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Abundant geographical divergence of *Clostridioides difficile* infection in China: a prospective multicenter cross-sectional study

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Abstract

Clostridioides difficile is the predominant pathogen in hospital-acquired infections and antibiotic-associated diarrhea. Dedicated networks and annual reports for *C. difficile* surveillance have been established in Europe and North America, however the extensive investigation on the prevalence of *C. difficile* infection (CDI) in China is limited. In this study, 1528 patients with diarrhea were recruited from seven geographically representative regions of China between July 2021 and July 2022. The positivity rate of toxigenic *C. difficile* using real-time fluorescence quantitative PCR test of feces was 10.2% (156/1528), and 125 (8.2%, 125/1528) strains were successfully isolated. The isolates from different geographical areas had divergent characteristics after multilocus sequence typing, toxin gene profiling, and antimicrobial susceptibility testing. No isolate from clade 2 were found, and clade 1 was still the main clade for these clinical isolates. Interestingly, clade 4, especially ST37, previously known as the characteristic type of China, showed a strong geographical divergence. Clade 3, although rare in China, has been detected in Hainan and Sichuan provinces. Most *C. difficile* isolates (76.8%, 96/125) were toxigenic. Clindamycin, erythromycin, and moxifloxacin were the top three antibiotics to which resistance was observed, with resistance rates of 81.3%, 63.6%, and 24.0%, respectively. Furthermore, 34 (27.2%, 34/125) multidrug-resistant (MDR) strains were identified. All the strains were sensitive to metronidazole, vancomycin, and meropenem. The genotype of *C. difficile* varies greatly among the different geographical regions in China, and new types are constantly emerging. Therefore, comprehensive, longitudinal, and standardized surveillance of *C. difficile* infections is needed in China, covering typical geographical areas.

Clinical trial number

Not applicable.

Keywords *Clostridioides difficile* infections, Molecular epidemiology, Multilocus sequence typing, Toxin gene profile, Antimicrobial susceptibility

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Introduction

Clostridioides difficile is the most common pathogen causing hospital-acquired and antibiotic-associated diarrhea and continues to be recognized as an urgent public health threat, resulting in a significant economic burden on public health [1]. The medical costs of *C. difficile* infection (CDI) in the United States amounted to USD 6.3 billion in 2020 [2]. The associated medical costs in Europe are approximately € 3 billion annually [3]. Although primarily categorized as nosocomial pathogens, the prevalence of community-acquired *C. difficile* infection (CA-CDI) appears to be increasing, accounting for more than half of all CDI cases [4]. The incidence of CA-CDI in the United States increased from 52.88 cases per 100,000 people in 2012 to 65.93 cases per 100,000 people in 2017 [5]. The European Center for Disease Prevention and Control (ECDC) annual report for 2022 showed that the proportion of CA-CDI increased from 13.6 to 32.7% [6]. In addition, the reported median age for CA-CDI and hospital-acquired CDI (HA-CDI) is 50 and 72 years, respectively, and the age of the CA-CDI population tends to be younger than that of HA-CDI [7].

The landscape of *C. difficile* epidemiology, including its molecular characteristics, continues to change worldwide. After several outbreaks in North America and Europe, the isolation rate of hypervirulent RT027/ST1 dropped from 25–9.4% [8]. Another hypervirulent isolate, RT078/ST11, was found to be more strongly associated with CA-CDI and economic animals [9, 10]. Currently, the epidemiological status of CDI in North America and Europe is relatively stable, with an incidence rate of 110.2 cases per 100,000 people and 3.48 cases per 10,000 patient-days, respectively [6, 11]. Although the incidence of CDI in Asia is generally regarded as infrequent, there are reports indicating that its incidence may be comparable to that observed in Western countries, approximately 5.3 cases per 10,000 patient days. In China, where research on CDI remains scarce and data regarding disease burden are lacking, the positive rate of toxigenic *C. difficile* stand at around 14% according to available studies; this figure aligns with the reported rates of 4–39% in Europe and 7–20% in the United States [12–14].

The population structure of *C. difficile* consists of five main clades and three additional, undefined clades [15]. Clade 1 is still the main source of clinical *C. difficile* worldwide, although the predominant ST types vary by region or country [16]. Regarding clade 4, RT017/ST37 and RT017/ST81 have been reported as characteristic types in China [17]. Clade3 is reported to be rare in China and worldwide. Globally, clinical *C. difficile* is highly resistant to erythromycin (ERY), clindamycin (CLI), and fluoroquinolones (FQs) [18]. *C. difficile* shows decreased susceptibility tometronidazole (MTZ) and vancomycin (VAN) in North America and Europe; however, in China, it is almost all susceptible [17, 19].

As an important part of the healthcare-associated infection-community interface, there are dedicated networks and annual reports for *C. difficile* surveillance in North America and Europe, from which one can easily see the changes in the epidemiology of *C. difficile* and the evolution and spread of this pathogen [4–6]. In contrast, although research on CDI in China has greatly increased in recent decades, it is still confined to limited geographical areas and even limited hospitals; to date, large-scale and sustained research on CDI has not been conducted in China, therefore, it is difficult to recognize the divergence of CDI in China in relation to its vast territory.

Therefore, we conducted this prospective surveillance of CDI in seven representative regions of China, from Jilin Province in the north to Hainan Province in the south and from Jiangxi Province in the east to Shaanxi Province in the west. This represents the first large-scale CDI study, encompassing extensive geographical regions and diverse economic levels across China, covering a total area of 1.71 million square kilometers and involving a population of approximately 300 million. This study aimed to provide a relatively comprehensive overview of the molecular divergence of CDI and highlight possible epidemiologic trends across China.We aspire for this study to ultimately facilitate the improvement of the CDI surveillance system in China and contribute valuable insights for developing effective management strategies.

Materials and methods

Study setting

This study was conducted at surveillance sites in seven geographically representative regions of China: Jilin, Jiangxi, Guangxi, Hainan, Sichuan, Yunnan, and Shaanxi. From July 2021 to July 2022, 1528 patients with diarrhea, defined as more than three bowel movements per day with altered fecal characteristics (loose, watery, or unformed stools), were recruited in the community and hospitals [20]. Due to high rates of *C. difficile* colonization in neonates and infants, individuals under 1 year of age were excluded from the study [21]. All fecal samples were detected for the *tcdB* toxin gene and *C. difficile* isolation. Patients with diarrhea whose stool specimens detected *C. difficile* toxin positive while ruling out other causes of diarrhea were defined as CDI [21]. Medical records were reviewed to determine whether it was hospital-acquired CDI (i.e., fecal collection occurred 48 h after hospitalization) or community-acquired CDI (i.e., fecal sampling occurred community or 48 h before hospitalization, and no admission was recorded 12 weeks prior to symptom onset) [22]. Molecular characterization of recovered *C. difficile* isolates was performed in National Institute for Communicable Disease Control and Prevention, Chinese

Center for Disease Control and Prevention. The research process is illustrated in Fig. 1.

Detection of virulent genes directly from fecal samples

The detection of virulence genes in fecal samples was according to the method of simon et al. [23]. DNA was extracted from all fecal samples using a fecal DNA extraction kit (Tiangen, China) according to the manufacturer's instructions. The DNA samples were stored at − 20 °C, and the virulence gene *tcdB* was detected by real-time fluorescence quantitative PCR (RT-qPCR) using the primers listed in Supplementary Table 1.

Isolation and identification

The isolation, culture, and identification of *C. difficile* were performed according to our previous research methods [24]. All fecal samples were treated with anhydrous ethanol and inoculated on selective cycloserine-cefoxitin-fructose agar plates (CCFA; Oxoid, UK) in an anaerobic chamber (DWS, UK) at 37 °C for 48 h. Suspected bacterial colonies were identified by the typical morphology and odor of *C. difficile*, while the 16 S rRNA (Supplementary Table 1) gene was amplified and the products were sanger sequenced to confirm *C. difficile*.

