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Original Article

Ribotypes and antimicrobial susceptibility profiles of clinical *Clostridioides difficile* isolates: A multicenter, laboratory-based surveillance in Taiwan, 2019—2021

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KEYWORDS
Clostridioides (*Clostridium*) *difficile*;
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Abstract *Background:* The clinical burden of *Clostridioides difficile* infections (CDIs) remains substantial globally. This study aimed to investigate the ribotypes (RTs) and antimicrobial susceptibility of *C. difficile* isolates collected in Taiwan.
Methods: C. difficile isolates were prospectively collected from four medical centers in Taiwan from 2019 to 2021. In a reference laboratory, *in vitro* susceptibility to clindamycin, moxifloxacin, metronidazole, vancomycin, fidaxomicin, and rifaximin were tested, and ribotyping was conducted to determine their genetic diversity.

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Fidaxomicin

Results: A total of 568 *C. difficile* isolates were included. Metronidazole resistance was not observed, and the susceptibility rate of vancomycin was 99.5 %. Clindamycin showed poor activity against these isolates, with a resistance rate of 74.8 %. Fidaxomicin exhibited potent activity and 97.4 % of isolates were inhibited at 0.25 μg/mL. Rifaximin MIC_{90} increased from 0.015 μg/mL in 2019 to 0.03 μg/mL in 2020 and 2021. Of 40 RTs identified, two predominant RTs were RT 078/126 (78, 14 %) and 014/020 (76, 13 %). RT 017, traditional harboring truncated *tcdA*, accounted for 3 % (20 isolates) and there was no isolate belonging to RT 027. The proportions of RT 078 increased from 11.2 % in 2019 to 17.1 % in 2021, and the predominance of RT 078/126 was more evident in central Taiwan.

Conclusions: Vancomycin, fidaxomicin, and metronidazole remain *in vitro* effective against clinical *C. difficile* isolates in Taiwan. The reservoirs and genetic relatedness of two major RTs with zoonotic potentials, RT 078/126 and 014/020, warrant further investigations.

Introduction

Clostridioides difficile is a Gram-positive, spore-forming bacterium that can cause severe diarrhea and colitis, and death.[1] In recent years, the incidence and severity of *C. difficile* infections (CDIs) have increased worldwide, posing a significant public health challenge.[2,3] Without exception in clinical isolates of *C. difficile*, there are growing concerns about the emergence of antimicrobial resistance, which can limit the effectiveness of current treatment options.[4] Fidaxomicin and rifaximin are two antibiotics that have been recently approved for the treatment of CDI. Rifaximin is a non-absorbable rifamycin derivative that has been shown to be effective in the treatment of recurrent CDI.[5] It is now considered a choice for treating metronidazole-unresponsive CDI, but there is still lack of solid evidence by a randomized trial[5] and a previous study showed decreased susceptibility with ribotype RT027.[6] Fidaxomicin is a macrocyclic antibiotic with a narrow spectrum of activity. When ingested orally, Fidaxomicin shows minimal absorption into the bloodstream. It acts as a bactericidal agent and specifically eliminates harmful *C. difficile* while causing minimal disturbance to the diverse range of bacterial species that constitute the natural and healthy intestinal microbiota. It has been shown to be non-inferior to oral vancomycin in the treatment of CDI in randomized controlled trials,[7,8] but to be associated with a lower recurrence rate, as compared with oral vancomycin. In a multicenter collection of 403 clinical *C. difficile* isolates from 2005 to 2010 in Taiwan, there was universal susceptibility to metronidazole and vancomycin, and fidaxomicin had potent i*n vitro* antibacterial activity. However, 11.6 % of *C. difficile* isolates had a rifaximin MIC of ≥128 μg/mL[9]. Another multicenter study from 2015 to 2016 showed decreased doxycycline and tigecycline susceptibilities among ribotype 078 isolates.[4]

