Original Article

The emergence of *Clostridioides difficile* PCR ribotype 127 at a hospital in northeastern Taiwan

Bo-Yang Tsai [a], Chun-Chih Chien [b], Shu-Huan Huang [c], Jun-Yuan Zheng [d], Chih-Yu Hsu [e], Yau-Sheng Tsai [f], Yuan-Pin Hung [g,h], Wen-Chien Ko [h,i,*], Pei-Jane Tsai [a,e,j,k,**]

[a] Institute of Basic Medical Sciences, College of Medicine, National Cheng Kung University, Tainan, Taiwan
[b] Department of Laboratory Medicine, Chang Gung Memorial Hospital at Kaohsiung, Kaohsiung, Taiwan
[c] Department of Laboratory Medicine, Chang Gung Memorial Hospital at Kee-Lung, Keelung, Taiwan
[d] Division of Infectious Diseases, Department of Internal Medicine, Chang Gung Memorial Hospital at Kee-Lung, Kee-Lung, Taiwan
[e] Department of Medical Laboratory Science and Biotechnology, National Cheng Kung University, Tainan, Taiwan
[f] Institute of Clinical Medicine, National Cheng Kung University, Tainan, Taiwan
[g] Departments of Internal Medicine, Tainan Hospital, Ministry of Health & Welfare, Tainan, Taiwan
[h] Department of Internal Medicine, National Cheng Kung University Hospital, College of Medicine, National Cheng Kung University, Tainan, Taiwan
[i] Department of Medicine, College of Medicine, National Cheng Kung University, Tainan, Taiwan
[j] Center of Infectious Disease and Signaling Research, National Cheng Kung University, Tainan, Taiwan
[k] Department of Pathology, National Cheng Kung University Hospital, College of Medicine, National Cheng Kung University, Tainan, Taiwan

Abstract *Background*: Several studies have highlighted the incidence of *Clostridioides difficile* infections (CDIs) in Taiwan and certain ribotypes have been related to severe clinical diseases. A study was conducted to investigate the polymerase chain reaction (PCR) ribotypes and genetic relatedness of clinical *C. difficile* strains collected from January 2009 to December

* Corresponding author. Department of Internal Medicine, National Cheng Kung University Hospital, No. 138, Sheng Li Road, Tainan, 70403, Taiwan. Fax: +886 6 2752038.
** Corresponding author. Department of Medical Laboratory Science and Biotechnology, College of Medicine, National Cheng Kung University, No. 1, University Road, Tainan, 70101, Taiwan. Fax: +886 6 2363956.
E-mail addresses: anemoday@hotmail.com (B.-Y. Tsai), jessica0307@cgmh.org.tw (C.-C. Chien), huan201904@gmail.com (S.-H. Huang), jercheng4@hotmail.com (J.-Y. Zheng), hsudengsiang@gmail.com (C.-Y. Hsu), yaustsai@mail.ncku.edu.tw (Y.-S. Tsai), yuebin16@yahoo.com.tw (Y.-P. Hung), winston3415@gmail.com (W.-C. Ko), peijtsai@mail.ncku.edu.tw (P.-J. Tsai).

2015 at a hospital in northeastern Taiwan.

Material and methods: A modified two-step typing algorithm for *C. difficile* was used by combining a modified 8-plex and 3′-truncated *tcdA* screening PCR. In addition, MLVA typing was adopted for investigation of bacterial clonality and transmission.

Results: Among a total of 86 strains, 24 (28%) were nontoxigenic and 62 (72%) had both *tcdA* and *tcdB* (A + B+). No *tcdA*-negative and *tcdB*-positive (A⁻B⁺) strains were identified. Binary toxin (CDT)-producing (*cdtA+/cdtB+*) strains were started to be identified in 2013. The 21 (34%) A⁺B⁺ clinical strains with binary toxin and *tcdC* deletion were identified as RT127 strains, which contained both RT078-lineage markers and fluoroquinolone (FQ)-resistant mutations (Thr82Ile in *gyrA*). Multiple loci variable-number tandem repeat analysis (MLVA) for phylogenetic relatedness of RT127 strains indicated that 20 of 21 strains belonged to a clonal complex that was identical to a clinical strain collected from southern Taiwan in 2011, suggestive of a clonal expansion in Taiwan.

Conclusion: A two-step typing method could rapidly confirm species identification and define the toxin gene profile of *C. difficile* isolates. The clonal expansion of RT127 strains in Taiwan indicates monitoring and surveillance of toxigenic *C. difficile* isolates from human, animal, and environment are critical to develop One Health prevention strategies.

Introduction

Clostridioides difficile is a Gram-positive and spore-forming bacterium that was recently reclassified into the *Clostridioides* genus after 16S rRNA phylogenetic analysis.[1] *C. difficile* infection (CDI), one of common nosocomial infection, mostly occurs in patients exposed to antibiotic treatment and ranges from mild diarrhea to pseudomembranous colitis (PMC), toxic megacolon, or death.[2–9] Such manifestations.[10] However, the incidence of community-acquired CDI (CA-CDI) has almost doubled in the last decade, mainly in younger population without a history of antibiotic exposure or hospitalization.[11,12]

Since 2000 several hospitals reported a dramatic increase in severe CDIs caused by certain epidemic strains, including polymerase chain reaction (PCR) ribotype 027 (RT027) and ribotype 078 (RT078) across Europe and North America[13–15] and ribotype 017 (RT017) in Asia.[16] RT027 and RT078 isolates produce both major toxin A/B (encoded by *tcdA/tcdB*) and a third binary toxin (*C. difficile* transferase [CDT], encoded by *cdtA/cdtB*), and are considered "hypervirulent" or "hypertoxigenic" strains (A⁺B⁺CDT⁺).[17] These hypervirulent strains are often characterized by fluoroquinolone (FQ) resistance (Thr82Ile in *gyrA*),[18] efficient sporulation, and higher toxin production because of *tcdC* mutations (Δ117A/18-bp deletion in RT027 or C184T/39-bp deletion in RT078).[19,20] Several ribotypes highly related to RT078, including RT126 and RT127 (A⁺B⁺CDT⁺), RT033 (A⁻B⁻CDT⁺), RT045, and RT066, are categorized as the clade 5 ST-11, which has been linked to severe symptoms and a high mortality rate.[20–23] In addition, RT078 and related ribotypes were found to contain a lineage-specific marker[24] and are highly associated with livestock, particularly piglets.[22,25] However, the direct transmission of RT078 lineage strains from animals, food, or the environment to humans has not been demonstrated. RT017 (A⁻B⁺CDT⁻) strains harbor toxin B and wild-type *tcdC*, but no toxin A (because of the 3′-end deletion of *tcdA*) or binary toxin.[16,26]

In Taiwan until 2014 the first case of CDI caused by a FQ-susceptible RT027 strain was identified.[27–29] During the same period, a multicenter surveillance in Taiwan revealed that RT017 and RT078-lineage strains were predominant,[6,30,31] which differed from the major ribotypes distributing in China or Japan.[2,8,32] In addition, RT126 in Taiwan could lead to severe PMC and recurrent CDI.[33] Moreover, clinical isolates of the RT078 lineage in Taiwan were genetically related to swine isolates, indicative of the zoonotic potential.[29,30] These findings support the need of molecular surveillance of *C. difficile* ribotypes in human, animal, and environment isolates in Taiwan.

Since most ribotypes isolates have been reported in clinical *C. difficile* isolates from southern Taiwan, the temporal dynamics of toxin genotypes and ribotypes of clinical *C. difficile* isolates from diarrheal patients at a hospital over seven years in northeastern Taiwan were studied.

