct resistant mechanisms against *C. difficile*. These include competition for niches and nutrients and the production of antimicrobials (48, 49).

The role of the immune system: innate immunity

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

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

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

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

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

The role of the immune system: adaptive immunity

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

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

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

SOURCES OF *C. DIFFICILE* - HUMAN

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

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

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

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

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

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

Food

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

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

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

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

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

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

Environment

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

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

In summary, *C. difficile* has been isolated from animals, retail food and the environment. Using ribotyping and whole genome sequencing techniques, there appears to be interspecies and environmental transmission but the directionality of the transmission remains to be elucidated.

EPIDEMIOLOGY OF ASYMPTOMATIC COLONIZATION

After having discussed possible sources of *C. difficile* and underlying mechanisms of colonization, a description of the epidemiology of colonization, including the prevalence of colonization rates among different populations, is essential.

Infants (0-24 months)

Asymptomatic colonization rates in neonates and infants (<2 years) are widely reported as high, but range between 4-71% (18, 104-108). Although the clinical relevance of *C. difficile* colonization in infants is considered as less significant, due to low rates of disease in this population (109), its potential as a transmission reservoir for adult populations remains.

An early study researching the prevalence of *C. difficile* in the neonate population found that approximately 30% of all newborns were asymptomatically colonized within their first month of life (18). However, these data included four specimens deemed positive with no identifiable organism, only toxin. Nonetheless, the transient nature of colonization at this early stage was highlighted with only 4 of 10 babies who were culture positive in the first week of life remaining positive at 14 and 28 days. A more recent review corroborated these 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

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

Children (2-16 years)

Meta-analysis of studies examining pediatric *C. difficile* epidemiology reported asymptomatic colonization in children older than 1 year at 15%, with prevalence reducing to 5% in those greater than 2 years of age (117). One explanation for the reduction in colonization rates after infancy is that by 12 months the distribution of gut flora begins to 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* asymptomatically (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 asymptomatically
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

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

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

Patients at admission to a hospital

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

but nonetheless accounted for the second most prevalent method of *C. difficile* introduction, due to their greater numbers (71). A major Canadian study of over 5000 admissions demonstrated a lower *C. difficile* prevalence rate, with 4.05% asymptomatically colonized (133); this rate was very similar in a more recent large-scale study (4.8%) (134). Kong et al. suggested that these low rates may be due to regional distribution, as the majority of *C. difficile* colonized patients in this multi-institution study were based in hospitals with higher proportions of NAP1-associated CDI (133).

A recent meta-analysis of studies reporting toxigenic *C. difficile* colonization rates upon hospital admissions, reported a rate of 8.1% among almost 9000 patients (135). Although this overall rate provides a strong insight into the prevalence of toxigenic *C. difficile* colonization, the meta-analysis excluded certain large studies due to methodology differences, in order to attain maximum compatibility of data sets. Such exclusions may well have impacted on the reported colonization rates.

Two considerably smaller studies have reported higher *C. difficile* colonization rates, highlighting the potential for sampling bias. Hung et al. found that 20% of 441 patients admitted to a Taiwanese hospital were *C. difficile* positive, with two thirds carrying toxigenic *C. difficile* (11), whilst Alasmari and colleagues reported a rate of 21.2% (n=259), with almost 75% harboring toxigenic strains (127). Prior healthcare exposure was very common and not statistically different between patients colonized with a toxigenic strain and non-colonized patients (prevalence of prior healthcare exposure 90% and 85%, respectively). However, 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).

Hospitalized patients

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

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

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

Patients in long-term care facilities

Previous reports of *C. difficile* colonization rates amongst residents of long-term healthcare facilities (LTHF) have ranged widely (4-51%) (139-142). A major caveat in the study reporting the highest colonization rate was that it was conducted during a CDI outbreak (143). Furthermore, two studies that found high rates examined relatively small cohorts (n=68 (143) and n=32 (141)). Interestingly, the data from Riggs and colleagues showed 37% of colonized residents harbored the outbreak strain (RT027/NAP1) asymptomatically, whilst Rea and O'Sullivan also isolated a range of outbreak-associated strains from the asymptomatic group, including RT027/NAP1, 078/NAP7, 018, 014/NAP4 and 026 (142). These rates must be considered with caution, as the presence of an epidemic strain in a given community is likely to inflate asymptomatic colonization rates. For example, the asymptomatic colonization rate before and post a CDI outbreak was reported to be 6.5% and 30.1%, respectively (p=0.01) (144).