Multilocus sequence typing

Multilocus sequence typing (MLST) was performed on all isolates using primers and methods developed by Griffiths et al. [25]. Seven housekeeping genes (*adk*, *atpA*, *dxr*, *glyA*, *recA*, *sodA*, and *tpi*) (Supplementary Table 1) were amplified, and the amplified products were sent to Sangon (China) for bidirectional sequencing. The sequences obtained were imported into the PubMLST database (https://pubmlst.org/cdifficile) for compariso n to obtain allele and ST assignments. The nucleotide diversity π values of all genes ranged from 0.0016 to 0.0081, and the ratio of non-synonymous to synonymous substitutions (dn/ds) was consistently below 1, indicating the predominance of purifying selection preferentially (Supplementary Table 2). A total of 17 reference strains from various sources were added (Supplementary Table 3). The concatemerized sequences (3587 bp) of each strain were aligned by MEGA11 using the maximum likelihood method with 1,000 bootstrap replicates to generate a phylogenetic tree. The evolutionary tree was established using the online editor Evolview (https://www.evolgenius.info/).

Detection of toxin gene profile in *C. Difficile* isolates

The toxin gene profile was detected using the method described in our previous study [24]. The bacterial template DNA for toxin analysis was prepared by centrifuging the bacterial solution and removing the precipitate in a 5% (wt/vol) Chelex-100 resin solution (Bio-Rad, USA). All isolates were analyzed using PCR for toxin A (*tcdA*), toxin B (*tcdB*), and binary toxin (*cdtA* and *cdtB*) genes (Supplementary Table 1). The PCR products were run on

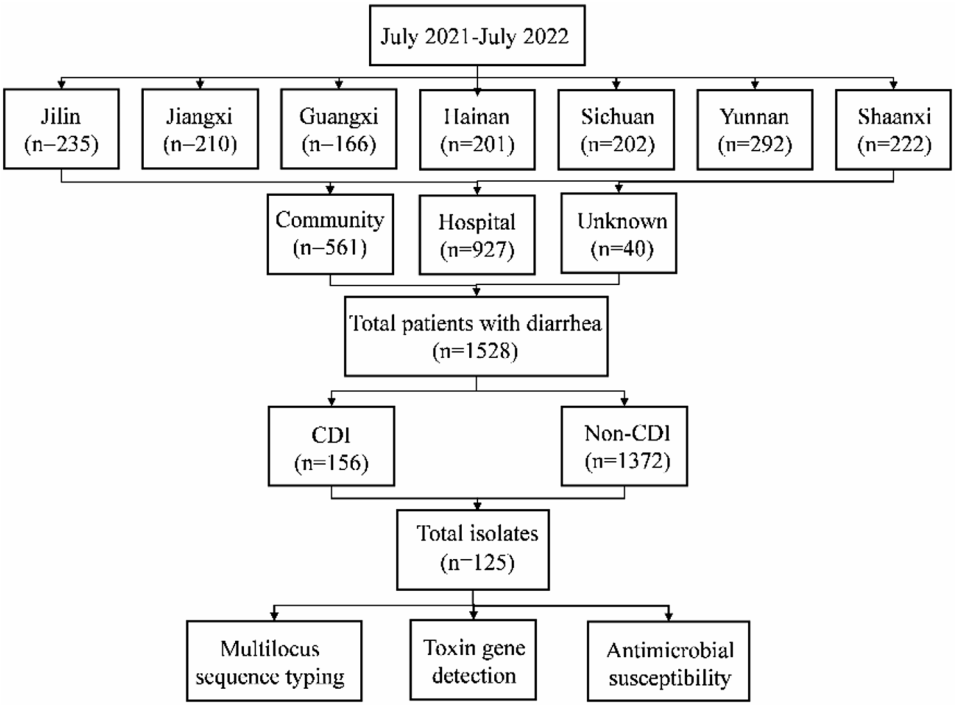

Fig. 1 Flow Chart indicating the recruitment and isolation of *C. difficile* in patients with diarrhea in China between 2021 and 2022

a QIAxcel capillary electrophoresis platform (QIAxcel Advanced DNA Screening Cartridge, QIAGEN).

Antimicrobial susceptibility testing

The minimum inhibitory concentration (MIC) was determined using an E-test strip (Biomerieux, France, and Liofilchem, Italy). The bacterial suspension was prepared in Brucella broth (Oxoid, UK) and adjusted to the 1 McFarland standard, and swabbed onto Brucella AGAR plates (Oxoid, UK) supplemented with 1 mg/L Vitamin K1, 5 mg/L heme chloride, and 5% defibrinated sheep blood. Results were read following anaerobic incubation at 37 °C for 48 h. A total of nine antibacterial, including CLI, ERY, moxifloxacin (MXF), rifampicin (RIF), tetracycline (TET), chloramphenicol (CHL), meropenem (MEM), VAN and MTZ, were detected. *C. difficile* ATCC700057 was used as a control. The MICs for MXF, CLI, TET, CHL, MEM, and MTZ were determined according to recommendations of the Clinical and Laboratory Standards Institute (CLSI) M100-S28, the breakpoints for VAN were established based on European Committee on Antimicrobial Susceptibility Testing (EUCAST) Version 9.0 criteria, and the breakpoints for ERY and RIF were determined according to a previous study [26]. According to CLSI recommendations, multidrug resistance (MDR) was defined as an isolate resistant to at least three or more antibiotics [27].

Statistical analysis

IBM SPSS software (version 19.0; IBM Corp., Armonk, NY, USA) was used for the statistical analysis. The $\chi 2$ test was used to compare groups, and statistical significance was set at $P < 0.05$.

Results

Patient demographics and prevalence rates of CDI

A total of 1528 patients with diarrhea from Jilin, Jiangxi, Guangxi, Hainan, Sichuan, Yunnan, and Shaanxi provinces in China were recruited for the study (Fig. 2A). Most patients were recruited from Yunnan (19.1%, 292/1528), the least number of patients was recruited from Guangxi (10.9%, 166/1528), and the number of patients recruited from other regions is shown in Fig. 2B. The age of the patients ranged from 1 to 95 years, and most patients (579) were aged ≥ 60 years (37.9%, 579/1528) (Fig. 2C). More than half (60.7% (927/1528) of the patients were from the hospital, and 36.7% (561/1528) of the patients were from the community. Information was missing for the remaining 40 patients. Patients with regional sources are shown in Fig. 2D. A total of 156 feacl samples were found to be positive for *C. difficile tcdB* gene by RT-qPCR (10.2%, 156/1528). The highest prevalence rates of *C. difficile* were found in participants from Guangxi (13.9%, 23/166) and Shaanxi (13.1%, 29/222), and the lowest was found in those from Jilin (5.5%, 13/235). The prevalence

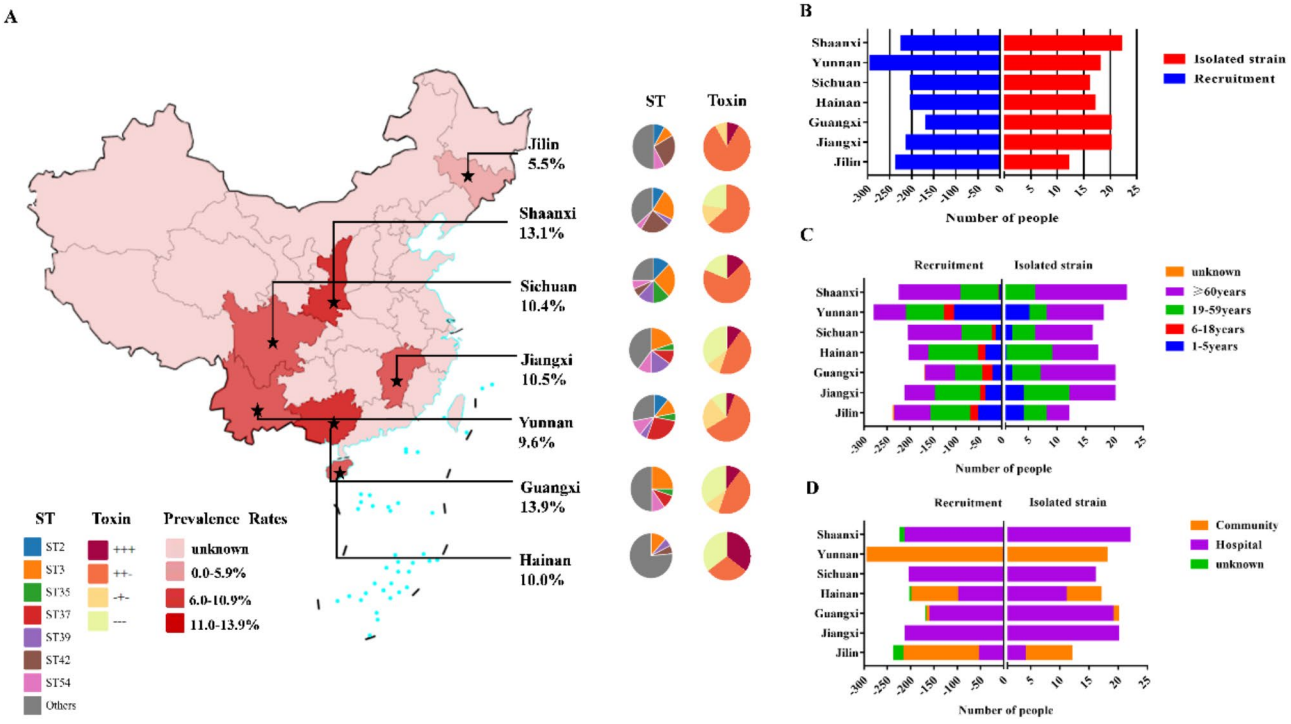

Fig. 2 The prevalence rates of CDI in seven geographically representative regions of China between July 2021 and July 2022 (**A**). Number of recruits and isolates from seven geographically representative regions of China (**B**). Composition of age groups in the recruited population and isolates (**C**). Composition of regional sources in the recruited population and isolates (**D**)

