

# Hype or hypervirulence

## A reflection on problematic *C. difficile* strains

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*Clostridium difficile* infections (CDI) have emerged as a major cause of healthcare associated disease, and recent epidemiological evidence also suggests an important role in community-acquired diarrhea. This increase is associated with specific types, especially PCR ribotypes 027 and 078, which are sometimes referred to as “hypervirulent”. Over the past years major advances have been made in our understanding of *C. difficile* pathogenicity, with the identification and characterization of the major clostridial toxins TcdA and TcdB. However, the relation between the toxins, their regulation, and “hypervirulence” remain unclear. Here I review our current understanding of *C. difficile* pathogenicity and argue that “hypervirulent” is an inadequate term to describe PCR ribotypes 027 and 078, that the ability of *C. difficile* to cause problematic infections is a consequence of a multifactorial process that extends beyond toxins, sporulation, and antimicrobial resistance, and that vigilance is in order toward types that are closely related to ribotypes 027 and 078, but are currently not considered problematic.

### Introduction

*Clostridium difficile* is a gram-positive anaerobic bacillus that was originally identified in the normal colonic flora of infants.<sup>1</sup> Later, *C. difficile* was identified as the causative agent for human disease, most notably antibiotic-associated diarrhea and pseudomembranous colitis.<sup>2</sup> The history of CDI is comprehensively reviewed in refs. 3 and 4.

Different strains of *C. difficile* can be distinguished by molecular methods such as PCR ribotyping, which is based on different banding patterns obtained by amplifying the 16S-23S rDNA intergenic regions.<sup>5</sup> A pan-European survey revealed 65 different circulating PCR ribotypes during the sampling period.<sup>6</sup>

Since 2004, a marked increase in cases of CDI was noted. Most of these were identified as PCR ribotype 027 (RT027; BI/NAP01). This type caused healthcare-associated outbreaks in North America, the UK, and mainland Europe and is associated with increased morbidity and mortality.<sup>7–11</sup>

Risk factors for CDI include (advanced) age, antibiotic treatment, and hospitalization, and for that reason it has long been

regarded as a mere nosocomial disease. However, recently an increase in community-acquired CDI has been noted.<sup>12,13</sup> At least in Europe PCR ribotype 078 (RT078) is the dominant type identified in CA-CDI cases,<sup>6</sup> and as for RT027 this type is associated with an increased severity of CDI and higher attributable mortality.<sup>14</sup> It is of note that RT078 is also the dominant strain of *C. difficile* in livestock, such as pigs, suggesting zoonotic potential.<sup>15–17</sup>

Together, RT027 and RT078 are often referred to as “hypervirulent” types of *C. difficile*. Though technically the term indicates undefined increase in virulence, it is generally used to signify strains that cause outbreaks, with morbidity and mortality that is significantly higher than that of a common reference. However, the term deserves more careful consideration. The success of epidemic strains (fitness) not necessarily reflects a mere increase in virulence. Such strains are not only characterized by their ability to cause disease (virulence) but also their ability to transmit from one host to the other. Fitness is a concept from evolutionary biology, describing the probability of a particular genotype or phenotype to survive and reproduce. For pathogenic bacteria, lower virulence might favor host survival and thus transmission to new susceptible hosts, illustrating a fine balance between virulence and fitness. Interestingly, the index case for the problematic RT027 *C. difficile*, R20291, isolated during an outbreak in Stoke-Mandeville (UK) is located on a stunted branch of the RT027 evolutionary tree.<sup>18</sup> It may therefore represent a hypervirulent, but not fit, example of this type as it caused a severe outbreak but did not spread widely. It is likely that other successful RT027 strains, in fact, demonstrate lower virulence than R20291, and are more representative of the whole epidemic lineage.

### Toxins and Virulence

The major virulence factors of *C. difficile* are the toxins TcdA and TcdB.<sup>4,19</sup> The genes encoding these proteins are contained in a 19 kb genomic region called the pathogenicity locus or PaLoc, which also encodes a sigma factor that is required for toxin expression (TcdR), a holin like protein (TcdE), and a putative anti-sigma factor (TcdC). Though it is agreed upon that at least one of the toxins is required for pathogenicity, the individual contributions of the toxins remain subject of debate.<sup>20–23</sup> It is noteworthy that some pathogenic strains are found to have deletions in their PaLoc that abrogate the production of toxin A or toxin B (e.g., PCR RT017, RT033, RT047) but not its capacity to cause disease.<sup>24,49</sup>

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In vitro TcdC has been shown to act as an anti-sigma factor, antagonizing the function of TcdR in a manner that is not yet fully understood.<sup>25,26</sup> Indeed, introduction of TcdC into strains that do not normally express TcdC can lead to reduction in toxin levels under certain conditions.<sup>27</sup> Interestingly, epidemic RT027 and RT078 both carry characteristic mutations that lead to a frameshift and/or a premature stop codon, as well as deletions in the *tcdC* gene<sup>14,28</sup> and this has been exploited to identify epidemic strains in the clinic.

