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Multilocus Sequence Typing of *Clostridium difficile*▽

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A robust high-throughput multilocus sequence typing (MLST) scheme for *Clostridium difficile* was developed and validated using a diverse collection of 50 reference isolates representing 45 different PCR ribotypes and 102 isolates from recent clinical samples. A total of 49 PCR ribotypes were represented overall. All isolates were typed by MLST and yielded 40 sequence types (STs). A web-accessible database was set up (http://pubmlst.org/cdifficile/) to facilitate the dissemination and comparison of *C. difficile* MLST genotyping data among laboratories. MLST and PCR ribotyping were similar in discriminatory abilities, having indices of discrimination of 0.90 and 0.92, respectively. Some STs corresponded to a single PCR ribotype (32/40), other STs corresponded to multiple PCR ribotypes (8/40), and, conversely, the PCR ribotype was not always predictive of the ST. The total number of variable nucleotide sites in the concatenated MLST sequences was 103/3,501 (2.9%). Concatenated MLST sequences were used to construct a neighbor-joining tree which identified four phylogenetic groups of STs and one outlier (ST-11; PCR ribotype 078). These groups apparently correlate with clades identified previously by comparative genomics. The MLST scheme was sufficiently robust to allow direct genotyping of *C. difficile* in total stool DNA extracts without isolate culture. The direct (nonculture) MLST approach may prove useful as a rapid genotyping method, potentially benefiting individual patients and informing hospital infection control.

Clostridium difficile is an obligate anaerobic Gram-positive bacillus carried asymptomatically in the gut of approximately 2 to 7% of healthy human adults (28, 35). Nosocomial acquisition of *C. difficile* in humans is common, and symptoms ranging from mild diarrhea to severe pseudomembranous colitis can develop during antibiotic treatment or shortly afterwards (1, 28, 40). Symptoms are caused by toxins A and B encoded by the *tcdA* and *tcdB* genes located within the pathogenicity locus (PaLoc) and potentially an additional binary toxin (reviewed in reference 7).

The rate and severity of nosocomial infections increased between the years 2000 and 2008 (27, 46, 47), coincident with the emergence of a hypervirulent fluoroquinolone-resistant clone, designated PCR ribotype 027 (31, 34). Outbreaks of *C. difficile* infection (CDI) caused by the 027 clone have been reported in North America and throughout Europe (15, 18, 23, 31, 32). This strain is now endemic, causing 36% of cases in England, United Kingdom, from April 2008 to March 2009 (10). Mortality can range from 6 to 15% (2), and the economic burden of CDI is substantial, with an estimated direct cost of over $6,000 per case in the United States (37).

Studies of global epidemiology require easily comparable genotyping data for large numbers of bacterial isolates. Genotyping methods in common use for *C. difficile* include PCR ribotyping, pulsed-field gel electrophoresis (PFGE), and restriction-endonuclease analysis (REA) (4, 10, 26, 41). These techniques are generally labor- and resource-intensive, not easily adapted to very high-throughput, and often restricted to reference laboratories. Furthermore, interlaboratory comparison of data can be difficult when they are based on gel banding patterns. Multilocus sequence typing (MLST) is a microbial genotyping method facilitating isolate discrimination using nucleotide sequences of housekeeping gene fragments (24). Each unique combination of alleles is assigned a sequence type (ST) number. The MLST technique is scalable, according to the question to be addressed or the resources available, and amenable to automation using very-high-throughput robotic systems (12). Searchable Internet-accessible MLST databases (as at http://pubmlst.org/) allow laboratories performing MLST to maintain ownership of their data. However, having a single laboratory as curator of the database avoids the potential confusion that arises when new allele and ST numbers are assigned (14). MLST data are also a powerful tool for studying

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the population biology of bacterial species (25, 42). An MLST scheme for *C. difficile* has been described (20) but has not been widely adopted. This may be partly because the *ddl* locus was a null allele and failed to amplify in certain strains (L. Lemee, personal communication). Furthermore, a curated Internet-accessible database was not available.

Our aim was to develop a robust MLST scheme for *C. difficile* and to set up an Internet-accessible database to allow the simple depositing, retrieval, and comparison of data. The scheme was validated using two collections of isolates, one representing a wide range of PCR ribotypes and another consisting of isolates cultured from recent clinical samples. The scheme was also sufficiently robust to allow direct MLST typing of *C. difficile* in total DNA extracts from stool samples, avoiding the need for isolate culture and enabling rapid genotyping (3.5 days for 24 samples) to be performed.

MATERIALS AND METHODS

***C. difficile* stools and culture.** A total of 215 human stool specimens submitted to the Clinical Microbiology Laboratory, John Radcliffe Hospital, Oxford, United Kingdom, between 12 July and 17 October 2008 were included in this study. Stools were from both hospital and community patients. Stools were chosen so that half were sequential, enzyme-linked immunosorbent assay (ELISA) positive ($n = 107$) with sufficient stool remaining, and half were ELISA negative ($n = 108$) submitted during the same time period (Premier Toxins A&B Enzyme Immunoassay; Meridian Bioscience Europe, Villa Cortese, Italy). All stool samples underwent culture for *C. difficile*. Industrial methylated spirits ([IMS] 0.5 ml) was added to a 0.5-ml fecal sample (pea-sized portion if the stool was formed), and the sample was vortex mixed and incubated at room temperature for 1 h. A loopful was then cultured onto modified Brazier's cycloserine-cefoxitin-egg yolk (CCEY) agar (CCEY agar base containing cycloserine-cefoxitin supplement and 5% defibrinated horse blood), and the plates were incubated anaerobically at 37°C for up to 7 days. A single colony was subcultured onto a Columbia blood agar (CBA) plate and incubated for 48 h, after which colonies giving the characteristic odor and fluorescence under UV illumination were obtained. For long-term storage, isolates were emulsified in nutrient broth containing 10% glycerol and stored at −80°C.