rates in other regions were 10.5% in Jiangxi (22/210), 10.4% in Sichuan (21/202), 10.0% in Hainan (20/201) and 9.6% in Yunnan (28/292) (Fig. 2A). There was no statistically significant difference in prevalence rates between the different regions ($P > 0.05$). Finally, 125 *C. difficile* strains were successfully isolated (8.2%, 125/1528). The highest number of isolated strains was 22 (17.6%, 22/125) in Shaanxi, and the lowest number was 12 (9.6%, 12/125) in Jilin (Fig. 2B). More than half of the strains were isolated from the ≥60-year-old age group (55.2%, 69/125). This was followed by 38 (30.4%, 38/125) isolates from the 19–59-year-old age group and 18 (14.4%, 18/125) isolates from the 1–5-year-old age group ($P < 0.05$). No strains were isolated from the 6–18-year-old age group (Fig. 2C). Moreover, the isolation rate of HA-CDI in China was 7.2% (67/927), while that of CA-CDI was 5.2% (29/561), which was statistically significant ($P < 0.05$) (Fig. 2D). The isolation rate of CA-CDI in Yunnan was 5.4% (16/292), considering that the patients were mainly recruited from the community. Interestingly, the isolation rate of CA-CDI in Guangxi was 14.3% (1/7); however, there is a risk of bias in this result, which might be related to the small sample size included. In addition, the patients recruited in Shaanxi, Jiangxi, and Sichuan were mainly from hospitals, and the HA-CDI isolation rates were 8.1% (17/211), 6.7% (14/210), and 6.4% (13/202), respectively.

Multilocus sequence typing (MLST) of *C. Difficile* isolates

According to the MLST gene profile, 125 isolates were successfully sequenced, and 38 STs were assigned, comprising 35 known and three novel STs (ST1202, ST1045, and ST1066). The 35 known STs in this study could be categorized into four clades (clades 1, 3, 4 and 5), within which clade 1 (67.2%, 84/125) was still the predominant clade. Isolates from clade 4 accounted for 20.8% (26/125) of the total, whereas there were three strainsin clades 3 and 5, each accounting for 2.4% (3/125), and no strain was detected from clade 2 (Fig. 3). The results showed that ST3 (18.4%, 23/125), ST42 (8.0%, 10/125), ST37 (7.2%, 9/125), ST54 (7.2%, 9/125), ST39 (6.4%, 8/125), ST2 (5.6%, 7/125), and ST35 (4.0%, 5/125) were the most prevalent types in China ($P < 0.05$) (Fig. 4A). Fortunately, the highly virulent strains, such as ST1 (BI/NAP1/027) and ST11 (RT078), were not detected. The same ST type may encompass various toxin gene profiles. For instance, the toxin gene profile of ST3 covers the majority of toxin gene profiles, including 17 toxigenic *C. difficile* isolates (73.9%, 17/23) and 6 non-toxigenic *C. difficile* (NTCD) isolates (26.1%, 6/23), and its predominant toxin gene profile is *tcdA* + *tcdB* + *cdtA/B-* (56.5%, 13/23). Additionally, the predominant toxin gene profile of ST42 was *tcdA* + *tcdB* + *cdtA/B-* (80.0%, 8/10), and that of ST37 was *tcdA-tcdB* + *cdtA/B-*(77.8%, 7/9) (Fig. 3).

The molecular characteristics of isolates from different geographical areas differed considerably (Fig. 4B). For example, ST3 was the predominant genotype in Sichuan (25.0%, 4/16), Guangxi (25.0%, 5/20), Shaanxi (22.7%, 5/22), and Jiangxi (20.0%, 4/20). ST42 was the predominant genotype in Jilin (25.0%, 3/12) and Shaanxi (22.7%, 5/22) provinces. ST37 (27.8%, 5/18) was the predominant genotype in Yunnan Province. Notably, the novel ST type, ST1202, was the predominant genotype in Guangxi (25.0%, 5/20). No statistically significant differences existed between the dominant types in different regions ($P > 0.05$). Interestingly, the distribution of ST types in Hainan was discrete, and there was no obvious dominant clonal group. Notably, clade 3 is rare in China. Of the three strains of clade 3 (ST5) found in this study, two were from Hainan, and one was from Jilin, which was not found in other regions (Figs. 4B and 5A and B). Moreover, nine strains (7.2%, 9/125) were detected as novel ST types, which were mainly found in Guangxi and Hainan (Fig. 5A, D). In addition to the above-mentioned five strains of ST1202 in Guangxi, three strains of ST1045 were also found in Guangxi and one strain of ST1066 in Hainan (Fig. 5A, D).

Toxin gene profiles of *C. Difficile* isolates

Among all isolates, 96 fecal samples were positive for toxin B by RT-qPCR, and the rest of the samples were negative, which corresponded one-to-one to the toxin gene profiles of the isolates (Fig. 3). Among the 125 isolates tested for virulent genes, the majority of *C. difficile* isolates (76.8%, 96/125) were toxigenic, with correspondingly 23.2% (29/125) non-toxigenic. Among the toxigenic strains, 14 (11.2%, 14/125) expressed all toxin genes (*tcdA* + *tcdB* + *cdtA/B+*). More than half of the strains (56.8%, 71/125) expressed *tcdA* and *tcdB* without binary toxin genes (*tcdA* + *tcdB* + *cdtA/B-*). In addition, 11 (8.8%, 11/125) strains contained only *tcdB* genes (*tcdA-tcdB* + *cdtA/B-*) ($P < 0.05$) (Fig. 4C).

The profiles of the toxin genes in different geographical areas were compared (Fig. 4D). The strains expressing *tcdA* and *tcdB* (*tcdA* + *tcdB* + *cdtA/B-*) were dominant in Jilin (83.3%, 10/12), Sichuan (68.8%, 11/16), Shaanxi (63.6%, 14/22), Yunnan (61.1%, 11/18), Jiangxi (55.0%, 11/20), and Guangxi (45.0%, 9/20), other than Hainan (29.4%, 5/17). The strains expressing all toxin genes (*tcdA* + *tcdB* + *cdtA/B+*) were the most common in Hainan (35.3%, 6/17), least common in Jilin(8.3%, 1/12) and Yunnan (5.6%, 1/18), and absent in Shaanxi. The strains expressing only tcdB genes (*tcdA-tcdB* + *cdtA/B-*) were found in the highest number in Yunnan (22.2%, 4/18), and in the lowest number in Jilin(8.3%, 1/12) and Jiangxi (5.0%, 1/20), but not in Hainan and Sichuan. The number of NTCD isolates was highest in Guangxi (35.0%, 7/20), and lowest in Yunnan (11.1%, 2/18), and no NTCD