Materials and methods

The collection of *C. difficile* isolates

This study was conducted from January 2009 to December 2015 in a 1089-bed hospital in northeastern Taiwan. In the study hospital, clinical *C. difficile* isolates were obtained from stool specimens of diarrheal patients by plating on selective cycloserine–cefoxitin-fructose agar (CCFA) and maintained at 37 °C in an anaerobic cabinet for 48 h incubation. Yellow ground-glass colonies on CCFA plate identified as *C. difficile* were stored at −80 °C in BHIS broth containing 15% glycerol. These isolates were submitted to the research laboratory of Prof. Tsai for storage, culture, and further molecular testing.

Genomic DNA preparation

A single colony of *C. difficile* isolates was grown in Brain Heart Infusion broth (BD, Franklin Lakes, USA) supplemented with

5 mg/mL yeast extract (Condalab, Madrid, Spain) and 0.1% L-cysteine (Sigma—Aldrich, St. Louis, USA), at 37 °C anaerobically for 16 h. After harvesting, genomic DNA was extracted using a genomic DNA mini kit (Geneaid, Ltd., Taiwan).

Molecular toxin gene typing and RT078-lineage screening

To rapidly screen for the toxin gene profile of *C. difficile* isolates, We modified a multiplex PCR methodology, which involved the combination of eight primer sets to detect *C. difficile* genes, including *16S* rDNA (bacterial internal control), *tcdA*, *tcdB*, *cdtA/cdtB*, and *tcdC* isoforms, as described previously.[34] The *tcdB* primers were optimized by degenerating sequences to cover the large diversity in *tcdB* (*tcdB*-F5670M: 5′-CCAAARTGGARTSTTACAAACAGGTG-3′ and *tcdB*-R6079M: 5′-GCATTTCTCCRTTYTCAGCAAAGTA-3′).[35,36] Furthermore, a primer set (forward: 5′-ATTTACAGGA-GAAGTTTCACCTCT-3′ and reverse: 5′-GCCCAGATTGGCTCA-TATGCAAC-3′) was used as a *C. difficile*-specific marker (300-bp targeting triosephosphate isomerase, *tpi*),[37,38] and also a RT078-lineage marker proposed by Knetsch et al. was used.[24] The final amplification products were analyzed by gel electrophoresis. The strains with truncated *tcdC* variants were further examined through full-length *tcdC* sequencing.[19] Since there were variant forms of *tcdA*, including entire deletion or internal deletions, the primer binding sites of *tcdA* may lead misinterpretations in the toxin profiling of *C. difficile* isolates.[39,40] Our modified 8-plex PCR will detect all *tcdA* variants, and the signature of 3′-end truncation in *tcdA* needs to be confirmed by a secondary primer set.[38]

PCR ribotyping and identification

A two-step analysis was performed to validate the ribotypes of *C. difficile* isolates. First, the QIAxcel-based platform[41] and primers as previously described[42] were used. After PCR amplification, the products were concentrated by a Gel/PCR DNA Fragments Extraction Kit (Geneaid, Ltd, Taiwan) and separated by a QIAxcel system (Qiagen, Hilden, Germany), QX Alignment Marker 15 bp/3 kb, and QX Size 50—800 bp Marker (Qiagen, Hilden, Germany) for high-resolution visualization. The findings were compared with our reference database. The isolates not aligning with the reference database were further typed by the WEBRIBO database (http://webribo.ages.at/) using a published method with minor modifications.[43—45] PCR fragments were analyzed in ABI 3730XL DNA Analyzer with a 50 cm capillary loaded with a POP7 gel. A 20-to-600-bp LIZ600 ladder was used as the internal marker for each sample. The size of each peak was determined using the Peak Scanner software 1.0 (Applied Biosystems) and submitted into the WEBRIBO. PCR ribotypes were confirmed based on the analysis algorithm in the database.[46]

Mutations in quinolone-resistance determining region (QRDR)

FQ resistance in *C. difficile* was examined through the detection of mutations in *gyrA*-or *gyrB*-encoding DNA gyrase subunits in the quinolone-resistance determining region (QRDR). The primers used for the QRDR mutations were suggested by Spigaglia et al.[47] After amplification and sequencing, the sequences were analyzed using the BioEdit 7.2.5 software package and the ClustalW program to align with the published *gyrA/B* genes.[47]

Multi-locus variable-number tandem repeat analysis (MLVA)

Seven tandem repeat loci (A6, B7, E7, G8, C6, F3, and H9) from published MLVA schemes were individually singleplex-amplified using specific PCR primers.[48] The forward primers of PCR were labeled with fluorescent dye at the 5′-end. The dyes used were as follows: 6-carboxyfluorescein (6-FAM) for A6 and E7, PET (unpublished proprietary dyes of Applied Biosystems company, California) for C6 and F3, 2′-chloro-7′-phenyl-1,4-dichloro-6-carboxyfluorescein (VIC) for B7 and H9, and 2′-chloro-5′-fluoro-7′,8′-fused phenyl-1,4-dichloro-6-carboxyfluorescein (NED) for G8. PCR products were referred to multicolored capillary electrophoresis in the ABI 3730XL DNA Analyzer with a LIZ600 marker as an internal marker for each sample. The size of each PCR fragment was estimated by the GeneScan software (Applied Biosystems). Repeat numbers of the seven loci were separately calculated using the BioNumerics software ver. 7.6 (Applied Maths, Belgium). The minimum-spanning tree (MST) was generated based on the categorical coefficient, and all selected loci were weighted equally, regardless of the number of repeats.[49]

Data analysis

The genetic relatedness between two isolates was determined by the summed tandem repeat difference (STRD) with the Manhattan coefficient algorithm. Phylogenetic microevolution was evaluated based on the MST results and the interpretation criteria proposed by Baines et al.[50] and Marsh et al.[51] An STRD of ≤ 2 was regarded as a clonal complex (CC) and an STRD of ≤ 10 as a genetically related cluster (GC).[52]

Results

Clinical *C. difficile* isolates

A total of 86 *C. difficile* isolates were obtained from the patients with diarrhea. From 2009 to 2013, annual clinical isolate number of *C. difficile* fluctuated between 5 and 10 (6, 5, 9, 7, and 10 in 2009, 2010, 2011, 2012, and 2013, respectively) but dramatically increased in 2014 and 2015 (23 and 26, respectively).

Distribution of *C. difficile* toxin genotype and emerging RT078-lineage

Different *C. difficile* ribotype strains with well-characterized toxin genes were used as reference strains (Fig. 1A). In the interpretation of data from the 8-plex PCR, a positive result of *tcd*A does not exclude the presence of 3′-truncated variants. Therefore, *tcd*A + strains will be screened by a primer set of *tcd*A 3′-end truncation (Fig. 1B), and the interpretation of 2-step *tcd*A PCR results was depicted in Fig. 2. All 86

strains were positive for the *C. difficile*-specific marker, *tpi*. Twenty-four (27.9%) strains were nontoxigenic (A⁻B⁻) and 62 (72.1%) toxigenic (A⁺B⁺). Of 62 toxigenic strains, 21 (33.9%) strains, accounting for 24.4% of all *C. difficile* strains, had binary toxin (A⁺B⁺CDT⁺) and *tcdC* deletion, and no A⁻B⁺CDT⁻ and A⁻B⁻CDT⁺ variants were identified. Of note, *tcdC* deletion was identified in one A⁺B⁺CDT⁻ strain. The deletion and mutation of *tcdC* were confirmed by sequencing. All 21 A⁺B⁺CDT⁺ strains carried a 39-bp deletion, a C184T mutation in *tcdC*, and a RT078-lineage marker. The only A⁺B⁺CDT⁻ strain with an 18-bp in-frame deletion in *tcdC* belongs to ribotype 705 (RT705). A summary of *C. difficile* toxin profiles is presented in Table 1. The proportions of different toxin profile groups (toxin A, B, and CDT) by year were shown in Fig. 3, and CDT⁺ strains were first identified in 2013.