An additional 50 isolates were obtained from a collection held at Leeds General Infirmary (reference laboratory for the *C. difficile* Ribotyping Network for England and Northern Ireland). They represented 45 different PCR ribotypes (plus five duplicates) chosen to represent the overall genetic diversity of *C. difficile* (determined by PCR ribotyping) and were used to validate the MLST scheme.

Extraction of total stool DNA. Total DNA was extracted from stool samples using a FastPrep homogenizer (MP Biomedicals Europe, Illkirch, France) to lyse cells and spores, followed by DNA purification using a FastDNA Spin kit for soil (MP Biomedicals). The manufacturer's protocol was followed with the following refinements. Stool samples (100 μl or equivalent volume if the stool was formed) were added to 978 μl of sodium phosphate buffer in impact-resistant 2.0-ml tubes containing matrix E, which comprises 1.4-mm ceramic spheres, 0.1-mm silica spheres, and one 4-mm glass bead in buffer. MT buffer (MP Biomedicals) (122 μl) was added, and stools were homogenized for 40 s at a speed of 6.0 m per s. The lysate was clarified by centrifugation at 13,000 rpm for 15 min. Proteins were precipitated using 250 μl of protein precipitation solution (PPS) and removed by centrifugation for 10 min. The supernatant was mixed with 1 ml of silica binding matrix for 2 min to take up DNA, and then the matrix was allowed to settle for 5 min. The binding matrix was transferred to a SPIN filter (MP Biomedicals) and washed using 500 μl of SEWS-M (salt-ethanol wash solution). After the sample was air dried at room temperature, DNA was eluted from the matrix in 100 μl of DNA elution solution ([DES] DNase and pyrogen-free water).

Preparation of chromosomal DNA from cultured *C. difficile* isolates. Isolates were cultured onto CBA and incubated anaerobically for 48 h. A few colonies were emulsified in TE (Tris-EDTA) buffer (Sigma-Aldrich Co., Ltd., Gillingham, United Kingdom) and heated at 100°C for 10 min. Debris was removed by centrifugation at 13,500 rpm for 2 min, and the supernatant was removed for use in MLST. DNA was stored at −20°C.

***C. difficile* nucleotide sequence alignment and choice of candidate loci for MLST.** Ten publicly available *C. difficile* genome sequences, including six of PCR ribotype 027, were aligned using Mauve alignment software (5). The annotated *C. difficile* 630 genome (36) was included as a reference. This alignment contained several large gaps and was refined using BLAST to position fragments of each genome that were left unaligned by Mauve. Candidate regions for MLST were determined from the refined alignment as follows. Using windows of 500 bp (10 to 60 variable sites), the numbers of variable sites and the numbers of gaps were calculated. MLST loci were chosen such that there was a significant degree of divergence across the 500 bp, and no gaps were present. Fragments were annotated according to their orthologues in *C. difficile* 630 to ensure that they spanned housekeeping genes. Candidate fragments were tested *in silico* for suitability for primer design. The standard BLASTn search for "short nearly exact matches" was used, and this search is equivalent to BLASTn with the following parameters: word size, 7; low-complexity filter (DUST) off; expect value, 1,000. The database was the entire GenBank nonredundant database (nr). Details are found at: http://www.ncbi.nlm.nih.gov/blast/producttable.shtml#shortn. PCR was carried out on a subset of *C. difficile* isolates to verify amplification efficiency of the new MLST fragment. The seven loci and primers chosen for MLST are shown in Table 1.

***C. difficile* high-throughput multilocus sequence typing.** MLST was performed as described below by setting up PCR and sequencing reactions in 24-, 48-, or 96-well plates (Fig. 3 shows the procedure for 24 samples, but it can easily be scaled up to 48 samples). Seven PCR amplicons were obtained for each isolate using the primers shown in Table 1. Each 50-μl PCR mixture contained 39.75 μl of molecular biology-grade water (Sigma-Aldrich Co., Ltd.), 5 μl of 10× PCR buffer (Qiagen Ltd., Crawley, United Kingdom), 1 μl of a 10 μM concentration of each forward and reverse primer, 1 μl of 10 mM deoxynucleoside triphosphate (dNTP) mix (Invitrogen Corp., Paisley, United Kingdom), 0.25 μl of HotStart *Taq* DNA polymerase (Qiagen Ltd.), and 2 μl of *C. difficile* chromosomal DNA (approximately 10 ng) or extracted total stool DNA. The amplification conditions were 95°C for 15 min, followed by 35 cycles of 94°C for 30 s, 50°C for 40 s, and 72°C for 70 s, with a final extension at 72°C for 5 min and storage at 15°C. The amplification products were purified by precipitation with 20% polyethylene glycol (molecular weight, 8,000) and 2.5 M NaCl, and their nucleotide sequences were determined on each DNA strand using the amplification primers and BigDye Ready Reaction Mix (Applied Biosystems, Warrington, United Kingdom) as follows. Each 10-μl sequencing reaction mixture comprised 2 μl of PCR amplicon, 4 μl of a 1:15 dilution of either forward or reverse PCR primer (0.66 μM), 0.25 μl of BigDye Ready Reaction Mix, 1.875 μl of 5× sequencing buffer (20 ml of stock solution comprised 200 μl of 1 M $MgCl_2$, 8 ml of 1 M Tris-HCl, pH 9, and 11.8 ml of molecular biology-grade water [all from Sigma-Aldrich Co., Ltd.]), and 1.875 μl of molecular biology-grade water. Dilution of the BigDye Ready Reaction Mix using 5× sequencing buffer reduces the cost of high-throughput sequencing without any compromise in sequence quality. The reaction conditions were 30 cycles of denaturation at 96°C for 10 s, annealing at 50°C for 5 s, and extension at 60°C for 2 min. Unincorporated dye terminators were removed by precipitation of the termination products with 2 volumes of ethanol and 0.1 volume of 3 M sodium acetate (pH 5.2), followed by centrifugation, and the resulting pellet was then washed with 70% ethanol. The reaction products were separated and detected using a 3730 XL DNA analyzer (Applied Biosystems). For each sample, the program STARS (Sequence Typing Analysis Retrieval System [http://pubmlst.org/software/assembly/]) was used to rapidly collate paired reads, determine sequences, and identify alleles. The data for *C. difficile* alleles and STs were deposited in a newly developed *C. difficile* MLST database, which is accessible at http://pubmlst.org/cdifficile. Phylogenetic analysis was performed using the program MEGA, version 4 (Molecular Evolutionary Genetics Analysis [http://www.megasoftware.net/]).

