**Abstract**

**Objective.** The aim of this study was to perform molecular characterization for and determine the antimicrobial susceptibility profiles of *Clostridium difficile* isolated from hospitals during a 4-year period (2009–2013) in China.

**Methods.** Strains of toxigenic *C. difficile* were isolated from patients with diarrhea, and this was followed by typing using multilocus sequence typing (MLST) and testing for susceptibility to 10 antimicrobials by using the E-test. The mechanisms of resistance to moxifloxacin, erythromycin, clindamycin and tetracycline were investigated by PCR.

**Results.** A total of 405 non-duplicate toxigenic *C. difficile* isolates were identified, while 31 sequence types (STs) were identified. A predominant type, ST-54, accounted for 20.2 % of the STs, followed by ST-35 (16.3 %) and ST-37 (13.6 %). We found that 6.2 % of the isolates were binary toxin genes-positive, and 83.7 % of these belonged to ST-5. All of the isolates demonstrated 100 % susceptibility to first-line *Clostridium difficile* infection (CDI) therapies (i.e. metronidazole and vancomycin), while the resistance rates varied for the other antibiotics tested. Two hundred and ninety three (72.3 %) isolates were susceptible to moxifloxacin. All 112 moxifloxacin-resistant isolates had mutations resulting in an amino acid substitution in gyrA and/or gyrB. The *ermB* gene was detected in 86.7 % (241/278) of the erythromycin- and clindamycin-resistant isolates, while the *tetM* gene was present in 97.1 % (85/87) of the tetracycline-resistant isolates.

**Conclusion.** MLST typing revealed a wide variety of STs causing CDI, while ST-54 was the most common ST. All of the isolates were susceptible to metronidazole and vancomycin, while the resistance rates varied for the other antibiotics tested. There were no changes in the trends for the STs and antibiotic susceptibility profiles over 4 years.

**INTRODUCTION**

*Clostridium difficile* is the main pathogen causing healthcare-associated diarrhea in hospitals across Europe and North America, and it causes an estimated 453,000 cases and 29,300 deaths each year in the USA alone [1]. The Centers for Disease Control and Prevention (CDC) also named *C. difficile* as one of the three most urgent health threats related to drug resistance [2]. Antibiotic use, which disrupts the normal flora of the gastrointestinal tract, is one of the most common risk factors for developing *C. difficile* infection (CDI) [3]. Furthermore, *C. difficile* has acquired resistance to antimicrobial agents as well as enhanced virulence and survival properties that may be contributing to dissemination of the pathogen in the environment [4, 5]. Metronidazole and vancomycin are the first-line therapies for treating CDI, but several reports have described reduced susceptibility or resistance to both antibiotics [6, 7]. It is important to know the antimicrobial susceptibility profiles of clinical *C. difficile*, not only with respect to the emergence of epidemic clones, but also with regard to the persistence of specific types over time in hospitals or regions.

Various methods have been used to type *C. difficile* isolates. While in North America pulsed-field gel electrophoresis (PFGE) is the predominant method, in Europe ribotyping by PCR is the most common technique. Multilocus sequence typing (MLST) was developed some years ago, and has allowed easier inter-laboratory comparison of data. In this study, we used MLST to type clinical isolates of...
C. difficile. The molecular epidemiology and antibiotic resistance patterns of C. difficile over the past decade in China have previously been reviewed [8]. Compared with the numerous reports of the epidemiology of CDI in Europe and North America, studies in China have employed smaller sample sizes. In this study, we characterized the basic molecular epidemiology and antimicrobial resistance profiles of toxigenic C. difficile isolates collected in a region of China during 2009–2013. The main aim of this study was to perform molecular characterization for and determine the antimicrobial susceptibility profiles of C. difficile collected from hospitals during a 4-year period in China.