The prevalence of *C. difficile* ribotypes (RTs) varies across different regions and countries. A prior study in North America[10,11] and Europe[10,11] found that RT 027, a hypervirulent clone, was associated with clinical grave outcomes and healthcare-associated outbreaks in these regions. In contrast, RT 017, a prevalent RT in Asia, has been associated with not only community-acquired CDI and hospital-acquired CDI, and has now disseminated globally.[12,13] A multicenter study in Japan in 2014—2015 found that RT 018/018, 014, and 002 were the most prevalent RTs in patients with CDI.[14] In Taiwan, the RT 078 lineage was noticed to be predominant among clinical toxigenic isolates with binary toxin.[4] However, the full picture of RT distribution of clinical *C. difficile* isolates in Taiwan was not reported yet.

Currently, in Taiwan, there are only a few nationwide surveys on the trends in changing molecular epidemiology such as RTs and emerging antibiotic resistance of *C. difficile*. Based on the research gap, the present study aimed to investigate the RTs and *in vitro* antimicrobial susceptibility of *C. difficile* isolates collected from four tertiary medical centers in Taiwan from 2019 to 2021.

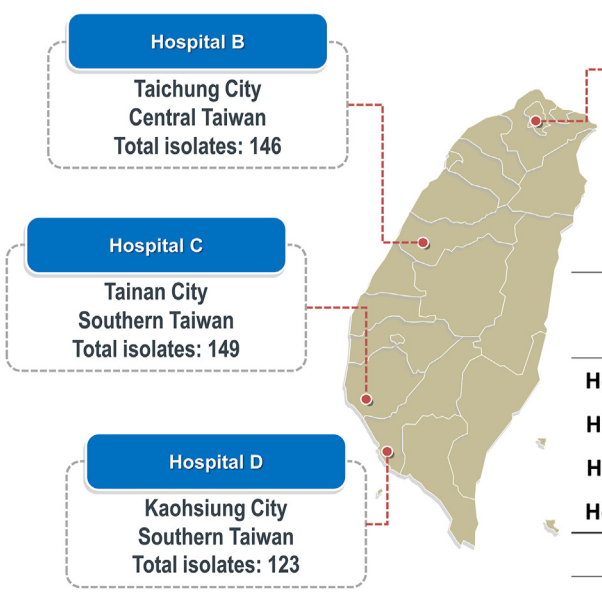
Material & methods

Collection of *C. difficile* isolates

C. difficile isolates were collected from toxin-positive stool samples using standard procedures in four tertiary medical centers in Taiwan (Hospital A to D, shown in Fig. 1) from 2019 to 2021. During the study period, *C. difficile* was isolated and identified by the automated system (Vitek ANC card, bioMérieux, France) in Hospital A, and matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) in the other three hospitals. Only the first *C. difficile* isolate form each patient during the collection period was collected. The yielded *C. difficile* isolates were frozen in trypticase soy broth (TSB) with glycerol at −80 °C and sent to the International Health Management Associates (IHMA, Schaumburg, Illinois, U.S.A.). After re-confirmation of species identification by MALDI-TOF mass spectrometry (Bruker Daltonics, Bremen, Germany), all isolates were then stored at −80 °C in TSB.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing for clindamycin, moxifloxacin, metronidazole, vancomycin, fidaxomicin, and rifaximin was performed by the agar dilution method following the protocols of Clinical and Laboratory Standards Institute (CLSI).[15,16] Brucella agar plates with serially



Sites	Years of collection			Percent of total isolates
	2019	2020	2021	
Hospital A	50	50	50	26.4%
Hospital B	47	49	50	25.7%
Hospital C	50	50	49	26.2%
Hospital D	23	50	50	21.7%
Total	170	199	199	100%

Figure 1. Study hospitals and collection years of 568 clinical *Clostridioides difficile* isolates included in this study.

diluted concentrations of antimicrobial agent and heat-labile supplements (hemin 5 μg/mL, vitamin K 1 μg/mL, and laked sheep blood 5 % v/v) were prepared. The 0.5 McFarland bacterial suspension was diluted 1:10 in sterile Mueller-Hinton broth to obtain a concentration of 10^7 colony forming units (CFU)/mL. An aliquot of each suspension was placed into the plates in a replicator inoculum block, the final inoculum on the agar was approximately 10^5 CFU per spot. Plates were incubated at 36 ± 1 °C under anaerobic conditions for 48 h. Minimal inhibitory concentrations (MICs) were recorded as the lowest concentration of antimicrobial that completely inhibited growth.

