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Note

Novel multiplex format of an extended multilocus variable-number-tandem-repeat analysis of *Clostridium difficile* correlates with tandem repeat sequence typing

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ABSTRACT

Subtyping of *Clostridium difficile* is crucial for outbreak investigations. An extended multilocus variable-number tandem-repeat analysis (eMLVA) of 14 variable number tandem repeat (VNTR) loci was validated in multiplex format compatible with a routine typing laboratory and showed excellent concordance with tandem repeat sequence typing (TRST) and high discriminatory power.

Clostridium difficile is a Gram-positive, anaerobic, spore-forming rod, which has emerged as a main cause of antibiotic-associated diarrhea, nosocomial diarrhea and pseudomembranous colitis (Bartlett and Gerding, 2008; Loo et al., 2005). Epidemic strains including PCR ribotype 027 (resembles tr027) have increased the incidence and severity of *C. difficile* infections during the past decade (Kuijper et al., 2008; McDonald et al., 2005).

Genotyping is essential to identify outbreaks, elucidate routes of transmission, for phylogenetic characterizations and for molecular epidemiological studies (Sabat et al., 2013). Several typing techniques are available for characterization of *C. difficile*, including PCR ribotyping, tandem repeat sequence typing (TRST), pulsed-field gel electrophoresis (PFGE), restriction enzyme analysis (REA), multilocus sequence typing (MLST), and multilocus variable-number tandem-repeat analysis (MLVA) each having both benefits and limitations (Killgore et al., 2008; Zaiss et al., 2009). PCR ribotyping is commonly used in Europe (Knetsch et al., 2013), and has been shown very useful especially for characterization of epidemic strains. The method however has the disadvantage of being gel-based requiring person dependent interpretation of data making interlaboratory comparability difficult. Although capillary-based PCR ribotyping has improved reproducibility and interpretation, the need for protocol standardization persists (Knetsch et al., 2013). TRST is a sequence analysis of two repetitive loci (TR6 and TR10) shown to have comparable discriminatory ability to PCR ribotyping (Zaiss et al., 2009). The advantages of a sequence-based typing technique compared to a gel-based method are higher reproducibility, ease of interpretation of data and exchangeability between laboratories. MLVA is a highly discriminatory method useful in tracking person-to-person transmission of *C. difficile* (Knetsch et al., 2013). A fifteen singleplex eMLVA scheme has been shown to be discriminatory for subtyping of *C. difficile* PCR ribotypes (Manzoor et al., 2011). Yet a fifteen singleplex scheme is time consuming for application to a routine typing setting.

The objective of this study was therefore to evaluate the comparability of an eMLVA scheme in multiplex format to TRST in a routine typing laboratory.

A total of 541 *C. difficile* isolates collected between 2006 and 2011 from the Region of Zealand, Denmark and 30 previously TRST typed isolates from a *C. difficile* strain-collection were analyzed. Isolates were recovered on 5% horse blood agar with yeast (SSI Diagnostica, Hillerød Denmark) incubated anaerobically at 37 °C for 24–48 h. DNA-extraction was performed using PrepMan® Ultra Sample Preparation Reagent (Applied Biosystems®, Foster City, CA, USA) according to the manufacturer's instructions. The sequence-based TRST of two tandem repeat loci TR6 and TR10 was performed according to Zaiss et al. (2009). Briefly, the two loci were amplified separately using the following primers in a 0.5 μM final concentration TR6-F (5′-TTTCAACTTGTCCA GTTTTTAAGTC-3′); TR6-R (5′-ATGACATAGCGTTTGTGGAAT-3′); TR10-F

