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Challenges for Standardization of *Clostridium difficile* Typing Methods

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Typing of *Clostridium difficile* facilitates understanding of the epidemiology of the infection. Some evaluations have shown that certain strain types (for example, ribotype 027) are more virulent than others and are associated with worse clinical outcomes. Although restriction endonuclease analysis (REA) and pulsed-field gel electrophoresis have been widely used in the past, PCR ribotyping is the current method of choice for typing of *C. difficile*. However, global standardization of ribotyping results is urgently needed. Whole-genome sequencing of *C. difficile* has the potential to provide even greater epidemiologic information than ribotyping.

Clostridium difficile, a Gram-positive spore-forming anaerobe, is the leading cause of infectious diarrhea in the industrialized world. *C. difficile* is known to express up to three toxins: toxin A (TcdA), toxin B (TcdB), and, less commonly, a third toxin called binary toxin (CDT). These toxins cause extensive colonic inflammation, epithelial tissue damage, and cell death (1). *C. difficile* has a highly dynamic genome which readily undergoes genetic exchange. Disease-causing *C. difficile* strains have arisen independently from a common ancestor that dates back millions of years (2–4).

The development of typing methods, in particular, ribotyping, has facilitated understanding of the epidemiology of *C. difficile* since the late 1990s. Strains of *C. difficile* with apparently increased virulence, especially ribotype 027, also known as North American pulsed-field gel electrophoresis type 1 (NAP1) or restriction endonuclease analysis group BI, have been described in the last 10 years. The increased virulence of the NAP1/027/BI strain may be due to increased expression of toxins A and B (5) as well as production of CDT, although this remains controversial. This strain has emerged in many regions of the world and, although the evidence is somewhat inconsistent, may be associated with increased severity, recurrence, and significant mortality (1, 5–7).

In an extensive European study, PCR ribotype 027 was the sixth most common ribotype, accounting for 5% of all toxigenic *C. difficile* isolates, with ribotype 014/020 being the most prevalent (16%), followed by ribotype 001 (10%) and ribotype 078 (8%) (8). In the United States, the prevalence of ribotype 027 has been described to be as high as 14% in a recent study (6). New ribotypes are frequently detected (1), including ribotypes 176, 198, and 244, which appear to have evolved from the 027 lineage. These newly identified PCR ribotypes may prove to be just as problematic as the ribotype 027 strain, highlighting the importance of methods to demonstrate strain relatedness. A recent study showed that isolates of ribotypes 176, 198, and 244 had erroneously been assigned to ribotype 027 by restriction endonuclease analysis (REA) typing (9). Additionally, earlier reports showed that not all NAP1 isolates are of PCR ribotype 027 (10), emphasizing the need for a globally standardized typing method for *C. difficile* (9, 10).

C. DIFFICILE TYPING METHODS APPLIED TODAY

Various typing methods for *C. difficile* are currently used: restriction endonuclease analysis (REA), pulsed-field gel electrophoresis (PFGE), multilocus sequence typing (MLST), repetitive-element PCR typing, toxinotyping, and PCR ribotyping as well as multilocus variable-number tandem-repeat analysis (MLVA). Serotyping has been replaced by surface-layer protein A-encoding gene (*slpA*) typing (11), and, with the recent advances in DNA sequencing technology, whole-genome sequencing (WGS) (12) may be the way of the not-too-distant future. Each of these methods is briefly reviewed (Table 1).

RESTRICTION ENDONUCLEASE ANALYSIS

REA of *C. difficile* makes use of whole-genome DNA, which is digested by the frequently cutting restriction enzyme HindIII. The restriction fragments are resolved by agarose gel electrophoresis (13). This technique was first used to track the spread of *C. difficile* in a hospital setting in 1986 (14). REA has high discriminatory power and stability, but the method and interpretation are technically demanding and the data are difficult to exchange between laboratories.

