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Short Communication

Hypervirulent *Clostridioides difficile* RT078 lineage isolates from the river: A potential reservoir for environmental transmission

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Abstract This is the first report to discover *Clostridiodes difficile* (*C. difficile*) ribotype RT126 and RT598 (both ribotypes belong to RT078-lineage) in a river water system in southern Taiwan. Fluoroquinolone resistance was also found. The connection between clinical isolates and those from the environment needs further investigation.

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Introduction

Clostridioides difficile (C. difficile), an anaerobic gram-positive, spore-forming, toxin-producing bacillus, is the major cause of *C. difficile* infection (CDI). CDI causes a variety of gastrointestinal diseases, from asymptomatic carriage, pseudomembranous colitis with uncontrolled diarrhea, to fulminant disease with toxic megacolon or even gut perforation.[1] In the last two decades, hypervirulent *C. difficile* strains, including ribotype (RT) 027 and 078, have afflicted many developed countries. These hypervirulent strains produce toxin A, toxin B, and binary toxin, and often present fluoroquinolone resistance.[2] In Taiwan, most of the clinical toxigenic *C. difficile* isolates belong to the RT078 lineage.[3] Literature demonstrated that toxigenic *C. difficile* of clinical importance can be found in the rivers.[4] In this study, we described the first complete report about toxigenic, hypervirulent *C. difficile* strains collected from the river system in Taiwan.

Methods

Isolation of *C. difficile* from the river water sample

The samples were collected from river water in Chiayi, Taiwan during different seasons in 2016. The exact locations for water sampling are shown in Fig. 1. Some of the sampling sites were close to animal farms and hospitals. At each sampling site, a water sample of approximately 1000 mL was collected for pathogen detection and was transported to the laboratory at 4 °C within 24 h for analysis. The water sample was subsequently filtered with a 0.45 μm sterile syringe filter. The filtered samples were inoculated on the culture medium with cycloserine, cefoxitin, fructose, and egg yolk broth (CCFB agar) at 37 °C in an anaerobic condition for 48 h. In the second selective isolation, the isolates were cultured in cycloserine, cefoxitin, fructose, and egg yolk agar (CCFA) at 37 °C for 24—48 h, and then the colonies of the *C. difficile* isolates were confirmed by multiplex polymerase chain reaction (PCR) as described below.

Multiplex PCR and ribotyping

A multiplex PCR detecting *tcdA* (encoding toxin A), *tcdB* (encoding toxin B), *tcdC* (a negative regulatory gene for *tcdA* and *tcdB*), *cdtA* and *cdtB* (encoding binary toxin) as previously described[5] were performed to confirm the *C. difficile* isolates. If the strain contains a truncated *tcdC*, it was then examined through *tcdC* sequencing, as previously described.[6] Detection of gyrase gene mutation in the quinolone-resistance determining region (QRDR) was performed by the method as mentioned in a prior study.[7] Sequence analysis of gyrase A (*gyrA*) and gyrase B (*gyrB*) was performed as previously described.[8] The DNA region was amplified using the primer pairs, *gyrA1* + *gyrA2* for *gyrA* and *gyrB1* + *gyrB2* for *gyrB*. PCR products were purified and sequenced. Pairwise alignments of DNA sequences were carried out using the BLAST server of the National Center for Biotechnology Information. Ribotyping was also performed by using the Gel/PCR DNA Fragments Extraction Kit (Geneaid Biotech, New Taipei, Taiwan) and separated with the QIAxcel capillary electrophoresis system (Qiagen, Hilden, Germany) using the "OM500" method and QX Alignment Marker 15 bp/3 kb (Qiagen, Hilden, Germany). The PCR ribotypes were confirmed by the WEBRIBO database (http://webribo.ages.at).

Antibiotic susceptibility testing

The minimal inhibitory concentrations (MICs) were determined by the agar dilution method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (M11-A8). Metronidazole and vancomycin were tested at concentrations of 0.03—512 mg/L, and MIC breakpoints for resistance are defined as $\geq$32 and $\geq$ 16 mg/L, respectively. MIC breakpoints for resistance to doxycycline and tigecycline are defined as $\geq$8 and $\geq$ 0.5 mg/L, respectively, according to previous reports[3] and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) version 6.0.16.

