


# In vitro Studies of Non-Diphtheriae *Corynebacterium* Isolates on Antimicrobial Susceptibilities, Drug Resistance Mechanisms, and Biofilm Formation Capabilities

Wei Sun\*, Liyan Ma\*, Yana Li, Ying Xu, Jingjuan Wei , Lei Sa, Xinxin Chen, Jianrong Su

Department of Clinical Laboratory, Beijing Friendship Hospital, Capital Medical University, Beijing, 100050, People's Republic of China

\*These authors contributed equally to this work

Correspondence: Jianrong Su, Department of Clinical Laboratory, Beijing Friendship Hospital, Capital Medical University, No. 95, Yong'an Road, Xicheng District, Beijing, 100050, People's Republic of China, Email youyilab@163.com

**Objective:** This study aimed to investigate the antimicrobial susceptibilities, drug resistance mechanisms, and biofilm formation capacities of non-diphtheriae *Corynebacterium* strains isolated from sterile midstream urine of hospitalized patients with clinical urinary tract infections (UTIs).

**Methods:** A total of 45 non-diphtheriae *Corynebacterium* isolates were recovered from sterile midstream urine. The available data of 45 patients were collected. Minimum inhibitory concentrations (MICs) of 10 commonly used antibiotics were determined. Meanwhile, the molecular resistance mechanisms of each agent were performed through PCR with specific primers. Moreover, the biofilm formation capability of each isolate on abiotic surfaces was detected with the MTT method.

**Results:** In this study, the most prevalent three species were *C. striatum* (15/45, 33.3%), *C. glucuronolyticum* (9/45, 20.0%) and *C. urealyticum* (8/45, 17.8%). These three species also accounted for most renal and ureteral calculi cases. Male patients older than 50 years, especially those with underlying diseases, were more susceptible to non-diphtheriae *Corynebacterium* infection. All the 45 isolates were 100% susceptible to vancomycin and linezolid, but highly resistant to macrolide–lincosamide–streptogramin B (MLSB), fluoroquinolones, tetracyclines and  $\beta$ -lactams with corresponding mechanisms. The detection rate of multidrug-resistant (MDR) non-diphtheriae *Corynebacterium* is 91.1%. All isolates are able to form biofilm on abiotic surfaces, except those of *C. urealyticum*, *C. tuberculostearicum* and *C. jeikeium*. Isolates of *C. glucuronolyticum* and *C. Striatum* possessed the strongest biofilm formation capacity. *C. amycolatum* could form biofilm, but varied greatly among different isolates.

**Conclusion:** *C. striatum*, *C. glucuronolyticum* and *C. urealyticum* were the most prevalent species relevant to UTIs. The high occurrence of MDR isolates and high diversities in resistance profiles, and the distinctive abilities of biofilm formation highlighted the urgency for identification to species level. We should pay more attention to the drug resistance profiles of non-diphtheriae *Corynebacterium*, which would help improve empirical antibiotic therapy and reduce drug resistance transmission.

**Keywords:** non-diphtheriae *Corynebacterium*, antimicrobial susceptibility, resistance, mechanism, biofilm

## Introduction

Urinary tract infections (UTIs) are a severe public health issue and are usually caused by a range of pathogens, including Gram-negative and Gram-positive bacteria, as well as certain fungi.<sup>1</sup> Increased antibiotic resistance and recurrence rates of uropathogens pose a substantial economic burden worldwide.<sup>2–6</sup> Although a wide variety of Gram-positive bacteria are common inhabitants of the human urinary tract, there are increasingly presented evidence that Gram-positive bacteria-related infections might be more prevalent than previously anticipated and might be important uropathogens in their own right.<sup>2,7</sup> Some species might be easily overlooked because routine diagnostic methods usually miss them.<sup>8–10</sup>

The gram-positive rod-shaped coryneform bacteria, other than *Corynebacterium diphtheriae* are such kind of gram-positive microorganisms, which have successfully attracted people's attention because of their relevance with various infections in recent years.

Previous studies have identified that non-diphtheriae *Corynebacterium* strains can cause superficial, as well as invasive infections, in both immunocompromised and immunocompetent patients.<sup>2,7,11–16</sup> There are also increasing reports on non-diphtheriae *Corynebacterium*-related UTIs, but most studies were case reports or focused on one species only.<sup>8,14,17–21</sup> Very few studies have identified non-diphtheriae *Corynebacterium* involving infections at the species level.<sup>13,22</sup> The increase of clinical relevance, unpredictable antimicrobial susceptibilities to the currently available agents, as well as little understanding of potential drug resistance mechanisms all make it necessary to move forward in this line of research.