METHODS

Bacterial isolates

This epidemiological study was conducted between 1 September 2009 and 31 August 2013 in three different tertiary hospitals in Zhejiang province. Hospital A is a tertiary teaching hospital with 2500 beds in Hangzhou City, Zhejiang province, while hospital B has 1500 beds and is located in the same city, and hospital C has 1300 beds and is located in Taizhou City, Zhejiang province. Unformed stool samples from adult patients suspected by clinicians to have a CDI were sent to the same clinical microbiology laboratory. Duplicate samples were excluded during the study period. Approximately 0.5 ml (0.5 g) of sample was mixed with 0.5 ml of 95 % ethanol for 30 min at room temperature. Cycloserine–cefoxitin–taurocholate agar (CCFA-TA; Oxoid) supplemented with 7 % sheep blood was used as the selective media, which was prereduced for 30 min in an anaerobic atmosphere before culture. Samples were cultured for 48 h at 35 °C and the isolates were confirmed as C. difficile by MALDI-TOF MS analysis using the Bruker Daltonics Microflex LT system (Bruker Daltonik GmbH, Bremen, Germany) using a published protocol [9].

Our survey was categorized as an ‘active surveillance study’ and therefore did not require ethical approval. None of the test results were used to alter individual care. Each of the participants submitted demographic data anonymously.

Definitions

Diarrhoea was defined as three or more loose stools within 24 h. Patients with diarrhoea, whose stool samples were positive for both C. difficile culture and toxin gene tests, and without other pathogens, were diagnosed as having a CDI [10]. An outbreak of CDI is defined as two or more cases related in time and place over a defined period (28 days) [11].

Detection of toxin genes

Single-colony DNA was extracted by the simplified alkaline lysis method according to a previously described protocol [12]. All of the isolates were tested for the presence of tcdA and tcdB genes by PCR as described previously [13]. The presence of both binary toxin genes, cdtA and cdtB, was detected as described by Stubbs et al. [14].

Multilocus sequence typing (MLST) and analysis

In order to investigate the population structure of the isolates, MLST was performed on all toxigenic isolates according to previously described protocols [15]. Allele designations were obtained through the C. difficile PubMLST batch profile query page (http://pubmlst.org/cdifficile/).

Antibiotic susceptibility testing and mechanisms of resistance

Antibiotic susceptibility testing was performed on subcultures of the isolates of C. difficile for the antibiotics metronidazole, vancomycin, clindamycin, erythromycin, tetracycline and linezolid with E-test strips (bioMérieux, Marcy-l’Étoile, France) ranging from 0.016 to 256 µg ml⁻¹, and for moxifloxacin, levofloxacin, ciprofloxacin and rifampicin with E-test strips ranging from 0.002 to 32 µg ml⁻¹. The E-test strips were applied to the Brucella agar (containing 1 µg ml⁻¹ vitamin K, 5 µg ml⁻¹ hemin and 5 % sheep red blood cells) surface and the plates were incubated in an anaerobic atmosphere for 24–48 h at 35 °C. The MICs were determined in line with the Clinical and Laboratory Standards Institute (CLSI) guidelines [16]. The resistance breakpoints determined by The European Committee on Antimicrobial Susceptibility Testing (EUCAST) was used for vancomycin (≥2 µg ml⁻¹), linezolid (≥4 µg ml⁻¹) and rifampicin (≥32 µg ml⁻¹) (http://www.eucast.org/clinical_breakpoints/), as there are no CLSI recommendations for these antibiotics. C. difficile ATCC 700507 was used as a control. Multidrug resistance was defined as resistance to at least three classes of antibiotics.

The mechanisms of resistance to moxifloxacin, erythromycin, clindamycin and tetracycline were investigated in more detail. The mutations in the gyrA and gyrB genes were tested on the isolates that were resistant to moxifloxacin according to previously described methods by Spigaglia et al. [17]. The erythromycin- and clindamycin-resistant isolates were tested for the presence of ermB, and the tetracycline-resistant isolates were tested for the presence of tetM genes, according to previous publications by Spigaglia et al. [18, 19].

RESULTS

In total, 405 (8.9 %) non-duplicate toxigenic C. difficile isolates were identified from 4550 patients suffering from diarrhoea in 3 hospitals. Among these isolates, 79.0 % were positive for both toxin A and toxin B genes (A+B), while 14.8 % were toxin A gene-negative and toxin B gene-positive (A−B+). Of the isolates, 6.2 % were toxin A−, toxin B− and binary toxin genes-positive (A+B+CDT+) (Table 1).

