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The perils of PCR-based diagnosis of Clostridioides difficile infections: painful lessons from clinical trials

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Introduction

Clostridioides difficile, the leading infective cause of healthcare-associated diarrhoea, continues to be an important source of morbidity and mortality for hospitalized patients. Since its emergence as a public health priority following outbreaks in the early 2000s, diagnostic tests favoured to detect C. difficile infections (CDI) have undergone successive changes, as our understanding of their characteristics evolved over time. Enzyme linked immunoassays (EIAs) detecting toxins A/B were frequently used as single tests, until polymerase chain reaction (PCR)/nucleic acid amplification-based tests became widely available, with the advantage of increased sensitivity. However, the problem of over-diagnosis with PCR testing, which detects the gene coding for toxin production, rather than the presence of actual free toxins, was soon highlighted and evidenced in several key studies [1-3]. Because PCR may detect both colonization and infection, a two-step diagnostic algorithm combining a sensitive screening assay (e.g. PCR or glutamate dehydrogenase (GDH)) followed by a toxin-based assay (e.g. toxin EIA) has been public health policy in the United Kingdom since 2012 [4]. This algorithm was then formally recommended in European guidelines in 2016 [5], and recently Infectious Diseases Society of America guidelines were also updated to reflect this approach [6]. The importance of accurate detection of patients with true CDI has thus been emphasized in the clinical setting. Here, we discuss the parallel of the clinical trial setting, using several examples from which important lessons have been learned on the key influence of the choice of diagnostic method.

Clinical trials

Bezlotoxumab

Bezlotoxumab and actoxumab are human monoclonal antibodies that bind and neutralize toxin B and toxin A, respectively, developed as potential therapies (when given alongside standard of care CDI antibiotics) to prevent recurrent CDI (rCDI) through passive immunity. MODIFY I (NCT01241552) and MODIFY II (NCT01513239), two global, randomized, double-blinded, placebo-controlled, phase 3 trials, evaluated the efficacy and safety of bezlotoxumab and actoxumab in the prevention of rCDI [7]. Patients were randomized to receive (in addition to standard of care CDI antibiotics) bezlotoxumab alone, bezlotoxumab and actoxumab, placebo, or in MODIFY I only, actoxumab alone [7]. In both trials, bezlotoxumab was found to be protective against rCDI, with lower rates of recurrent infection compared with placebo through 12 weeks of follow-up [7]. In contrast, enrolment in the actoxumab group stopped after interim analyses revealed higher recurrence rates, deaths and serious adverse events; and the combination of bezlotoxumab and actoxumab did not confer additional benefit over bezlotoxumab alone [7]. CDI diagnosis in these trials was based on presence of diarrhoea and various laboratory methods, and the impact of testing method on efficacy outcome measures has been evaluated in a post hoc analysis [8].

Accepted testing methods used to diagnose baseline CDI episodes included cell cytotoxin neutralisation assays (CCNA), stool culture with strain typing or subsequent toxin detection from the isolate (toxigenic culture), commercially available EIAs against toxins A/B, and commercially available PCRs for detection of toxin genes [7]. Examining pooled data from MODIFY I and II, 781 patients were randomized to the bezlotoxumab group and 773 to the placebo group, for a total of 1554 patients [8]. Overall, toxin EIAs were the most frequently used test for baseline CDI diagnosis (757/1554, 48.7%), followed by PCR (694/1554, 44.7%), toxigenic culture (87/1554, 5.6%), and CCNA (16/1554, 1.1%) [8]. These proportions of tests used tended to reflect the prevalence of assays in routine use during the MODIFY studies (2011-15). The proportions were similar between the bezlotoxumab group

and placebo group: 372/781 (47.6%) and 357/781 (45.7%) of patients in the bezlotoxumab group were diagnosed with a toxin EIA and PCR, respectively, compared with 385/773 (49.8%) and 337/773 (43.6%) in the placebo group [8].