Fluoroquinolone resistance—associated QRDR mutations

FQ resistance in *C. difficile* isolates might be contributed by the spread of A⁺B⁺CDT⁺ epidemic strains, such as RT027 and RT078.[53] We investigated QRDR mutations in *gyrA* and *gyrB* in all included strains (Table 1), and ribotyping of the strains with relevant mutations in *gyrA* or *gyrB* was performed. All 22 toxigenic strains harbored Thr82Ile in *gyrA* and no mutation in *gyrB*. Of 24 nontoxigenic strains, mainly RT010 (6 strains) and RT039/2 (9), *gyrA* or *gyrB* mutation was observed in 16 (66.7%) strains, as illustrated in Table 2.

Ribotypes and genetic relatedness of RT127 strains

We identified 21 RT127 strains among all 21 RT078-lineage marker-harboring A⁺B⁺CDT⁺ strains, based on the capillary electrophoresis results (Fig. 4. No RT027 or RT078 strains were found in this study. To clarify the genetic relatedness of RT127 strains in Taiwan, we utilized MLVA to compare these 21 strains and three distinct RT127 strains from three
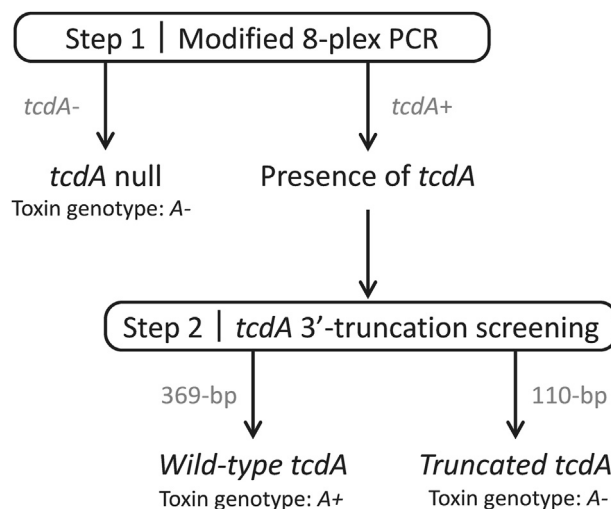

Figure 2. An interpretation algorithm of the results of the 2-step typing method.

patients in southern Taiwan in 2011 (*i.e.,* TNHP7, TNHP17, and TNHP92).[30] The MST revealed that all RT127 strains, except TNHP7, formed a genetic related complex (STRD ≤10) (Fig. 5). All but one (KLCG54 in 2013) of 21 RT127 strains and another strain from southern Taiwan (TNHP17) could be clustered into a clonal complex (STRD ≤2). In the clonal complex, 20 RT127 strains from the study hospital demonstrated microevolution with single-locus variants, which differed from the double-locus variant, TNHP17. These data indicated that a RT127 clonal expansion occurred at a hospital in 2014 and 2015 and could be linked to a clinical isolate from southern Taiwan in 2011 (Fig. 5).

Discussion

We describe herein a rapid and reliable molecular approach to investigate the toxin profile in clinical *C. difficile* strains.
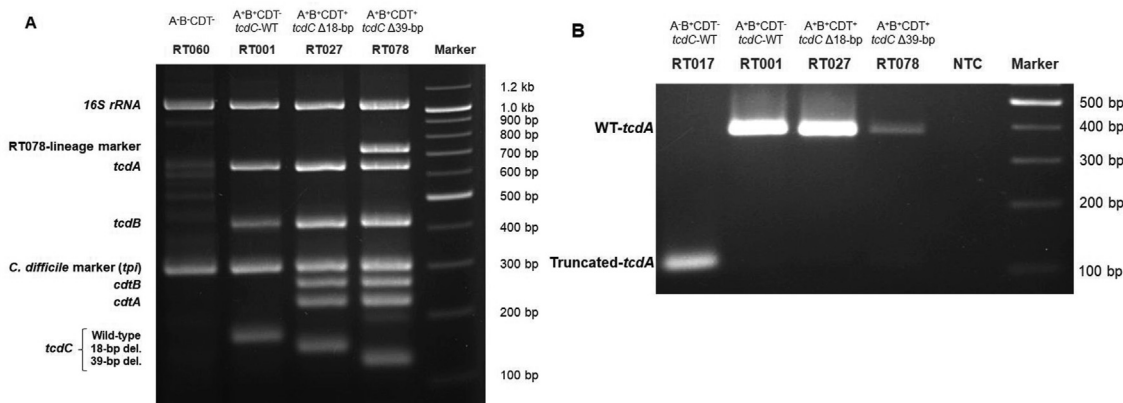
Figure 1. Polymerase chain reaction profiles of different *Clostridioides difficile* ribotypes (RTs) are determined by a modified 8-plex PCR (A) and a *tcdA* 3′-end truncation screening (B). CCUG 37780, a nontoxigenic RT060 strain (A⁻B⁻CDT⁻); CCUG 4938ᵀ, a toxigenic RT001 strain, harbors *tcdA*/*tcdB* and wild-type *tcdC* but no binary toxin (A⁺B⁺CDT⁻); ATCC BAA-1805, a hypertoxigenic RT027 strain (A⁺B⁺CDT⁺), harbors 18-bp deletion/Δ117A in *tcdC*; RT078, a hypertoxigenic strain (A⁺B⁺CDT⁺) from the Netherlands, harbors 39-bp deletion/C184T in *tcdC*; TNHP82, a RT017 strain (A⁻B⁺CDT⁻), harbors wild-type *tcdC*; Marker, 100-bp DNA ladder (Bioneer); NTC, no template control.

Table 1 Toxin gene typing and fluoroquinolone resistance-associated mutations of 86 clinical *Clostridioides difficile* strains between 2009 and 2015.

Toxin A/B gene (*tcdA*/*tcdB*)	Binary toxin gene (*cdtA*/*cdtB*)	*tcdC* type	RT078 lineage	*gyrA* mutation[a]	*gyrB* mutation[a]
A⁺B⁺, 62 (72.1%)	CDT⁺, 21 (33.9%)	39-bp del./C184T, 21 (100%)	21 (100%)	21 (100.0%)	0 (0%)
	CDT⁻, 41 (66.1%)	Wild-type, 40 (97.6%)	0 (0%)	1 (2.5%)	0 (0%)
		18-bp in-frame del., 1 (2.4%)	0 (0%)	0 (0%)	0 (0%)
A⁻B⁻, 24 (27.9%)	CDT⁻, 24 (100%)	None	0 (0%)	12 (50%)	4 (16.7%)

[a] Fluoroquinolone-resistant mutations of *C. difficile* identified by Spigaglia et al. (2010).
Note: The numeric data are strain numbers (percentages); del. = deletion.
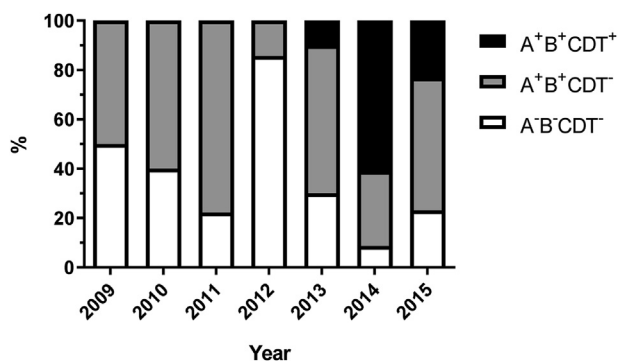
Figure 3. Annual distribution of 86 *Clostridioides difficile* strains from diarrheal patients assigned into three groups based on the profiles of toxin A, B, and CDT toxin.

