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Research paper
Clostridioides difficile (including epidemiology)

Correlation between restriction endonuclease analysis and PCR ribotyping for the identification of *Clostridioides* (*Clostridium*) *difficile* clinical strains

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ABSTRACT

Restriction endonuclease analysis (REA) and PCR ribotyping are two typing systems that have been frequently utilized for molecular epidemiologic characterization of *Clostridioides* (*Clostridium*) *difficile*. To correlate typing data obtained from each method, we performed both REA and PCR ribotyping on a large and diverse set of historical and contemporary *C. difficile* infection clinical isolates. Eighty isolates were selected from each reference laboratory in the United States (Microbiology Reference Laboratory, Hines VA Medical Center) and United Kingdom (*Clostridium difficile* Network for England and Northern Ireland laboratory, University of Leeds). The 160 isolates were assigned to 82 unique ribotypes and 51 unique REA groups (116 unique REA types). In general, concordance between typing methods was good. Dendrogram analysis of PCR ribotype band patterns demonstrated close genetic relationships among strain types with discordant REA and ribotype assignments. While REA typing was more discriminatory, several REA types in this study were further discriminated by PCR ribotyping, indicating that discriminatory value of these typing methods may be strain dependent. These data will assist with molecular epidemiologic surveillance of strains identified by these two commonly used *C. difficile* typing systems.

1. Introduction

Clostridioides (*Clostridium*) *difficile* has emerged as an important public health threat that is associated with considerable morbidity, mortality, and increased healthcare expenditures [1]. The emergence and global dissemination of *C. difficile* has been associated with the spread of antibiotic-resistant and potentially hypervirulent epidemic strains. The most notable strain, which caused outbreaks of severe *C. difficile* infection (CDI) first in North America [2,3] and later in the UK [4], is the strain identified as group BI by restriction endonuclease analysis (REA), ribotype 027 by polymerase chain reaction (PCR) ribotyping, and NAP1 by pulsed-field gel electrophoresis (PFGE). However, the molecular epidemiology of *C. difficile* is dynamic and oftentimes highly variable among different regions of the world; BI/NAP1/027 is declining in both the US [5] and UK [6], and new strain types are emerging. Thus, rigorous surveillance and investigation of the molecular epidemiology of CDI is an important public health responsibility. Identification of epidemic strains has guided research to better understand *C. difficile* pathogenesis [1], human transmission [7], global dissemination [8], and identification of novel potential reservoirs for *C. difficile*, such as animals and food [4].

However, molecular epidemiologic investigation of CDI has presented several challenges, in particular the lack of a single portable typing system that is shared amongst the public health

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and academic communities. Several typing methods are in use, each with unique benefits and limitations [9]. PCR ribotyping and REA are two typing systems that have been frequently utilized for molecular epidemiologic characterization of *C. difficile*. However, particularly for REA, very few laboratories perform these analyses, and generated data are not portable, presenting challenges for cross-typing of strains analyzed by these methods. Several previous studies have compared REA and PCR ribotyping data (in addition to other typing methods), but these prior studies are limited by small sample size and restricted strain diversity [10], omission of several epidemiologically important ribotypes [11], or lack of inclusion of strains collected outside of North America [12]. The objective of this study was to correlate REA and PCR ribotyping performed by two highly experienced reference laboratories for identification of a diverse collection of clinical *C. difficile* strains obtained from a multinational group of patients predominantly in the US and UK.

2. Materials and methods

2.1. Study isolates

This study included isolates collected from two reference laboratories: the Microbiology Reference Laboratory (MRL) at Hines VA Medical Center, Hines, Illinois, USA, where REA [13] is performed; and the *Clostridium difficile* Network for England and Northern Ireland (CDRN) laboratory at University of Leeds, Leeds, UK, where PCR ribotyping [14] is performed. Clinical isolates were predominantly from patients in the US and UK, although the CDRN laboratory also occasionally receives strains from other European countries. Saved clinical *C. difficile* isolates from each reference laboratory were selected to undergo both REA and PCR ribotyping. From each site, a diverse collection of historical and contemporary strain types known to commonly cause CDI in humans was selected. These isolates were originally collected between 1982 and 2009. From the Hines MRL, 80 unique isolates were selected. Eighty isolates were also selected from the Leeds CDRN laboratory, and this subset included a reference panel of 70 well-characterized unique isolates and 5 pairs of duplicate isolates [14,15]. The isolates selected for inclusion in this study were de-identified, and a waiver of informed consent was granted by the institutional review board.