MLST types

After MLST analysis, we identified 31 C. difficile sequence types (STs) (Table 1). The most prevalent type was ST-54 (20.2 %, 82/405), followed by ST-35 (16.3 %, 66/405) and ST-37 (13.6 %, 55/405). The hypervirulent C. difficile strain ST-1 (NAP1/B1/027) was not identified in our cohort. During the study period, there was no significant change of ST types over the 4 years (Fig. 1).
Antimicrobial resistances

The distribution of the MICs of the antimicrobial agents against *C. difficile* isolates is shown in Table 2. Metronidazole and vancomycin both showed *in vitro* activity against all of the *C. difficile* isolates within a narrow range (Table 2). Six isolates showed resistance to linezolid with an MIC above 4 µg ml⁻¹; four of these were isolated from hospital A and two were isolated from hospital B. Most of the isolates that were resistant to moxifloxacin were from hospital A (79.4 %, 89/112), while 17.9 % (n=20) were from hospital B, and 2.7 % (n=3) were from hospital C. In total, 55.6 % (n=225) of the isolates were resistant to at least three classes of antibiotics, and we defined these as being multidrug-resistant (MDR). Among these MDR isolates, 146 isolates were from hospital A, 48 were from hospital B and 31 were from hospital C, and these accounted for 58.6 %, 47.1 % and 55.6 % of the total isolates of each hospital, respectively. The MDR isolates belonged to 18 different STs, for which the predominant types were ST-37 (29.5 %), ST-54 (21.9 %) and ST-35 (20.5 %). Of the 40 *C. difficile* isolates that were resistant to rifampin, 31 belonged to ST-17. The isolates with binary toxin genes showed lower MICs to the tested antibiotics and were all susceptible to moxifloxacin or tetracycline. The trends in antibiotic susceptibility testing remained similar over the 4 years (Fig. 2).

**Gene mutations**

The *ermB* gene was present in 86.7 % (241/278) of the erythromycin- and clindamycin-resistant isolates, while the *tetM* gene was present in 97.1 % (85/87) of the tetracycline-resistant isolates. All 112 moxifloxacin-resistant isolates had mutations resulting in an amino acid substitution in gryA or gyrB. Out of the moxifloxacin-resistant isolates, 69.6 % (78/
112) only had mutations in \( \text{gyrA} \); 11.6% (13/112) only had mutations in \( \text{gyrB} \) and 18.8% (21/112) had mutations in both \( \text{gyrA} \) and \( \text{gyrB} \). Overall, four kinds of amino acid substitutions in \( \text{gyrA} \) (Thr82/Ile, Thr82/Val, Asp71/Val and Asp81/Asn) and three different substitutions in \( \text{gyrB} \) (Asp426/Asn, Asp426/Val, Arg447/Lys) were identified.

Thr82/Ile was the most common amino acid substitution and was found in various STs.

**DISCUSSION**

In this study, we characterized 405 clinical isolates of toxigenic \( \text{C. difficile} \) from 3 different tertiary hospitals over a 4-year period (2009–2013). MLST typing revealed a wide variety of types causing CDI. ST-54 (PCR ribotype 010) was the most common ST, followed by ST-35 (PCR ribotype 017) and ST-37 (PCR ribotype 017). Looking at reports from a previous study [9], it is interesting to note that ST-54 is virtually unique to China, although the reason for this is unknown. Although neither ST-1 (NAP1/B1/027) nor ST-11 (NAP7.8/BK/078) were detected during this study, 25 (6.2%) isolates with binary toxin genes (cdtA/cdtB) were found, and 83.7% (21/25) of these isolates belonged to ST-5 (PCR ribotype 023), which is among the 10 most prevalent ribotypes in Europe [20]. Compared with the rate of binary toxins (21%) found among isolates (21/100) in a study in Spain [21], the rate in the current study was lower, but it was higher than that found in a previous study at our hospital [9].