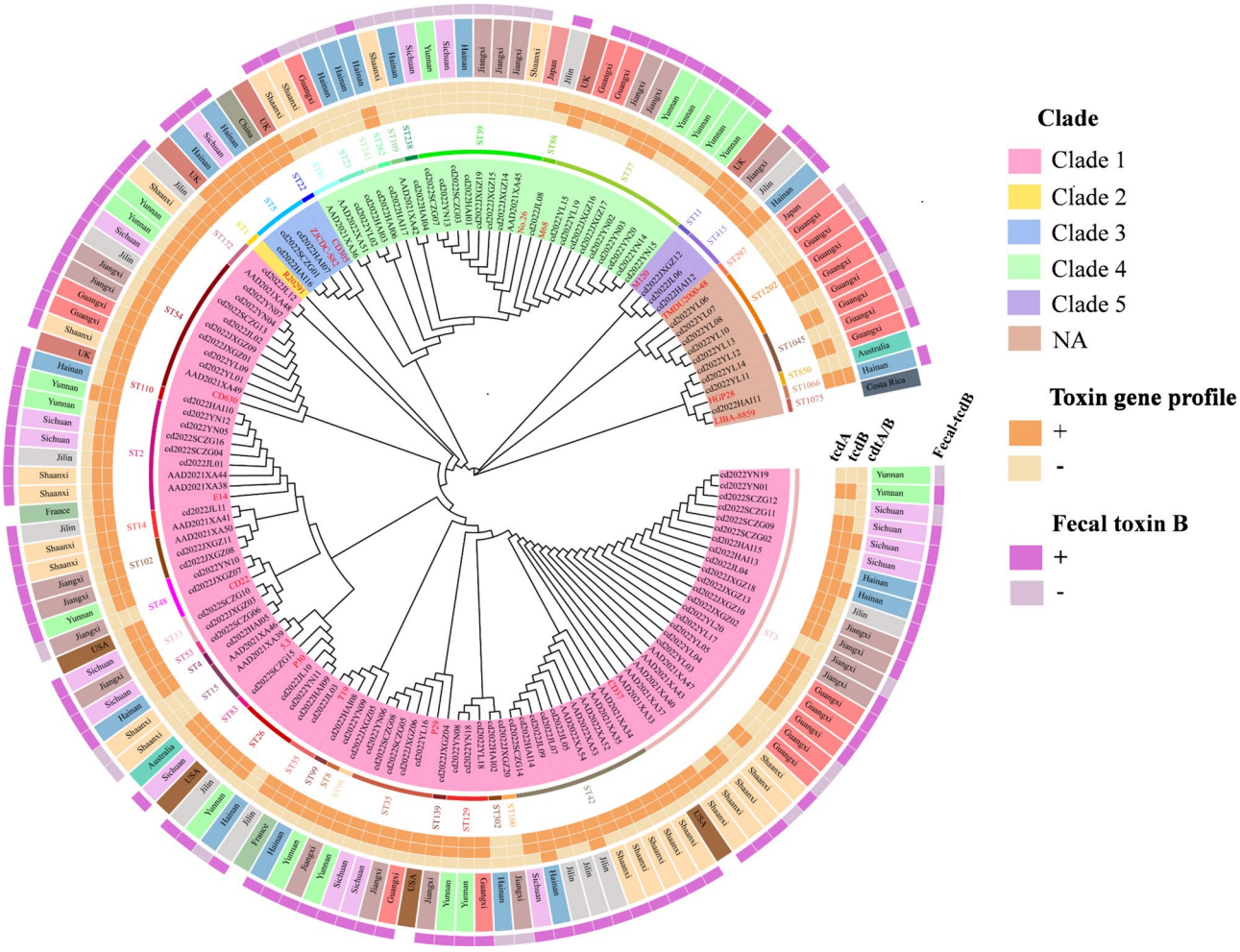

Fig. 3 Phylogenetic trees of *C. difficile* isolates were generated by the maximum likelihood method with 1000 bootstrap replicates. Reference strains are highlighted in red. Starting from the inner circle, the first ring represents clade branching, followed by ST genotypes in the second ring, *tcdA* and *tcdB* in the third and fourth rings respectively, binary toxin spectrum in the fifth ring, the regional source of the isolates in the sixth ring, and finally the seventh ring represents toxin B detection results of corresponding fecal samples

isolates were found in Jilin. No statistically significant differences existed in the toxin gene profiles between the different regions ($P > 0.05$).

Antibiotic resistance of *C. Difficile* isolates

All the strains were sensitive to MTZ, VAN, and MEM. Among toxigenic strains, CLI, ERY, and MXF were the top three antibiotics to which resistance was observed, with resistance rates of 81.3% (78/96), 63.6% (61/96), and 24.0% (23/96), respectively ($P < 0.05$), followed by TET (7.3%, 7/96) and RIF (5.2%, 5/96). The resistance rate to CHL (4.2%, 4/96) was the lowest ($P < 0.05$). In addition, 10 (10.4%, 10/96) strains were sensitive to all nine antibiotic classes (Fig. 6; Table 1). Among the three antibiotic classes (CLI, ERY, and MXF) with the highest resistance rates, genotypes ST3 (100%, 17/17), ST42 (100%, 9/9), and ST54 (100%, 9/9) were frequently associated with CLI resistance ($P > 0.05$) (Fig. 7A). Genotypes ST3 (94.1%, 16/17) and ST54 (88.9%, 8/9) were frequently associated with ERY resistance ($P < 0.05$) (Fig. 7A). Genotypes ST3 (64.7%, 11/17) had higher rates of resistance to MXF than other genotypes ($P < 0.05$) (Fig. 7A). Owing to the small number of isolates, the branches of clades 3 and 5 were not included in the analysis of the relationship between antibiotic resistance and molecular genotyping. The strains expressing only *tcdB* (*tcdA-tcdB + cdtA/B-*) had the highest resistance rate to MXF (54.5%, 6/11), and the strain expressing *tcdA* and *tcdB* (*tcdA + tcdB + cdtA/B-*) had the highest resistance rate to CLI (84.5%, 60/71) ($P < 0.05$) (Fig. 7A). In addition, the NTCD isolates had higher rates of resistance to ERY (86.2%, 25/29) than the other isolates with toxin gene profiles ($P > 0.05$).

We further analyzed the resistance rate in different geographical areas and found that the resistance rate of CLI was more than half in all regions, with the highest rate in Jiangxi (100%, 14/14) and the lowest in Hainan (63.6%,

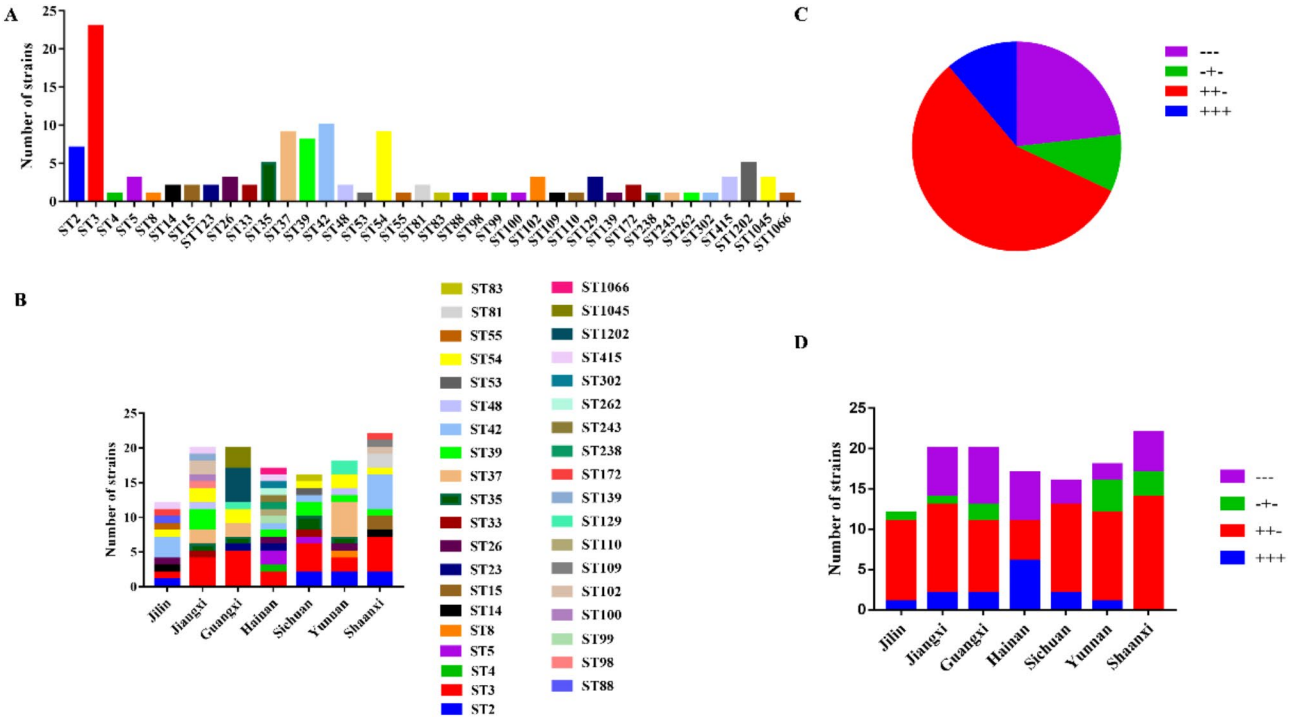

Fig. 4 The number of different ST genotypes for all isolates (**A**). The number of different ST genotypes for isolates in seven geographically representative regions (**B**). The toxin gene profiles of all isolates are represented as follows: purple indicates the *tcdA-tcdB-cdtA/B-* (---), green indicates the *tcdA-tcdB + cdtA/B-* (-+-), red indicates the *tcdA + tcdB + cdtA/B-* (++-), and blue indicates the *tcdA + tcdB + cdtA/B+* (+++) (**C**). Toxin gene profiles of isolates from different regions, the color coding conveys the same meaning as depicted in Fig. 4C (**D**)