This molecular typing method revealed the emergence of FQ-resistant hypertoxigenic RT127 strains, which caused the clonal transmission of CDI at a hospital in northeastern Taiwan between 2009 and 2015. Furthermore, the same clonal complex found in *C. difficile* strains from different geographic areas in Taiwan suggests microbiological surveillance is required to further disclose the clonal complexes circulating in the environment, community, and hospitals in Taiwan.)

A modified molecular typing algorithm of toxin genes was utilized in this study. The toxin genes of *C. difficile* isolates can be concurrently typed by an 8-plex PCR and a *tcdA* 3′-end truncation screening. This typing system also can be applied directly to fresh fecal DNA samples (unpublished data). Furthermore, since the RT078-lineage is the predominant toxigenic type in Taiwan,[31] we included a RT078-lineage marker in the multiplex PCR. However, the attempt to incorporate the detection of truncated *tcdA* into a single reaction fails, and a two-step algorithm is recommended.

In the present work, the number of RT127 strains increased over the study period. Valiente et al. proposed that an emerging ribotype might originate from a hypervirulent lineage, such as RT027 or RT078, because of the similar genetic background and niche adaptation.[29,54,55] RT127 belongs to the RT078-lineage and sequence type (ST)-11 of the clade 5 by MLST, and has been highly associated with livestock, especially swine and calves.[56,57] Although FQ-resistant RT127 strains were widespread in humans,[30,31] swine,[58,59] and slaughterhouses[60] in Taiwan, zoonotic transmission to human has not been directly demonstrated. The common sources and reservoirs in the food chain and environment may facilitate the transmission to human, because the RT078 lineage isolates (RT078/126/127) have been isolated from seafood, vegetables, soil, and wastewater.[61] Here, the clonal spread potential of RT078 lineage

Table 2 Ribotype distribution and *gyr* mutations of 38 fluoroquinolone-resistant *Clostridioides difficile* strains.

C. difficile, strain number	Gene	Amino acid substitution	Strain number (%)	Ribotype	Strain number (%)
Toxigenic (A⁺B⁺), 22	*gyrA*	Thr82Ile	22 (100%)	RT127	21 (95%)
				RT633	1 (5%)
Non-toxigenic (A⁻B⁻), 16	*gyrA*	Thr82Ile	12 (75%)	RT010	2 (17%)
				RT039/2	9 (75%)
				RT596	1 (8%)
	gyrB	Asp426Asn	4 (25%)	RT010	4 (100%)
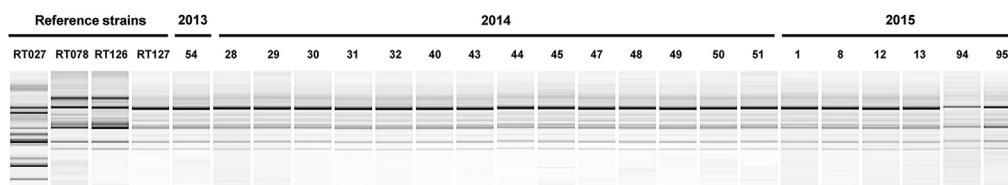
Figure 4. The ribotyping results of 21 A⁺B⁺CDT⁺ clinical strains of *Clostridioides difficile* from 2013 to 2015.
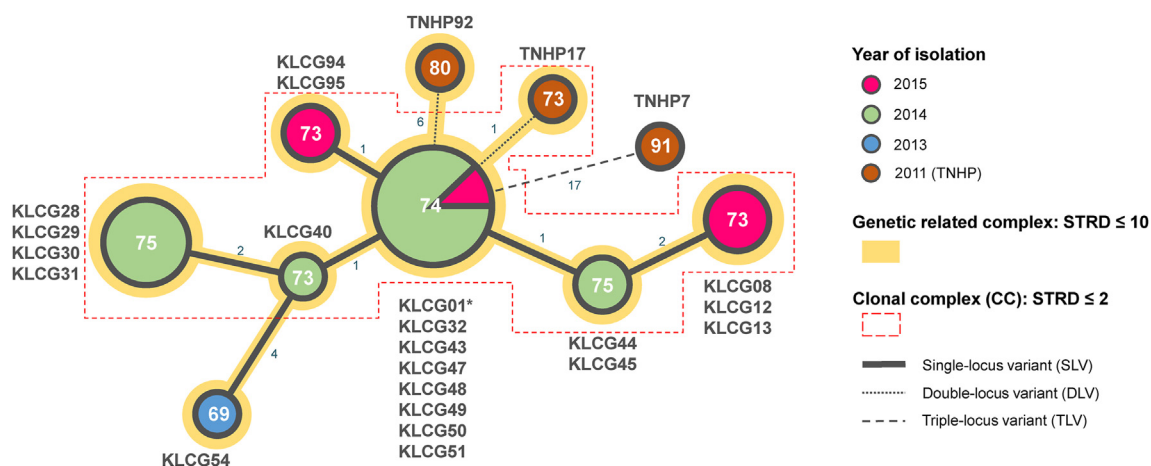
Figure 5. Delineation of microevolution within RT127 strains by multi-locus variable-number tandem repeat analysis. Minimum spanning tree (MST) analyses based on seven microsatellite loci on 21 clinical RT127 strains and 3 distinct RT127 strains (TNHP7, TNHP17, and TNHP92) obtained from southern Taiwan in 2011. 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). The asterisk (*) indicates the isolation year of the strain differs from the others in the same STR-profile group.

was further supported by the genetic linkage between a clinical strain in southern Taiwan in 2011 and a collection of clinical strains in northeastern Taiwan from 2013 to 2015.

It's notable that RT127 isolates in the RT078 lineage rarely infect humans in Asia apart from Taiwan.[62] To date, few studies focus on RT127 and its detailed clinical or microbiological characterization is not well studied. Although RT127 has the same toxin gene profile ($A^+B^+CDT^+$) and a PCR-ribotyping pattern similar to that of RT078 or RT126,[23,24] the analysis of whole genome sequence shows that RT126, but not RT127, has the closest similarity with RT078 leading to the formulation of a genetic complex, i.e., RT078/126.[63] It is likely that these differences in genomic composition between RT127 and the RT078/126 complex may affect the adaptive competition within the RT078 lineage. However, the variations of clinical pathogenicity and ecological distribution within the RT078 lineage warrant further investigations.

FQ resistance in *C. difficile* is generally mediated by chromosomal alteration in drug targets (i.e., DNA gyrase subunits, *gyrA* and *gyrB*).[64,65] Because FQ is not used as antimicrobial therapy for CDI, the selection and maintenance of FQ-resistant *C. difficile* isolates are not directly relevant to clinical outcome. Herein, FQ resistance-associated *gyrA/B* mutations were also observed in nontoxigenic RT010 and RT039 strains. There are two implications for these findings. First, the mutations identified in FQ-resistant strains displayed no in vitro fitness cost, suggesting that the resistant phenotypes can be maintained in the nature, despite the absence of antibiotic pressure,[66] and such antimicrobial-resistant strains may have competitive and adaptive advantage in the hosts after antibiotic exposure. Second, FQ resistance may be a zoonotic origin and be related to antibiotic use in agriculture and veterinary fields.[20] Enrofloxacin has been administered in poultry and livestock in Taiwan, and the FQ-resistant population may subsequently enter the ecosystem.[67] Our previous studies demonstrated that the RT078 lineage, esp. RT127, was predominant in swine and often had *gyrA* mutations.[58,59] Moreover, an international

investigation also revealed RT010 and RT039 could be isolated from cattle, poultry, and pets.[25]

In spite of no inclusion of *C. difficile* isolates from the animals or environments in northern Taiwan into the present study, the possibility of interconnected *C. difficile* transmission pathways among humans, animals, and environment shall not be ignored.[62] Thus, the implementation of antibiotic stewardship in the hospitals and the restriction of agricultural antibiotic use are urgently required.[68] In Australia, the use of FQs is not approved in the meat and livestock industry, and is limited in agriculture and clinical medicine, which may explain the rarity of FQ-resistant RT027 isolates.[20] Therefore, epidemiological monitoring programs should be continued and the origins of clinical RT127 strains should be surveyed in Taiwan. The virulence of the prevalent *C. difficile* isolates and their correlation of clinical disease severity and outcomes warrant further investigations.