Therefore, this study aimed to systematically investigate the characteristics of antimicrobial susceptibilities and related drug resistance mechanisms, as well as the biofilm formation capacities among different species of non-diphtheriae *Corynebacterium* isolated from sterile midstream urine.

## Materials and Methods

### Organism Collection and Identification

During 2018–2020, 45 strains of non-diphtheriae *Corynebacterium* were isolated from clinical midstream urine specimens, submitted to a university hospital's microbiology laboratory for routine culture. Those grown in significant numbers ( $>3.0 \times 10^4$  CFU/mL) (colony-forming unit, CFU) and in pure culture were collected and stored in skim milk at  $-80^\circ\text{C}$  for further investigation. All non-duplicate isolates were identified by VITEK 2 Compact using the Anaerobe and *Corynebacterium* Identification Card (BioMérieux, France). Species identification was confirmed through the matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) (BioMérieux, France) by following the manufacturer's instructions. All strains were routinely cultured on Columbia agar supplemented with 5% sheep blood (OXOID) at  $37^\circ\text{C}$  for 24 h unless otherwise specified.

### Antimicrobial Susceptibility Testing

Antimicrobial susceptibility profiles were determined by minimum inhibitory concentration (MIC) using broth micro-dilution method.<sup>23</sup> Ten antibiotics tested in this study include gentamicin (1–32  $\mu\text{g/mL}$ ), rifampicin (0.5–64  $\mu\text{g/mL}$ ), erythromycin (0.25–64  $\mu\text{g/mL}$ ), clindamycin (0.5–64  $\mu\text{g/mL}$ ), ciprofloxacin (1–64  $\mu\text{g/mL}$ ), penicillin (0.12–32  $\mu\text{g/mL}$ ), cefotaxime (1–64  $\mu\text{g/mL}$ ), vancomycin (0.12–2  $\mu\text{g/mL}$ ), linezolid (0.12–2  $\mu\text{g/mL}$ ) and tetracycline (2–16  $\mu\text{g/mL}$ ). The antimicrobial susceptibility test and result interpretation were performed according to the third edition of the Clinical and Laboratory Standards Institute guidelines M45.<sup>24</sup> MDR was defined as acquired non-susceptibility to at least one agent in three or more antimicrobial categories.<sup>25</sup> In this study, the cation adjusted Mueller–Hinton broth (CAMHB, OXOID) supplemented with 5% sterile lysed horse blood was used to prepare bacterial inoculums and dilute various antibiotics. Plates were incubated aerobically at  $37^\circ\text{C}$  and examined for growth at 24h and 48 h, due to the slow growth of some species (*C. jeikeium* and *C. tuberculostrictum*). ATCC25922 (American Type Culture Collection) and ATCC49619 were used as controls. All experiments were conducted three times on different days.

### PCR Amplification of Drug Resistance Genes

Bacterial genomic DNA was extracted from pure colonies growing on 5% sheep blood agar plates using a Bacterial Genomic DNA Extraction Kit (Takara, Japan). The genomic DNA was stored at  $-20^\circ\text{C}$  until used for PCR experiments.

The drug resistance relevant genes *ermX*, *ermB*, *mef(A-E)*, *aac(3)-XI*, *bla*, *ampC*, *gyrA*, *tetA*, *tetB* and *rpoB* were amplified by PCR using specific primer pairs (Table 1).<sup>26–28</sup> DNA amplification by PCR was carried out in a reaction volume of 25  $\mu\text{L}$ , with 2  $\mu\text{L}$  of template DNA, 1  $\mu\text{L}$  each of 10 pmol/ $\mu\text{L}$  forward and reverse primer, 12.5  $\mu\text{L}$  of 2×Multiplex PCR Mix, and 8.5  $\mu\text{L}$  of distilled water. The PCR was performed using a ABI7500 DNA Analyzer (Applied Biosystems, USA) with the following program: Initial denaturation step of 5 min at  $95^\circ\text{C}$ , followed by 35 cycles of 30s at  $94^\circ\text{C}$ , 1 min at annealing temperature, and 1 min at  $72^\circ\text{C}$ . The final extension was performed for 10 min at  $72^\circ\text{C}$ . The

