Clostridium difficile Mixed Infection and Reinfection

David W. Eyres, A. Sarah Walker, David Griffiths, Mark H. Wilcox, David H. Wyllie, Kate E. Dingle, Derrick W. Crook, and Tim E. A. Peto

NIHR Oxford Biomedical Research Centre, John Radcliffe Hospital, Oxford, United Kingdom; MRC Clinical Trials Unit, London, United Kingdom; Department of Microbiology, The General Infirmary, Old Medical School, Leeds, United Kingdom; and Leeds Institute of Molecular Medicine, University of Leeds, Leeds, United Kingdom

Isolates from consecutive Clostridium difficile infection (CDI) fecal samples underwent multilocus sequence typing. Potential reinfections with different genotypes were identified in 88/560 (16%) sample pairs taken 1 to 1,414 days (median, 24; interquartile range [IQR], 1 to 52 days) apart; odds of reinfection increased by 58% for every doubling of time between samples. Of 109 sample pairs taken on the same day, 3 (3%) had different genotypes. Considering samples 0 to 7 days apart as the same CDI, 7% of cases had mixed infections with >1 genotype.

The prevalence of multiple Clostridium difficile strains within the same patient either simultaneously (mixed infection) or over time (reinfection) has important implications for understanding Clostridium difficile infection (CDI) epidemiology.

For example, relatively small studies (<100 patients) of recurrent CDI have found that 25% (10) to ~50% (1, 5, 7, 11) of recurrences are actually reinfections, with later recurrences possibly more likely due to reinfection (1, 5).

This study exploited a large, long-term population-based study of all CDI in Oxfordshire (~600,000 people) from September 2006 to March 2011 to estimate rates of reinfection and mixed infection. The Oxford Radcliffe Hospitals (ORH) microbiology laboratory provides routine clinical C. difficile testing for all stool samples from hospital and community patients by enzyme immunoassay (EIA) (Meridian Bioscience, Cincinnati, OH). EIA-positive samples submitted for routine clinical testing with sufficient sample remaining were cultured, a single colony was subcultured, and the resulting isolate undergoes discriminatory genotyping with multilocus sequence typing (MLST) (4).

During the study, local infection control policy was that any inpatient with diarrhea (≥3 unformed stools in 24 h) should be tested for C. difficile. From May 2007, laboratory policy was to test all unformed stool samples from those aged >65 years for C. difficile, whether or not this had been requested. Patients were considered CDI negative only after three EIA-negative samples. Given the delay (albeit short) between sample submission and test results becoming available, multiple positive samples were sometimes obtained from the same patient on the same day (or shortly afterwards), allowing an estimate of mixed-infection rates from MLST. Similarly, samples taken further apart from patients with symptomatic recurrences or persistent diarrhea allow rates of reinfection due to new strains to be estimated.

All patients with ≥2 samples from which genotyped isolates were obtained were included in analyses, which were stratified according to whether or not the first CDI was with the epidemic PCR-ribotype-027/NAP-1/sequence type (ST)-1. Logistic regression was used to estimate the relationship between pairs of consecutive isolates having different genotypes and the elapsed days between samples, using fractional polynomial models to reflect non-linearity (8). Stata 11.2 was used for all analyses, which were conducted by A. S. Walker.

Of 3,258 EIA-positive samples, 2,906 (89%) were retrieved for culture. C. difficile was recovered in 2,235/2,906 (77%) and successfully genotyped in 2,231 (1,549 patients). A total of 79 distinct sequence types were identified, with ST-1 (PCR-ribotype-027/NAP-1) the most prevalent (30%); other STs each accounted for <8% of isolates, and 22 STs were isolated once. Most (1,356; 61%) isolates were from clade 1 (3).