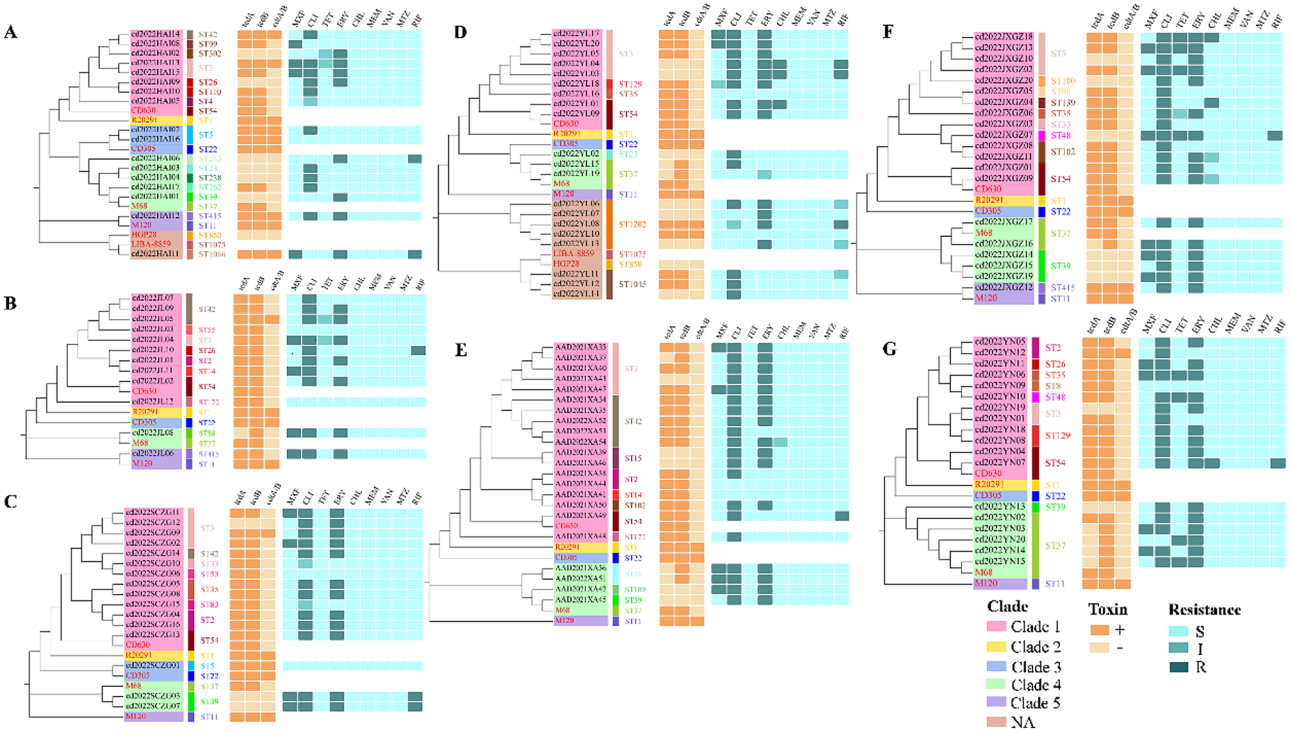

Fig. 5 Molecular characteristics, toxin gene profile, and antibiotic sensitivity of isolates from Hainan Province, reference strains are highlighted in red (**A**). Isolates from Jilin Province (**B**). Isolates from Sichuan Province (**C**). Isolates from Guangxi Province (**D**). Isolates from Shaanxi Province (**E**). Isolates from Jiangxi Province (**F**). Isolates from Yunnan Province (**G**)

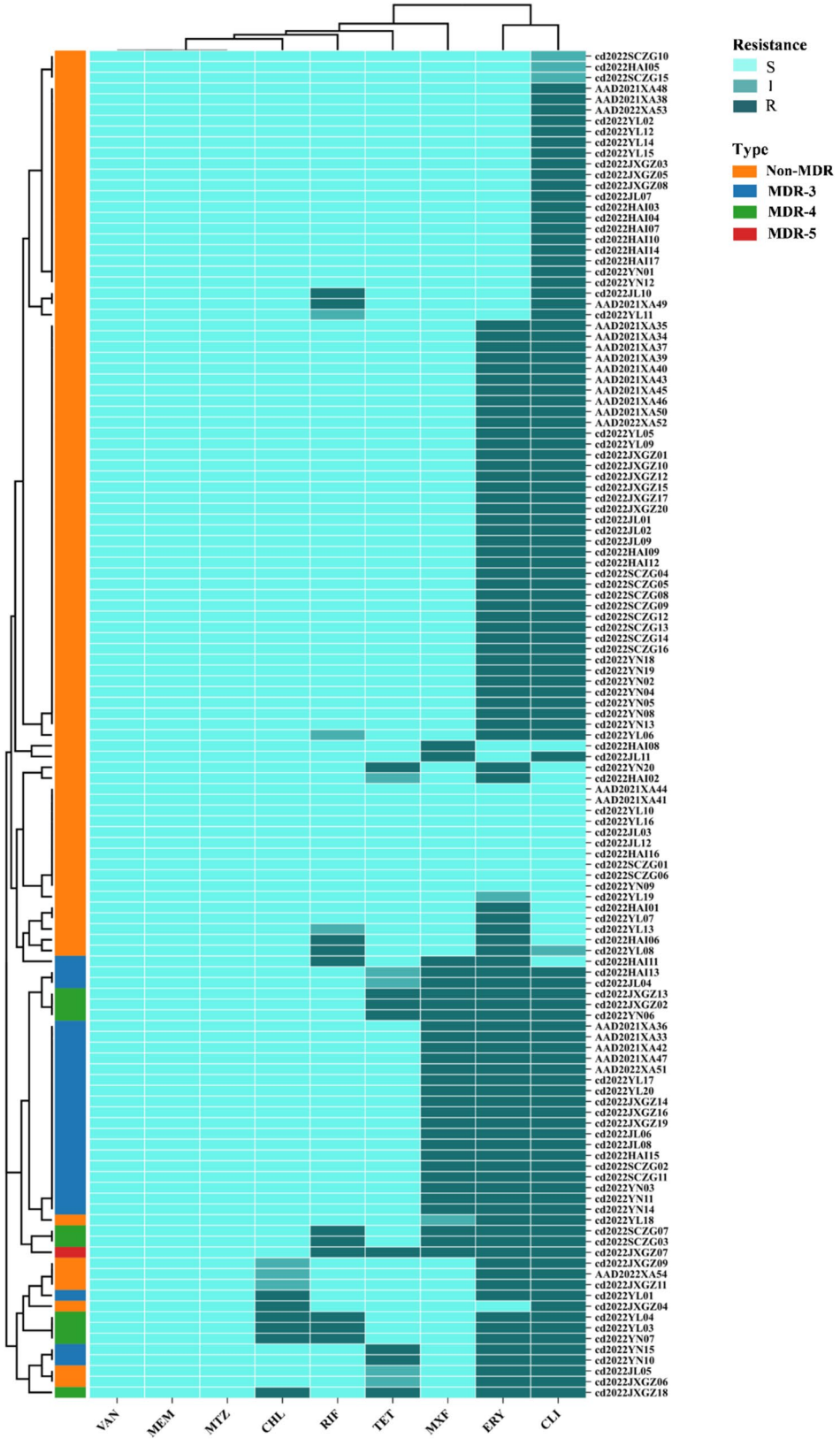

Fig. 6 Antibiotic resistance test results for all isolates, vancomycin (VAN), meropenem. (MEM), metronidazole (MTZ), chloramphenicol (CHL), rifampicin (RIF), tetracycline. (TET), moxifloxacin (MXF), erythromycin (ERY), clindamycin (CLI)