Conclusion

This study used a modified two-step molecular typing system for clinical *C. difficile* isolates to rapidly confirm species identification and disclose the toxin gene profile, and further demonstrated the emergence of a FQ-resistant RT127 clone causing a clonal transmission of CDI at a hospital in northeastern Taiwan. The potential sources and the spread extent of this prevalent clone are warranted to be explored further.

Author contributions

B.Y.T., C.C.C., S.H.H., J.Y.Z., and C.Y.H. researched data, contributed to the discussion, and wrote the manuscript. Y.S.T. and Y.P.H. contributed to the discussion and reviewed/edited the manuscript. W.C.K. and P.J.T. contributed to the original concept, discussion, and wrote, reviewed, and edited the manuscript.

Declaration of competing interest

The authors declare no conflicts of interest in this work.

Acknowledgments

References

1. Lawson PA, Citron DM, Tyrrell KL, Finegold SM. Reclassification of Clostridium difficile as Clostridioides difficile (Hall and O'Toole 1935) prevot 1938. *Anaerobe* 2016;**40**:95—9.

2. Zhang RF, Man YX, Bai YY, Shao CH, Liu CM, Wang CH, et al. Molecular characterization of Clostridioides difficile ribotype 027 in a major Chinese hospital. *J Microbiol Immunol Infect* 2021;**54**:1179—83.

3. Lee JC, Hung YP, Tsai BY, Tsai PJ, Ko WC. Severe Clostridium difficile infections in intensive care units: diverse clinical presentations. *J Microbiol Immunol Infect* 2020;**54**:1111—7.

4. Chang TH, Hsu WY, Yang TI, Lu CY, Hsueh PR, Chen JM, et al. Increased age and proton pump inhibitors are associated with severe Clostridium difficile infections in children. *J Microbiol Immunol Infect* 2020;**53**:578—84.

5. Hung YP, Lee JC, Tsai BY, Wu JL, Liu HC, Liu HC, et al. Risk factors of Clostridium difficile-associated diarrhea in hospitalized adults: vary by hospitalized duration. *J Microbiol Immunol Infect* 2019;**54**:276—83.

6. Hung YP, Tsai CS, Tsai BY, Tsai PJ, Lee YT, Lee JC, et al. Clostridioides difficile infection in patients with hematological malignancy: a multicenter study in Taiwan. *J Microbiol Immunol Infect* 2021;**54**:1101—10.

7. Lin TC, Hung YP, Ko WC, Ruan JW. Fecal microbiota transplantation for Clostridium difficile infection in Taiwan: establishment and implementation. *J Microbiol Immunol Infect* 2019;**52**:841—50.

8. Wu KS, Syue LS, Cheng A, Yen TY, Chen HM, Chiu YH, et al. Recommendations and guidelines for the treatment of Clostridioides difficile infection in Taiwan. *J Microbiol Immunol Infect* 2020;**53**:191—208.

9. Chang FC, Liu CP, Sun FJ, Lin CC. Optimizing laboratory workflow for the diagnosis of Clostridiodes difficile infection in a medical center in Northern Taiwan. *J Microbiol Immunol Infect* 2021;**54**:284—9.

10. Peng Z, Ling L, Stratton CW, Li C, Polage CR, Wu B, et al. Advances in the diagnosis and treatment of Clostridium difficile infections. *Emerg Microb Infect* 2018;**7**:15.

11. Ofori E, Ramai D, Dhawan M, Mustafa F, Gasperino J, Reddy M. Community-acquired Clostridium difficile: epidemiology, ribotype, risk factors, hospital and intensive care unit outcomes, and current and emerging therapies. *J Hosp Infect* 2018;**99**:436—42.

12. Guh AY, Adkins SH, Li Q, Bulens SN, Farley MM, Smith Z, et al. Risk factors for community-associated Clostridium difficile infection in adults: a case-control study. *Open Forum Infect Dis* 2017;**4**:ofx171.

13. He M, Miyajima F, Roberts P, Ellison L, Pickard DJ, Martin MJ, et al. Emergence and global spread of epidemic healthcare-associated Clostridium difficile. *Nat Genet* 2013;**45**:109—13.

14. Goorhuis A, Bakker D, Corver J, Debast SB, Harmanus C, Notermans DW, et al. Emergence of Clostridium difficile infection due to a new hypervirulent strain, polymerase chain reaction ribotype 078. *Clin Infect Dis* 2008;**47**:1162—70.

15. Fatima R, Aziz M. The hypervirulent strain of Clostridium difficile: NAP1/B1/027 - a brief overview. *Cureus* 2019;**11**:e3977.

16. Imwattana K, Knight DR, Kullin B, Collins DA, Putsathit P, Kiratisin P, et al. Clostridium difficile ribotype 017-characterization, evolution and epidemiology of the dominant strain in Asia. *Emerg Microb Infect* 2019;**8**:796—807.

17. Smits WK. Hype or hypervirulence: a reflection on problematic C. difficile strains. *Virulence* 2013;**4**:592—6.

18. Spigaglia P. Recent advances in the understanding of antibiotic resistance in Clostridium difficile infection. *Ther Adv Infect Dis* 2016;**3**:23—42.

19. 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;**45**:215—21.

20. Brown AWW, Wilson RB. Clostridium difficile colitis and zoonotic origins-a narrative review. *Gastroenterol Rep (Oxf)* 2018;**6**:157—66.

21. Dingle KE, Didelot X, Quan TP, Eyre DW, Stoesser N, Marwick CA, et al. A Role for tetracycline selection in recent evolution of agriculture-associated Clostridium difficile PCR ribotype 078. *mBio* 2019;**10**. e02790-18.

22. Knight DR, Riley TV. Genomic delineation of zoonotic origins of Clostridium difficile. *Front Public Health* 2019;**7**:164.

23. Stabler RA, Dawson LF, Valiente E, Cairns MD, Martin MJ, Donahue EH, et al. Macro and micro diversity of Clostridium difficile isolates from diverse sources and geographical locations. *PLoS One* 2012;**7**:e31559.

24. Knetsch CW, Hensgens MP, Harmanus C, van der Bijl MW, Savelkoul PH, Kuijper EJ, et al. Genetic markers for Clostridium difficile lineages linked to hypervirulence. *Microbiology* 2011;**157**:3113—23.

25. Janezic S, Zidaric V, Pardon B, Indra A, Kokotovic B, Blanco JL, et al. International Clostridium difficile animal strain collection and large diversity of animal associated strains. *BMC Microbiol* 2014;**14**:173.

26. Cairns MD, Preston MD, Hall CL, Gerding DN, Hawkey PM, Kato H, et al. Comparative genome analysis and global phylogeny of the toxin variant Clostridium difficile PCR ribotype 017 reveals the evolution of two independent sublineages. *J Clin Microbiol* 2017;**55**:865—76.

27. Hung YP, Cia CT, Tsai BY, Chen PC, Lin HJ, Liu HC, et al. The first case of severe Clostridium difficile ribotype 027 infection in Taiwan. *J Infect* 2015;**70**:98—101.

28. Suzuki H, Tomita M, Tsai PJ, Ko WC, Hung YP, Huang IH, et al. Comparative genomic analysis of Clostridium difficile ribotype 027 strains including the newly sequenced strain NCKUH-21 isolated from a patient in Taiwan. *Gut Pathog* 2017;**9**:70.