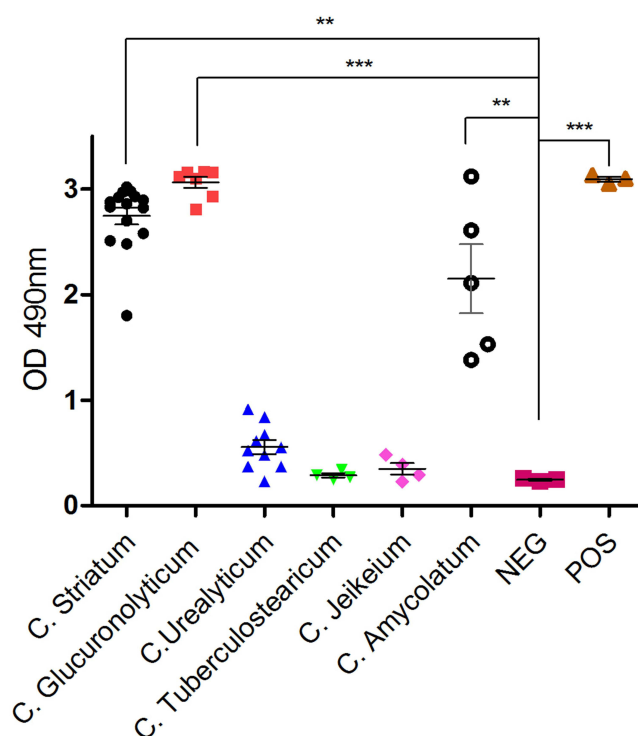
**Table I** Primers for Analysis of Resistance Genes Used in This Study

Target Genes	Related Resistance	Primers	DNA Sequence (5'–3')	T <sub>m</sub> (°C)	Size (bp)	Reference
<i>erm(X)</i>	Erythromycin, Clidamycin	<i>erm(X)</i> -F <i>erm(X)</i> -R	AACCATGATTGTGTTTCTGAACG ACCAGGAAGCGGTGCCCT	57	560	[24]
<i>erm(B)</i>	Erythromycin, Clidamycin	<i>erm(B)</i> -F <i>erm(B)</i> -R	GAAAAGGTACTCAACCAAATA AGTAACGGTACTTAAATTGTTTAC	52	639	[24]
<i>mef(A-E)</i>	Erythromycin, Clidamycin	<i>mef(A-E)</i> -F <i>mef(A-E)</i> -R	GCAAATGGTGTAGGTAAGACAAC TAAACAAATGTAGTGACTA	52	399	[24]
<i>aac(3)-XI</i>	Gentamicin	<i>aac(3)-XI</i> -F <i>aac(3)-XI</i> -R	ATGACTACAACCAACGAGATC CTAAAGCTCCCGGATGTAGAG	52	452	[25]
<i>bla</i>	Penicillin, Cefotaxime	<i>bla</i> -F <i>bla</i> -R	CAGTCTAGCCACTTCGCCAAT TGACTGCACGGATGGAGATGG	55	808	[22]
<i>ampC</i>	Penicillin, Cefotaxime	<i>ampC</i> -F <i>ampC</i> -R	CAATCGGATTCTCGGTCGCT TGGTTCGCGTGATGTTTTTCG	55	965	[22,26]
<i>gyrA</i>	Ciprofloxacin	<i>gyrA</i> -F <i>gyrA</i> -R	GCGGCTACGTAAAGTCC CCGCCGGAGCCGTTTCA	60	337	[27]
<i>tetA</i>	Tetracycline	<i>tetA</i> -F <i>tetA</i> -R	TTAGCGTTCGGCGACCTGG GGTGGTCTTGCTGCCCTCA	60	552	[28]
<i>tetB</i>	Tetracycline	<i>tetB</i> -F <i>tetB</i> -R	ACGGTGTTCAACGCCCTGTT AACTGGGTGCCTTCAGGGTC	59	506	[28]
<i>rpoB</i>	Rifampin	<i>rpoB</i> -F <i>rpoB</i> -R	CTGATCCAGAACCAGGTCCG GACGTACTCCACCACACCAG	55	811	[28,29]

PCR products (5µL) were visualized by electrophoresis on a 1.5% agarose gel with a 2000-bp ladder (DL2000, Takara, Japan) as the molecular size marker. The 16S rRNA was used as a positive control for indicating the successful DNA extraction. The PCR products of *gyrA* gene and *ropB* gene were sequenced for mapping mutations associated with ciprofloxacin and rifampicin resistance, respectively (Sangon Biotech, Beijing, China). The obtained nucleotide sequences were analyzed and compared with corresponding susceptible ones through BLAST searches against the NCBI database ([www.ncbi.nlm.nih.gov/BLAST](http://www.ncbi.nlm.nih.gov/BLAST)).