The total number of isolates (n), MIC_{50} (μg/mL), MIC_{90} (μg/mL), MIC ranges, and percentage (%) of susceptible, intermediate, and resistant strains were determined for all antimicrobial agents tested using the available CLSI breakpoints. As there are no CLSI breakpoints of vancomycin for *C. difficile*, vancomycin susceptibility is based on the epidemiological cut-off value (ECV) of European Committee on Antimicrobial Susceptibility Testing (EUCAST), *i.e.*, ≤ 2 μg/mL.[17]

Ribotyping

RTs were determined for all confirmed *C. difficile* isolates. PCR to generate RT (i.e., ribosomal gene) amplicon patterns was performed at Creighton University (Omaha, Nebraska, U.S.A.) using the protocol described by Stubbs et al.[18] and modified by Svenungsson et al.[19] In brief, the targeted DNA region is the 16S–23S rRNA intergenic spacer. Specific primers, including primers 5′-GTGCGGCTGGATCACCTCCT-3' (16S) and 5′-CCCTGCACCCTT-AATAACTTGACC-3' (23S), as previously described,[20] binding to conserved regions of the 16S and 23S rRNA genes were used to amplify the intergenic spacer between them. The resulting DNA fragments are separated by gel electrophoresis to create a banding pattern or RT profile, with subsequent comparison to the database provided by *C. difficile* ribotyping network (CDRN) service, Public Health England.[21]

The study was approved by the Research Ethics Committees of National Taiwan University Hospital (201810105RSA), China Medical University Hospital (CMUH107-REC1-157), Institutional Review Board of National Cheng Kung University Hospital (B-ER-107-352), and Kaohsiung Medical University Hospital (KMUHIRB-E(I)-20180328). The informed consent was waived. This study was conducted according to the principles expressed in the Declaration of Helsinki.

Results

From 2019 to 2021, a total of 580 *C. difficile* isolates were collected from four tertiary medical centers in Taiwan. Of them, 568 isolates were confirmed as *C. difficile* by MALDI-TOF. The locations and isolate numbers of the four study sites were shown in Fig. 1. There was no metronidazole resistance among all 568 *C. difficile* isolates, and the susceptibility rate of vancomycin was 99.5 %. As expected, the *in vitro* activity of clindamycin against *C. difficile* was poor, with a resistance rate of 74.8 %. The trend in moxifloxacin resistance did not rise during the study period.

Fidaxomicin exhibited potent *in vitro* activity against *C. difficile*, with an MIC_{90} of 0.25 μg/mL (Table 1), and 97.4 % of all *C. difficile* isolates were inhibited at 0.25 μg/mL. The percentage of the isolates with a MIC of metronidazole ≤0.25 μg/mL for 2019, 2020, and 2021 was 98.8 %, 96.5 %, and 97.0 %, respectively. A total of 99.8 % of *C. difficile* isolates were inhibited by fidaxomicin at 0.5 μg/mL (Fig. 2A). The MIC_{90} of rifaximin increased from 0.015 μg/mL in 2019 to 0.03 μg/mL in 2020 and 2021. Of all isolates, 94.3 % had a rifaximin MIC of ≤0.03 μg/mL (Fig. 2B). Notably, 2.6 % of isolates displayed a rifaximin MIC of >8 μg/mL (Fig. 2B), and the ribotypes of these isolates were RT009 (4 isolates), RT015 (1), RT017 (4), RT039 (2), RT046 (1), RT050 (2), RT404 (1), respectively.

Table 1 *In vitro* activity of six antimicrobial agents against 568 clinical *Clostridioides difficile* isolates.