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

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

Healthcare workers

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

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

Longitudinal studies of Japanese healthy populations have followed asymptomatic carriers among students, employees and hospital workers. Kato et al. performed a longitudinal surveillance on 38 asymptomatic carriers for 5-7 months and determined 12 (31.6%) remained *C. difficile* positive during this time (124). Half of these remained with the same PFGE type, whilst five had acquired a new strain. The remaining participant retained the original strain and acquired a new type. Therefore, only 18.4% of participants retained the same strain after six months, again implying a high rate of transient colonization. Nonetheless, analysis of a single, six-month follow up sample does not permit in-depth analysis of the dynamics of carriage and it remains unclear if carriage was lost after a few days, weeks or months. Testing of 18 asymptomatic subjects in three-month intervals, over one year period found that ten participants (55.6%) only tested positive for *C. difficile* on a single sampling occasion, indicating loss of carriage within three months; only three (16.7%) were persistently colonized throughout (123). This further supports the suggestion that intestinal colonization in healthy adults is largely a transient phenomenon. Of those testing positive on three or four instances, five harbored the same strain on consecutive sampling

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

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

Ribotype specific differences

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

In the context of outbreaks, colonization rates by hyper-virulent strains appear to be markedly increased. Loo et al. and Riggs et al. found very similar (asymptomatic) 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 asymptomatically 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 asymptomatically 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_{cr1},
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 (<= 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/

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

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

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

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

Risk factors for colonization by toxigenic versus non-toxigenic strains

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

C. DIFFICILE COLONIZATION AND SUBSEQUENT CDI

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

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

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

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

INFECTION CONTROL AND ANTIMICROBIAL STEWARDSHIP IMPLICATIONS FOR ASYMPTOMATIC CARRIERS

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

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

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

CONCLUDING REMARKS AND FUTURE DIRECTIONS

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

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

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

Conflicts of interest

MHW has received: consulting fees from Actelion, Astellas, bioMerieux, MedImmune, Merck, Pfizer, Qiagen, Sanofi-Pasteur, Seres, Summit, Synthetic Biologics and Valneva; lecture fees from Alere, Astellas, Merck & Pfizer; and grant support from Actelion, Astellas, bioMerieux, Da Volterra, Merck, Sanofi-Pasteur, Seres and Summit.

VGL has received consulting fees from Merck.

MJC, JJV, LYK, SP, EJK: no conflicts of interest

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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
Direct culture	*C. difficile*	Yes	Does not differentiate between colonization or infection by CD, does not differentiate between tCD and ntCD
Enrichment culture	*C. difficile*	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
GDH EIA	GDH	Yes	Does not differentiate between colonization or infection by CD, does not differentiate between tCD and ntCD
Toxigenic culture	Toxigenic *C. difficile*	Yes	Does not differentiate between infection and colonization by tCD
PCR of toxin genes	*tcdA*, *tcdB*, binary toxin genes	Yes	Does not differentiate between infection and colonization by tCD
Toxin A/B EIA	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
CCNA	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.