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(5′-TGCATCAAATTGGTCAAGACTC-3′) and TR10-R (5′-TGAAATCATTGACTATAAAGCAAAA-3′). Successful amplification was verified by E-gel® electrophoresis (Applied Biosystems). Primary PCR products were purified by using an illustra™ ExoProStar™ 1-step (GE Healthcare, Buckinghamshire, UK). Sequence PCR was done with the primers given above and BigDye® Terminator v3.1 Sequencing Standard (Applied Biosystems). PCR products were purified according to the BigDye® XTerminator™ Purification Kit protocol (Applied Biosystems) and sequenced on a 3130*xl* Genetic Analyzer (Applied Biosystems). Sequence alignments and analyses were performed using BioNumerics Software (v7.1; Applied Maths, Sint-Martens-Latem, Belgium). Alleles for TR6 and TR10 were assigned using the publically available TRST database (pubtrst.org). For eMLVA, previously published primer sequences were used (Manzoor et al., 2011) and optimized to a multiplex format in this study. Fifteen variable number tandem repeat (VNTR) loci were amplified by PCR in four multiplex reactions (A–D) (Table 1); 2 μl DNA-extract in a final volume of 25.0 μl containing 0.7 μl $MgCl_2$ (25 mM; Roche Applied Science, Mannheim, Germany), 2.5 μl 5× Q-Solution® (QIAGEN® GmbH, Hilden, Germany), 7.3 μl primer mix (A, B, C or D, Table 1) and 12.5 μl 2× QIAGEN Multiplex PCR Master Mix (QIAGEN).

The polymerase was activated at 95 °C for 15 min followed by 35 cycles at 95 °C for 1 min, 56 °C for 1 min and 72 °C for 1 min ending at 72 °C for 10 min using a 2720 Thermal Cycler (Applied Biosystems). PCR products were diluted in sterile H_2O (3:170) and 1 μl dilution was mixed with diluted (1:12) GeneScan™ 1200LIZ™ Size Standard (Applied Biosystems). Denature was performed on a 2720 Thermal Cycler (Applied Biosystems) at 96 °C for 3 min. PCR product sizes were quantified by multicolored capillary electrophoresis on a 3130*xl* Genetic Analyzer (Applied Biosystems) and analyzed with GeneMapper Software (v4.0; Applied Biosystems). Strain NL2 (tr027) served as control standard in all PCR runs and eMLVA analyses. In order to convert fragment sizes into alleles a scheme for each VNTR loci was designed in Excel in accordance with fragment size data and the known repeat size of the loci (Marsh et al., 2006; van den Berg et al., 2007). Fragment sizes were assigned an arbitrary allele number. One locus (CDTR5) was unstable in multiplex format, and was excluded from analyses. CDTR5 has previously been reported to be generally invariant across five REA types (Marsh et al., 2010) and invariant in PCR ribotype 027 (Tanner et al., 2010) and the exclusion should have minor significance on the discriminatory ability of the method. Minimum spanning tree (MST) analysis based on the eMLVA data was carried out using BioNumerics Software (v7.1; Applied Maths). The discriminatory abilities of MLVA and TRST were assessed using Simpson's index of diversity (*D*) according to Hunter and Gaston (1988). Individual VNTR loci diversity indices were calculated in BioNumerics (v7.1; Applied Maths).

A total of 46 different TRST types were identified and 250 unique MLVA types were assigned for the 571 *C. difficile* isolates, reflecting a higher discriminatory power of the multiplex eMLVA scheme ($D = 0.984$ for eMLVA; $D = 0.734$ for TRST) (Fig. 1).

Each TRST type was divided into several MLVA types and closely related MLVA types had the same TRST type. An excellent agreement was observed for MLVA types and TRST types; only a single MLVA type (080) included more than one TRST type, tr027 and tr108 (Fig. 1). The TR6 locus of tr027 and tr108 was identical but the TR10 locus of tr108 contained one additional repeat (N004, N023) when compared to tr027. Furthermore, the tr027 and tr108 isolates, assigned as MLVA-080, originated from the same patient, indicating that tr027 and tr108 have a very close common ancestor. The two TRST types, tr051 and tr052, were grouped together (Fig. 1), sharing the same TR6 and deviating by one single nucleotide in TR10 only supporting that these TRST types are indeed closely related.