2.2. Restriction endonuclease analysis

As previously described [13], REA was performed by analyzing unique electrophoretic DNA band patterns from extracted whole genomic DNA after restriction digestion with the *Hin*dIII. Isolates were typed based on manual comparison of electrophoretic band patterns of the isolate to the band patterns of a large collection of reference isolate band patterns. Band patterns with a 90% similarity index are assigned to a REA group (letter designation) and unique patterns are given a specific REA type (number designation). Thus, an REA type is assigned to an isolate band pattern that is identical to an existing REA type in the reference isolate library. REA groups and types are categorized in chronological order as new groups/types are identified. Currently, >120 REA groups (i.e., REA groups A through DS) and >600 REA types have been identified.

Potentially new REA types (i.e., those isolates with as few as a single band difference from all isolates in the reference library) [16] identified in this study were confirmed only for REA groups of known epidemiologic and/or clinical significance. For those types with subtle band differences, isolates were further assessed to determine whether the band difference represents a new REA type or an isolate of an existing REA type that also contains a plasmid. Plasmid preparations of the strain of interest and closely related reference strains were prepared as previously described [17]. On the same gel, electrophoresis was performed as described above on whole genomic DNA and plasmid preparations of the strain of interest and closely related reference strains.

2.3. PCR ribotyping

As previously described [14], PCR ribotyping was performed by analyzing capillary electrophoresis banding patterns from PCR products of the 16S–23S rRNA intergenic spacer region. Unique PCR ribotypes were identified based on the patterns of major peaks in fluorescent signal obtained from PCR product analysis of each isolate. BioNumerics v5.1 (Applied Maths, Sint-Martens-Latum, Belgium) was used to discriminate PCR ribotypes based on inter-comparison of isolate peak profiles. PCR ribotype identities are assigned to isolates following basic maximum similarity scoring against a validated reference library. New PCR ribotypes (i.e., those isolates with as few as a single peak difference [>5 base-pairs in length], when compared with all profiles in the reference library) were further tested for pattern stability and reproducibility before new library assignments were opened. New PCR-ribotypes are categorized in chronological order as new ribotypes are identified. Currently, >900 distinct PCR ribotypes are present in the library.

2.4. Comparison of REA and PCR ribotyping

To correlate REA and PCR ribotyping data, each site provided the other site with 80 previously typed *C. difficile* isolates. Each site subsequently analyzed those 80 isolates by the typing method performed at their reference laboratory. Thus, 155 unique isolates (plus 1 duplicate of each of 5 strains) underwent both REA and PCR ribotyping. Both sites were initially blinded to the previous typing results obtained by the other laboratory. To assess REA precision, the 5 pairs of duplicate isolates provided by the Leeds CDRN laboratory underwent REA. The Hines MRL was blinded to the identification of the duplicate isolates. After REA and PCR ribotyping were completed, the investigators were unblinded to the identification of the duplicate isolates. Discordance between anticipated REA groups/types and ribotypes in three of the duplicate pairs prompted reassessment to confirm the preliminary typing data. REA groups/types reported here are the final typing data assigned after unblinding.

3. Results

In total, 160 isolates underwent both REA and PCR ribotyping (155 unique isolates, and 5 additional duplicates among those 75 unique isolates provided by the Leeds CDRN laboratory). Among these, strains were assigned to 82 unique ribotypes and 51 unique REA groups (further characterized into 116 unique REA types). There were an additional 15 potential newly identified REA types, but because of the unclear epidemiologic and/or clinical significance, they were only identified to the level of REA group and a specific REA type was not assigned. PCR ribotypes and the corresponding REA identifications, and vice versa, are provided in Tables 1 and 2, respectively. In general, REA typing provided greater discrimination of strain types. The genetic relationships between PCR ribotypes and REA groups/types are illustrated in the PCR ribotype dendrogram aligned by ribotype pattern similarity (Fig. 1- dendrogram separated into sections for print; Figure S1- complete dendrogram online).

Of the 27 ribotypes represented by multiple unique isolates per ribotype, all 27 of these ribotypes were further distinguished into distinct REA groups and/or types (Table 1). However, the discriminatory value of PCR ribotyping was also identified. Of the 17 REA