Vancomycin and metronidazole are the only two antimicrobial agents used to treat \( \text{C. difficile} \) infection in China. There are some reports regarding metronidazole heteroresistance and reduced susceptibility in clinical \( \text{C. difficile} \) [22, 23], while isolates with reduced susceptibility to vancomycin were reported in Asia and Europe [6, 24]. However, it is important to note that reduced susceptibility to vancomycin and metronidazole has not been associated with adverse clinical outcomes, likely because the luminal concentrations still far exceed the MIC values. In this study, we did not observe isolates with resistance to metronidazole or vancomycin. The highest MIC values for vancomycin and metronidazole (0.38/0.75 and 0.047/0.125 µg ml\(^{-1}\), respectively) were similar to those previously reported in China [25].

Linezolid has been reported to reduce the toxin levels of \( \text{C. difficile} \) [26]. Resistance to linezolid has occasionally been found in clinical isolates of \( \text{C. difficile} \) [27]. Although linezolid-resistant \( \text{C. difficile} \) isolates are not common, their selection and transmission could lead to an increase in \( \text{C. difficile} \) infection, mainly in clinical settings where linezolid is used frequently. In a report from Spain, nine isolates (1%) showed high linezolid MIC values (6 µg ml to 16 µg ml\(^{-1}\)) and six of these belonged to RT017 (ST-37) [28]. In our study, six isolates (1.5%) from six different patients were resistant to linezolid and three of these belonged to ST-37 in the same hospital (A), but there was no evidence to indicate that these ST-37 isolates belonged to one clonal cluster, since each of these isolates had different linezolid MIC values (6 µg ml\(^{-1}\), 16 µg ml\(^{-1}\) and ≥256 µg ml\(^{-1}\), respectively). As Marin et al. suggested, the cfr gene may be a possible mechanism of resistance to linezolid in \( \text{C. difficile} \) [28], and the mechanisms of linezolid resistance in our isolates need to be further studied.

The acquisition of fluoroquinolone resistance is associated with the emergence of hypervirulent isolates, such as BI/NAP1/027 [29]. Our data showed that all of the isolates were resistant to ciprofloxacin, with the highest MICs being...
### Table 2. Antimicrobial resistance rates of toxigenic *C. difficile* isolates for the common STs

