Increased Rate of DNA Recovery from United Kingdom Epidemic Clostridium difficile PCR Ribotype 1 Strains Stored Cryogenically

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We noted that some Clostridium difficile isolates are nonrecoverable after frozen storage and so used molecular typing analysis to characterize DNA from these strains. The recovery rate of Clostridium difficile PCR ribotype 1 was statistically significantly greater than that of other strains. This observation has implications for Clostridium difficile epidemiological studies.

Clostridium difficile infection (CDI) is an important nosocomial pathogen commonly associated with elderly hospital inpatients following antibiotic administration (7). Various molecular typing methods for C. difficile have been described and applied in clinical settings (1). Such studies have demonstrated that CDI can be caused by many strains, although certain ribotypes predominate. For example, C. difficile PCR ribotype 1 was present in 33/58 hospitals in England and Wales and is also causing concern in the United States (1, 6). Studies of CDI in elderly medical patients within our own institution have also causing concern in the United States (1, 6). Studies of CDI, generating a large collection of C. difficile isolates that were stored frozen at 37°C for 48 h, were conducted (2). More recently, we conducted a hospital-wide study of CDI, generating a large collection of C. difficile isolates that were stored frozen at −70°C to allow retrospective molecular typing. Random amplified polymorphic DNA (RAPD) was used to fingerprint C. difficile and identify subtypes within the PCR ribotype 1 group (3). We observed that a high proportion of isolates could not be recovered from storage and were concerned that this would affect the quality of data obtained. A protocol was devised to allow molecular typing of nonviable bacteria from the storage vial to ensure that an accurate representation of CDI within the hospital was obtained.

The study included 316 C. difficile isolates collected from hospital inpatients at the Leeds Teaching Hospitals Trust between June 2001 and April 2002. Isolates were recovered from C. difficile cytotoxin-positive diarrheal fecal specimens, as demonstrated by Vero cell cytotoxicity assay. C. difficile was cultured on cycloserine-cefoxitin egg yolk agar (CCEY; PHLS Media Services, Leeds, United Kingdom) and anaerobic culture (Oxoid, Basingstoke, United Kingdom) at 37°C for 48 h. If there were signs of growth in broth, then subculture was made on CCEYL and Columbia blood agar (PHLS Media Services). DNA was extracted from C. difficile colonies as previously described (2). For isolates that could not be recovered, 200 μl Protect cryopreservation fluid was centrifuged to pellet the bacterial cells. Cells were washed twice with phosphate-buffered saline and resuspended in 50 μl sterile distilled water. DNA was then extracted as described above. RAPD profiling was performed as described previously (2). Of 316 stored C. difficile isolates, 219 (69%) were recoverable. One hundred ninety-four of these had RAPD profiles corresponding to PCR ribotype 1 as determined in an earlier study (3), 22 had other DNA profiles, and 3 were nontypeable. Of the 97 stored isolates that could not be cultured, 44 had PCR ribotype 1 profiles, 50 had other profiles, and 3 were nontypeable (Fig. 1). The recovery of PCR ribotype 1 C. difficile isolates from cryogenic storage was statistically significantly greater than that of other types (χ² [3 df] = 66.93; P < 0.001).

We used a commercially available system to cryostore C. difficile strains. Cryobeads are commonly used in diagnostic laboratories for microorganism storage (4). Each vial contains approximately 20 individual beads together with a cryopreservative liquid. Microorganisms readily adhere to the surface of beads because of their porosity. When a fresh culture is required, a single bead can be removed from the frozen vial and used to directly inoculate a suitable bacteriological medium. We have no reason to believe that this system is inferior to other storage methods for C. difficile. Data collated by the manufacturer indicate that the Protect system is suitable for the storage of C. difficile. Indeed C. difficile has been successfully stored in this way for up to 96 months at −20°C (manufacturer's database; no data available for storage at −70°C). We modified the use of the Protect system by retaining the approximate 1 ml of cryopreservative fluid, and this subsequently allowed centrifugation of bacterial DNA. We do not
believe that this was detrimental to bacterial viability. Notably, the attempted recovery of bacterial isolates described here was the first manipulation of the stored cryovials, and we made strenuous efforts to achieve bacterial growth.

Using *C. difficile* PCR ribotype 1, we have found that storage temperature and multiple cycles of freezing or refrigeration/thawing have minimal effects upon the viability of the bacterium or its spores (5). We have previously demonstrated that *C. difficile* PCR ribotype 1 has a greater sporulation and spore germination capacity than other ribotypes (8; J. Freeman and M. H. Wilcox, unpublished data). For example, the epidemic *C. difficile* PCR ribotype 1 strain produced approximately three- to fivefold more spores in vitro than nonprevalent strains (*P* < 0.05) (8). These attributes may partly explain the enhanced clinical virulence of *C. difficile* PCR ribotype 1. Furthermore, such observations may account for the differences in recovery following long-term cryogenic storage seen in the present study. We did not observe bacteria for spores, so we cannot comment on the presence or absence of spores for each culture at the time of storage.

Our findings have implications for studies of the epidemiology of *C. difficile* isolates and infection, particularly retrospective analyses. Studies using frozen stored isolates may overestimate the true burden of *C. difficile* PCR ribotype 1 disease or potentially that caused by other types. Indeed within this collection of isolates, data based on recoverable isolates would have shown the proportion of *C. difficile* PCR ribotype 1 isolates in our institution to be 89% compared with the more accurate figure of 75%. It is important that future studies take into account this potential source of bias. Improved rates of recovery may be achievable by extended incubation of culture plates prior to harvesting of cells, thereby increasing the number of *C. difficile* spores. Alternatively, we have shown that recovery of DNA from samples of nonculturable stored isolates followed by molecular fingerprinting allows an accurate assessment of *C. difficile* types to be made.

REFERENCES