Some VNTR loci were not detected within specific TRST types (Table 2). For locus A6Cd, the absence has previously been reported in tr067 and tr070 (resembles PCR ribotypes 066 and 078 respectively) (Zaiss et al., 2009), and the primer annealing sites for loci B7Cd and C6Cd reported to have mismatches in PCR ribotype 078 (Bakker et al., 2010), which could explain the lack of amplification of these loci in tr070. In our scheme the additional loci compared to Bakker et al. (2010) were however sufficient for discrimination without the need for modification. In agreement, Manzoor et al. reported no limitations of the protocol for specific PCR ribotypes (Manzoor et al., 2011). Both tr067 and tr070 are binary toxin positive strains with two single nucleotide mutations in *tcdC* in common (A117T and C184T) and were grouped closely together. The two TRST types shared the same TR10 locus and deviated only by five extra repeats in TR6 for tr070 suggesting a common ancestor.

The stability of the 14 MLVA loci was analyzed for each TRST type when represented by more than ten isolates (Table 2). The most diverse TRST type was tr014 resembling the heterogenic PCR ribotypes 014/020/077 that usually are difficult to separate with PCR ribotyping. This TRST type included isolates with up to nine loci differences and where identified in four distinct groups (Fig. 1) allowing a higher discrimination with eMLVA than with TRST and PCR ribotyping.

The VNTR loci from earlier MLVA schemes (Tanner et al., 2010; van den Berg et al., 2007), especially A6Cd and C6Cd, were most variable (Table 2). The additional VNTR loci from the scheme by Manzoor et al. (designated CDxxx) however were highly stable within a given TRST type, especially loci CD44 and CD105 (Table 2), and variable across different TRST types. Hence, the stable loci seem to establish the phylogeny, whereas the variable loci provide discriminatory power. The discriminatory indices were higher for the variable loci compared to

Table 1
VNTR loci details, primer sequences and final concentrations used for PCR amplification of the loci in the eMLVA scheme.

Mix	Locus[a]	Repeat size	Primer sequence (5′–3′)		Final conc. (nM)
			Forward	Reverse	
A	A6Cd*	6	FAM-TTAATTGAGGGAGAATGTTAAA	AAATACTTTTCCCACTTTCATAA	180
	CDR60†	17	NED-AGTTTGTAGGGAAGTGTGTAAATAGAT	CGCATTAAATTTCACTCCTCAT	90
	CD105‡	45	PET-TCAGCAACAGCAGAGGAAAG	CATTTGTCTCATACTCGGTTCAA	90
	CD12‡	42	VIC-TGACCCTTACAATAGCCAATCA	ATCTGGCAGTTGATTCAGCA	45
B	G8Cd†	8	FAM-TGTATGAAGCAAGCTTTTTATT	AATCTAATAATCCAGTAATTTAAATT	260
	CD14‡	45	NED-TTTCATAAAAGATTCCTTTCCTGT	TGTGGTTGTTCCTGAAGTTTT	195
	CD19‡	76	PET-AATTGGTAAGCAATCTGGACTTT	TGCAGCTGGATATGTATCAGTTTA	195
	B7Cd*	7	VIC-CTTAATACTAAACTAACTCTAACCAGTAA	TTATATTTTATGGGCATGTTAAA	195
C	CD35‡	41	FAM-TTGGTGAAGCATTAATGGATGT	CGGCAATGCTTTGAACTATG	140
	CD102‡	50	NED-GCGTACAAGAGGTCGGAGTC	CCACCCTCACTTTATTCAAACC	210
	CDR5•	8	PET-AGCCATTTTTATCAATCCTTTCTAT	AATTTTAAGTTAACGTTTTTCTACAT	420
	E7Cd*	7	VIC-TGGAGCTATGGAAATTGATAA	CAAATACATCTTGCATTAATTCTT	210
D	CD9‡	42	FAM-AAATAGAGAAATTGTTGTAGCACAAAG	GTAAGGTGAGAAGCGGACT	110
	C6Cd*	6	NED-GTTTAGAATCTACAGCATTATTTGA	ATTGGAATTGAATGTAACAAAA	220
	CD44‡	42	PET-TGCTGCGATAAGCTCTGCTA	TCATTTCCATTCAAATTATGTGACTAT	275

[a] *, van den Berg et al. (2007); †, Tanner et al. (2010); ‡, Manzoor et al. (2011); •, Marsh et al. (2006). The locus was excluded from analyses.