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Comparison of a newly developed binary typing with ribotyping and multilocus sequence typing methods for *Clostridium difficile*

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ABSTRACT

Clostridium difficile is the causative pathogen for antibiotic-related nosocomial diarrhea. For epidemiological study and identification of virulent clones, a new binary typing method was developed for *C. difficile* in this study. The usefulness of this newly developed optimized 10-loci binary typing method was compared with two widely used methods ribotyping and multilocus sequence typing (MLST) in 189 *C. difficile* samples. The binary typing, ribotyping and MLST typed the samples into 53 binary types (BTs), 26 ribotypes (RTs), and 33 MLST sequence types (STs), respectively. The typing ability of the binary method was better than that of either ribotyping or MLST expressed in Simpson Index (SI) at 0.937, 0.892 and 0.859, respectively. The ease of testing, portability and cost-effectiveness of the new binary typing would make it a useful typing alternative for outbreak investigations within healthcare facilities and epidemiological research.

1. Introduction

Clostridium difficile is the most commonly recognized cause of infectious nosocomial diarrhea (Simmerlein et al., 2016; Cohen et al., 2010). Illnesses associated with *C. difficile* range from mild diarrhea to pseudomembranous colitis and toxic megacolon (Maccioni et al., 2015; Tang and Stone, 2017; Moudgal and Sobel, 2012). To understand the spread of this pathogen and identify clones with increased virulence, several molecular typing methods such as pulsed-field gel electrophoresis (PFGE), multilocus sequence typing (MLST), multilocus variable-number tandem-repeat analysis (MLVA), and amplified fragment length polymorphism (AFLP) have been developed to investigate *C. difficile* outbreaks (Tenover et al., 2011; Killgore et al., 2008; Huber et al., 2013; Griffiths et al., 2010; Bidet et al., 1999). However, the application of these typing methods for *C. difficile* has been hampered by some of their limitations: long turn-around time, high cost, low typeability, poor-reproducibility, difficulties in portability and data analysis.

Binary typing, a new typing technique used successfully to study the epidemiology of *Campylobacter jejuni*, may provide a better typing solution for *C. difficile* (Cornelius et al., 2010; Price et al., 2006; Huang et al., 2013). This technique is based on the idea that every binary target (gene present in some strains but not in others) could divide strains into two different groups. Using an array of binary targets, a strain could be characterized with a binary profile (Huang et al., 2013). The typing capability of the binary typing method for *C. jejuni* has been compared with those of serotyping, PFGE, and MLST. The binary typing has showed advantages in turn-around time, simplicity, cost, discriminatory ability, and portability (Cornelius et al., 2010).

In view of finding a better typing method, we report here the development of a novel binary typing scheme for *C. difficile*. The usefulness of the binary typing was compared with two commonly used methods, PCR-ribotyping and MLST in *C. difficile* typing.

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Table 1
Primers used in optimized binary typing.

Binary loci.	Binary genes selected (bp)	Forward primer (5′→3′)	Reverse primer (5′→3′)	References	Description
CDB1	CDBI1_02275 (200)[a]	GCAACAATACACCACCATAG	TCACTGCCTTTCTGGATATG	This study	Hypothetical protein
CDB2	*tetM* (1080)	TGGAATTGATTTATCAACGG	TTCCAACCATACAATCCTTG	Collins et al., 2013; Spigaglia et al., 2005; Marchese et al., 1998	Tetracycline resistance protein Tn5397
CDB3	CD630_20070-1 (196)	TGGAACATCTGTGGTATGG	TGCGTCTGACATCTATCTG	This study	Ribosomal RNA adenine N-6-methyltransferase
CDB4	23S_rRNA (223)	GCTACGGCATCAGTAATGA	CCTGTTGTCCATCACCTAC	This study	23S ribosomal RNA
CDB5	CDBI1_02830 (449)	AGTCTCTGTTTGGATTGCTA	CCTGCTTCTCTTCTATCGTA	This study	Membrane-associated metalloprotease
CDB6	CDBI1_09860 (354)	GTATGTGGACACTGATTACTG	TCCTATTACTGCGGACAATT	This study	Hypothetical protein
CDB7	CD630_31520 (173)	TCCATATACAAGGGAATCCT	GAATGAAGAAGAAGCACAAG	This study	Conserved hypothetical protein
CDB8	*tcdR* (300)	AAAAGCGATGCTATTATAGTCAAA	CCTTATTAACAGCTTGTCTAGAT	Spigaglia et al., 2006	Alternative RNA polymerase sigma factor
CDB9	CDBI1_01190 (113)	GGTGAGTGTAGTTGGTATGA	AGTGGTCTTKGAGCCTTAC	This study	dtdp-4-Dehydrorhamnose reductase
CDB10	CDBI1_03750 (237)	ATATTCACGACAAGCACCA	AATCCACGGCACAATTTCAC	This study	Cytidine/deoxycytidylate deaminase family