Detection of PaLoc genes by PCR. The oligonucleotide primers used to detect the *tcdA* (encoding toxin A), *tcdB* (encoding toxin B), and *tcdC* (encoding a negative regulator of toxins A and B) sequences found within the pathogenicity locus operon (PaLoc) are summarized in Table 1. The *tcdA* assay was published by Lemee et al. (21) and amplifies a 369-bp amplicon for toxin A-positive B-positive (A^+ B^+) strains and a 110-bp amplicon for A-negative (A^-) B^+ strains, which contain a deletion in the *tcdA* gene. The reaction conditions were 95°C for 15 min, followed by 35 cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 40 s, with a final extension at 72°C for 5 min and storage at 15°C. The *tcdB* primers amplify a 688-bp amplicon under the same reaction conditions used for *tcdA*, except an annealing temperature of 50°C for 40 s and extension of 72°C for 70 s were used. The *tcdC* primers (33) amplify the 5′ region of the *tcdC* gene, giving a 475-bp amplicon under the same conditions used for *tcdA*. The absence of the PaLoc was demonstrated using primers lok1 and lok3 (3), which amplify

TABLE 1. Nucleotide sequences of the oligonucleotide primers used in the study[a]

Locus	Primer name[b]	Primer sequence (5′–3′)	Amplicon size (bp)	Locus size (bp)[c]	Reference or source
adk	adk1F adk1R	TTACTTGGACCTCCAGGTGC TTTCCACTTCCTAAGGCTGC	635	501	This study
*atp*A	atpA1F atpA1R	TGATGATTTAAGTAAACAAGCTG AATCATGAGTGAAGTCTTCTCC	674	555	This study
dxr	dxr3F dxr4R	GCTACTTTCCATTCTATCTG CCAACTCTTTGTGCTATAAA	525	411	This study
*gly*A	glyA1F glyA1R	ATAGCTGATGAGGTTGGAGC TTCTAGCCTTAGATTCTTCATC	625	516	This study
*rec*A	recA2F recA2R	CAGTAATGAAATTGGGAGAAGC ATTCAGCTTGCTTAAATGGTG	705	564	This study
*sod*A	sodA5F sodA6R	CCAGTTGTCAATGTATTCATTTC ATAACTTCATTTGCTTTTACACC	585	450	This study
tpi	tpi2F tpi2R	ATGAGAAAACCTATAATTGCAG TTGAAGGTTTAACACTTCCACC	640	504	This study
*tcd*A	tcdA-F tcdA-R	AGATTCCTATATTTACATGACAATAT GTATCAGGCATAAAGTAATATACTTT	369 (A^+ B^+)[d] 110 (A^- B^+)[d]	NA	21
tcdB	tcdB1 tcdB2	TGATGAAGATACAGCAGAAGC TGATTCTCCCTCAAAATTCTC	688	NA	This study
*tcd*C	tcdC-F(−17) tcdC-R(+462)	AAAAGGGAGATTGTATTATGTTTTC CAATAACTTGAATAACCTTACCTTCA	479	NA	33
cdd1 *cdu1*	lok1(*cdd1*) lok3 (*cdu1*)	AAAATATACTGCACATCTGTATAC TTTACCAGAAAAAGTAGCTTTAA	769	NA	3

[a] Primers were used (i) to perform *C. difficile* MLST, (ii) to detect the presence of three loci within the pathogenicity locus (*tcdA*, *tcdB*, and *tcdC*), and (iii) to confirm the absence of the pathogenicity locus in nontoxigenic strains (lok1/lok3).
[b] In the primer names F indicates forward, and R indicates reverse.
[c] NA, not applicable.
[d] The size of the amplicon varies with the strain genotype.

a 769-bp amplicon in strains without the PaLoc. The reaction conditions were the same as those for *tcdB* above.