Multiple locus variable number tandem repeat analysis (MLVA)

MLVA *C. difficile* have seven repeats loci ($A6_{Cd}$, $B7_{Cd}$, $C6_{Cd}$, $E7_{Cd}$, $F3_{Cd}$, $G8_{Cd}$, and $H9_{Cd}$), or so-called variable-number tandem-repeat (VNTR) loci. Seven regions with short tandem repeats spread over the genome. The samples were analyzed by agarose gel electrophoresis (Major Science) and all PCR products were STR sequencing by sequence company (Genomics). The genetic relatedness between two isolates was determined by the summed tandem repeat difference (STRD) with the Manhattan coefficient algorithm. An STRD of $\leq$2 was regarded as a clonal complex and an STRD of $\leq$10 as a genetically related cluster. The minimum-spanning tree was depicted by the Bionumerics version 7.6 (Applied Maths, Austin, USA). The detailed methods of MLVA in this research were mentioned in a previous study.[9]

Results

There were 29 water samples collected from rivers near animal farms and hospitals in Chiayi (shown in Fig. 1), and 7 of them (24.1%) yielded *C. difficile*, as summarized in the Table. Two samples (1IM02 and 4IM16) were collected near the hospital wastewater sewage system, while the other five (1G043, 1GM12, 1NP03, 3GM16 and 3NG01) were collected near the animal farms. All *C. difficile* isolated in this study were toxigenic and positive for *tcdA* and *tcdB*. The PCR ribotyping demonstrated that two of the *C. difficile* isolates were RT633 (1IM02) and RT002 (1G043), and one isolate (4IM16) could not be determined. The rest of the four (1GM12, 1NP03, 3GM16, and 3NG01) possessed binary toxin with *tcdC* gene 39 bp deletion (C184T), suggesting they were hypervirulent strains. Among the four hypervirulent strains, three shared the same ribotype RT126 (1NP03, 3GM16, and 3NG01), and one was RT598 (1GM12). Both RT126 and RT598 belong to RT078 lineage, which has been known as the predominant strains in animals worldwide.[10]