If baseline CDI episodes were over-estimated in those diagnosed by PCR, the effect that might be observed from the intervention might plausibly be diminished if some patients did not have true infection to begin with. When examining rates of recurrence, bezlotoxumab-treated patients diagnosed by PCR had a higher rate of rCDI compared with those diagnosed by toxin EIA (19.6% vs. 14.5% respectively) [8]. However, patients receiving placebo had similar rates of rCDI regardless of diagnostic method [8]. Thus, the relative reduction in rCDI rates of bezlotoxumab over placebo in patients diagnosed with toxin EIA was almost double that seen in those diagnosed with PCR (47% versus 25%) [8].

Episodes of rCDI were diagnosed based on testing at local laboratories, using the aforementioned accepted tests, and confirmatory testing at a central laboratory using stool culture with subsequent toxin detection [8]. Overall, 220/335 (65.6%) rCDI episodes tested positive in both the local and central laboratories [8]. Of 278 episodes of rCDI diagnosed at local laboratories, 140 (50.4%) were tested with PCR, and 119 (42.8%) with a toxin EIA. More patients in the bezlotoxumab group (57/102, 55.9%) diagnosed with rCDI at local laboratories were tested using PCR compared with those in the placebo group (83/176, 47.2%). If a proportion of the PCR-diagnosed rCDI were misdiagnoses, and there were in fact fewer true recurrences, the magnitude of rCDI reduction found could plausibly have been greater.

A significant protective effect was found for bezlotoxumab overall regardless of the testing method in MODIFY I and II, but the post hoc analysis showed that the degree of the effect found could vary depending on the diagnostic test chosen. Initial CDI misdiagnoses

included as true CDI in the analyses could plausibly have diluted the effect of the intervention. Similarly, subsequent over-diagnoses of recurrences could have masked the true magnitude of reduction in rCDI achieved by the intervention.

SER-109

SER-109, a novel microbiome therapeutic developed to prevent rCDI, is an oral capsule formulation composed of Firmicutes spores fractionated from stool specimens of healthy human donors [9]. It is postulated to exert its effect via metabolic competition between spore-forming organisms in SER-109 and C. difficile, augmenting a host deficit in colonisation resistance, and thereby preventing pathogen proliferation (and toxin production) [9]. A phase 1b trial evaluating the efficacy and safety of SER-109 in preventing rCDI showed promising results, with 29 of 30 participants achieving clinical resolution of rCDI at 8 weeks of follow-up [9].

However, the phase 2 trial for SER-109 (NCT02437487) did not confirm these positive findings, leading the investigators to conduct an analysis of potential contributors to these results [10, 11]. It was noted that 72/89 (81%) of study participants with CDI were enrolled based on a diagnosis by PCR [10]. These samples were not available for retesting to determine the presence of free toxin [10]. However, among participants in an open label extension of the phase 2 trial, only 15 of 31 patients who tested positive by PCR also tested positive for the presence of C. difficile free toxin upon retesting [10]. In addition, when reanalysing data from the phase 2 trial using toxin assays to determine recurrences, it was evident that ~25-50% of rCDIs determined by PCR could have been misdiagnoses [10].

Thus, potential overestimates of both the initial episodes and subsequent recurrences due to the use of PCR as a standalone diagnostic method likely contributed to reducing the observed efficacy of SER-109 to prevent rCDI in this phase 2 trial. As a result of these

112 considerations, the design of a phase 3 trial of SER-109 vs. placebo (NCT03183128) was
113 tailored to specify diagnosis based on a C. difficile toxin test, with recruitment ongoing [12].

114 **Surotomycin**

115 Surotomycin is an oral lipopeptide antibiotic with minimal systemic absorption and selective
116 action against Gram-positive bacteria that was evaluated as a potential alternative to the
117 current first-line drugs for CDI treatment. In a phase 2 study of surotomycin vs. vancomycin
118 (NCT01085591) involving 209 patients, surotomycin was found to be non-inferior to
119 vancomycin with similar clinical cure rates at 2 days after end of therapy [13]. Furthermore,
120 recurrence rates were lower with surotomycin 250mg twice daily compared with vancomycin,
121 postulated to result from surotomycin's minimal disruption of the microbiota [13]. CDI
122 episodes in this study were diagnosed using toxin detection assays [13].