Year (n)	Drug	Range	MIC_{50}	MIC_{90}	% Sus	% Int	% Res
All (568)	Fidaxomicin	≤0.015–1	0.12	0.25	N/A	N/A	N/A
	Clindamycin	≤0.03 - >8	8	>8	7.4	17.8	74.8
	Metronidazole	≤0.06–1	0.25	0.5	100	0	0
	Moxifloxacin	≤0.06 - >8	2	>8	76.4	0.7	22.9
	Rifaximin	≤0.002 - >8	0.015	0.03	N/A	N/A	N/A
	Vancomycin	≤0.25–8	1	1	99.5	N/A	0.5
2019 (170)	Fidaxomicin	≤0.015–0.5	0.12	0.25	N/A	N/A	N/A
	Clindamycin	≤0.06 - >8	8	>8	14.7	18.2	67.1
	Metronidazole	≤0.06–1	0.5	0.5	100	0	0
	Moxifloxacin	0.5 - >8	2	>8	77.6	0.6	21.8
	Rifaximin	≤0.002 - >8	0.008	0.015	N/A	N/A	N/A
	Vancomycin	≤0.25–2	1	1	100	N/A	N/A
2020 (199)	Fidaxomicin	≤0.015–0.5	0.12	0.25	N/A	N/A	N/A
	Clindamycin	≤0.03 - >8	8	>8	3	16.6	80.4
	Metronidazole	≤0.06–1	0.25	0.5	100	0	0
	Moxifloxacin	≤0.06 - >8	2	>8	72.4	1	26.6
	Rifaximin	≤0.008 - >8	0.015	0.03	N/A	N/A	N/A
	Vancomycin	≤0.25–2	0.5	1	100	N/A	N/A
2021 (199)	Fidaxomicin	≤0.015–1	0.06	0.25	N/A	N/A	N/A
	Clindamycin	≤0.03 - >8	8	>8	5.5	18.6	75.9
	Metronidazole	≤0.06–1	0.25	0.5	100	0	0
	Moxifloxacin	0.5 - >8	2	>8	79.4	0.5	20.1
	Rifaximin	0.008 - >8	0.015	0.03	N/A	N/A	N/A
	Vancomycin	≤0.25–8	1	1	98.5	N/A	1.5

Percent susceptible defined by 2022 CLSI guidelines where available except for vancomycin, which is based on EUCAST 2022 estimated cut-off values (ECV); MIC, minimal inhibitory concentration. Sus, susceptible. Int, intermediate. Res, resistant. N/A, no breakpoint available.

Of 568 isolates, forty RTs were identified, but there were 94 isolates without an identified RT (Fig. 3A). There were six major RTs (*i.e.*, RT 078/126, 014/20, 001, 002, 007 and 106) and each RT included at least 40 isolates. RT 078/126 and 014/020 were two most common RTs (Fig. 3B). During the study period, there was an increasing trend in the proportions of RT 078, from 11.2 % in 2019 to 17.1 % in 2021 (Fig. 3B). Of note, the predominance of RT 078/126 was more evident in hospital B (21.9 %) which is in central Taiwan than that in other hospitals (hospital A: 6.7 %; hospital C: 10.7 %; hospital D: 16.3 %, $P = 0.001$) (Fig. 3C).