PULSED-FIELD GEL ELECTROPHORESIS

PFGE was one of the first molecular typing methods to be applied to *C. difficile*, particularly in North America, where it has been used for analysis of food-borne disease outbreaks caused by various bacterial species for many years. It is still considered the standard in Canada and the United States and continues to be used elsewhere for the investigation of epidemiologically related isolates and in population studies. The technique resolves large fragments of DNA generated from whole-genome macrorestriction with an infrequently cutting restriction enzyme. For *C. difficile*, this is usually SmaI (10), but SacII (15) and other restriction enzymes have also demonstrated good discriminatory power against

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TABLE 1 Summary table of *C. difficile* typing methods applied currently

Typing method	Technique applied	Benefit(s)	Challenge(s)
Restriction endonuclease analysis (REA)	Whole-genome restriction and detection by agarose gel electrophoresis	High discriminatory power and stability	Method is technically demanding; data are difficult to exchange between laboratories
Pulsed-field gel electrophoresis (PFGE)	Whole-genome restriction and detection by pulsed-field gel electrophoresis	High discriminatory power	The method is laborious; results cannot easily be compared between laboratories
Multilocus sequence typing (MLST)	PCR amplification and sequencing of parts of housekeeping genes	Stability and transferability of data	Costs are high
Repetitive-element PCR typing (Diversilab typing)	Repetitive-element PCR system	High discriminatory power	Interlaboratory reproducibility needs to be assessed
Toxinotyping	PCR amplification followed by restriction enzyme digestion of 10 regions of the pathogenicity locus	Highly reproducible while giving a clear view of the toxin status, excluding binary toxin	Less resolution power than other typing methods
PCR ribotyping	PCR amplification of the 16S-23S intergenic spacer region		
Agarose-based PCR ribotyping	PCR amplification followed by agarose gel electrophoresis	A globally used reference library has been established	Data are not easily interchangeable between laboratories
Sequencer-based PCR ribotyping	PCR amplification followed by sequencer-based capillary separation	Highly accurate, reproducible, and interchangeable digital data are obtained	No standardization of ribotype nomenclature
Qiaxcel-based PCR ribotyping	PCR amplification followed by Qiaxcel-based capillary separation	Accurate and reproducible digital data are obtained at low cost	Not widely used in laboratories; normalization across capillaries may be inaccurate
Multilocus variable-number tandem-repeat analysis	Multiple singleplex PCR amplifications followed by sequencer-based capillary separation	Digital data with high resolution power and the possibility to determine phylogenetic relationships are obtained	Relatively costly and labor-intensive compared with PCR ribotyping
Surface-layer protein A-encoding gene (*slpA*) typing	PCR amplification of the variable region of the *slpA* gene, followed by DNA sequencing	Relevance to vaccine development	Method is not widely used
Whole-genome sequencing	Whole-genome sequencing by methods such as Sanger, Roche 454, and Illumina sequencing	Accurate and reproducible digital data with very high discriminatory power are obtained	Informatics expertise necessary

C. difficile. Early use of this method was problematic due to difficulty lysing spores and DNA degradation among some strains, but, with the use of younger cultures and the addition of thiourea to the electrophoresis buffer, 100% typeability can be achieved (16). Problems remain in the speed of the method, with completion of analyses taking several days, in the requirement of specialist equipment for the PFGE technique (however, the equipment can also be used for typing other microorganisms), and in interpretation and exchange of data between laboratories. The latter shortcoming could be improved through the provision of a standardized methodology, ladders, and software for interpretation as well as an accessible database of "fingerprints" similar to the CDC's PulseNet program (www.cdc.gov/pulsenet).

MULTILOCUS SEQUENCE TYPING

MLST involves the partial amplification and sequencing of a selection of housekeeping genes. The unambiguous sequence data generated may easily be compared between laboratories. In 2010, a MLST typing scheme with discriminatory power similar to that of agarose gel-based PCR ribotyping was developed for *C. difficile* (17) and is most useful for population studies. However, MLST is relatively costly compared with PCR ribotyping (10, 18).

rep-PCR

Repetitive-element PCR (rep-PCR) typing is based on polymorphisms of repetitive elements that exist in multiple copies in the *C. difficile* genome. The DiversiLab system (bioMérieux, Marcy l'Etoile, France) is a commercially available rep-PCR system that has higher discriminatory power than agarose-based ribotyping in typing *C. difficile* (19).