Table 1 Antibiotic resistance rates of *C. Difficile* isolates excluding NTCD isolates in China

		Jilin isolates	Shaanxi isolates	Jiangxi isolates	Sichuan isolates	Guangxi isolates	Yunnan isolates	Hainan isolates	Total
Antimicrobial agent	**Breakpoint(μg/mL)**	**Percentage resistance (% (*n*/*N*))**	**Percentage resistance (% (*n*/*N*))**	**Percentage resistance (% (*n*/*N*))**	**Percentage resistance (% (*n*/*N*))**	**Percentage resistance (% (*n*/*N*))**	**Percentage resistance (% (*n*/*N*))**	**Percentage resistance (% (*n*/*N*))**	**Percentage resistance (% (*n*/*N*))**
Clindamycin	≥ 8	83.3% (10/12)	88.2% (15/17)	100% (14/14)	84.6% (11/13)	69.2% (9/13)	87.5% (14/16)	63.6% (7/11)	81.3% (78/96)
Erythromycin	≥ 8	58.3% (7/12)	64.7% (11/17)	71.4% (10/14)	69.2% (9/13)	53.8% (7/13)	81.3% (13/16)	36.4% (4/11)	63.6% (61/96)
Moxifloxacin	≥ 8	33.3% (4/12)	23.5% (4/17)	21.4% (3/14)	15.4% (2/13)	15.4% (2/13)	25.0% (4/16)	36.4% (4/11)	24.0% (23/96)
Tetracycline	≥ 16	0 (0/12)	0 (0/17)	21.4% (3/14)	0 (0/13)	0 (0/13)	25.0% (4/16)	0 (0/11)	7.3% (7/96)
Rifampicin	≥ 4	8.3% (1 /12)	5.9% (1/17)	0 (0/14)	0 (0/13)	7.7% (1/13)	6.3% (1/16)	9.1% (1/11)	5.2% (5/96)
Chloramphenicol	≥ 32	0 (0/12)	0 (0/17)	14.3% (2/14)	0 (0/13)	7.7% (1/13)	5.6% (1/16)	0 (0/11)	4.2% (4/96)
Meropenem	≥ 16	0 (0/12)	0 (0/17)	0 (0/14)	0 (0/13)	0 (0/13)	0 (0/16)	0 (0/11)	0 (0/96)
Vancomycin	> 2	0 (0/12)	0 (0/17)	0 (0/14)	0 (0/13)	0 (0/13)	0 (0/16)	0 (0/11)	0 (0/96)
Metronidazole	≥ 32	0 (0/12)	0 (0/17)	0 (0/14)	0 (0/13)	0 (0/13)	0 (0/16)	0 (0/11)	0 (0/96)

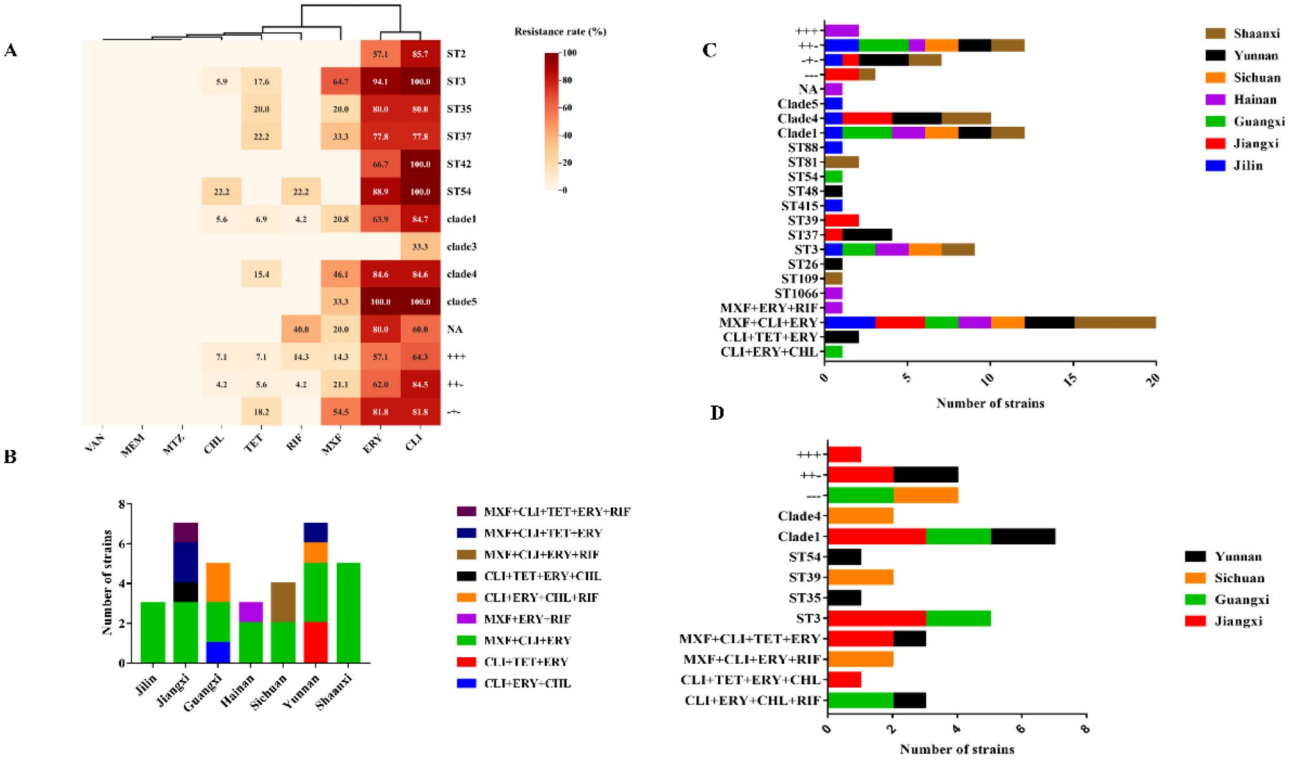

Fig. 7 Antibiotic resistance rate of different molecular genotype and toxin genotype isolates. (**A**). The number of multidrug-resistant isolates in different regions (**B**). The number of regional distribution, molecular genotypes, and toxin gene profiles of multidrug-resistant isolates which resistant to three classes of antibiotics. The order of toxin gene profiles is presented as follows: *tcdA* + *tcdB* + *cdtA/B*+ (+++), *tcdA* + *tcdB* + *cdtA/B*- (++-), *tcdA-tcdB* + *cdtA/B*- (-+-), *tcdA-tcdB-cdtA/B*- (---) (**C**). The number of regional distribution, molecular genotypes, and toxin gene profiles of multidrug-resistant isolates which resistant to four classes of antibiotics. The order of toxin gene profiles is presented as follows: *tcdA* + *tcdB* + *cdtA/B*+ (+++), *tcdA* + *tcdB* + *cdtA/B*- (++-), *tcdA-tcdB-cdtA/B*- (---) (**D**)

7/11). The resistance rate to ERY was highest in Yunnan (81.3%, 13/16) and lowest in Hainan (36.4%, 4/11). The resistance rate of MXF was highest in Hainan (36.4%, 4/11) and lowest in Sichuan (15.4%, 2/13) and Guangxi (15.4%, 2/13). The TET resistance rates in Yunnan and Jiangxi were 25.0% and 21.4%, respectively, whereas isolates from other regions were susceptible. The resistance rate of RIF was highest in Yunnan (9.1%, 1/11) and lowest in Shaanxi (5.9%, 1/17). In addition, two strains from Jiangxi, one from Guangxi and one from Yunnan, were resistant to CHL, while all isolates from the other regions were susceptible (Table 1). There was no statistically significant difference in antibiotic resistance rates between the different regions ($P > 0.05$).