Intuitively, the above suggests that there is a clear correlation between *tcdC* status, toxin levels, and virulence. Although this was initially reported in a study comparing strains from different toxinotypes,<sup>29</sup> later studies failed to show such a correlation.<sup>30,31</sup> Similarly, the deletion of *tcdC* from strains that normally do encode it, or introduction of various *tcdC* alleles into a RT027 strain was not found to affect toxin levels.<sup>32,33</sup> Therefore, one has to conclude that the levels of TcdA and TcdB, potentially regulated by TcdC, are insufficient to explain the epidemic nature of RT027 and RT078 strains.

One potential explanation lies in the efficacy of the toxins. TcdB toxin from a RT027 shows increased toxicity compared with TcdB from the lab strain 630 and this may contribute to the increased mortality of RT027-related cases of CDI.<sup>34,35</sup>

In addition to TcdA and TcdB, the epidemic types also encode a binary toxin.<sup>36,37</sup> As for the major toxins, the binary toxin (CDT) is encoded by the genes *cdtA* and *cdtB* on a particular genomic locus (CdtLoc), which also encodes its positive regulator CdtR.<sup>38</sup> The role of binary toxin in CDI remains poorly understood, though it was found to cause fluid accumulation in a rabbit ileal loop assay,<sup>39</sup> to increase adherence of bacteria to epithelial cells,<sup>40,41</sup> and to induce clustering of its receptor LSR into lipid rafts.<sup>42,43</sup> The findings that binary toxin positive strains may be associated with an increased severity of CDI,<sup>44</sup> higher case fatality rate,<sup>45</sup> and recurrence of CDI<sup>46</sup> are not undisputed<sup>47</sup> and the data should therefore be interpreted with care. Notwithstanding, binary toxin is also found in RT023 strains, which was found in a retrospective analysis to be associated with severe CDI, similar to RT027<sup>48</sup> and in many strains closely related to RT027 and RT078.<sup>49</sup> However, as binary toxin is also found in strains that are so far considered non-epidemic (e.g., RT058, RT131, and others),<sup>49</sup> the precise contribution to CDI severity remains to be established.

A major limitation of most studies on the relation between toxin production and strain type is that they are based on in vitro assays, or single round infections. As host passage can affect virulence of pathogens,<sup>50,51</sup> these results should be interpreted with care. Of note, based on BLAST homology searches the receptor for CdtAB<sup>42</sup> appears to be absent from the Syrian Golden Hamster, a commonly used animal model for CDI,<sup>52</sup> suggesting that this model is not suitable to study the effects of binary toxin in vivo.

### Sporulation Efficiency and Virulence

*C. difficile* is a strict anaerobic bacterium. In order to survive the oxygen-containing environment outside the host, it is capable of

forming highly resistant endospores. These spores are also metabolically inactive, rendering them insensitive to most classes of antimicrobials. Together with its multidrug resistance, these features are crucial for *C. difficile* to outgrow and colonize the host gut after treatment with antimicrobials. Sporulation is dependent on the key regulator Spo0A.<sup>53,54</sup> Direct evidence for a role for spores in CDI came from experiments using a RT027 *spo0A* knockout strain that showed that Spo0A is important for transmission and persistence in a mouse model.<sup>55</sup> Interestingly, the same study found that a *spo0A* mutant of a RT027 caused more fulminant disease due to overproduction of toxins once animals were colonized. Though this was in contrast with a previous report,<sup>56</sup> an independent study also showed no positive effect of Spo0A on toxin production.<sup>57</sup> Thus, Spo0A plays both a positive (formation of spores) and negative (toxin expression) role in the virulence of *C. difficile*.

It has been reported that RT027 strains are highly transmissible because of an increase in sporulation frequency and/or spore resistance.<sup>30,58</sup> Others, however, have found that the within-type variation in sporulation and germination is as large or larger than the between-type variation,<sup>59–61</sup> suggesting that there is no unambiguous relation between sporulation and virulence. One should note that—as for many toxin determinations—these studies were all done in vitro, and it is unknown how the findings relate to in vivo sporulation frequencies, or the antibiotic-associated induction of a supershedder state.<sup>62</sup> Thus, strains that demonstrate similar properties in an in vitro system may behave differently in vivo.

### Resistance

*C. difficile* is a multidrug-resistant organism, in part due to resistance determinants carried on the many mobile genetic elements in the mosaic genome.<sup>63,64</sup> As a result, treatment of CDI consists of a limited set of antimicrobials, including metronidazole, vancomycin, and fidaxomicin.<sup>65</sup> Moderate resistance to metronidazole has been reported,<sup>66</sup> but is uncommon and not associated with RT027 or RT078 strains of *C. difficile* specifically. Moreover, most epidemic strains show substantial differences in resistance patterns.<sup>67</sup>

So far the only indication of involvement of resistance mechanisms in epidemicity of *C. difficile* comes from a whole genome sequencing effort directed at RT027.<sup>18</sup> It was found that two independently acquired, but identical, mutations leading to fluoroquinolone resistance (FQR) are associated with the global spread of this type.