We analyzed all 560 consecutive same-patient pairs of EIA-positive, culture-positive samples with STs taken ≥1 day apart (from 388 patients) for evidence of reinfection with a new strain. Consecutive samples were taken a median 24 (interquartile range [IQR], 1 to 52; range, 1 to 1,414) days apart, 203 (36%) within 7 days; 88/560 (16%) pairs had different genotypes, with the samples taken a median 54 (IQR, 30 to 132) days apart, compared with 21 (IQR, 1 to 43) days for pairs sharing the same ST (P < 0.0001). A total of 31 pairs had >6 months between consecutive samples, of which 13/31 (42%) shared the same ST, as did 5/14 (36%) pairs with >1 year between samples. Of 194 pairs where the first isolate was the most common ST-1/PCR-ribotype-027, 18 (9%) had a second isolate of a different ST (median, 70 days) versus 70/366 (19%) in pairs where the first isolate was not ST-1 (median, 53 days) (P = 0.002) (Fig. 1A). The chance of a subsequent sample having a different ST increased 5-fold, from 1 to 11 days and overall increased by 58% (odds ratio [OR]) (95% confidence interval [CI], 40 to 79%) for every doubling of the time between samples (P < 0.0001), allowing for differences in the underlying prevalence of ST-1 versus other STs (Fig. 1B).

On 109 occasions more than one EIA-positive, culture-positive isolate was obtained from different fecal samples from the same patient on the same day (97 patients with 2 samples, 11 with 3 samples, 1 with 4 samples). On 3/109 (2.8%; 95% CI, 0.6 to 5.7%) of cases analyzed, the second isolate had a different ST (one was ST-1/PCR-ribotype-027, one was ST-11/PCR-ribotype-027, and one was ST-11/PC RB 030) compared with 34/360 (9.5%; 95% CI, 6.4 to 12.6%) if the first isolate was not ST-1 (P = 0.001) (Fig. 1B).
occasions, involving 2 different patients, >1 ST was isolated from the same patient on the same day. If multiple samples taken within 0 to 7 days were considered as the same CDI, >1 genotype was isolated on 21/292 (7.2%; 95% CI, 4.5 to 10.8%) occasions; similarly, rates for samples 14 days apart (21/305) were 6.9% (95% CI, 4.3 to 10.3%).

This study provides clear evidence that as the time between fecal samples increases there is a significant and marked increase in the rate of isolation of different C. difficile strains from individual patients. However, as with other infectious diseases such as tuberculosis (6), the timings of same-strain recurrence and new reinfections overlap to some extent. Indeed, we found a reasonable minority of patients in whom relatively uncommon STs were reisolated >2 months after the original CDI, in some cases over 1 year later. Whether bowel flora in these patients particularly encourage long-term carriage or these episodes represent reinfections with a different lineage of the same (albeit uncommon) ST may be resolved with whole-genome sequencing of isolates recovered via repeat sampling. Similarly, while MLST offers reasonable discriminatory power to detect different strain infections (4), the dominance of common STs, particularly ST-1, which accounted for 30% of cases, may result in an underestimate of reinfections and mixed infections with different lineages of the same ST.

Assessment of CDI associated with the presence of multiple strains by opportunistic sampling of patients on the same day found rates of 2.8%. We are unable to determine whether the multiple strains are true mixed infection or whether one strain is dominant. However, our data suggest that the chance of identifying a new ST infection increases most rapidly during the first days after the initial positive sample, and they indicate that strains identified during the first 7 to 14 days are most likely to reflect original, possibly mixed, CDIs. Our measured mixed CDI rate (~7% based on multiple EIA-positive, culture-positive isolates obtained over up to 7 days) is consistent with those from previous investigations.

![Figure 1](https://example.com/fig1.png)
(9 to 13%) that examined multiple *C. difficile* colonies from single fecal samples (2, 9, 10, 12).

As finer genotyping schemes and whole-genome sequencing become more widespread in clinical research and practice, it is inevitable that increasing diversity will become apparent. These results emphasize the importance for careful interpretation of typing data when determining the transmission and recurrence of CDI.

**ACKNOWLEDGMENTS**

This work was supported by the NIHR Biomedical Research Centre, Oxford, United Kingdom.

The institution of D.W.C. and T.E.A.P. received per-case funding from Optimer Pharmaceuticals to support fidaxomicin trial patient expenses. D.W.C. and T.E.A.P. also received honoraria from Optimer Pharmaceuticals for participation in additional meetings related to investigative planning for fidaxomicin. M.H.W. has received honoraria for consultancy work, financial support to attend meetings, and research funding from bioMerieux, Optimer, Novacta, Pfizer, Summit, The Medicines Company, and Viropharma. No other author has a conflict of interest.

**REFERENCES**