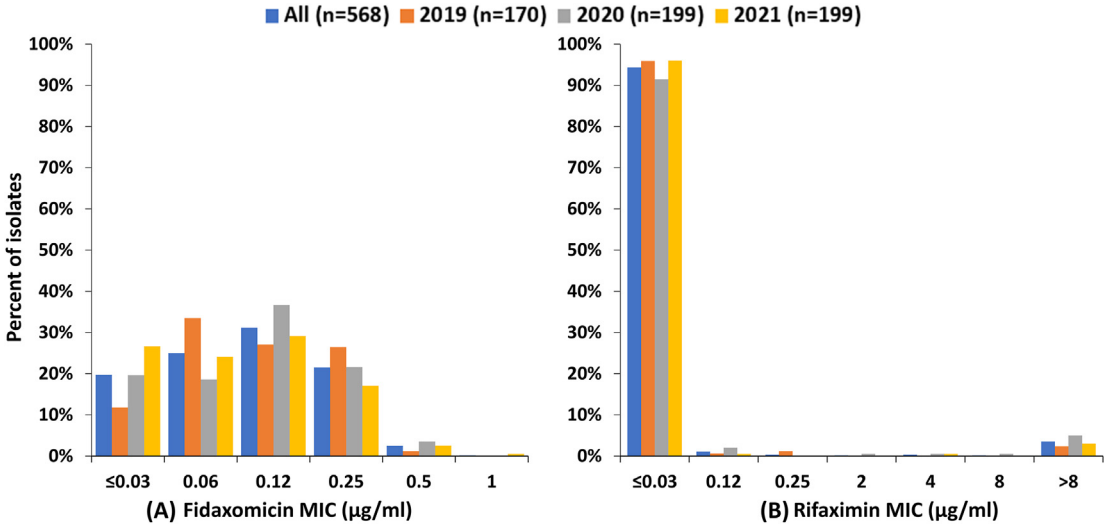


Figure 2. Minimal inhibitory concentration (MIC) distribution of fidaxomicin (A) and rifaximin (B) against *Clostridioides difficile* isolates.

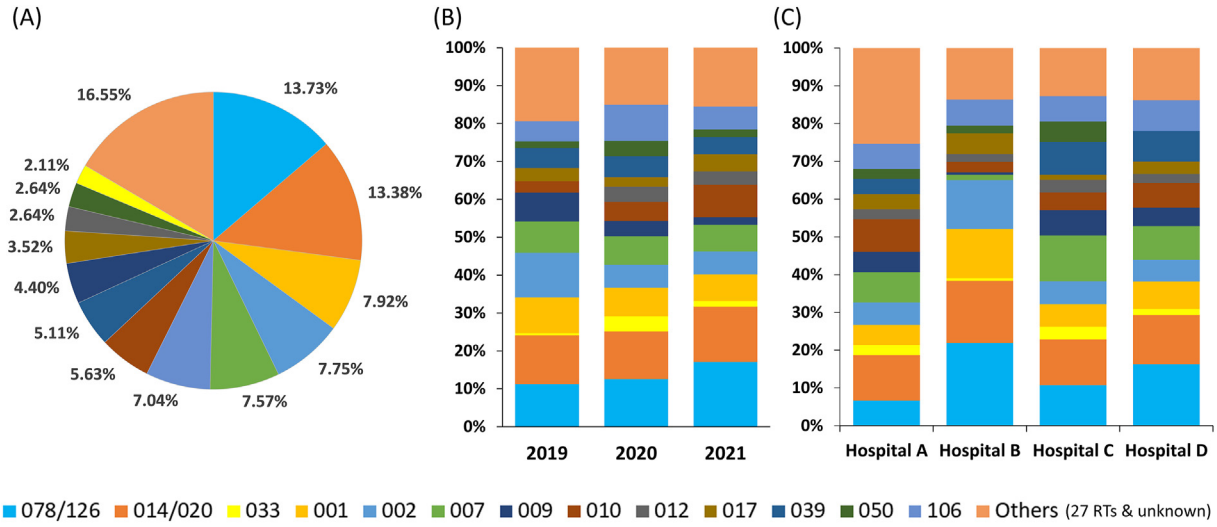


Figure 3. Ribotype distribution of 568 clinical *Clostridioides difficile* isolates (A), and stratified by years (B) or hospitals (C).