## Biofilm Formation

Biofilm formation capacities of non-diphtheriae *Corynebacterium* isolates were detected with the MTT method in 96-well microtiter plates as previously reported with minor modifications.<sup>29,30</sup> Cell suspensions were prepared with Tryptic Soy Broth (TSB) using pure colonies with inoculum equivalent to a 0.5 McFarland standard ( $1.5 \times 10^8$  CFU/mL). After proper dilutions, 200 µL of bacterial suspension was added to each well with the final concentration of approximately  $3 \times 10^5$  colony-forming units (CFU)/mL. Wells containing TSB only were set as the negative control, whereas wells containing ATCC 43300 grown in TSB were set as the positive control. After incubation for 48 h at 37°C, the content of each well was removed carefully, and next all wells were gently washed twice with 200 µL of PBS (0.01M, pH 7.2). Then, 90 µL of TSB and 10 µL of MTT reagent were added to each well for continued 4 h incubation at 37°C. Then, the supernatant of all wells was discarded, and 110 µL of formazan solution was added to each well. After shaking on a low-speed shaking table for 10 minutes, the fully dissolved crystals were measured for the absorbance value of each well at 490 nm using an enzyme immunosorbent assay reader (BioRad, model 550). Results were obtained by subtracting the negative controls' average ODs from experimental wells' average ODs. And biofilm formation abilities of various species of the 45 isolates are listed in Figure 1. Each experiment was carried out in triplicate and repeated three times on different days.



**Figure 1** Biofilm formation capabilities of different species of non-diphtheriae *Corynebacterium* isolates. \*\* $P < 0.01$  and \*\*\* $P < 0.001$  vs the negative control. **Abbreviations:** NEG, negative control; POS, positive control.

## Statistical Analysis

Statistical analyses were carried out by using IBM SPSS Software Version 20.0 (IBM, Armonk, NY). The chi-square or Fisher's exact test was used to assess categorical variables, which are presented as frequencies and percentages. Normally distributed continuous variables were presented as means  $\pm$  SDs (standard deviations), and analyzed by one-way ANOVA. Continuous variables that were not normally distributed were shown as medians and interquartile ranges (IQRs). A p-value of less than 0.05 ( $p < 0.05$ ) was regarded as statistically significant for differences in the data. A particular case's incomplete data were excluded.

## Results

### Clinical Features of Different Species of Non-Diphtheriae *Corynebacterium* isolates

Data for age, gender, and underlying diseases were obtained from the available medical records of the 45 patients, as listed in Table 2. Among non-diphtheriae *Corynebacterium* strains, *C. striatum* was the most prevalent species (15/45, 33.3%), followed by *C. glucuronolyticum* (9/45, 20.0%), and *C. urealyticum* (8/45, 17.8%). The remaining three species occupied a relatively small proportion, *C. tuberculostearicum* (4/45, 8.9%), *C. jeikeium* (4/45, 8.9%) and *C. amycolatum* (5/45, 11.1%). Elderly patients, especially those older than 50 years, were more susceptible to non-diphtheriae *Corynebacterium* infections, such as *C. striatum* (13/15, 84.4%), *C. tuberculostearicum* (4/4, 100%), *C. jeikeium* (3/4, 75%), *C. amycolatum* (4/5, 80%). However, there was no apparent aggregation among different age groups for *C. glucuronolyticum* and *C. urealyticum* isolates. Interestingly, from the clinical data, we noticed that *C. striatum* (5/15, 33.3%) isolates were more related to renal calculi, while *C. glucuronolyticum* (4/9, 44.4%) and *C. urealyticum* (7/8, 87.5%) isolates were more relevant to ureteral calculi. Meanwhile, it was shown that male patients had a higher likelihood of contracting non-diphtheriae *Corynebacterium* strains (>60%), with the exception of *C. amycolatum* (40%). Additionally, *C. jeikeium* was entirely (100%) isolated from male patients.

### Antimicrobial Susceptibility Analysis

As shown in Table 3, all 45 isolates of non-diphtheriae *Corynebacterium* were susceptible to vancomycin and linezolid. Considering the MIC<sub>90</sub> values, the most active compound was vancomycin (MIC<sub>90</sub> = 0.5  $\mu$ g/mL), followed by linezolid