<table>
<thead>
<tr>
<th>STs (no. of isolates)</th>
<th>Vancocin (µg ml⁻¹)</th>
<th>Metronidazole (µg ml⁻¹)</th>
<th>Linezolid (µg ml⁻¹)</th>
<th>Clindamycin (µg ml⁻¹)</th>
<th>Rifampicin (µg ml⁻¹)</th>
<th>Giprofloxacin (µg ml⁻¹)</th>
<th>Levofloxacin (µg ml⁻¹)</th>
<th>Monixoflacin (µg ml⁻¹)</th>
<th>Tetracycline (µg ml⁻¹)</th>
<th>Erythromycin (µg ml⁻¹)</th>
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<td><strong>Total isolates (n=405)</strong></td>
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<td>n (%)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>6 (1.5)</td>
<td>29.3 (72.8)</td>
<td>40 (9.9)</td>
<td>405 (100)</td>
<td>350 (86.4)</td>
<td>112 (27.7)</td>
<td>87 (21.5)</td>
<td>274 (67.7)</td>
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<td>≤0.016–1.5</td>
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<td>0.094–256</td>
<td>≤0.002–32</td>
<td>≥32</td>
<td>0.016–32</td>
<td>0.094–32</td>
<td>0.016–256</td>
<td>0.047–256</td>
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<td><strong>Hospital A (n=249)</strong></td>
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<td>n (%)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>4 (1.6)</td>
<td>177 (71.1)</td>
<td>40 (16.1)</td>
<td>249 (100)</td>
<td>196 (78.7)</td>
<td>89 (35.7)</td>
<td>61 (24.5)</td>
<td>165 (66.3)</td>
</tr>
<tr>
<td>Range</td>
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<td>≤0.016–1.5</td>
<td>≤0.016–256</td>
<td>0.094–256</td>
<td>≤0.002–32</td>
<td>≥32</td>
<td>0.016–32</td>
<td>0.094–32</td>
<td>0.016–256</td>
<td>0.064–256</td>
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<td><strong>Hospital B (n=102)</strong></td>
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<td>n (%)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>2 (2.0)</td>
<td>80 (78.4)</td>
<td>0 (0)</td>
<td>102 (100)</td>
<td>98 (96.1)</td>
<td>20 (19.6)</td>
<td>20 (19.6)</td>
<td>66 (64.7)</td>
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<td>≤0.016–1.5</td>
<td>≤0.016–4</td>
<td>0.19–256</td>
<td>≤0.002–0.002</td>
<td>≥32</td>
<td>0.5–32</td>
<td>0.125–32</td>
<td>0.032–256</td>
<td>0.047–256</td>
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<tr>
<td><strong>Hospital C (n=54)</strong></td>
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<tr>
<td>n (%)</td>
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<td>0 (0)</td>
<td>0 (0)</td>
<td>36 (66.7)</td>
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<td>54 (100)</td>
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<td>43 (79.6)</td>
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<td>0.094–256</td>
<td>≤0.002–0.002</td>
<td>≥32</td>
<td>0.38–32</td>
<td>0.094–32</td>
<td>0.047–256</td>
<td>0.125–256</td>
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<td>n (%)</td>
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<td>0 (0)</td>
<td>0 (0)</td>
<td>73 (89.0)</td>
<td>0 (0)</td>
<td>82 (100)</td>
<td>72 (87.8)</td>
<td>16 (19.5)</td>
<td>7 (8.5)</td>
<td>66 (80.5)</td>
</tr>
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<td>≤0.023–1</td>
<td>0.25–256</td>
<td>≤0.002–0.002</td>
<td>≥32</td>
<td>1.5–32</td>
<td>0.094–32</td>
<td>0.064–256</td>
<td>0.064–256</td>
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<td><strong>ST-35 (n=66)</strong></td>
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<tr>
<td>n (%)</td>
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<td>0 (0)</td>
<td>2 (3.0)</td>
<td>60 (90.9)</td>
<td>6 (9.5)</td>
<td>66 (100)</td>
<td>59 (89.4)</td>
<td>23 (34.8)</td>
<td>29 (43.9)</td>
<td>59 (89.4)</td>
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<td>Range</td>
<td>0.064–1.5</td>
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<td>0 (0)</td>
<td>3 (5.5)</td>
<td>47 (85.5)</td>
<td>31 (56.4)</td>
<td>55 (100)</td>
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<td>37 (77.1)</td>
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<td>n (%)</td>
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<td>0 (0)</td>
<td>0 (0)</td>
<td>18 (49.9)</td>
<td>0 (0)</td>
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<td>0.94–256</td>
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<td>≥32</td>
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<td><strong>CD with binary toxin (n=25)</strong></td>
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<td>0 (0)</td>
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<td>3 (12.0)</td>
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<td>0.25–1</td>
<td>1.5–256</td>
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<td>≥32</td>
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<td>n (%)</td>
<td>0.38</td>
<td>0.38</td>
<td>0.38</td>
<td>1.5</td>
<td>≤0.002</td>
<td>≥32</td>
<td>1.5</td>
<td>0.75</td>
<td>≥32</td>
<td>0.38</td>
</tr>
</tbody>
</table>

n (%) are the number and percentage of resistant isolates, respectively.

MIC, minimum inhibitory concentration; MIC⁵⁰/⁹⁰, MIC for 50 and 90 % of the isolates, respectively.
The proportion of resistance to moxifloxacin (27.7%) is in agreement with that in other studies [30], but does contrast significantly with a report from Australia [31]. As Riley et al. suggested, the low proportion of fluoroquinolone resistance in Australia is likely due to a lack of epidemic fluoroquinolone-resistant ST-1 (NAP1/B1/027) and a conservative policy on fluoroquinolone use [32]. The principal mechanism of moxifloxacin resistance in C. difficile has been determined to be amino acid substitutions in GyrA and GyrB. All moxifloxacin-resistant isolates carried a mutation in either gyrA and/or gyrB. All moxifloxacin-resistant isolates carried a mutation in either gyrA and/or gyrB. However, the gyrA mutation occurred more frequently than the gyrB one, which is a similar finding to those in other reports [17]. There was no evidence to indicate that isolates with double mutations in gyrA and gyrB showed higher MICs for moxifloxacin than isolates with only one mutation. In comparison with a study from Shanghai, China from 2009 [33], the resistant rates we observed were lower for moxifloxacin (27.7 vs 61.8%) but higher for levofloxacin (86.4 vs 66.4%).