Though fluoroquinolones are not used as a treatment for CDI, FQR of epidemic strains explains at least in part the previous observation that treatment with fluoroquinolones is a risk factor for CDI.<sup>8,10,68–70</sup>

Whether the relation between FQR and fitness of *C. difficile* extends beyond this remains to be established, but it is interesting that the expression of certain major cell surface proteins as well as toxin levels may be affected by sub-inhibitory concentrations of fluoroquinolone antimicrobials in certain strains.<sup>71,72</sup>

Fluoroquinolone resistance is common in RT078 strains,<sup>14,73</sup> but is also found in other clinically relevant PCR ribotypes

that are common but not considered epidemic (e.g., RT001, RT014/020).<sup>74,75</sup> This suggests that there may be a link with clinical isolates in general, rather than epidemic strains specifically.

A limitation of whole genome sequencing single nucleotide polymorphism typing such as the RT027 study is that the analyses are based on a conserved core genome. The contribution of the accessory genome, including horizontally acquired elements, to resistance and virulence has so far largely been unexplored.

### Evolutionary Relationships

Typing is an essential tool in clinical practice to identify and characterize *C. difficile* isolates. The fact that infections with RT027 and RT078 strains are associated with increased morbidity and mortality can be used to guide physicians in choosing the most appropriate course of treatment and manage infections. However, it is important to put the current emphasis on typing in perspective, as illustrated below.

One specific ribotype can contain both epidemic and non-epidemic strains. For instance, CD196 is a historic isolate of RT027 that is not considered epidemic,<sup>76,77</sup> but in current clinical practice would be classified as a hypervirulent strain. One might argue that as a result of the increased fitness of epidemic strains the non-epidemic isolates of the same ribotype are likely underrepresented in clinical diagnoses, and may therefore be disregarded from a precautionary principle.

It should be noted that in literature the terms historic and hypervirulent have also incorrectly been applied to two different ribotype strains, 630 (RT012) and R20291 (RT027), respectively.<sup>34,35</sup> This has led to the proposition that differences in toxicity between the TcdB toxins might explain the rise of hypervirulent RT027 strains. A careful comparison between the sequences of the TcdB proteins of CD196 and R20291, however, reveal no differences. Therefore, though it may (partially) explain the difference in CDI severity between different ribotype strains, it does not explain the rise of epidemic isolates of RT027 from its non-epidemic ancestors.

Any typing method balances discriminatory power vs. ease. For instance, whole genome sequencing has superior power of discrimination but so far is too expensive, too time consuming and too labor intensive to have found its way into the clinic for routine diagnostics. Multi-locus sequence typing (MLST) and PCR ribotyping are more widely applied because of their ease of use. They have similar discriminatory power and show good general concordance, with a few notable exceptions. MLST has revealed that multiple PCR ribotypes are highly related to RT027 (i.e., RT016,

RT036, RT176; all sequence type 1/clade 2) and RT078 (RT033, RT045, RT066, RT126, RT193; all sequence type 11/lineage 5).<sup>49,64,78-80</sup> In fact, some of these are indistinguishable from the epidemic ribotypes in a phylogenetic reconstruction based on the core genome. As these types so far are not very common, insufficient data is available to determine the morbidity and mortality vs. RT027, RT078, or other ribotypes. It should be noted however that outbreaks of RT176 and fulminant disease from a non-characterized RT027-like strain have been reported.<sup>81,82</sup>

Though a more careful consideration of the virulence characteristics of these strains is required, it is clear that there is an epidemic potential for strains that are not regarded with special attention in current clinical practice. Together with the fact that several ribotypes are as common or more common than RT027 and RT078,<sup>6</sup> care should be taken to not place an unbalanced emphasis on the clinical importance of the epidemic ribotypes.

### Concluding Remarks

The picture that emerges from our current understanding of the pathogenicity of *C. difficile* is that no single factor (toxins, sporulation, or resistance) is responsible for the increased virulence of epidemic strains, though at least one of the major clostridial toxins is required.<sup>4</sup> What then determines the successfulness of *C. difficile* as a pathogen?

Cell surface proteins (including surface layer proteins, flagellae and other membrane/wall-associated factors) directly interact with the host immune system and are highly relevant for adherence and colonization.<sup>13,30,83-86</sup> It is to be expected that these are major contributors to the virulence of *C. difficile* but it is unlikely that a single surface protein is responsible for epidemicity.

The multifactorial nature of virulence makes it a fluid phenotype and it will be a challenge for the future to determine characteristics of *C. difficile* that affect its propensity to become epidemic. Until such time, vigilance for all types—not merely RT027 or RT078—is appropriate.

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No potential conflicts of interest were disclosed.

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