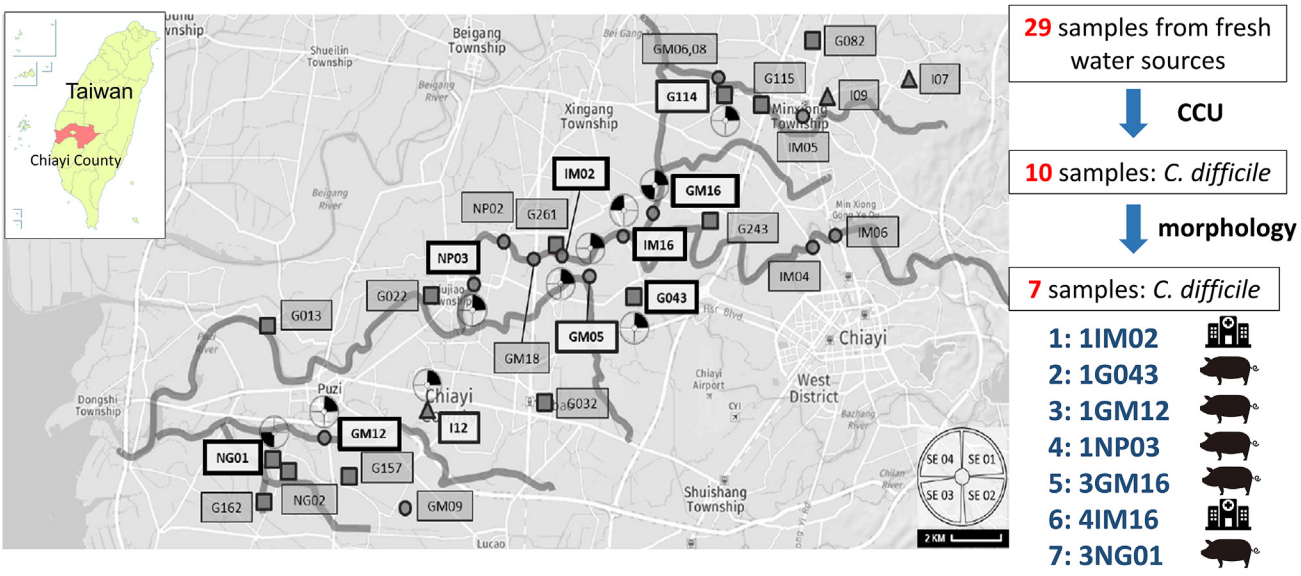

Figure 1. The sites of river water sampling in this study. Foot note: the river water samples were collected in four seasons (SE01～04: spring, summer, fall, and winter, respectively) in the drainage area of Puzi River (thick grey line), which passes through Chiayi County and flows into the Taiwan Strait. Each sample was given a code for identification. The codes begin with G, NG, GM, or NP represent water samples collected near the animal farms, while those start with I or IM indicate samples collected near the hospitals. The sampling sites were marked by small circles (surface water in Puzi River), small squares (near the livestock sewage system), and small triangles (near the hospital sewage system). The sampling sites with positive *C. difficile* culture results were represented by the codes with heavy black frames.

The results of antibiotic susceptibility testing of the seven *C. difficile* isolates were summarized in Table 1. Two strains (1GM12 and 1NP03) showed resistance to fluoroquinolones including ciprofloxacin, levofloxacin, and moxifloxacin. Further examinations of drug-resistant genes were performed, and both isolates revealed amino acid substitution Thr82Ile in the *gyrA* gene and Ser416Ala in the *gyrB* gene. No antimicrobial resistance to metronidazole, doxycycline, or vancomycin was found among the seven *C. difficile* isolates.

MLVA of the seven *C. difficile* isolates were subsequently performed. The minimum-spanning-tree (Fig. 2A) showed 3GM16 and 3NG01 (both RT126) were in the same clonal complex (MLVA number 77, STRD = 0), formed a genetic cluster with 1GM12 (RT598, MLVA number 67, STRD = 10), but were not linked to 1NP03 (RT126, MLVA number 54). MLVA of the rest of the four isolates showed they were not genetically related. Further comparison (Fig. 2B) showed the seven *C. difficile* isolates were not associated with the 28 reference RT126 isolates from pigs and CDI patients.[11]

Discussion

To our knowledge, *C. difficile* RT598 had never been identified in Taiwan until we performed this study, and it was the first time to discover hypervirulent *C. difficile* RT126 in the river water in Taiwan. Both RT126 and RT598 belong to the RT078 lineage; the former has been found in the nationwide surveillance of toxigenic *C. difficile* isolates from CDI patients and animals in Taiwan,[3] while the latter has only been reported in studies focusing on livestock and poultry.[12]

Since this study focused on the transmission of toxigenic and even hypervirulent *C. difficile* strains, a one-step multiplex PCR including *tcdA*, *tcdB*, *cdtA* and *cdtB* was

Table 1 Characteristics of *C. difficile* isolates from river water samples in this study.

Strain code	Ribotype	Toxin gene			Amino acid substitution		MIC, mg/L				
		tcdA	*tcdB*	*cdtA/cdtB*	GyrA	GyrB	MX	LVX	CIP	MZ	VA
1IM02	RT633	+	+	−	−	−	1.0	2.0	4.0	0.38	0.19
1G043	RT002	+	+	−	−	−	1.0	2.0	6.0	0.064	0.38
1GM12	RT598	+	+	+	Thr82Ile	Ser416Ala	>32	>32	>32	0.125	0.75
1NP03	RT126	+	+	+	Thr82Ile	Ser416Ala	>32	>32	>32	0.125	0.75
3GM16	RT126	+	+	+	−	Ser416Ala	0.38	1.5	4.0	0.094	1.0
4IM16	Unknown	+	+	−	−	−	0.5	1.5	4.0	0.094	0.25
3NG01	RT126	+	+	+	−	Ser416Ala	0.38	1.5	4.0	0.125	0.75

MX, moxifloxacin. LVX, levofloxacin. CIP, ciprofloxacin. MZ, metronidazole. VA, vancomycin.