**Table 2** Demographic and Clinical Characteristics of Non-Diphtheriae *Corynebacterium* Isolates

Patient Characteristics	<i>C. striatum</i> (n=15) Median (P25, P75)	<i>C. glucuronolyticum</i> (n=9) Median (P25, P75)	<i>C. urealyticum</i> (n=8) Median (P25, P75)	<i>C. tuberculostearicum</i> (n=4) Median (P25, P75)	<i>C. jeikeium</i> (n=4) Median (P25, P75)	<i>C. amycolatum</i> (n=5) Median (P25, P75)	P value
Age median (P25, P75)	68 (56, 91)	32 (30, 54)	46 (37, 64)	77 (57, 94)	54 (36, 93)	71 (67, 77)	0.011
Range (years)	4–93	6–85	35–64	57–101	36–95	48–94	
≤30	1 (6.7%)	2 (22.2%)	0	0	0	0	
30–40	1 (6.7%)	4 (44.4%)	2 (25.0%)	0	1 (25.0%)	1 (20.0%)	
40–50	0	0	3 (37.5%)	0	0	0	
50–60	4 (26.7%)	1 (11.1%)	1 (12.5%)	1 (25.0%)	1 (25.0%)	0	
>60	9 (60.0%)	2 (22.2%)	2 (25.0%)	3 (75.0%)	2 (50.0%)	4 (80.0%)	
Male, %	13 (86.7%)	8 (88.9%)	5 (62.5%)	3 (75.0%)	4 (100%)	2 (40.0%)	0.174
Admission Diagnosis							
Renal calculi	5 (33.3%)	1 (11.1%)	0	1 (25.0%)	0	0	0.210
Ureteral calculi	0	4 (44.4%)	7 (87.5%)	0	0	1 (20.0%)	0.0001
Renal diseases except calculi	1 (6.7%)	0	0	1 (25.0%)	0	1 (20.0%)	0.405
Urinary tract infection	2 (13.3%)	1 (11.1%)	1 (12.5%)	0	3 (75.0%)	0	0.027
Hydronephrosis	1 (6.7%)	1 (11.1%)	0	0	0	0	0.836
Cardiovascular diseases	2 (13.3%)	0	0	0	0	1 (20.0%)	0.509
Malignancy	3 (20.0%)	1 (11.1%)	0	1 (25.0%)	1 (25.0%)	1 (20.0%)	0.784
Edema	0	1 (11.1%)	0	1 (25.0%)	0	1 (20.0%)	0.326
Dementia	1 (6.7%)	0	0	0	0	0	0.843

**Table 3** Antimicrobial Susceptibilities of 45 Non-Diphtheriae *Corynebacterium* Against ten Antimicrobial Agents

Antibiotics	MIC range (µg/mL)	All <i>Corynebacterium</i> spp. (n=45)			<i>C. striatum</i> (n=15)			<i>C. glucuronolyticum</i> (n=9)			<i>C. urealyticum</i> (n=8)			<i>C. tuberculostrictum</i> (n=4)			<i>C. jeikeium</i> (n=4)			<i>C. amycolatum</i> (n=5)		
		MIC <sub>50</sub>	MIC <sub>90</sub>	R%	MIC <sub>50</sub>	MIC <sub>90</sub>	R%	MIC <sub>50</sub>	MIC <sub>90</sub>	R%	MIC <sub>50</sub>	MIC <sub>90</sub>	R%	MIC <sub>50</sub>	MIC <sub>90</sub>	R%	MIC <sub>50</sub>	MIC <sub>90</sub>	R%	MIC <sub>50</sub>	MIC <sub>90</sub>	R%
Penicillin	≤0.12 to ≥4	64	>64	66.7	>32	>32	80.0	2	8	22.2	32	>64	75.0	64	>128	100	>64	>64	100	2	>64	40.0
Cefotaxime	≤1 to ≥4	8	>64	53.3	>64	>64	80.0	<1	<1	0	2	>64	50.0	16	64	75.0	>64	>64	100	2	>64	20.0
Gentamicin	≤4 to ≥16	8	32	22.2	4	8	6.7	4	32	22.2	16	64	50.0	<4	<16	0	4	>64	50.0	32	32	20.0
Erythromycin	≤0.5 to ≥2	>64	>64	93.3	>64	>64	86.7	>64	>64	88.9	>32	>32	100	>32	>32	100	>32	>32	100	>32	>32	100.0
Ciprofloxacin	≤1 to ≥4	32	>64	93.3	32	64	93.3	32	>64	100	32	64	87.5	4	32	75.0	32	>64	100	16	>64	100.0
Tetracycline	≤4 to ≥16	<4	64	37.8	4	64	33.3	32	64	77.8	16	32	37.5	<4	<4	0	4	>64	25.0	16	32	20.0
Clindamycin	≤0.5 to ≥4	16	>64	91.1	16	>64	80.0	>64	>64	88.9	>64	>64	87.5	16	>64	100	16	>64	100	16	>64	100.0
Rifampin	≤1 to ≥4	<1	32	13.3	1	4	13.3	<1	<1	0	<1	8	12.5	<4	16	50.0	<1	32	25.0	<1	<4	0.0
Linezolid	≤2	1	2	0	0.5	0.5	0	0.5	2	0	0.12	1	0	2	2	0	1	2	0	1	1	0.0
Vancomycin	≤2	<0.12	0.5	0	0.5	0.5	0	0.5	0.5	0	<0.12	<0.12	0	<0.12	<0.12	0	<0.12	<0.12	0	<0.12	<0.12	0.0

Notes: R%: Resistance Rate.