123 Subsequently, two parallel phase 3 trials of surotomycin 250mg twice daily vs.
124 vancomycin were conducted [14, 15]. These studies followed identical protocols, and
125 enrolled patients diagnosed with CDI based on toxin EIA, PCR, or CCNA assays [14, 15]. In
126 the first trial (NCT01597505), 570 subjects were randomized to receive either surotomycin or
127 vancomycin. Surotomycin neither met the non-inferiority criteria for clinical cure at end of
128 therapy compared with vancomycin, nor did it demonstrate superiority over vancomycin for
129 sustained clinical response, clinical response over time or rate of recurrent infection [14]. The
130 second trial (NCT01598311) included 577 randomized patients [15]; although surotomycin
131 did reach the primary endpoint of non-inferiority in clinical cure rates, it did not demonstrate
132 superiority vs. vancomycin in key secondary endpoints, including rCDI rate [15].
133 Consequently, the surotomycin development programme was discontinued [15].

134 Notably, overall only 41% of patients in both phase 3 trials were diagnosed by toxin
135 detection [16]. The authors of the first trial observed that clinical cure rates at end of therapy

were lower overall for both treatment groups in patients diagnosed by toxin EIAs, compared with those diagnosed by PCR [14]. This observation may be explained by the higher likelihood of toxin-positive patients to have true CDI, and possibly more severe CDI; detection of any difference between treatment arms within the group of toxin-positive patients may be limited by the smaller number of patients. Without retesting and reanalysing data, it is difficult to estimate what proportion of these patients diagnosed by PCR were actually toxin-positive, what their relative distributions between the two treatment groups were, and how this might change the study results. Similarly, interpretation of results for the key secondary endpoint of rCDI could have been hampered by potential overestimates of recurrences.

C. difficile vaccine

A final example of how diagnostic method impacts C. difficile clinical research may be found in the vaccine development field. There are four vaccine candidates that have entered phase 2 or later trials, the most advanced of which was the Cdiffense vaccine, a toxoid vaccine composed of chemically detoxified toxins A and B, developed by Sanofi/Pasteur [17]. After several phase 1 and 2 trials showing promising immunogenicity and safety profiles, a phase 3 trial was launched in October 2013 (NCT01887912) [17]. The investigators defined the primary outcome measure as the efficacy of the vaccine in preventing symptomatic, primary CDI after one injection in up to 3 years after vaccination; the diagnosis of CDI was defined by a positive PCR test. Secondary endpoints also included diagnoses of CDI, based on PCR, after different vaccine doses [18]. After enrolment of over 9000 patients, a planned interim analysis showed that the primary objective was unlikely to be achieved, and the trial (and the C. difficile vaccine development programme) was terminated [17]. Overestimates of incident CDI cases in vaccinated subjects because PCR was used as a standalone test could certainly have been a factor in this outcome. Inclusion of patients with differing risks for developing

CDI was another potential contributor, as the investigators included patients hospitalized for elective surgery, which is a low-CDI risk population.

The only other vaccine candidate currently being evaluated in a phase 3 trial is a recombinant toxoid vaccine developed by Pfizer (NCT03090191) [17]. Since launching in March 2017, the study has enrolled more than 17000 patients, and notably relies on the diagnosis of CDI by toxin detection, with results likely forthcoming in 2020 [19].

Conclusion

Drug and vaccine development are laborious and costly processes, where accurate measurements of efficacy hinges upon choosing the optimal diagnostic methods to determine the primary (and secondary) outcomes. For C. difficile infection in particular, this is a crucial and evolving issue. We have highlighted the perils of using PCR alone in studies involving different aspects of C. difficile clinical research, including immunotherapies, microbiome-based therapies, treatments, and vaccines. Of the four clinical examples presented, diagnostic issues could feasibly have contributed to all three of the clinical trials that failed to meet their primary outcomes. In addition, there is a theoretical possibility that, had all CDIs examined in the phase 3 clinical trials of bezlotoxumab been diagnosed by PCR alone, the primary outcome, a significant reduction versus placebo in the risk of rCDI, could have been at risk. This scenario would not have been because the monoclonal antibody was non-efficacious, but instead because the use of a poorly predictive CDI diagnostic test masked the true therapeutic benefit. The importance of designing C. difficile clinical trials with careful consideration to the diagnostic testing method to accurately detect true infection, rather than colonization, cannot be overemphasized.

Declarations of interest

Ling Yuan Kong has no declarations of interest.

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