This high resolution may be useful for investigating outbreaks within a hospital. Interlaboratory reproducibility has not yet been assessed for the genotyping of *C. difficile* by the DiversiLab system in an international setting (19).

TOXINOTYPING

C. difficile toxins A (TcdA) and B (TcdB) are encoded on a well-defined chromosomal region of 19.6 kb called the pathogenicity locus (PaLoc) (20). Toxinotyping is performed by PCR amplification and subsequent restriction enzyme digestion of 10 regions of the PaLoc. Toxinotyping has very high reproducibility. Although the discriminatory power is not as good as it is for PFGE and ribotyping, toxinotyping gives a good picture of the toxigenic status of *C. difficile* strains (18), excluding binary toxin.

PCR RIBOTYPING

PCR ribotyping, which is based on the amplification of the 16S-23S intergenic spacer region (ISR) (21), is the preferred typing method in Europe as well as Australia (15, 18, 22). PCR ribotyping has also been recently employed in the United States (6).

Bacterial rRNA (rrn) operons are usually organized in the order 16S *rrnA*-ISR-23S *rrnA*-ISR-5S *rrnA*, and their copy numbers can range between 1 and 15. The published whole genome of *C. difficile* strain 630 contains 11 *rrn* operons (2), but the number of *rrn* operons is known to be subject to variation in *C. difficile*. Compared with other bacterial species, *C. difficile* also has considerable intraspecific diversity in the ISRs (22), making it suitable for PCR ribotyping.

The concept of making use of the heterogeneity of the intergenic 16S-23S rRNA spacer regions in *C. difficile* was first described in 1993 by Gürtler (23). In order to obtain smaller fragments for improved analysis on agarose gels, new primers closer to the spacer region were designed by O'Neill et al. (21). This approach has since been routinely used by the Anaerobe Reference Laboratory in Cardiff, United Kingdom, where a widely used library has been established (18).

SEQUENCER-BASED RIBOTYPING

A sequencer-based PCR ribotyping method making use of a Web-based database was developed in 2008, enabling data comparison between laboratories. PCR amplification was performed using the same primers as for agarose gel-based ribotyping except that one of the primers was labeled with a fluorescent label. The amplicon sizes were determined using an ABI genetic analyzer, and a database was established (24). This Web-based database allows adjustment of different sequencer settings as well as of the main primer pairs used. Known ribotypes can be determined by simply uploading sequencer data files (http://webribo.ages.at/). Capillary sequencers are widely used in clinical laboratories (15, 24). However, standardization of the ribotype nomenclature remains a challenge (25).

QIAXCEL-BASED RIBOTYPING

Recently, QIAxcel (Qiagen, Hilden, Germany) has become available as an alternative to agarose slab gel-based PCR ribotyping. QIAxcel does not require the use of a fluorescein-labeled primer, and the cost is around 3 to 4 dollars per isolate versus 6 to 7 dollars if a DNA sequencer is used. However, although it is a vast improvement over agarose-based ribotyping (25), QIAxcel-based ribotyping has poorer resolution than sequencer-based ribotyping. The limit of discrimination for QIAxcel is 3 to 5 bp for amplicons in the range of 100 to 500 bp in length when separated with the high-resolution cartridge, and for fragments between 500 bp and 1 kb, the resolution decreases to 50 bp. Also, in contrast to agarose- and sequencer-based ribotyping, it is not possible to include an internal ladder.

Therefore, normalization across capillaries may be inaccurate, with the analysis instead relying on single alignment markers at either end of the electrophoresis pattern.