(MIC<sub>90</sub> = 2 µg/mL). Moreover, most isolates were also susceptible to rifampin (86.7%), gentamicin 77.8%), and tetracycline (>62.2%). However, the majority of isolates were resistant to erythromycin (93.3%), ciprofloxacin (93.3%), clindamycin (91.1%) and penicillin (66.7%), respectively.

Forty-four isolates of all non-diphtheriae *Corynebacterium* isolates were resistant to at least one of the tested antibiotics. MDR was observed in 41 strains (91.1%) in this study. The most frequently encountered resistance phenotype was erythromycin–clindamycin–ciprofloxacin. Thirty of the 41 MDR isolates exhibited resistance to five or more antimicrobial drugs from the classes of macrolides, lincosamides, fluoroquinolones, penicillins, and cepheems.

Notably, the most prevalent species of *C. striatum* among all non-diphtheriae *Corynebacterium* isolates exhibited a much greater resistance rate (>66.7%) against most of the 10 antibiotics tested, with the exception of rifampin (13.3%) and gentamicin (22.2%). Meanwhile, sensitivities of the other species to 10 antibiotics also varied greatly and the isolates of *C. tuberculostearicum* and *C. jeikeium* were completely resistant to penicillin, erythromycin, and clindamycin. *C. jeikeium* displayed 100% resistance to penicillin and cefotaxime. *C. glucuronolyticum* isolates were the least resistant to penicillin (22.2%) and gentamicin (22.2%), and none of them was resistant to cefotaxime or rifampin. In addition, none of the *C. tuberculostearicum* was resistant to tetracycline, gentamicin, or ciprofloxacin. Every control results fell within the established reference ranges.

## Molecular Analysis of the Drug Resistance Mechanisms

Molecular resistance mechanisms to different antibiotics were determined with specific primers listed in Table 1. Drug-resistant genes corresponding to macrolides were investigated by amplification of *erm(X)* gene, *erm(B)* gene, and *mef(A-E)* gene. In this study, the *erm(X)* gene was key in mediating MLSB resistance, which was detected in 91.1% of all non-diphtheriae *Corynebacterium* isolates. In contrast, neither *erm(B)* gene nor *mef(A-E)* gene was detected in any tested isolates (data not shown in Table 4).

In non-diphtheriae *Corynebacterium* strains, aminoglycoside modifying enzyme (AME) genes were frequently present, 29 out of 45 isolates have the *aac(3)-XI* gene in them (64.4%). However, only 10 of the 29 *aac(3)-XI* gene-detective isolates were gentamicin-resistant when compared with the phenotypic antimicrobial susceptibility test results; the other 19 isolates were either sensitive or intermediate to gentamicin. Therefore, the *aac(3)-XI* gene may only be partially responsible for gentamicin resistance.

Resistance to fluoroquinolones of *Corynebacterium* spp. is commonly caused by mutations in the quinolone-resistance-determining region (QRDR) of the *gyrA* gene encoding the enzyme gyrase A subunit. Therefore, the QRDR at *gyrA* gene was amplified and sequenced as previously described.<sup>8,28,31,32</sup> In this study, the sequenced data of the *gyrA* gene from 42 ciprofloxacin-resistant isolates were compared to those from susceptible ones. Table 5 summarizes the associations between ciprofloxacin MICs and mutations in the *gyrA* QRDRs of ciprofloxacin-resistant isolates. In 18 of the 42 ciprofloxacin-resistant isolates, an increase of MICs until 32µg/mL was related to a double non-conservative mutations, at positions 87 and 91. 21 strains with a single mutation at positions 87 were still resistant to ciprofloxacin but with lower MICs (in the range of 4–16µg/mL). There were still three ciprofloxacin-resistant strains with no changes in their QRDRs, indicating other mechanisms might mediate their resistance. However, three ciprofloxacin-resistant