Thirty-four isolates (27.2%, 34/125) were multidrug-resistant (MDR) (Fig. 6). They were distributed in all geographically representative regions, with Yunnan (38.9%, 7/18) and Jiangxi (35.0%, 7/20) having the highest MDR isolation rates, while Shaanxi (22.7%, 5/22) and Hainan (17.6%, 3/17) had the lowest ($P > 0.05$) (Fig. 7B). Notably, the MDR isolation rates of clade 4 was 46.2% (12/26)(Figs. 3 and 7C and D). Among the MDR isolates, 24 (19.2%, 24/125) strains were resistant to three classes of antibiotics and were distributed across all geographical areas (Fig. 7C). The predominant resistance profile was MXF + CLI + ERY (83.3%, 20/24), the main molecular typing was concentrated in clade 1 (50.0%, 12/24) and clade 4 (41.7%, 10/24) ($P < 0.05$), especially ST3 (37.5%, 9/24) and ST37 (16.7%, 4/24) ($P < 0.05$). Furthermore, the main toxin profile was positive for *tcdA* and *tcdB* genes (*tcdA* + *tcdB* + *cdtA/B*-) (50.0%, 12/24) and *tcdB* genes only (*tcdA*-*tcdB* + *cdtA/B*-) (29.2%, 7/24) ($P < 0.05$). Nine (7.2%, 9/125) strains were resistant to four classes of antibiotics distributed in Jiangxi, Guangxi, Sichuan, and Yunnan (Fig. 7D). The main resistance profiles were CLI + ERY + CHL + RIF (33.3%, 3/9) and MXF + CLI + TET + ERY (33.3%, 3/9), which were mainly distributed in ST3 (55.6%,5/9)/clade 1 (77.8%,7/9) ($P > 0.05$), and the main toxin profile was positive for *tcdA* and *tcdB* genes (*tcdA* + *tcdB* + *cdtA/B*-) (44.4%, 4/9) ($P > 0.05$). In addition, only one (0.8%, 1/125) strain from Jiangxi was resistant to five classes of antibiotics (MXF + CLI + TET + ERY + RIF), which was an NTCD isolate of ST48/clade 1 (Fig. 5E).

Discussion

This is the first geographically comprehensive, multicenter, and prospective report on the epidemiology and molecular characteristics of CDI in China. In this study, the prevalence rate was 10.2% (156/1528), which is comparable to the results of a recent meta-analysis conducted in China (11.4%) [28]. At the same time, the study mentioned above showed regional differences within China, with a higher CDI prevalence rate observed in the north (13.6%) than in the south (11.0%) [28]. However, it is noteworthy that within our selected representative regions, Shaanxi and Jilin in the northern region showed a prevalence range of 5.5–13.1%, while the remaining five southern regions showed a prevalence range of 9.6–13.9%. These results suggest that CDI prevalence is changing in all parts of China and that continuous surveillance is needed. As is well known, old age is the main risk factor for CDI. Accordingly, more than half of the isolates in our study were from an elderly population (age ≥ 60 years) (55.2%, 69/125). The lack of strains in the 6–18-year-old age group may be attributed to the relatively limited recruitment numbers. Moreover, annual reports of CDI in North America and Europe suggest that the incidence of CA-CDI is increasing each year, while in our study, the isolation rate of CA-CDI was 5.2% (29/561), which was consistent with a survey of CA-CDI in southwest China from 2013 to 2016 (5.62%), indicating that the incidence of CA-CDI in China has not changed significantly in recent years [29]. Interestingly, the elderly population (age ≥ 60 years) accounted for 59.7% (40/67) of HA-CDI isolates, whereas they accounted for only 41.4% (12/29) of CA-CDI isolates, suggesting that CA-CDI is more common in younger adults than HA-CDI, which is consistent with a previous study [30]. However, considering that some regions only report CA-CDI cases (i.e. Yunnan) and some regions only report HA-CDI cases (i.e. Shaanxi, Jiangxi, and Sichuan), which may affect the results of this study to some extent, we will design a more reasonable protocol in future studies.

The molecular epidemiological features of these *C. difficile* isolates were diverse and included clade 1, clade 4, clade 3, and clade 5. Clade 1 was still the dominant group of most clinical isolates, with ST3 (18.4%, 23/125), ST42 (8.0%, 10/125), and ST54 (7.2%, 9/125) being the top three types. In addition to ST42, other genotypes were also among the dominant clonal groups in previous studies conducted in China [17]. ST42/RT106/clade 1 is frequently isolated in the United Kingdom and the United States, especially in adults, but is rarer in other countries in the world [31]. However, a high proportion (8.0%) of ST42 was detected in this study, mainly originating from Shaanxi and Jilin, which has not been fully recognized in previous domestic studies. ST42/clade 1 was reported to produce higher levels of toxins compared with other prevalent ST types, such as ST2, ST4, ST6, ST13, and ST37, with the general characteristic being the lack of binary toxins(*tcdA* + *tcdB* + *cdtA/B*-), and resistance to CLI and ERY (Figs. 3 and 7A) [32]. The significant number of ST42 indicates that the molecular characteristics of *C. difficile* isolates in China are undergoing continuous change. Hence, it is essential to perpetually enhance routine surveillance in the future, which is also a crucial

means to comprehend the evolutionary characteristics of *C. difficile* isolates.

In addition, clade 4 was dominated by ST37 (7.2%, 9/125) and ST39 (6.4%, 8/125), which is consistent with a previous study [17]. ST37, in particular, has reportedly caused several outbreaks internationally and has become a common pathogenic type of CDI [33]. However, as a typical characteristic type in China, ST37 was still the dominant type in clade 4 in this study, although it was isolated only from Yunnan (five strains), Jiangxi (two strains), and Guangxi (two strains). Because ST37 strain can give rise to a broad spectrum of clinical CDI infections, and its clinical symptoms are reported to be as severe as those induced by the highly virulent RT027, we must persistently heighten our focus on this type of surveillance [34]. In addition, previous studies have shown that ST81 is dominant in first-tier cities such as Beijing and Shanghai and is considered a representative strain of the clade 4 group [17]. However, in this study, only two strains of ST81 were found in Shaanxi Province (Figs. 3 and 5F). This indicates that the molecular typing characteristics of *C. difficile* in China show large regional differences. In the future, special monitoring programs should be formulated based on the molecular characteristics of different regions to cope with the occurrence of CDI.

Moreover, in a retrospective study of CA-CDI in Yunnan from 2013 to 2016, no ST37 type clade 4 was found in 55 *C. difficile* isolates. However, 5 years later, ST37 was found to be the predominant genotype in Yunnan (Figs. 4B and 5G). This shift is likely attributable to the increased frequency of domestic and international exchanges, which facilitate the dissemination of pathogens. This shows that the molecular characteristics of *C. difficile* in the Yunnan region have changed significantly over time, emphasizing the need for continuous surveillance in this region.

Notably, clade 3 was detected in Hainan (two strains) and Sichuan (one strain) in this study, which was previously reported as a rare clade in China and worldwide. It is distinguished from other clades by the clade-specific pathogenicity locus (PaLoc) feature of a Tn6218 insertion [35]. In this study, all strains in clade 3 were ST5, and all toxin types were toxin gene-positive (*tcdA* + *tcdB* + *cdtA/B*+) (Fig. 3). It has been reported that ST5/RT023 is a well-characterized, highly virulent lineage that can cause CDI of the same severity as RT027 and can even cause outbreaks [36]. Previously, ST5 was found in a study in Sichuan, and ST5 was also found in Sichuan in this study, suggesting that attention should be paid to hypervirulent genotypes in the same region [35]. Simultaneously, the emergence of clade 3 should also raise our vigilance, and it is necessary to expedite the improvement of CDI management strategies to cope with the outbreak of CDI. In addition, this study found that most of the new ST types were concentrated in Guangxi (eight strains) and Hainan (one strain). As border areas and free trade ports in China, these two areas have extremely frequent population movements, suggesting that we need to focus on monitoring these areas to understand the evolutionary characteristics of *C. difficile* isolates in a timely manner.

The toxigenic strain was the predominant strain isolated in this study, which was consistent with the global epidemic trend. Notably, NTCD strains accounted for 23.2% of the total population in this study, which is consistent with reports of high NTCD strain colonization rates (10.4–28.6%) in Asian countries [37]. In contrast, NTCD strains are rare in other regions, such as Australia, where NTCD has a prevalence of only 2% [38]. Studies have shown that NTCD strains can reduce the risk of recurrent CDI infections in clinical trials, which could be the reason for the lower prevalence of CDI and even fewer outbreaks in Asia-Pacific countries [39]. However, testing this hypothesis requires more comprehensive epidemiological data.