MULTILOCUS VARIABLE-NUMBER TANDEM-REPEAT ANALYSIS

MLVA involves using capillary electrophoresis, usually employing a sequencer, to determine fragment sizes, and hence the number of repeat sequences, at multiple PCR-amplified loci. The numbers of repeats can differ between strains at each locus. MLVA has a number of advantages over PCR ribotyping. The increased discriminatory power of MLVA allows outbreaks to be tracked more efficiently, and, in contrast to PCR ribotyping, it has the potential to determine phylogenetic relationships. Several MLVA schemes have been described for typing *C. difficile*. Recently, a MLVA scheme was developed which could subtype clinically significant ribotypes while still clustering isolates in concordance with PCR ribotyping (26). However, 15 PCRs are required to be performed for this MLVA scheme, making the approach relatively laborious and expensive. Compared with traditional PCR ribotyping, MLVA has the additional advantage of producing digital data with a decreased turnaround time.

These advantages may be due to the fact that, in contrast to the use of slab-gel electrophoresis in traditional PCR ribotyping, capillary electrophoresis is employed (26, 27).

SEROTYPING

Serotyping has traditionally been performed by slide agglutination or enzyme-linked immunosorbent assays, using specific antisera. These methods allow the differentiation of 10 major serogroups (A, B, C, D, F, G, H, I, K, and X). Strains of the A serogroup have a flagellar antigen in common that is responsible for cross-agglutination on slides but can be divided into 20 subgroups by polyacrylamide gel electrophoresis (11).

SURFACE-LAYER PROTEIN A-ENCODING GENE (*slpA*) TYPING

The *C. difficile* cell surface contains the so called S-layer, consisting of an immunodominant protein encoded by the *slpA* gene. *slpA* typing was originally performed using PCR amplification of the variable region of the *slpA* gene, followed by restriction fragment length polymorphism (RFLP) analysis and DNA sequencing.

With the exception of serogroup A, the *slpA* nucleotide sequences were found to be 100% identical within a given serogroup and different between serogroups. *slpA* typing may therefore replace serotyping (11). Some studies suggest a correlation between ribotype and *slpA* type. However, a recently published study that included a large number of genomes showed that isolates of the same ribotype or MLST type may differ in *slpA* types. S-layer-based typing may therefore have greater relevance to vaccine development than conventional genotyping (12).

WHOLE-GENOME SEQUENCING (WGS)

The first *C. difficile* whole-genome sequence, published in 2006, was generated by Sanger sequencing (2). In 2010, the evolutionary dynamics of *C. difficile* were shown using Sanger sequencing in combination with the next-generation sequencing technologies Roche 454 and Illumina. Phylogenetic analyses were performed on the 29 genome sequences generated (3). In two very recent studies, 57 and 151 *C. difficile* genome sequences were generated using Illumina sequencing, indicating that traditional genotyping methods may not always provide sufficient information for epidemiological purposes (12, 28).

CONCLUSIONS

In order to study the *C. difficile* epidemiology, a typing method with high discriminatory power and reproducibility is needed. Among all the *C. difficile* typing methods applied globally, PCR ribotyping has developed as the most widely used typing method. PCR ribotyping is a straightforward and affordable typing method

for *C. difficile* (25). However, although PCR ribotyping has been commonly used since 1995, standardization remains difficult (18, 25).

Ribotyping has traditionally been performed by agarose gel analysis, which does not allow the assignment of a ribotype unless the laboratory has the correct reference strains (15). Analysis of band sizes is hampered due to poor resolution of agarose-gel electrophoresis, and comparison of results between laboratories is unreliable.

Also, since the establishment of the globally used reference library by the Anaerobe Reference Laboratory in Cardiff, United Kingdom, PCR ribotyping has been further developed.

New primer pairs have been used, resulting in different amplicon lengths for the same PCR ribotype and posing additional challenges to standardization of ribotyping nomenclature (25, 29, 30).

DNA sequencers are widely used and have very high accuracy and resolution. Sequencer-based PCR ribotyping may therefore be the method of choice—however, there remains an urgent need for standardization of ribotype nomenclature for global epidemiology (24, 25). Toxinotyping and MLVA typing may be applied if further resolution is needed in addition to ribotyping results (18, 27). Whole-genome sequencing may become the method of choice for the investigation of *C. difficile* strains in the years to come (12, 28).

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