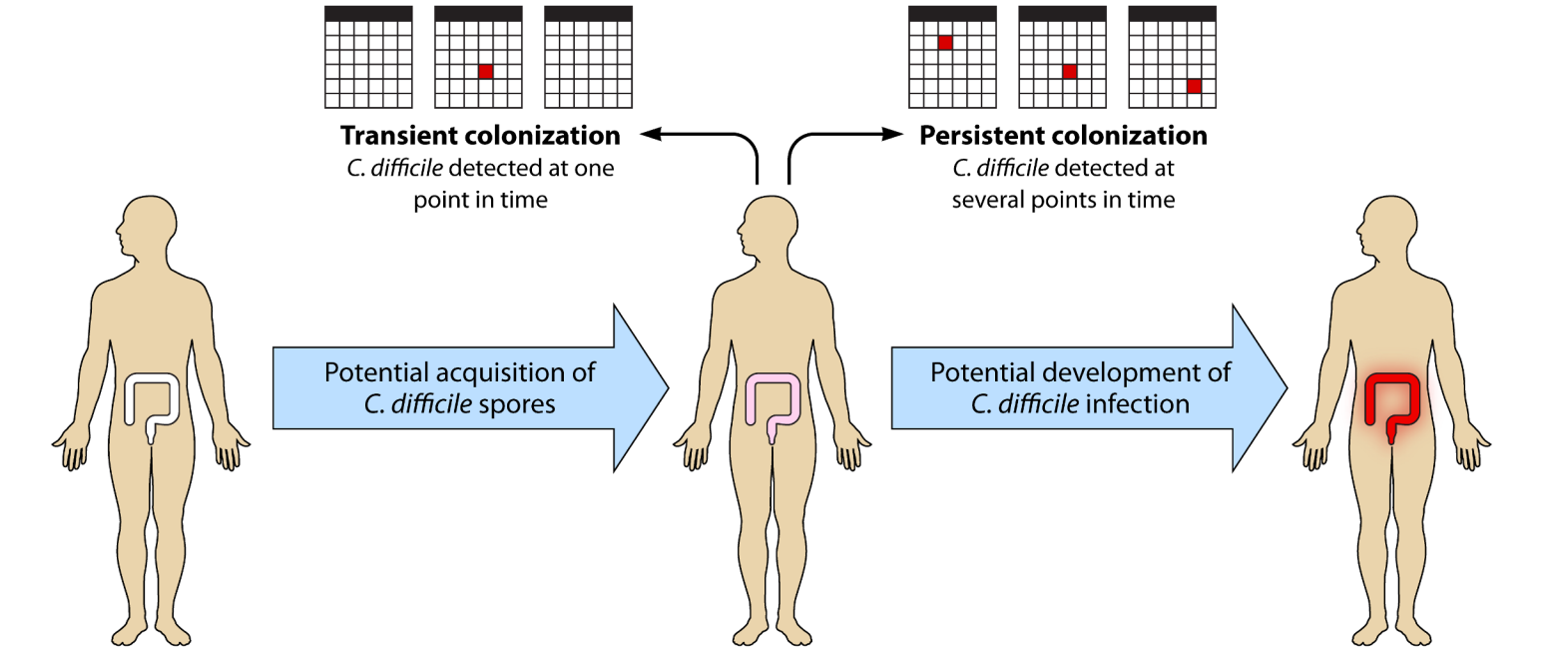
Condition	Identified risk factor	Reference
	Risk factors for colonization at admission	
CDC	previous hospitalization	133, 15
	previous CDI episode	133
	previous use of corticosteroids or other immunosuppressant medication	133, 15
	presence of antibodies against Toxin B	133
	current loose stools/diarrhea but not meeting CDI criteria	15
tCDC	previous hospitalization	129
	chronic dialysis	129
	use of corticosteroids	129
	Risk factors for acquiring colonization during admission	
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