We found that all *C. difficile* isolates were sensitive to MTZ and VAN, regardless of their evolutionary branching and molecular type, which is consistent with a previous report [17]. There are a few domestic reports of resistance or reduced susceptibility to MTZ or VAN. According to a 2017 CDI survey in eastern China, 15.7% of the isolates were resistant to MTZ [40]. Furthermore, in a survey of *C. difficile* in children in Shanghai, China, 30% of *C. difficile* isolates were resistant to MTZ, and 6% were resistant to VAN [41]. This suggests obvious regional differences in the distribution of antimicrobial resistance in China, which may be related to differences in antimicrobial prescriptions in different geographical regions or the methods used in different antimicrobial susceptibility testing studies. However, resistance to MTZ and VAN has long been reported internationally, and it has been suggested that plasmids may play an important role in resistance [42]. Studies in North America have shown that the VAN resistance is between 1.2% and 2.1%, which has increased over the last decade [43]. A 2022 ECDC report found reduced susceptibility of strain RT027 to MTZ [6]. The recent clinical failure of MTZ for CDI may be attributed to its decreased sensitivity [44]. Currently, MTZ has been adjusted in Western countries as an alternative treatment for CDI when VAN and Fidaxomicin are absent; however, it remains the first-line agent recommended by CDI clinical guidelines in China [45–47]. Therefore, there is a need to increase the surveillance of MTZ and VAN resistance as first-line agents for CDI. Notably, Fidaxomicin is still unavailable in China. However, the newly published CDI clinical guidelines have designated it as the preferred option for recurrent CDI. Once it is available in China, we will consider including Fidaxomicin in the scope of resistance

testing [47]. Furthermore, MEM demonstrated complete sensitivity across all isolates examined in this study, likely due to stringent management practices surrounding its use within China. The sensitivity detection of MEM has significantly contributed to the epidemiological analysis of CDI in China, and this task must continue in the future.

In our study, the resistance rates of CLI (81.3%), ERY (63.6%), and MXF (24.0%) were the highest, which is consistent with the results of previous studies [27, 48]. Widespread use and resistance to antibiotics have been identified as important factors driving the prevalence of CDI [18]. CLI has been reported to be associated with a higher risk of CDI than other antibiotics and has been linked to several CDI outbreaks in Europe and the United States [18]. Our results showed that ST3, ST42, and ST54 all had 100% resistance to CLI, emphasizing the need for increased surveillance to prevent CDI outbreaks (Fig. 7A). Furthermore, exposure to FQs is believed not only to enhance drug resistance but also to elevate the risk of CDI infections by disrupting gut microbiota balance; additionally, it is widely believed to play an important role in CDI transmission [49]. A typical example is the emergence and spread of the RT027 strain, which is associated with the widespread use of FQs and the development of resistance to these antibiotics [50]. In 2018, initiatives were launched in the United States advocating for a reduction in unnecessary FQs use to mitigate FQs resistance rates [49]. Therefore, we cannot ignore the threat of FQs resistance, even though the MXF resistance rate in our data (24.0%) is similar to that in previous studies (24.2%), which may be related to the strict antimicrobial management policy in China in recent years [48].

In addition, 27.2% (34/125) of MDR isolates were found in this study, which is slightly higher than the 17% previously reported in China but much lower than the 55% reported in Europe [48, 51]. Our previous study from 2010 to 2017 showed that the lowest MDR rate was 5.8% in southwestern China and that the highest rate was 43.75% in southern China compared with the rest of the country [48]. In contrast, the distribution of MDR in this study was high in the southwest (Yunnan 38.8%) and low in the south (Hainan 17.6%). This indicates that the prevalence of MDR isolates in different regions has changed significantly in recent years. Extremely high MDR isolation rates have been reported, especially in the clade 4 group, which can be as high as 97% [26]. However, as a typical branch of China, the MDR isolation rates of clade 4 in this study was only 46.2% (12/26) (Figs. 3 and 7C and D), which was far lower than the 97% reported in previous studies [17, 18]. This difference could be due to the recent implementation of a national antibiotic containment policy or the differences in antibiotic prescriptions in different regions. Nevertheless, continuous and comprehensive surveillance of *C. difficile* isolate resistance is essential to detect evolving antibiotic resistance patterns and promptly recognize CDI outbreaks.

As is well-known, a rational antimicrobial management plan can effectively reduce the consumption of antibiotics and alleviate the tense state of antibiotic resistance. To address this issue, China should continue to perform well in the antimicrobial management plan and strengthen the implementation of the policy nationwide. Additionally, with the increasing aging of the population, the incidence of CDI in China will also encounter a tense situation. Based on longitudinal results from the same region, this study reasonably hypothesized that the molecular characteristics of CDI in China might migrate to a certain extent over time in the future, but it might not change significantly in the short term. Continuous comprehensive monitoring is of great significance for characterizing the changing trend of CDI in China.

This study acknowledges certain limitations. Firstly, the basic information of enrolled patients was not comprehensively analyzed, which impeded a thorough analysis of risk factors for CDI in China in recent years. In fact, we planned to collect baseline characteristics of the patients, including antibiotic usage and underlying health conditions, in the original study design; however, due to the heterogeneity of the data collected at different surveillance sites and the large number of missing data, a systematic analysis was not possible. Secondly, there are no stringent criteria regarding the proportion of patient sources recruited, such as hospitalized patients versus community populations or various age groups, which may result in discrepancies in our estimation of the incidence of different types of CDI. Moving forward, we intend to conduct a more standardized, extensive, and continuous investigation and carry out special studies focusing on the disease burden of CDI in China.

Conclusions

In this study, seven representative geographical regions in China were selected for prospective CDI over 1 year. The detected *C. difficile* isolates showed significant differences in molecular characteristics, toxin gene profiles, and antibiotic resistance properties among different regions, and new molecular types were continuously discovered. A comparison based on longitudinal studies showed that molecular types and resistance also changed significantly over time in the same region. Therefore, China should establish a comprehensive, longitudinal, and standardized surveillance protocol for CDI from different sources.

Abbreviations

CDI	Clostridioides difficile infection
CA-CDI	Community-acquired Clostridioides difficile infection
HA-CDI	Hospital-acquired Clostridioides difficile infection

MDR	Multidrug-resistan
ECDC	European Center for Disease Prevention and Control
CLSI	Clinical and Laboratory Standards Institute
MLST	Multilocus sequence typing
ST	Sequence type
RT	Ribotype
RT-qPCR	Real-time fluorescence quantitative PCR
NTCD	Non-toxigenic Clostridioides difficile
ERY	Erythromycin
CLI	Clindamycin
MTZ	Metronidazole
VAN	Vancomycin
MXF	Moxifloxacin
RIF	Rifampicin
TET	Tetracycline
CHL	Chloramphenicol
MEM	Meropenem
FQs	Fluoroquinolones

Supplementary Information

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Supplementary Material 1: Supplementary table 1. Sequences of the primers used in the PCR, Supplementary table 2. Seven housekeeping genes in the MLST approach, Supplementary table 3. Details of all referenced strains in this study

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Author contributions
Lulu Bai and Yuan Wu designed the study and performed the data analyses. Lulu Bai and Telong Xu performed methodology, data analysis, and visualization and wrote the draft preparation. Lulu Bai, Wenzhu Zhang, and Yajun Jiang confirmed the data and revised the manuscript. Wenpeng Gu, Wei Zhao, Yang Luan, Yanfeng Xiong, Nianli Zou, Yalin Zhang, and Ming Luo were accountable for the collection of fecal samples. Jinxing Lu, Bike Zhang, and Yuan Wu supervised the study and contributed to reviewing and editing the manuscript. All authors reviewed the manuscript.

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Data availability
Data is provided within the manuscript or supplementary information files and all data generated and/or analyzed during the current study are available from the corresponding authors on reasonable request.

Declarations

Ethical approval and consent to participate
This experimental procedure adheres to the guidelines of the Helkishin Declaration and has received ethical approval from the Institutional Review Board of the National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention, and each testing hospital was approved by the institutional review board of its unit (IRB protocol No. [ICDC-202113]). All clinical information has been already collected in another project and doesn't involve any identifiable private information; consequently, the Institutional Review Board of the National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention, waived the requirement for informed consent.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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