[a] The figure within parentheses is the amplified PCR product size.

2. Materials and methods

2.1. Identification of candidate binary loci based on C. difficile genome sequence alignment

Four fully sequenced *C. difficile* genomes of R20291 (NC_013316), QCD-63q42 (NZ_CM000637), QCD-76w55 (NZ_CM000661), and 630 (NC_009089) were chosen randomly and downloaded from the GenBank Genomes. The genome alignment software Mauve 2.3.1 was used to compare the genome sequence of *C. difficile* 630 with the other three genomes of R20291, QCD-63q42, and QCD-76w55 (Huang et al., 2013; Doerks et al., 2002). The genes present and absent in either two of the four 4 strains were identified to be potential binary genes (loci). A total of 50 candidate binary loci were firstly selected for further analysis.

With a considerable number of fully sequenced *C. difficile* genomes available in GenBank, an *in silico* assessment of the distribution and usefulness of the selected binary loci could be conducted. A group of 50 *C. difficile* complete genomes were randomly selected downloaded from the NCBI genome database for the initial analysis. Each of the selected candidate binary loci was searched in the 50 *C. difficile* sample set with BLASTn alignment. The presence of the binary locus in a *C. difficile* genome was recorded as “1”, while the absence was recorded as “0” in an Excel file. The potential typing ability of these binary loci combination was measured using the Simpson's index (SI) described by Hunter and Gaston (1988). The SI of different binary loci combinations was calculated with the software Optimal Combination Finder (OCF) as described previously (Huang et al., 2013; Shao and Tu, 1995). The *in silico* assessment found that 28 binary loci were promising in *C. difficile* typing. Therefore, they were validated using collected *C. difficile* strains. An optimal binary combination was identified for future *C. difficile* typing.

2.2. C. difficile isolates

An ethical approval of the Institutional Review Board (IRB) was sought and granted from the Second Hospital of Hebei Medical University [Approval # 20101007, 2013L-18]. A written consent form was obtained from each of the participants and guardians of the children enrolled in this study.

In total, 189 *C. difficile* strains were used in this study, of which 8 were reference strains provided from Oxoid Ltd., Cambridge, UK (see Table 4). They were used as an unrelated sample set to assess the binary typing ability. The rest 181 strains were isolated from March 2010 to September 2014 from three cities in Hebei Province of China. A total of 54 strains were isolated from kindergartens (healthy children aged 1–8 years) in Cangzhou (25/54) and Xingtai (29/54) cities. The other 127 were from Shijiazhuang city. They included 63 strains from hospitalized children without diarrhea in the Second Hospital of Hebei Medical University, 25 strains from hospitalized children with diarrhea in the Children's Hospital of Hebei Province, 10 strains from hospitalized adult patients with diarrhea in the Forth Hospital of Hebei Medical University, and 29 from healthy adults in Shijiazhuang city including 28 community-dwelling people and 1 healthcare worker. These strains were identified as *C. difficile* using matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) and VITEK2 ANC card (bioMérieux, France). They were deposited in Hebei Provincial Bank for Medical Culture Collections (HBMCC).

2.3. DNA extraction

C. difficile strains were inoculated onto *Clostridium difficile* Moxalactam Norfloxacin (CDMN) agar and incubated for 48 h at 37 °C in an anaerobic chamber. DNA was extracted using a commercial kit (TIANamp Bacteria DNA kit, Beijing, China) per the manufacturer's instructions. Genomic DNA was stored at −80 °C until use.