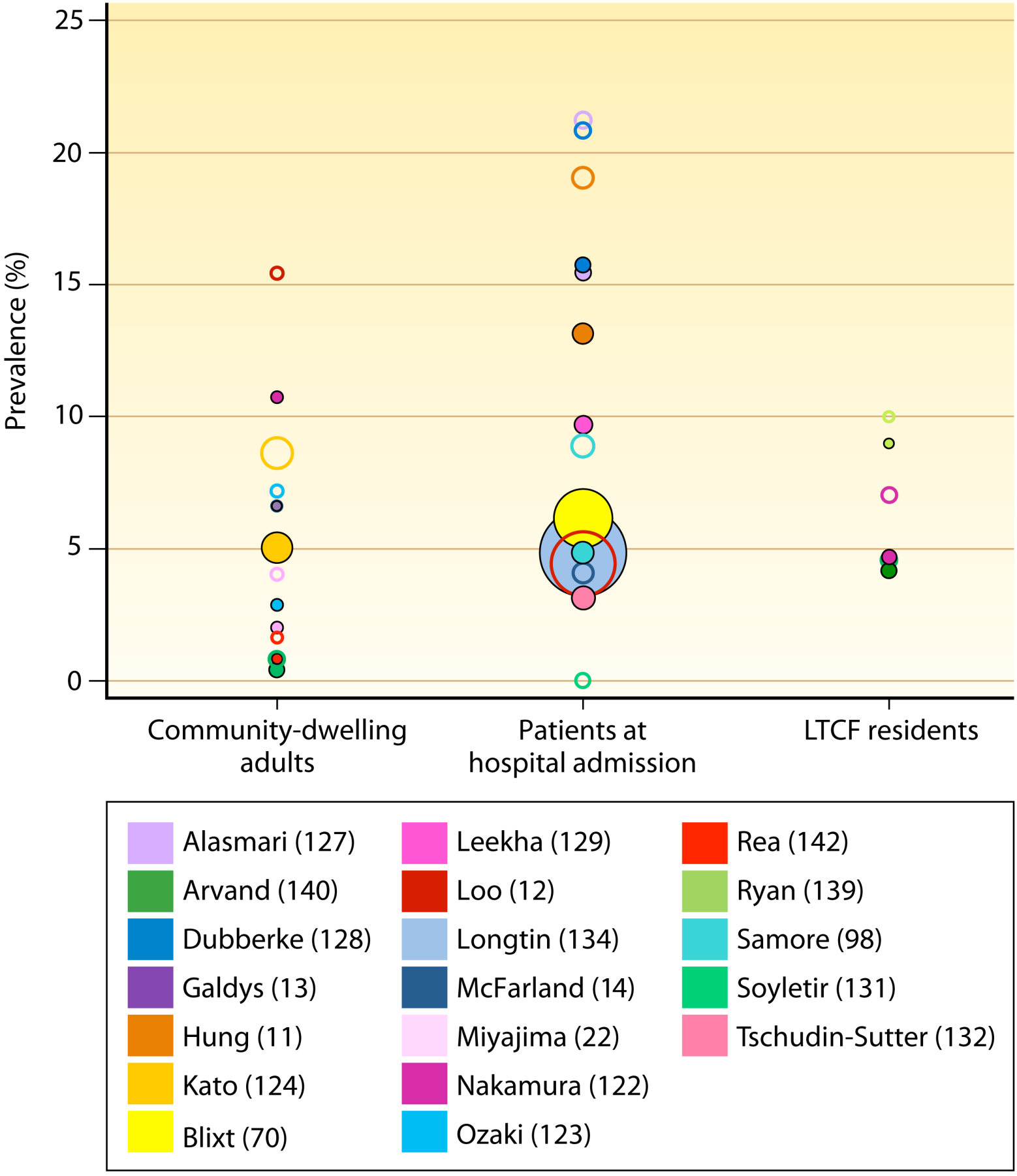
Table 3. Studies investigating the risk of development of CDI among patients with toxigenic *C. difficile* colonization on admission. Studies were included if: published since 1994, sample size > 100 patients, comparison of patients with toxigenic *C. difficile* colonization on admission with controls (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.

Study	Country and period	Setting and patients	Follow up period	Included patients (N)	Preva-lence tCDC (%)	CDI among tCDC (%)	CDI among controls (%)	RR for CDI (95% CI)	Remarks
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	213/3464 (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
Potential acquisition of C. difficile spores
Potential development of C. difficile infection
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


25
20
15
10
5
0
Prevalence (%)
Community-dwelling adults
Patients at hospital admission
LTCF residents
Alasmari (127)
Arvand (140)
Dubberke (128)
Galdys (13)
Hung (11)
Kato (124)
Blixt (70)
Leekha (129)
Loo (12)
Longtin (134)
McFarland (14)
Miyajima (22)
Nakamura (122)
Ozaki (123)
Rea (142)
Ryan (139)
Samore (98)
Soyletir (131)
Tschudin-Sutter (132)