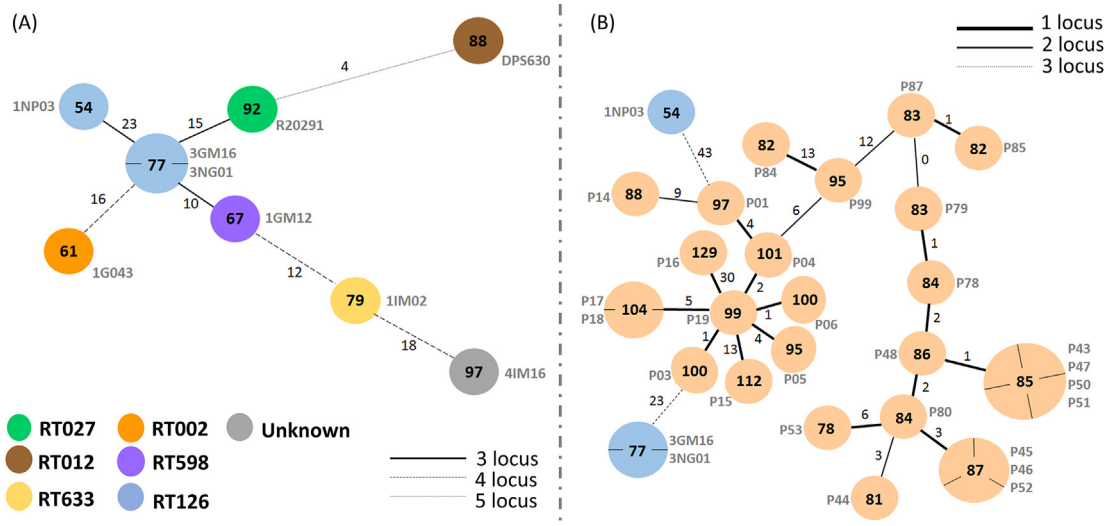

Figure 2. Minimum-spanning-tree analysis of multiple locus variable-number tandem repeat analysis (MLVA) of the *C. difficile* isolates from the river water samples in this study. Foot note: the numbers in circles are the sum of tandem repeats (STRs) and the numbers near the lines between circles are the summed tandem repeat differences (STRDs). (A) MLVA of the seven *C. difficile* isolates collected in the river water in this study. The isolates R20291 and DPS630 were the reference strains. (B) MLVA of the seven *C. difficile* isolates with other *C. difficile* isolates from animals and humans with similar ribotypes or lineages.

performed to confirm the yielded isolates from the CCFA and CCFB agars. Therefore, only toxigenic *C. difficile* isolates were collected for further analysis. Among the four hypervirulent isolates in this study, two (50%) presented fluoroquinolone resistance, with amino acid substitution of gyrase A (Thr82Ile), compatible with a previous investigation on RT078 lineage in Taiwan, in which Thr82Ile was detected among 58% of the 114 hypervirulent isolates collected from pig farms.[13]

Although three *C. difficile* isolates in this study were closely related, the MLVA results showed water samples from the same river basin could yield disparate *C. difficile* strains, even the same ribotype (RT126), since they may originate from different sources, similar to the results of a previous study.[14] Further analysis did not show a close genetic relationship between *C. difficile* from river water in our study and those from CDI patients as well as animals. It is possible because these isolates were not collected in the same period of time or the identical geographic region. Further investigation should be considered to compare *C. difficile* from the river with those from hospitals or animal farms in proximity.

In conclusion, we demonstrated the first report of hypervirulent *C. difficile* detected in river water in Taiwan. With the emerging threat of community-onset CDI, the route of transmission in the environment should be clarified. Our study results supported the evidence that CDI should be considered a "one-health" public health issue, and more investigations should be performed to examine the connection between clinical isolates and those from the environment shortly.