**Table 4** Drug-Resistant Gene Profiles of Non-Diphtheriae *Corynebacterium* Isolates

Species	No.	Prevalence	Drug Resistance Genes							
			<i>erm(X)</i>	<i>aac(3)-XI</i>	<i>bla</i>	<i>ampC</i>	<i>gyrA</i>	<i>tetA</i>	<i>tetB</i>	<i>rpoB</i>
<i>C. striatum</i>	15	33.3%	+	+	+	+	+	+	+	+
<i>C. glucuronolyticum</i>	9	20.0%	+	+	+	-	+	+	+	+
<i>C. urealyticum</i>	8	17.8%	+	+	+	-	+	+	+	+
<i>C. tuberculostearicum</i>	4	8.9%	+	+	+	-	+	-	+	+
<i>C. jeikeium</i>	4	8.9%	+	-	+	-	+	-	+	+
<i>C. amycolatum</i>	5	11.1%	+	-	+	-	+	+	+	+
Total	45	100.0%	41 (91.1%)	29 (64.4%)	20 (44.4%)	14 (31.1%)	45 (100%)	11 (24.4%)	31 (68.9%)	45 (100%)



**Table 5** Amino Acid Substitutions in *gyrA* Gene Associated with Resistance to Quinolones in the Non-Diphtheriae *Corynebacterium* Isolates

Species (Total No.)	No. of Ciprofloxacin-Resistant Isolates	Amino Acid Substitutions of <i>gyrA</i> Gene	
		Position 87	Position 91
<i>C. striatum</i> (15)	8	Ser→Val	Asp
	6	Ser→Phe	Asp→Ala
<i>C. glucuronolyticum</i> (9)	6	Ser→Tyr	Asp
	3	Ser→Tyr	Asp→Ala
<i>C. urealyticum</i> (8)	5	Ser→Tyr	Asp→Ala
	2	Ser→Val	Asp→Tyr
<i>C. tuberculostearicum</i> (4)	3	ND	ND
<i>C. jeikeium</i> (4)	2	Ser→Ile	Asp
	2	Ser→Ile	Asp→Tyr
<i>C. amycolatum</i> (5)	5	Ser→Arg	Asp
Total (45)	42		

**Abbreviation:** ND, not detected.

*C. tuberculostearicum* isolates failed to be sequenced due to insufficient PCR amplification. So there were actually 39 strains analyzed for the amino acid substitutions in *gyrA* gene.

Forty-five non-diphtheriae *Corynebacterium* isolates were tested for the presence of *bla* and *ampC* genes, which encode a class A and a class C  $\beta$ -lactamase, respectively, and, are involved in penicillin and cefotaxime resistance in *Corynebacterium* spp. In this study, the *bla* gene distributed in every *Corynebacterium* species, and 20 out of the 45 isolates were positive for *bla* gene. Whereas the *ampC* gene was only detected among *C. striatum* isolates, 14 out of the 15 *C. striatum* isolates were *ampC* positive. Furthermore, 12 out of the 14 *ampC*-positive isolates were resistant to penicillin and cefotaxime. Overall, the resistance rates of all *Corynebacterium* spp. to penicillin and cefotaxime were 66.7% and 53.3%, respectively. However, the *bla* gene and *ampC* gene were only detected in 44.4% and 31.1% of all isolates. As a result, other mechanisms that contribute to penicillin and cefotaxime resistance that were not investigated in this study could exist.

Genes involved in tetracycline transportation out of the membrane were amplified simultaneously. Both *tetA* gene and *tetB* gene were detected in all non-diphtheriae *Corynebacterium* species, except for *C. tuberculostearicum* and *C. jeikeium*. However, *tetB* gene (64.4%) was more common than *tetA* gene (24.4%).

Rifampicin is an antibiotic that inhibits transcription by binding to the  $\beta$ -subunit of the bacterial DNA-dependent RNA polymerase, which is encoded by the *rpoB* gene. Rifampicin is usually used in combination therapy for serious infections, and its resistance is caused by mutations in a highly conserved region of *rpoB* gene, known as the rifampicin resistance-determining region.<sup>33</sup> Polymerase chain reaction (PCR) and DNA sequencing were used in this study to look for mutations that cause rifampicin resistance in all of the selected isolates. We obtained six rifampicin-resistant *Corynebacterium* isolates (two *C. striatum* isolates, two *C. tuberculostearicum* isolates, one *C. urealyticum* isolate, and one *C. jeikeium* isolate), which showed low-level resistance to rifampicin with a minimal inhibitory concentration (MIC) less than or equal to 32  $\mu$ g/mL. *RpoB* gene sequences from rifampicin-resistant isolates and rifampicin-susceptible isolates of related species were aligned, but no amino acid substitution was observed.