29. Valiente E, Dawson LF, Cairns MD, Stabler RA, Wren BW. Emergence of new PCR ribotypes from the hypervirulent Clostridium difficile 027 lineage. *J Med Microbiol* 2012;**61**:49—56.

30. Hung YP, Huang IH, Lin HJ, Tsai BY, Liu HC, Liu HC, et al. Predominance of Clostridium difficile ribotypes 017 and 078 among toxigenic clinical isolates in southern Taiwan. *PLoS One* 2016;**11**:e0166159.

31. 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;**11**:1197—203.

32. Luo Y, Cheong E, Bian Q, Collins DA, Ye J, Shin JH, et al. Different molecular characteristics and antimicrobial resistance profiles of Clostridium difficile in the Asia-Pacific region. *Emerg Microb Infect* 2019;**8**:1553—62.

33. Hung YP, Lin HJ, Tsai BY, Liu HC, Liu HC, Lee JC, et al. Clostridium difficile ribotype 126 in southern Taiwan: a cluster of three symptomatic cases. *Anaerobe* 2014;**30**:188—92.

34. Persson S, Jensen JN, Olsen KE. Multiplex PCR method for detection of Clostridium difficile tcdA, tcdB, cdtA, and cdtB and internal in-frame deletion of tcdC. *J Clin Microbiol* 2011;**49**: 4299—300.

35. Li Z, Lee K, Rajyaguru U, Jones CH, Janezic S, Rupnik M, et al. Ribotype Classification of Clostridioides difficile isolates is not predictive of the amino acid sequence diversity of the toxin virulence factors tcdA and tcdB. *Front Microbiol* 2020;**11**:1310.

36. Shen E, Zhu K, Li D, Pan Z, Luo Y, Bian Q, et al. Subtyping analysis reveals new variants and accelerated evolution of Clostridioides difficile toxin B. *Commun Biol* 2020;**3**:347.

37. Dhalluin A, Lemee L, Pestel-Caron M, Mory F, Leluan G, Lemeland JF, et al. Genotypic differentiation of twelve Clostridium species by polymorphism analysis of the triosephosphate isomerase (tpi) gene. *Syst Appl Microbiol* 2003;**26**:90—6.

38. Lemee L, Dhalluin A, Testelin S, Mattrat MA, Maillard K, Lemeland JF, et al. Multiplex PCR targeting *tpi* (triose phosphate isomerase), *tcdA* (Toxin A), and *tcdB* (Toxin B) genes for toxigenic culture of Clostridium difficile. *J Clin Microbiol* 2004;**42**:5710—4.

39. Cao B, Zhang Y, Bian F, Cheng Y, Wang J, Jia H, et al. Detection of Clostridium difficile toxin genes by PCR: sequence variation may cause false-negative results. *J Med Microbiol* 2015;**64**:195—7.

40. Du P, Cao B, Wang J, Li W, Jia H, Zhang W, et al. Sequence variation in tcdA and tcdB of Clostridium difficile: ST37 with truncated tcdA is a potential epidemic strain in China. *J Clin Microbiol* 2014;**52**:3264—70.

41. Lim SK, Stuart RL, Mackin KE, Carter GP, Kotsanas D, Francis MJ, et al. Emergence of a ribotype 244 strain of Clostridium difficile associated with severe disease and related to the epidemic ribotype 027 strain. *Clin Infect Dis* 2014;**58**:1723—30.

42. Bidet P, Barbut F, Lalande V, Burghoffer B, Petit JC. Development of a new PCR-ribotyping method for Clostridium difficile based on ribosomal RNA gene sequencing. *FEMS Microbiol Lett* 1999;**175**:261—6.

43. Martinson JN, Broadaway S, Lohman E, Johnson C, Alam MJ, Khaleduzzaman M, et al. Evaluation of portability and cost of a fluorescent PCR ribotyping protocol for Clostridium difficile epidemiology. *J Clin Microbiol* 2015;**53**:1192—7.

44. Fawley WN, Knetsch CW, MacCannell DR, Harmanus C, Du T, Mulvey MR, et al. Development and validation of an internationally-standardized, high-resolution capillary gel-based electrophoresis PCR-ribotyping protocol for Clostridium difficile. *PLoS One* 2015;**10**:e0118150.

45. Janezic S, Strumbelj I, Rupnik M. Use of modified PCR ribotyping for direct detection of Clostridium difficile ribotypes in stool samples. *J Clin Microbiol* 2011;**49**:3024—5.

46. Dingle TC, MacCannell DR. Chapter 9 - molecular strain typing and characterisation of toxigenic Clostridium difficile. In: Sails A, Tang Y-W, editors. *Methods in microbiology*. Academic Press; 2015. p. 329—57.

47. 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;**24**:61—7.

48. van den Berg RJ, Schaap I, Templeton KE, Klaassen CH, Kuijper EJ. Typing and subtyping of Clostridium difficile isolates by using multiple-locus variable-number tandem-repeat analysis. *J Clin Microbiol* 2007;**45**:1024—8.

49. Bandelj P, Harmanus C, Blagus R, Cotman M, Kuijper EJ, Ocepek M, et al. Quantification of Clostridioides (Clostridium) difficile in feces of calves of different age and determination of predominant Clostridioides difficile ribotype 033 relatedness and transmission between family dairy farms using multilocus variable-number tandem-repeat analysis. *BMC Vet Res* 2018;**14**:298.

50. Baines SD, O'Connor R, Freeman J, Fawley WN, Harmanus C, Mastrantonio P, et al. Emergence of reduced susceptibility to metronidazole in Clostridium difficile. *J Antimicrob Chemother* 2008;**62**:1046—52.

51. 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;**44**:2558—66.

52. Krutova M, Wilcox MH, Kuijper EJ. A two-step approach for the investigation of a Clostridium difficile outbreak by molecular methods. *Clin Microbiol Infect* 2019;**25**:1300—1.

53. Zhang LJ, Yang L, Gu XX, Chen PX, Fu JL, Jiang HX. The first isolation of Clostridium difficile RT078/ST11 from pigs in China. *PLoS One* 2019;**14**:e0212965.

54. Valiente E, Cairns MD, Wren BW. The Clostridium difficile PCR ribotype 027 lineage: a pathogen on the move. *Clin Microbiol Infect* 2014;**20**:396—404.

55. Dawson LF, Valiente E, Wren BW. Clostridium difficile-a continually evolving and problematic pathogen. *Infect Genet Evol* 2009;**9**:1410—7.

56. Knight DR, Riley TV. Clostridium difficile clade 5 in Australia: antimicrobial susceptibility profiling of PCR ribotypes of human and animal origin. *J Antimicrob Chemother* 2016;**71**:2213—7.

57. Knight DR, Putsathit P, Elliott B, Riley TV. Contamination of Australian newborn calf carcasses at slaughter with Clostridium difficile. *Clin Microbiol Infect* 2016;**22**. 266.e1-7.

58. 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;**306**:115—22.

59. 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;**41**:125—30.

60. Wu YC, Chen CM, Kuo CJ, Lee JJ, Chen PC, Chang YC, et al. Prevalence and molecular characterization of Clostridium difficile isolates from a pig slaughterhouse, pork, and humans in Taiwan. *Int J Food Microbiol* 2017;**242**:37—44.

61. Lim SC, Knight DR, Riley TV. Clostridium difficile and one health. *Clin Microbiol Infect* 2019;**26**:857—63.

62. Collins DA, Riley TV. Clostridium difficile in Asia: opportunities for one health management. *Trav Med Infect Dis* 2018;**4**.

63. Knight DR, Kullin B, Androga GO, Barbut F, Eckert C, Johnson S, et al. Evolutionary and genomic insights into Clostridioides difficile sequence type 11: a diverse zoonotic and antimicrobial-resistant lineage of global one health importance. *mBio* 2019;**10**.