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References

1. Bartlett JG, Gerding DN. Clinical recognition and diagnosis of *Clostridium difficile* infection. *Clin Infect Dis* 2008 Jan 15; **46**(Suppl 1):S12—8.
2. Kuijper EJ, Coignard B, Tüll P. ESCMID study group for *Clostridium difficile*, EU member states, European Centre for Disease Prevention and Control. Emergence of Clostridium difficile-associated disease in North America and Europe. *Clin Microbiol Infect* 2006 Oct;**12**(Suppl 6):2—18.
3. Hung YP, Tsai PJ, Lee YT, Tang HJ, Lin HJ, Liu HC, et al. Nationwide surveillance of ribotypes and antimicrobial susceptibilities of toxigenic *Clostridium difficile* isolates with an emphasis on reduced doxycycline and tigecycline susceptibilities among ribotype 078 lineage isolates in Taiwan. *Infect Drug Resist* 2018 Aug 15;**11**:1197—203.
4. Zidaric V, Beigot S, Lapajne S, Rupnik M. The occurrence and high diversity of *Clostridium difficile* genotypes in rivers. *Anaerobe* 2010 Aug;**16**(4):371—5.
5. Chankhamhaengdecha S, Hadpanus P, Aroonnual A, Ngamwongsatit P, Chotiprasitsakul D, Chongtrakool P, et al. Evaluation of multiplex PCR with enhanced spore germination for detection of *Clostridium difficile* from stool samples of the hospitalized patients. *BioMed Res Int* 2013;**2013**: 875437.
6. Curry SR, Marsh JW, Muto CA, O'Leary MM, Pasculle AW, Harrison LH. *tcdC* genotypes associated with severe *TcdC* truncation in an epidemic clone and other strains of *Clostridium difficile*. *J Clin Microbiol* 2007 Jan;**45**(1):215—21.
7. Spigaglia P, Carattoli A, Barbanti F, Mastrantonio P. Detection of *gyrA* and *gyrB* mutations in *Clostridium difficile* isolates by real-time PCR. *Mol Cell Probes* 2010 Apr;**24**(2):61—7.
8. Dridi L, Tankovic J, Burghoffer B, Barbut F, Petit JC. *gyrA* and *gyrB* mutations are implicated in cross-resistance to ciprofloxacin and moxifloxacin in *Clostridium difficile*. *Antimicrob Agents Chemother* 2002 Nov;**46**(11):3418—21.

9. Marsh JW, O'Leary MM, Shutt KA, Pasculle AW, Johnson S, Gerding DN, et al. Multilocus variable-number tandem-repeat analysis for investigation of *Clostridium difficile* transmission in hospitals. *J Clin Microbiol* 2006 Jul;**44**(7):2558—66.
10. Tsai CS, Hung YP, Lee JC, Syue LS, Hsueh PR, Ko WC. *Clostridioides difficile* infection: an emerging zoonosis? *Expert Rev Anti Infect Ther* 2021 Aug;**23**:1—10.
11. Wu YC, Lee JJ, Tsai BY, Liu YF, Chen CM, Tien N, et al. Potentially hypervirulent *Clostridium difficile* PCR ribotype 078 lineage isolates in pigs and possible implications for humans in Taiwan. *Int J Med Microbiol* 2016 Feb;**306**(2):115—22.
12. Schneeberg A, Neubauer H, Schmoock G, Baier S, Harlizius J, Nienhoff H, et al. *Clostridium difficile* genotypes in piglet populations in Germany. *J Clin Microbiol* 2013 Nov;**51**(11):3796—803.
13. Tsai BY, Ko WC, Chen TH, Wu YC, Lan PH, Chen YH, et al. Zoonotic potential of the *Clostridium difficile* RT078 family in Taiwan. *Anaerobe* 2016 Oct;**41**:125—30.
14. Janezic S, Potocnik M, Zidaric V, Rupnik M. Highly divergent *Clostridium difficile* strains isolated from the environment. *PLoS One* 2016 Nov 23;(11):11.