PCR ribotyping. All PCR ribotyping of reference isolates and cultured isolates described in the present study was performed at the reference laboratory for the *C. difficile* Ribotyping Network for England and Northern Ireland, Leeds General Infirmary. PCR ribotyping was performed as described previously, with modifications (30). Briefly, bacterial growth was harvested from cultures raised on modified Brazier's CCEY agar with the omission of egg yolk and addition of 5 mg/liter lysozyme (CCEYL) (Bio-Connections, Wetherby, United Kingdom) for 48 h at 37°C (44). Template DNA was prepared using a QIAxtractor automated nucleic acid extraction system (Qiagen Ltd). Amplification reactions were performed in 50-μl volumes containing 50 pmol of both forward and reverse primers, 25 μl of HotStart *Taq* Plus PCR Master Mix (Qiagen Ltd.), 19 μl of water, and 5 μl of DNA template. The reaction mixtures were activated by heating to 95°C for 5 min and then subjected to 30 cycles of 92°C for 1 min, 55°C for 1 min, and 72°C for 1.5 min. A final cycle of 95°C for 1 min, 55°C for 45 s, and 72°C for 5 min was added. The resultant amplimer was concentrated to a final volume of approximately 20 μl by heating the opened reaction tubes at 75°C for 30 min. Amplification products were subjected to electrophoresis using 3% Metasieve agarose (Flowgen Bioscience, Nottingham, United Kingdom) at a field strength of 7.5 V/cm for approximately 2.5 h. Agarose gels were imaged using a GeneGenius camera system (Syngene, Cambridge, United Kingdom) after ethidium bromide staining. DNA profiles were analyzed and identified against a library of known PCR ribotypes using BioNumerics, version 4.6, software (Applied Maths, Belgium).

ID. The index of discrimination (ID) for MLST and PCR ribotyping was calculated according to Hunter and Gaston (11). The ID expresses the average probability that two individuals in the collection will have the same MLST type.

RESULTS

***C. difficile* MLST.** The MLST scheme was designed to be technically robust, generating high amplicon yields for all *C. difficile* genotypes, under the same PCR conditions for all seven loci. Loci were chosen that were widely distributed around the chromosome (Fig. 1), had demonstrable discriminatory ability, and lacked insertions or deletions that caused changes in length. The longest possible sequence was used at each locus to maximize resolution, trim sites were located so that primer sequences were removed, and null alleles were avoided (as far as possible, given current information on the species) to ensure 100% typeability.

Validation of the *C. difficile* MLST scheme. (i) Typeability of all genotypes. The ability of the MLST primers to amplify and sequence all *C. difficile* genotypes was assessed using DNA from 152 cultured isolates. These included 50 isolates of 45 different PCR ribotypes (from a collection held at Leeds General Infirmary, the reference laboratory for the *C. difficile* Ri-

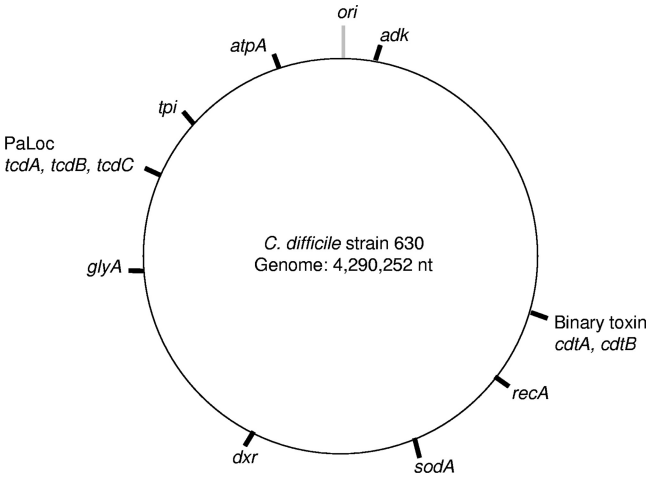

FIG. 1. The relative positions (to scale) of the seven housekeeping loci chosen for MLST on the *C. difficile* strain 630 chromosome, together with three genes of the pathogenicity locus (PaLoc) detected by PCRs referred to in this study and the genes encoding the binary toxin *cdtA* and *cdtB*.

botyping Network for England and Northern Ireland) representing the known diversity of the species (Fig. 2) and an additional 102 isolates from recent clinical samples (91 from 107 ELISA-positive stools and 11 from 108 ELISA-negative stools) (Tables 2 and 3). The clinical isolates were also PCR ribotyped, and consequently four additional PCR ribotypes were included, giving a total of 49 in the study as a whole (Fig. 2 and Tables 2 and 3). All isolates were successfully typed by MLST, and a total of 40 STs were identified (Fig. 2). These data have been made available at http://pubmlst.org/cdifficile/.

The MLST primers amplified DNA from both toxigenic and nontoxigenic strains. The 91 isolates from ELISA-positive stools were presumed to be toxigenic, and all were typed by MLST. The 11 isolates from ELISA-negative stools were assessed by PCRs designed to amplify fragments of three genes located within the PaLoc (Table 3). Nine of 11 isolates were toxigenic. The other two were nontoxigenic and were assigned a unique genotype, ST-15. They both yielded a 769-bp amplicon with the lok1/lok3 primer pair (Tables 1 and 3), confirming the absence of the PaLoc. The MLST primers have been further validated using a collection of 37 isolates (including 26 nontoxigenic strains) from healthy infants aged under 2 years and a large collection of ~1,600 clinical isolates collected at the John Radcliffe Hospital, Oxford, United Kingdom, between September 2006 and April 2009 (molecular epidemiology data are beyond the scope of this paper).