Antimicrobial susceptibility patterns of antimicrobial agents tested in six major RTs are shown in Fig. 4. For the proportion of *C. difficile* isolates with a fidaxomicin MIC of 0.06 or 0.12 μg/ml exceeds 50 % in each year of 2019, 2020, and 2021. For the six most common RTs (Figs. 4), 98.5 % had a MIC of ≤0.25 μg/ml. The vast majority (94.4 %) of *C. difficile* isolates had a rifaximin MIC of ≤0.03 μg/mL, but there were 89.5 % of RT 014/020 isolates with a rifaximin MIC of ≥0.12 μg/mL. The distribution of isolates by MIC value for clindamycin, moxifloxacin, and fidaxomicin is plotted in Fig. 5. Among the six major RTs, more than half had MIC >4 μg/mL against clindamycin, and RT 001 showed the least susceptible (Fig. 5A). RT 001 strains showed higher MICs against moxifloxacin than the other five RTs, with 48.9 % of them having MIC >4 μg/mL (Fig. 5B). In contrast, 80.0 % (36/45) of RT 001 strains had a MIC of ≤0.03 μg/mL for fidaxomicin, while 65.0 % (26/40) of RT 106 strains had a MIC of ≥0.25 μg/mL (Fig. 5C).

Discussion

Currently, multicenter surveillance aimed at investigating the burden of CDI remains infrequent in Taiwan.[4] This study assessed the *in vitro* activity of metronidazole, vancomycin, fidaxomicin, and rifaximin against *C. difficile* isolates in Taiwan. The MIC_{90} values for these agents in 2019—2021 were similar, and no metronidazole- or vancomycin-resistant isolates were identified. In a previous multicenter study in Taiwan during 2015—2016, resistance rates to metronidazole and vancomycin were 0.6%—3.3 % and 1.1%—4.6 %, respectively. The MIC_{90} values for metronidazole and vancomycin were 1 μg/mL each. These rates and values were higher than those observed in our study.[4] This disparity may be attributed to the enrollment of only the first *C. difficile* isolate per patient in our study, whereas the previous study included recurrent or relapsing isolates.[4] Variations in inclusion and exclusion criteria between studies could impact antimicrobial resistance results. Many current studies on *C. difficile* suggest an increasing resistance to metronidazole. Our research focused on the first episode of CDI, demonstrating that *C. difficile* isolates did not exhibit high resistance to metronidazole. This finding may also impact clinicians in their choice of empirical antibiotics when managing patients with the first episode of CDI.

Molecular epidemiology, which includes ribotyping and analysis of antimicrobial resistance, plays a vital role in comprehending and combating CDI. Molecular techniques like ribotyping facilitate the exploration of genetic diversity among strains, unveiling the intricate transmission dynamics of *C. difficile* within hospitals, communities, and the environment. This insight aids in outbreak identification, source tracing, and targeted control implementation. Furthermore, analyzing antimicrobial resistance profiles provides crucial understanding of resistance mechanisms, guiding appropriate antibiotic stewardship. Integrating nationwide molecular epidemiology, ribotyping, and antimicrobial resistance analysis assists in detecting emerging strains, monitoring infection control effectiveness, and shaping national public health strategies against *C. difficile* infection. Another multicenter surveillance in Taiwan during 2005—2010 revealed that 10.9 % of 403 *C. difficile* isolates were resistant to rifaximin (MIC >128 μg/mL) and lacked binary toxins.[9] Our study identified only 2.6 % of 568 isolates with rifaximin MIC >8 μg/mL, primarily RT 009, 017, 039, 050, and others. Despite varying rifaximin susceptibility, MIC_{90} of fidaxomicin remained 0.25 μg/mL. Further susceptibility surveillance, especially involving recurrent or refractory CDI cases, is necessary to unveil clinical utility of oral rifaximin.

The hypervirulent RT 078 lineage, encompassing RT 078 and 126, was found in CDI patients and associated with livestock and river water in Taiwan. Not only was a higher rate of fluoroquinolones found among the strains belonging to the RT078 lineage, but the result of genetic fingerprinting also showed the potential for zoonotic transfer.[4,22,23] The present study during 2019—2021 detected an increasing proportion of the RT 078 lineage, predominantly in the central Taiwan hospital. This emphasizes the need for clinical and environmental surveys to gauge their prevalence in healthcare and community settings.