64. Banawas SS. Clostridium difficile infections: a global overview of drug sensitivity and resistance mechanisms. *BioMed Res Int* 2018;**2018**:8414257.

65. Peng Z, Jin D, Kim HB, Stratton CW, Wu B, Tang YW, et al. Update on antimicrobial resistance in Clostridium difficile: resistance mechanisms and antimicrobial susceptibility testing. *J Clin Microbiol* 2017;**55**:1998—2008.

66. Wasels F, Kuehne SA, Cartman ST, Spigaglia P, Barbanti F, Minton NP, et al. Fluoroquinolone resistance does not impose a cost on the fitness of Clostridium difficile in vitro. *Antimicrob Agents Chemother* 2015;**59**:1794—6.

67. Hsieh CY, Liaw ET, Fan KM. Removal of veterinary antibiotics, alkylphenolic compounds, and estrogens from the Wuluo constructed wetland in southern Taiwan. *J Environ Sci Health A Tox Hazard Subst Environ Eng* 2015;**50**:151—60.

68. Donskey CJ. Fluoroquinolone restriction to control fluoroquinolone-resistant Clostridium difficile. *Lancet Infect Dis* 2017;**17**:353—4.

Apppendix

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Cd isolates in this study.

Year	KLCG	Bacterial DNA internal control	C. difficile marker	RT078-lineage	Toxin A		Toxin B	Binary toxin CDT		Toxin negative regulator			QRDR of gyrA (213 bp)		QRDR of gyrB (390 bp)		Ribotyping
		16S rRNA (1062 bp)	tpiA (340 bp)	Marker (707 bp)	tcdA (629 bp)	Wild-type (369 bp) or truncation (110 bp)	tcdB (410 bp)	cdtA (221bp)	cdtB (262 bp)	tcdC (162 bp)	Deletions	Mutations	Allele types	Mutations	Allele types	Mutations	
2015	1	+	+	+	+	Wild-type (369 bp)	+	+	+	Truncated	39 bp	C184T	A5	Thr82 to Ile	B1		RT127
	2	+	+	−	+	Wild-type (369 bp)	+	−	−	Wild-type (162 bp)			A0		B0		
	3	+	+	−	+	Wild-type (369 bp)	+	−	−	Wild-type (162 bp)			A0		B0		
	4	+	+	−	+	Wild-type (369 bp)	+	−	−	Wild-type (162 bp)			A0		B0		
	5	+	+	−	−		−	−	−	−			A1	Thr82 to Ile	B0		RT039
	6	+	+	−	−		−	−	−	−			A0		B0		
	7	+	+	−	−		−	−	−	−			A0		B0		
	8	+	+	+	+	Wild-type (369 bp)	+	+	+	Truncated	39 bp	C184T	A5	Thr82 to Ile	B1		RT127
	9	+	+	−	+	Wild-type (369 bp)	+	−	−	Wild-type (162 bp)			A0		B0		
	11	+	+	−	+	Wild-type (369 bp)	+	−	−	Wild-type (162 bp)			A0		B0		
	12	+	+	+	+	Wild-type (369 bp)	+	+	+	Truncated	39 bp	C184T	A5	Thr82 to Ile	B1		RT127
	13	+	+	+	+	Wild-type (369 bp)	+	+	+	Truncated	39 bp	C184T	A5	Thr82 to Ile	B1		RT127
	14	+	+	−	+	Wild-type (369 bp)	+	−	−	Wild-type (162 bp)			A0		B0		
	15	+	+	−	+	Wild-type (369 bp)	+	−	−	Wild-type (162 bp)			A0		B0		
	16	+	+	−	−		−	−	−	−			A6		B6-like		
	18	+	+	−	+	Wild-type (369 bp)	+	−	−	Wild-type (162 bp)			A3-like	Without Thr82 to Ile	B0		
	19	+	+	−	−		−	−	−	−			A0		B4	Asp 426 to Asn	RT010

Year	ID														
	20	+	+	−	+	Wild-type (369 bp)	+	−	−	Wild-type (162 bp)		A0		B0	
	21	+	+	−	+	Wild-type (369 bp)	+	−	−	Wild-type (162 bp)		A0		B0	
	22	+	+	−	+	Wild-type (369 bp)	+	−	−	Wild-type (162 bp)		A0		B0	
	23	+	+	−	+	Wild-type (369 bp)	+	−	−	Wild-type (162 bp)		A0		B0	
	24	+	+	−	+	Wild-type (369 bp)	+	−	−	Wild-type (162 bp)		A0		B0	
	25	+	+	−	+	Wild-type (369 bp)	+	−	−	Wild-type (162 bp)		A0		B0	
	93	+	+	−	−		−	−	−	—		A1	Thr82 to Ile	B0	RT010
	94	+	+	+	+	Wild-type (369 bp)	+	+	+	Truncated 39 bp	C184T	A5	Thr82 to Ile	B1	RT127
	95	+	+	+	+	Wild-type (369 bp)	+	+	+	Truncated 39 bp	C184T	A5	Thr82 to Ile	B1	RT127
2014	26	+	+	−	+	Wild-type (369 bp)	+	−	−	Wild-type (162 bp)		A0		B0	
	27	+	+	−	+	Wild-type (369 bp)	+	−	−	Wild-type (162 bp)		A0		B0	
	28	+	+	+	+	Wild-type (369 bp)	+	+	+	Truncated 39 bp	C184T	A5	Thr82 to Ile	B1	RT127
	29	+	+	+	+	Wild-type (369 bp)	+	+	+	Truncated 39 bp	C184T	A5	Thr82 to Ile	B1	RT127
	30	+	+	+	+	Wild-type (369 bp)	+	+	+	Truncated 39 bp	C184T	A5	Thr82 to Ile	B1	RT127
	31	+	+	+	+	Wild-type (369 bp)	+	+	+	Truncated 39 bp	C184T	A5	Thr82 to Ile	B1	RT127
	32	+	+	+	+	Wild-type (369 bp)	+	+	+	Truncated 39 bp	C184T	A5	Thr82 to Ile	B1	RT127
	33	+	+	−	+	Wild-type (369 bp)	+	−	−	Wild-type (162 bp)		A0-like	Contain 1 new SNP	B0	
	35	+	+	−	+	Wild-type (369 bp)	+	−	−	Wild-type (162 bp)		A0		B0	
	36	+	+	−	+	Wild-type (369 bp)	+	−	−	Wild-type (162 bp)		A0		B0	
	37	+	+	−	+	Wild-type (369 bp)	+	−	−	Wild-type (162 bp)		A0		B0	
	40	+	+	+	+	Wild-type (369 bp)	+	+	+	Truncated 39 bp	C184T	A5	Thr82 to Ile	B1	RT127
	41	+	+	−	−		−	−	−	—		A0		B0	
	42	+	+	−	−		−	−	−	—		A0		B0	
	43	+	+	+	+	Wild-type (369 bp)	+	+	+	Truncated 39 bp	C184T	A5	Thr82 to Ile	B1	RT127