(ii) Discrimination among isolates. The level of discrimination achieved with MLST was compared to PCR ribotyping. The total number of isolates with both ST and PCR ribotype data available was 152, comprising 50 in the Leeds PCR ribotype collection, 91 from ELISA-positive stools (Table 2), and 11 from ELISA-negative stools (Table 3). Among the 152 isolates, 32/40 STs occurred with a single PCR ribotype, but 8 STs were associated with two or more PCR ribotypes (Fig. 2). Conversely, the PCR ribotype was not always predictive of the ST, with three PCR ribotypes being associated with more than one ST (Fig. 2). The multiple PCR ribotypes associated with single STs had a high degree of similarity in DNA profile. For example, when PCR ribotypes share an ST ($n = 8$), 6/8 STs were PCR ribotypes that differed by a single band. The other two were PCR ribotypes 023 and 063 (both ST-5) (Fig. 2), which differ by four bands, and PCR ribotype 009 (ST-3), which differs from the other PCR ribotypes associated with ST-3 by four bands.

The set of 102 clinical isolates was used to compare the discriminatory ability of the two methods. The IDs (11) for MLST and PCR ribotyping were 0.90 and 0.92, respectively, a difference that is unlikely to be statistically significant.

(iii) MLST performed directly on total stool DNA. The robustness of the MLST scheme was validated by typing strains contained in total DNA extracted from stool samples. *C. difficile* DNA isolated from cultured cells is a straightforward template for PCR amplification. However, when the same target is mixed in human feces, successful amplification requires efficient lysis of *C. difficile* cells and spores, PCR inhibitor removal (19, 29), and specific amplification from a complex mixture of DNAs (8, 22). To test the approach, MLST data were generated from both cultured isolates and total stool DNA extracts for 95 clinical samples (89/107 ELISA-positive and 6/108 ELISA-negative samples) (Tables 2 and 3). The direct and culture MLST data were generated by two different people, each without prior knowledge of the other's results. Direct MLST data were examined carefully for evidence of primer cross-reaction with other clostridial species in stools and coinfections with multiple *C. difficile* genotypes. Among 93 of the 95 stools, there was 100% agreement of the direct and culture MLST data. The two exceptions yielded occasional mixed peaks in the sequence chromatograms (2.1%), indicating a coinfection of more than one *C. difficile* genotype. This compares to 3/23 (13%) stools reported by Wroblewski et al. (45) using PCR detection of binary toxin gene(s) to detect heterogeneous isolate populations, and 2/23 (9%) patients with a first episode of CDI detected by van den Berg et al. (43) using PCR ribotyping. One additional stool DNA extract (Table 2, sample 104+) yielded very low levels of PCR amplicons, insufficient for sequencing at all seven loci.

Genetic variation and phylogenetic relationships among isolates. The relationships among the 40 STs were examined using the concatenated sequences of the seven MLST loci to construct a neighbor-joining tree (Fig. 2). The total number of variable nucleotide sites was 103/3,501 (2.9%), falling to 59/3,501 (1.7%) if the outlier ST-11 (078) was excluded. The comparable data for amino acid sites were 22/1,167 (1.9%) and 17/1,167 (1.5%). Despite the relatively low level of genetic diversity, the STs clustered into four groups with one outlier (ST-11) (Fig. 2). The majority of sequence types clustered in group 1 with very low internal bootstrap values. Group 2 contained ST-1 (027), the hypervirulent clone; group 3 contained two STs, both of which were associated with PCR ribotype 023; and group 4 contained the toxin A^- B^+ ST-37 (PCR ribotype 017). The outlier ST-11 is associated with PCR ribotype 078, which has been reported as an emergent hypervirulent clone.

DISCUSSION

MLST is a proven technology for understanding the molecular epidemiology and population biology of bacterial species