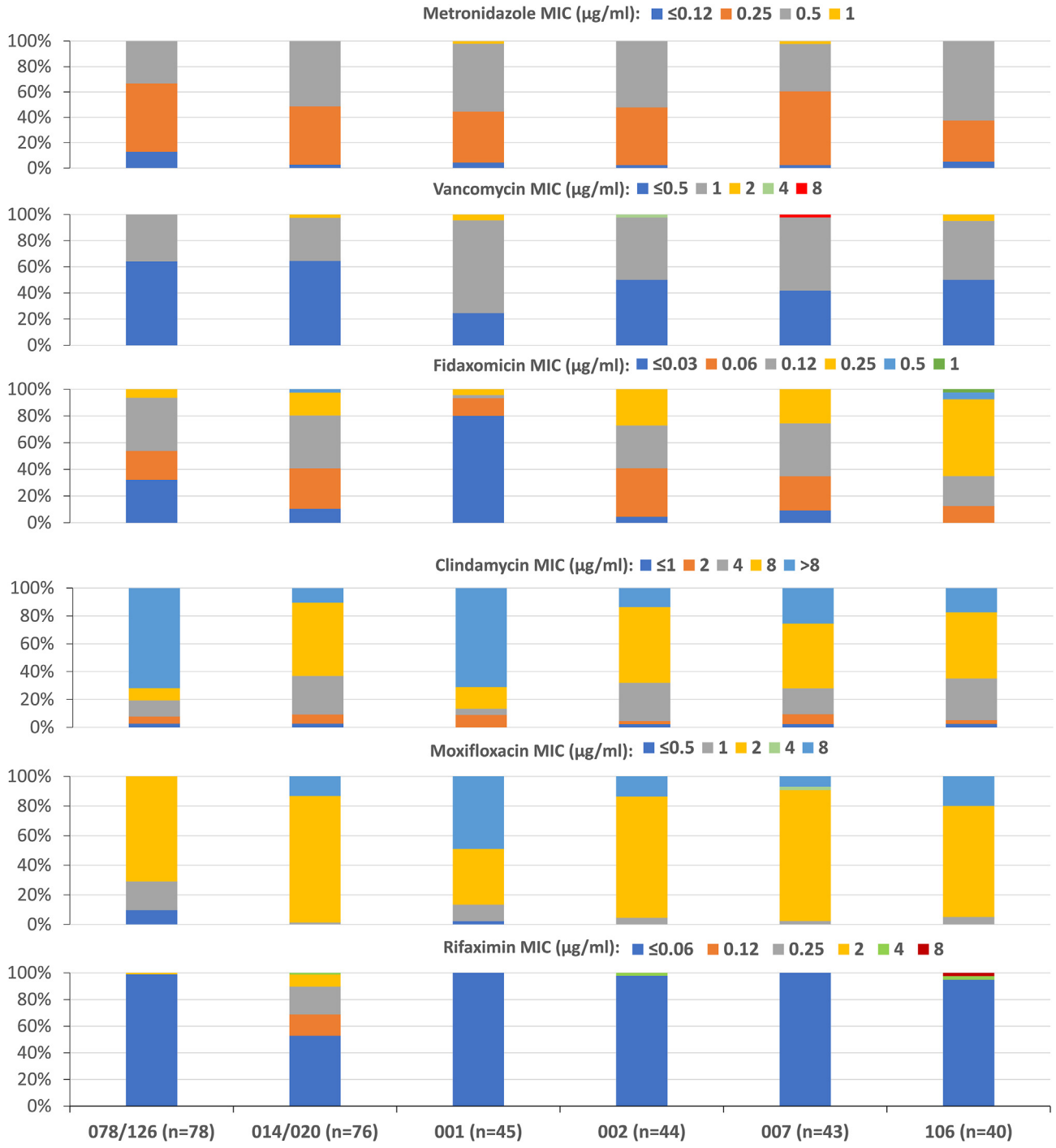


Figure 4. *In vitro* activity of antibiotics against six major ribotypes of *Clostridioides difficile* isolates.