Year	KLCG	Bacterial DNA internal control	C. difficile marker	RT078-lineage	Toxin A		Toxin B	Binary toxin CDT		Toxin negative regulator			QRDR of gyrA (213 bp)		QRDR of gyrB (390 bp)		Ribotyping
		16S rRNA (1062 bp)	*tpiA* (340 bp)	Marker (707 bp)	*tcdA* (629 bp)	Wild-type (369 bp) or truncation (110 bp)	*tcdB* (410 bp)	*cdtA* (221bp)	*cdtB* (262 bp)	*tcdC* (162 bp)	Deletions	Mutations	Allele types	Mutations	Allele types	Mutations	
	44	+	+	+	+	Wild-type (369 bp)	+	+	+	Truncated	39 bp	C184T	A5	Thr82 to Ile	B1		RT127
	45	+	+	+	+	Wild-type (369 bp)	+	+	+	Truncated	39 bp	C184T	A5	Thr82 to Ile	B1		RT127
	46	+	+	−	+	Wild-type (369 bp)	+	−	−	Wild-type (162 bp)			New-1	Without Thr82 to Ile	B0		
	47	+	+	+	+	Wild-type (369 bp)	+	+	+	Truncated	39 bp	C184T	A5	Thr82 to Ile	B1		RT127
	48	+	+	+	+	Wild-type (369 bp)	+	+	+	Truncated	39 bp	C184T	A5	Thr82 to Ile	B1		RT127
	49	+	+	+	+	Wild-type (369 bp)	+	+	+	Truncated	39 bp	C184T	A5	Thr82 to Ile	B1		RT127
	50	+	+	+	+	Wild-type (369 bp)	+	+	+	Truncated	39 bp	C184T	A5	Thr82 to Ile	B1		RT127
	51	+	+	+	+	Wild-type (369 bp)	+	+	+	Truncated	39 bp	C184T	A5	Thr82 to Ile	B1		RT127
2013	53	+	+	−	−		−	−	−	−			A1	Thr82 to Ile	B0		RT039
	54	+	+	+	+	Wild-type (369 bp)	+	+	+	Truncated	39 bp	C184T	A5	Thr82 to Ile	B1		RT127
	55	+	+	−	+	Wild-type (369 bp)	+	−	−	Truncated	18 bp	None	A0		B0		RT705
	56	+	+	−	−		−	−	−	−			A1	Thr82 to Ile	B0		RT039
	57	+	+	−	−		−	−	−	−			A1	Thr82 to Ile	B0		RT039
	58	+	+	−	+	Wild-type (369 bp)	+	−	−	Wild-type (162 bp)			A0		B0		
	59	+	+	−	+	Wild-type (369 bp)	+	−	−	Wild-type (162 bp)			A0		B0		
	60	+	+	−	+	Wild-type (369 bp)	+	−	−	Wild-type (162 bp)			A0		B0		
	61	+	+	−	+	Wild-type (369 bp)	+	−	−	Wild-type (162 bp)			A0		B0		
	62	+	+	−	+	Wild-type (369 bp)	+	−	−	Wild-type (162 bp)			A0		B0		

Year	No.										tcdC				RT
2012	63	+	+	−	+	Wild-type (369 bp)	+	−	−	Wild-type (162 bp)	A0		B0		
	64	+	+	−	−		−	−	−	−	A1	Thr82 to Ile	B0		RT010
	66	+	+	−	−		−	−	−	−	A1	Thr82 to Ile	B0		RT596
	67	+	+	−	−		−	−	−	−	New-1	Without Thr82 to Ile	B0		
	68	+	+	−	−		−	−	−	−	A3-like	Without Thr82 to Ile	B0		
	69	+	+	−	−		−	−	−	−	A1	Thr82 to Ile	B0		RT039
	70	+	+	−	−		−	−	−	−	A3-lke	Without Thr82 to Ile	B0		
2011	71	+	+	−	−		−	−	−	−	A1	Thr82 to Ile	B0		RT039
	72	+	+	−	−		−	−	−	−	A1	Thr82 to Ile	B0		RT039
	73	+	+	−	+	Wild-type (369 bp)	+	−	−	Wild-type (162 bp)	A0		B0		
	74	+	+	−	+	Wild-type (369 bp)	+	−	−	Wild-type (162 bp)	A0		B0		
	75	+	+	−	+	Wild-type (369 bp)	+	−	−	Wild-type (162 bp)	A0		B0		
	76	+	+	−	+	Wild-type (369 bp)	+	−	−	Wild-type (162 bp)	A0		B0		
	77	+	+	−	+	Wild-type (369 bp)	+	−	−	Wild-type (162 bp)	A0		B0		
	78	+	+	−	+	Wild-type (369 bp)	+	−	−	Wild-type (162 bp)	A0		B0		
	80	+	+	−	+	Wild-type (369 bp)	+	−	−	Wild-type (162 bp)	A1	Thr82 to Ile	B0		RT633
2010	81	+	+	−	−		−	−	−	−	A1	Thr82 to Ile	B0		RT039
	82	+	+	−	+	Wild-type (369 bp)	+	−	−	Wild-type (162 bp)	A0		B0		
	83	+	+	−	−		−	−	−	−	A1	Thr82 to Ile	B0		RT039
	84	+	+	−	+	Wild-type (369 bp)	+	−	−	Wild-type (162 bp)	A0		B0		
	85	+	+	−	+	Wild-type (369 bp)	+	−	−	Wild-type (162 bp)	A0		B0		
2009	87	+	+	−	+	Wild-type (369 bp)	+	−	−	Wild-type (162 bp)	A0		B0		
	88	+	+	−	−		−	−	−	−	A0		B4	Asp 426 to Asn	RT010
	89	+	+	−	+	Wild-type (369 bp)	+	−	−	Wild-type (162 bp)	A0		B0		

Year	KLCG	Bacterial DNA internal control	C. difficile marker	RT078-lineage	Toxin A		Toxin B	Binary toxin CDT		Toxin negative regulator			QRDR of gyrA (213 bp)		QRDR of gyrB (390 bp)		Ribotyping
		16S rRNA (1062 bp)	tpiA (340 bp)	Marker (707 bp)	tcdA (629 bp)	Wild-type (369 bp) or truncation (110 bp)	tcdB (410 bp)	cdtA (221bp)	cdtB (262 bp)	tcdC (162 bp)	Deletions	Mutations	Allele types	Mutations	Allele types	Mutations	
	90	+	+	−	+	Wild-type (369 bp)	+	−	−	Wild-type (162 bp)			A1-like	Without Thr82 to Ile	B0		
	91	+	+	−	−		−	−	−	−			A0		B4	Asp 426 to Asn	RT010
	92	+	+	−	−		−	−	−	−			A0		B4	Asp 426 to Asn	RT010

No.	Strain name	Year	A6	B7	C6	G8	H9	F3	E7	Summed tandem-repeat (STR)
1	TNHP92	2011	0	16	30	21	2	5	6	80
2	TNHP7	2011	0	22	36	20	2	5	6	91
3	TNHP17	2011	0	18	23	19	2	5	6	73
4	KLCG44	2014	0	16	27	19	2	5	6	75
5	KLCG47	2014	0	16	26	19	2	5	6	74
6	KLCG40	2014	0	15	26	19	2	5	6	73
7	KLCG94	2015	0	16	26	18	2	5	6	73
8	KLCG54	2013	0	15	22	19	2	5	6	69
9	KLCG32	2014	0	16	26	19	2	5	6	74
10	KLCG08	2015	0	16	27	17	2	5	6	73
11	KLCG30	2014	0	15	26	21	2	5	6	75
12	KLCG12	2015	0	16	27	17	2	5	6	73
13	KLCG45	2014	0	16	27	19	2	5	6	75
14	KLCG28	2014	0	15	26	21	2	5	6	75
15	KLCG13	2015	0	16	27	17	2	5	6	73
16	KLCG01	2015	0	16	26	19	2	5	6	74
17	KLCG43	2014	0	16	26	19	2	5	6	74
18	KLCG48	2014	0	16	26	19	2	5	6	74
19	KLCG51	2014	0	16	26	19	2	5	6	74
20	KLCG31	2014	0	15	26	21	2	5	6	75
21	KLCG49	2014	0	16	26	19	2	5	6	74
22	KLCG95	2015	0	16	26	18	2	5	6	73
23	KLCG29	2014	0	15	26	21	2	5	6	75
24	KLCG50	2014	0	16	26	19	2	5	6	74

MLVA profile of RT127 strains.