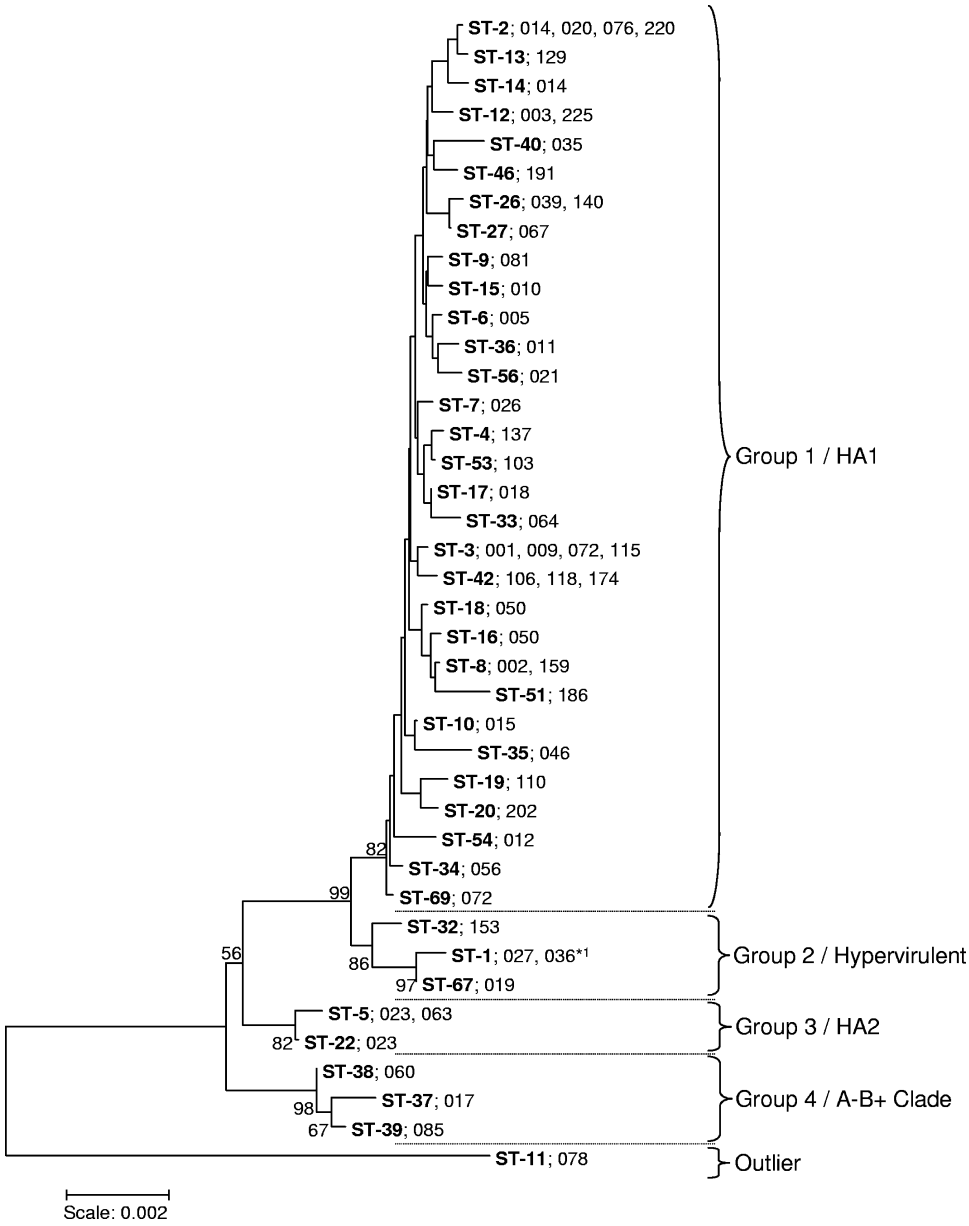

FIG. 2. Neighbor-joining tree constructed using the concatenated sequences (3,501 nucleotides) of the seven loci used in MLST. Bootstraps were generated using 1,000 replicates, and low values were removed for clarity. STs are shown in bold. The PCR ribotype(s) found in association with each ST are indicted. The STs cluster into four groups, designated 1 to 4, with bootstraps greater than 80 and one outlier (ST-11). These correspond to groups defined previously by microarray analysis of whole genomes (39) which were designated HA1 (human and animal 1), hypervirulent (containing the 027 strain), HA2, and the $A^- B^+$ clade (also containing other toxigenic types). The total number of variable sites was 103/3,501 (2.9%), and if the outlier ST-11 was excluded, the number was 59/3,501 (1.7%). ST-1 (indicated by *1) was associated with PCR ribotype 027 and also with a single PCR ribotype 036 isolate. DNA profiles for PCR ribotypes 036 and 027 are very similar and differ by only a single band.

(25). Although it has been applied to a diverse collection of *C. difficile* isolates (20), MLST has not been widely adopted for this organism, in contrast to the majority of clinically important bacterial species (25). Our aim was to further develop MLST for *C. difficile*, setting up a more robust method by the following steps: (i) replacing the null allele employed at one of the loci included in the previously published scheme (20) with an allele present in all strains, (ii) improving discrimination by using longer sequences for MLST, and (iii) establishing an Internet-accessible MLST database to allow straightforward accumulation of data over time and to simplify the comparison of data among laboratories. This MLST scheme for *C. difficile* was also sufficiently robust to allow typing to be performed directly on DNA extracted from stool, without culture. This could potentially be used to generate actionable genotyping data close to real time since the entire process can be completed for a batch of 24 isolates in 3.5 days (Fig. 3), at a consumables cost of £15 per stool (or $24.65 as of 29 October 2009) and the cost of one graduate-level member of staff.

The MLST scheme was sufficiently discriminatory to give

TABLE 2. MLST of *C. difficile* from ELISA-positive stool samples determined both directly using total stool DNA and using isolates cultured from the same stools