Recently, RT 014 emerged as a common RT for clinical *C. difficile* isolates causing community-acquired CDIs in developed countries,[14,24,25] and was also prevalent in our collection. Our study observed a similar prevalence of RT 014/20 isolates, which exhibited reduced rifaximin susceptibility compared to other RTs. A prior Australian study detected the RT 014 lineage in wastewater,[26] suggesting potential zoonotic transmission due to genetic relatedness between human and animal isolates.[27] Therefore, in Taiwan the local prevalence of RT 014/20 in animals and the environment demands attention.

There are limitations in this present study, which encompass the lack of clinical data, therapeutic interventions, and patient outcomes in this laboratory-based surveillance. The impact of different RTs on health remains unassessed. Additionally, only isolates from the first CDI episode were collected, potentially underestimating antimicrobial resistance. Susceptibility to other agents like

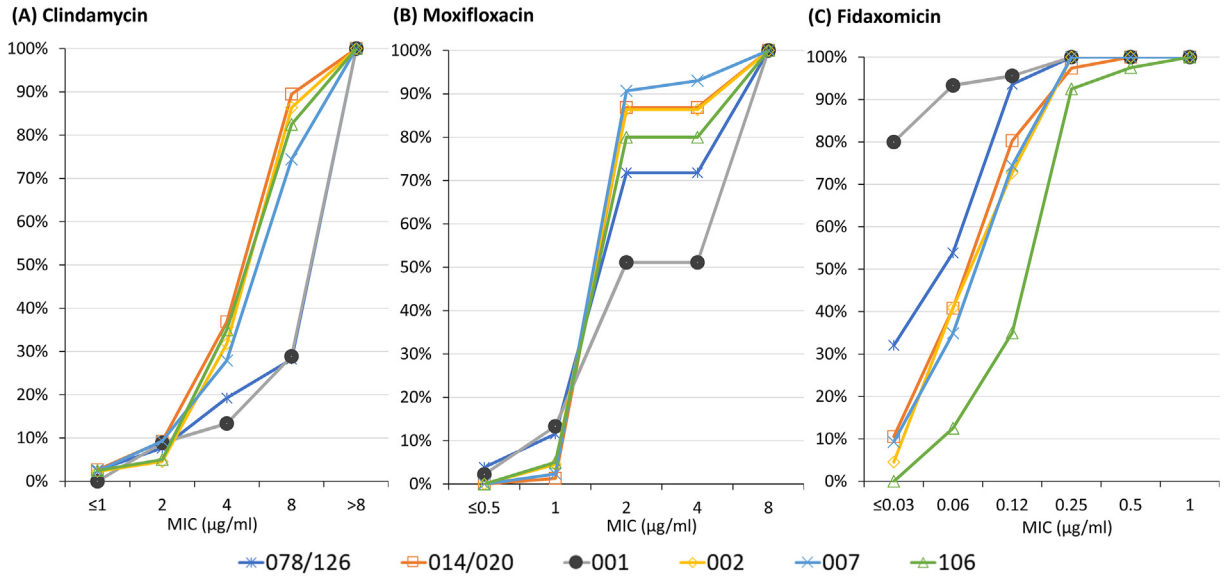


Figure 5. MIC distribution of clindamycin (A), moxifloxacin (B), and fidaxomicin (C) in clinical *Clostridioides difficile* isolates.

teicoplanin or tigecycline, and distribution of toxin genes, were unexplored. Genetic relatedness within the same RT and potential intra- or inter-hospital clone spread were not determined.

In conclusion, over a three-year period in Taiwan, recommended antimicrobial agents for CDI treatment, including fidaxomicin and vancomycin, maintained potent *in vitro* activity. Strains with zoonotic potential, such as RT 078/126 and 014/020, prevailed across study years and hospitals. Further research should concentrate on molecular epidemiology, encompassing ribotyping and antimicrobial susceptibility analysis, to detect emerging strains, monitor infection control efficacy, and inform national strategies for mitigating CDI burden.

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