Clindamycin was the first antimicrobial to be associated with the development of CDI in humans [34]. The mechanism and the pattern of resistance are similar to those for erythromycin [35]. Resistance to erythromycin and/or clindamycin is still the most common phenotype in C. difficile isolates isolated in China and other parts of the world [6, 33]. In this study, nearly 70% of the isolates were resistant to erythromycin and clindamycin. Ribosomal methylation is considered to be the most widespread mechanism of resistance, and is mediated by erm genes [36], while ermB is the most prevalent class. Although most of the isolates with resistance to erythromycin and clindamycin were positive for the ermB gene in this study, 10% of the isolates lacked it. Compared with other reports from Europe [35], the resistance rate to tetracycline was high in our study (21.5 vs 11.9%), but lower than in a report from a tertiary hospital in China [37]. These resistant isolates belonged to various STs, but the highest proportion of resistance was found among isolates of ST-35 and ST-37. Most of the resistant isolates were positive for the tetM gene, which is the most widespread gene coding for ribosomal protection proteins.

The binary toxin (CDT) produced by C. difficile may be an important virulence factor of C. difficile, however, the significance of which remains to be determined. Several clinical studies have suggested an association between the presence of binary toxin in infecting C. difficile isolates and increased patient mortality [38]. The prevalence of binary toxin genes in this study was lower than that of Danish (23%) and Taiwanese isolates (13%), but was similar to those in another report from mainland China [39]. Compared with the other most common STs, the isolates with CDT did not show higher resistance rates to the tested antibiotics.

The patterns of resistance of C. difficile to antimicrobial agents differed depending on the ST. Although ST-54 was the most common type, these isolates had higher rates of resistance to clindamycin (89%) and erythromycin (67.7%), but lower rates of resistance to rifampin and moxifloxacin compared with other common STs. However, the isolates of ST-37, which was the third most common type in this study, were resistant to multiple antibiotics, including clindamycin, fluoroquinolones, erythromycin, rifampin and tetracycline. A total of 78.2% of ST-37 isolates (43/55) were resistant to 3 or more classes of antibiotics.

There are some limitations to this study. Firstly, only one isolate was collected from each patient, as samples from patients with recurrence or readmission were excluded. This means that this study can offer no data on the isolates found in incidents of CDI recurrence. Secondly, the antibiotics employed in the susceptibility testing did not include cephalosporins due to a lack of the requisite E-test strips. Cephalosporin resistance, which is regarded as a risk factor for CDI, should be included in further studies. Thirdly, the E-test is an easy method to detect antimicrobial susceptibility, but it may not be the optimal method for assessing resistance to some drugs, such as metronidazole [40]. Fourthly, PCR ribotyping, which is considered to be the standard typing method in
Europe, was not performed in the study. There are several studies, such as that by Knetsch et al. [41], which have revealed a good correlation between PCR ribotyping and MLST. Lastly, fidaxomicin, which has been approved by the US Federal Drug Administration and the European Medicines Agency for the treatment of CDI [42], was not included in this study. In a recent report in China, fidaxomicin exhibited high antimicrobial activity against all of the tested C. difficile isolates, indicating its potential as a new drug for treating Chinese CDI patients [43].

**Conclusion**

In this study, we assessed the antibiotic resistance profiles and sequence types of 405 non-duplicate clinical isolates of *C. difficile* from three different hospitals in China. The most prevalent type was ST-54 (20.2%, 82/405), followed by ST-35 (16.3%, 66/405) and ST-37 (13.6%, 55/405). All of the isolates were susceptible to metronidazole and vancomycin, while the resistance rates varied for the other antibiotics tested. Compared with the other most common STs, the isolates with binary toxin did not show higher resistant rates than the tested antibiotics. A high rate of multidrug resistance was observed among ST-37 isolates (78.2%, 43/55). There were no changes in the trends for the STs and antibiotic susceptibility profiles over the 4 years.

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**Conflicts of interest**

The authors declare that there are no conflicts of interest.

**Ethical statement**

This article does not contain any studies with human participants or animals performed by any of the authors.

**References**