Sample no.[a]	ELISA OD[b]	MLST PCR result (direct)	Direct ST[c]	Culture result[i]	Culture ST[d]	PCR ribotype	Sample no.[a]	ELISA OD[b]	MLST PCR result (direct)	Direct ST[c]	Culture result[i]	Culture ST[d]	PCR ribotype
23+	+++++	+	1	+++	1	027	77+	2.427	+	8	+++	8	002
17+	0.353	+	1	++	1	027	96+	2.282	+	8	+++	8	002
2+	2.251	+	1	+	1	027	97+	2.379	+	8	+++	8	002
8+	+++++	+	1	+++	1	027	105+	2.136	+	8	+++	8	002
37+	1.216	+	1	+	1	027	73+	0.499	+	8	+++	8	002
43+	0.663	+	1	+	1	027	79+	1.163	+	9	+	9	081
72+	2.5	+	1	+++	1	027	7+	0.35	+	9	+	9	081
75+	2.5	+	1	++	1	027	80+	2.5	+	10	+++	10	015
85+	0.388	+	1	+	1	027	99+	0.796	+	10	+++	10	015
88+	5.584	+	1	+++	1	027	15+	0.202	+	10	+	10	015
89+	5.392	+	1	++	1	027	82+	2.5	+	11	++	11	078
94+	7.098	+	1	+++	1	027	4+	2.13	+	11	+++	11	078
98+	2.5	+	1	+++	1	027	61+	0.567	+	11	+	11	078
101+	1.208	+	1	+++	1	027	20+	+++++	+	12	+	12	003
107+	2.5	+	1	+++	1	027	103+	0.657	+	12	+++	12	225
60+	+++++	+	1	++	1	027	108+	2.5	+	13	+++	13	129
53+	1.8	+	2	+	2	014	22+	+++++	+	14	+++	14	014
58+	0.261	+	2	++	2	020	5+	1.658	+	14	+	14	014
27+	+++++	+	2	+++	2	020	33+	1.658	+	14	++	14	014
11+	1.614	+	2	+	2	020	42+	0.317	+	14	++	14	014
12+	0.968	+	2	+++	2	020	46+	0.385	+	14	+	14	014
13+	7.718	+	2	+++	2	020	62+	2.5	+	14	+++	14	014
14+	3.648	+	2	++	2	020	19+	2.257	+	14	+	14	014
31+	5.648	+	2	+	2	014	36+	1.09	+	14	++	14	014
41+	0.843	+	2	+	2	020	28+	0.447	+	19	++	19	110
44+	0.29	+	2	+++	2	020	6+	+++++	+	22	+	22	023
48+	1.003	+	2	++	2	076	49+	0.41	+	37	++	37	017
63+	2.5	+	2	+++	2	014	9+	+++++	+	42	+++	42	106
66+	2.5	+	2	+++	2	020	24+	0.182	+	42	+	42	174
67+	2.5	+	2	++	2	014	40+	+++++	+	42	++	42	106
84+	0.563	+	2	+++	2	020	56+	3.296	+	54	++	54	012
1+	+++++	+	2	++	2	076	104+	0.387	(+)[h]	ND[e]	+++	1	027
106+	1.049	+	2	+++	2	020	100+	0.67	+	Mixed[g]	+	5	023
52+	+++++	+	3	++	3	072	71+	2.322	+	Mixed[g]	+++	11	078
55+	+++++	+	3	++	3	001	45+	2.127	+	1	−		
57+	0.285	+	3	+	3	072	47+	6.186	+	1	−		
16+	+++++	+	3	++	3	001	50+	0.278	−		+	2	220
64+	0.434	+	3	+++	3	072	86+	0.369	−		+	7	026
65+	2.5	+	4	++	4	137	91+	3.578	−		+++	8	002
68+	1.322	+	4	++	4	137	90+	1.155	−		+	ND[f]	ND[f]
69+	2.294	+	5	+++	5	023	102+	0.885	−		+	ND[f]	ND[f]
76+	2.5	+	5	++	5	023	54+	0.26	−		−		
78+	2.5	+	5	++	5	023	21+	7.792	−		−		
95+	2.5	+	5	+++	5	023	25+	0.67	−		−		
59+	4.083	+	6	+	6	005	29+	3.475	−		−		
51+	+++++	+	6	+++	6	005	30+	0.875	−		−		
26+	1.155	+	6	+	6	005	18+	0.158	−		−		
70+	2.5	+	6	++	6	005	34+	1.487	−		−		
92+	1.896	+	6	+++	6	005	35+	0.435	−		−		
93+	0.7	+	6	+++	6	005	38+	0.270	−		−		
3+	0.679	+	7	+	7	026	81+	0.537	−		−		
32+	0.499	+	7	+	7	026	83+	0.475	−		−		
74+	2.5	+	7	+++	7	026	87+	0.391	−		−		
39+	0.27	+	8	+++	8	002							

[a] The plus sign indicates an ELISA-positive result.
[b] OD, optical density. +++++, value above the scale.
[c] MLST was performed directly from stool samples.
[d] MLST was performed from cultured isolates.
[e] ND, not done due to very low PCR amplicon yields or no amplicon.
[f] ND, not done because sample was negative on reculturing from frozen isolate stock.
[g] Evidence of more than one genotype present in stool due to mixed peaks in the nucleotide sequence chromatogram.
[h] Poor amplicon yield.
[i] Crude estimate of viable *C. difficile* load. +, <100 colonies; ++, 100 to 1,000 colonies; +++, >1,000 colonies.

TABLE 3. MLST of *C. difficile* from ELISA-negative stool samples determined both directly using total stool DNA and using isolates cultured from the same stools

Sample no.[a]	MLST PCR result (direct)	Direct ST[d]	Culture result[g]	Culture ST[e]	PCR ribotype	PaLoc PCR result	lok1/lok3 PCR
22−	+	6	+	6	005	+	−
23−	+	15	+	15	010	−	+[f]
36−	+	6	+	6	005	+	−
43−	+	8	+	8	002	+	−
69−	+	2	+	2	020	+	−
79−	+	2	+	2	020	+	−
15−	−		+	69	072	+	−
55−[b]	−		+	1	027	+	−
85−[c]	−		+	1	027	+	−
99−	−		++	15	010	−	+[f]
108−	−		+	8	002	+	−

[a] The minus sign indicates an ELISA-negative result.
[b] Patient provided a subsequent sample not included in the study (+7 days) that was ELISA positive and ST-1 by culture (sample not typed by direct MLST).
[c] Patient provided a subsequent sample not included in the study (+39 days) that was ELISA positive and ST-1 by culture (sample not typed by direct MLST).
[d] MLST was performed directly from stool samples.
[e] MLST was performed from cultured isolates.
[f] PCR yielded a769-bp amplicon, confirming the absence of the PaLoc.
[g] Crude estimate of viable *C. difficile* load. +, <100 colonies; ++, 100 to 1,000 colonies; +++, >1,000 colonies.

typing data which can be interpreted with confidence; according to Hunter and Gaston (11) an ID greater than 0.90 is desirable to meet this requirement. For our 102 clinical isolates, MLST and PCR ribotyping had comparable discriminatory abilities (ID of 0.90 for MLST and of 0.92 for PCR ribotyping). The differences between the methods were generally consistent with a simple genetic explanation; multiple ribotypes for the same ST usually had very similar profiles, and multiple STs for the same ribotype generally had very closely related STs. Capillary gel electrophoresis-based PCR ribotyping is a promising tool to study subtypes within ribotypes, and it may assist the explanation of such observations (13). They may also be consistent with limited recombination that may be characteristic of *C. difficile*.

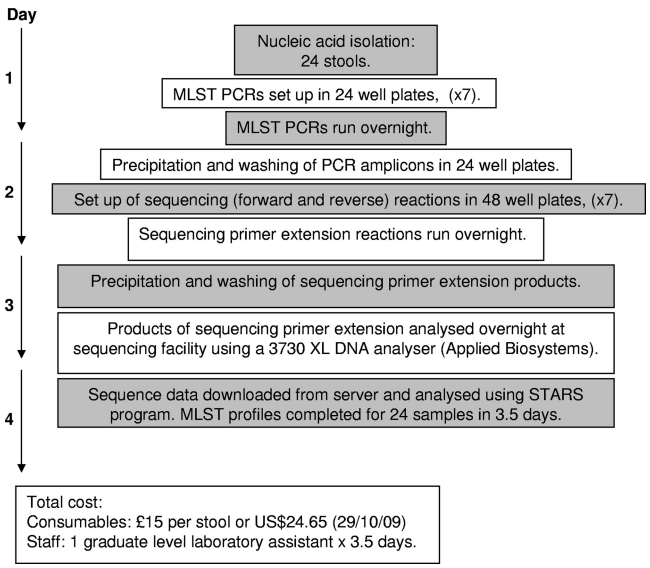

FIG. 3. Flow diagram summarizing the time required to perform the laboratory work and sequence data analysis in high-throughput MLST.

We calculated the ID as 0.958 for the previously published MLST scheme (34 STs, 62 PCR ribotypes, and 72 isolates) (20) and as 0.983 for PCR ribotyping for the same collection. However, this is not an entirely robust comparison since all the isolates were specifically chosen for their genetic diversity, and a true ID should reflect the capacity of a typing method to discriminate epidemiologically unrelated isolates within a population.

Pulsed-field gel electrophoresis is another genotyping technique widely used to characterize *C. difficile*. The IDs for PFGE, the previously published MLST scheme of Lemee et al. (20), and PCR ribotyping were found by Killgore et al. (17) to be 0.843 (PFGE), 0.699 (MLST), and 0.700 (PCR ribotyping) for a collection of 42 isolates from four countries representing epidemic strains and the next most commonly isolated strain types.

Despite the relatively low overall genetic diversity detected within these housekeeping loci, it was still possible to identify four different phylogenetic groups of *C. difficile* STs (Fig. 2). The majority of STs clustered in group 1, group 2 contained ST-1 (PCR ribotype 027), group 3 contained two STs associated with PCR ribotype 023, and group 4 contained toxin A^- B^+ ST-37 (PCR ribotype 017). A single outlier, ST-11, was associated with PCR ribotype 078. The previously described MLST scheme for *C. difficile* identified three divergent lineages, one containing the A^- B^+ isolates, which corresponds to our group 4 (20). Stabler et al. (39) used comparative genomics to identify four clades, and these appear to correlate with the four groups we have identified by MLST in the present study (Fig. 2). In that previous study HA1 (human and animal 1) (39) contained mainly human isolates with just a few animals, and this clade probably corresponds to our group 1, which contained the majority of our human isolate STs. HA2 probably corresponds to our group 3 as this contained mainly animal isolates (pig and bovine), with few isolates from humans. These data suggest that genotypes clustered by MLST may correlate with groups derived from whole-genome comparisons using DNA microarrays (39), implying that MLST

may be an accurate proxy for whole-genome analysis. The newly emergent ST-11 (PCR ribotype 078) hypervirulent clone was a genetically distinct outlier. This genotype causes infection in humans, pigs, and calves (6, 16) and has been found in cooked and raw meat products (38). Multilocus variable-number tandem-repeat analysis (MLVA) data confirmed a strong degree of genetic relatedness between human and animal isolates belonging to this genotype in The Netherlands (9). ST data presented here suggest that ST-11 (078) has emerged from a single, genetically distinct clade. The other four ST groups may represent different *C. difficile* clonal complexes, with the level of nucleotide sequence divergence between STs representing each group ranging from 11/3,501 (0.3%) to 60/3,501 (1.7%).

A robust MLST scheme can now be applied to studies of *C. difficile* epidemiology and population structure. Direct MLST of *C. difficile* in stool provides a rapid genotyping method which generates data that are easily compared among laboratories using an Internet-accessible database. It will now be possible to test in a clinical setting the utility of MLST for outbreak identification, detection of transmission events among patients, and the identification of emergent hypervirulent clones, thereby assessing the potential benefits of MLST to individual patients and hospital infection control.

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