## Biofilm Formation

The biofilm formation capacities of all 45 isolates in 96-wells microtiter plates are displayed in Figure 1. After 48 hours of MTT treatment, viable sessile bacterial cells were found. Overall, all *C. glucuronolyticum* isolates consistently showed the strongest ability to adhere to abiotic surfaces when compared with negative controls ( $p < 0.001$ ), while *C. striatum* was



the second species to demonstrate good biofilm forming capability ( $p < 0.01$ ). Although the biofilm formation ability of *C. amycolatum* varied a lot among different isolates, the average OD490 still showed stronger biofilm formation ability ( $p < 0.01$ ). However, *C. glucuronolyticum*, *C. urealyticum*, and *C. tuberculostearicum* could not form biofilm on the abiotic surfaces, just the same as negative controls ( $p > 0.05$ ).

## Discussion

For many decades, the pathogenic potential of non-diphtheriae *Corynebacterium* species was underestimated, owing to the difficulty distinguishing colonization from infection.<sup>8,15,34–36</sup> A growing number of studies have demonstrated the relevance of non-diphtheriae *Corynebacterium* pathogens in a variety of infectious processes, including UTIs, in both immunocompromised and immunocompetent patients.<sup>14,15,22,37</sup> The literatures strongly suggested that urologic diseases caused by gram-positive bacteria, including *Corynebacteria*, could be easily missed due to the limited culture-based assays used in hospital microbiology laboratories. Because of the slow growth characteristics of some species, such as *C. tuberculostearicum* and *C. jeikeium* in this study, the culture dishes were discarded before being observed for further operation.

One of the most serious problems in treating infections caused by non-diphtheriae *Corynebacterium* isolates is the difficulty in selecting optimal antibiotic therapy due to their diverse antimicrobial susceptibility profiles and MDR characteristics. The MDR isolates investigated in this study came from patients who had long hospital stays, were undergoing invasive surgical interventions, or had serious underlying comorbidities. Antibiotic resistance has spread rapidly, resulting in therapeutic failure and treatment options being limited. Nonetheless, antimicrobial susceptibility test for non-diphtheriae *Corynebacterium* isolates were not routinely performed in most Chinese hospitals. Furthermore, most previous research has focused solely on clinically important species, such as *Corynebacterium striatum*.<sup>15,29,30,38</sup> There was little information about other *Corynebacterium* species. Vancomycin and linezolid are currently the most effective in vitro antibiotics against *Corynebacteria*. The significant variability in antimicrobial agent resistance profiles emphasizes the importance of continuous antibiotic susceptibility monitoring for non-diphtheriae *Corynebacterium* isolates.

Usually,  $\beta$ -lactams are the most broadly used class of antimicrobials in clinical applications. Poor sensitivities to penicillin and cefotaxime were detected among non-diphtheriae *Corynebacterium* isolates, especially in *C. jeikeium*, *C. striatum*, *C. tuberculostearicum*, and *C. urealyticum*, with the MIC<sub>50</sub> and MIC<sub>90</sub> over than 32 $\mu$ g/mL and 64 $\mu$ g/mL, respectively. Of note, all *C. jeikeium* isolates were 100% resistant to penicillin and cefotaxime. Two  $\beta$ -lactamase-encoding genes, *bla* and/or *ampC* were detected in  $\beta$ -lactam-resistant isolates. The *bla* gene, encoding a class A  $\beta$ -lactamase, is distributed among all species with a 44.4% detection rate. The *ampC* gene, encoding a class C  $\beta$ -lactamase which was active on both penicillins and cephalosporins, was detected in 10 of the 12 penicillin-resistant *C. striatum* isolates but not detected in any other species. Data in this study revealed high resistance rates to  $\beta$ -lactams among non-diphtheriae *Corynebacterium* isolates. So  $\beta$ -lactam agents were not recommended for clinical therapy, especially for *C. jeikeium*, *C. striatum*, *C. tuberculostearicum*, and *C. urealyticum*.

Data in this study confirm the high prevalence of resistance to the MLSB group compounds among *Corynebacterium* spp., as previously reported.<sup>39</sup> Erythromycin and clindamycin were resistant to more than 91% of all non-diphtheriae *Corynebacterium* isolates. Significantly, isolates of *C. tuberculostearicum*, *C. jeikeium*, and *C. amycolatum* exhibited a 100% resistance rate. The MLS resistance in *Corynebacterium* spp. was usually mediated by two mechanisms: target-site modification is mediated by ribosomal RNA methylases encoded by the *erm* genes and active drug-efflux is mediated by a membrane efflux pump codified by the *mef(A-E)* gene.<sup>39</sup> Our results were consistent with previous studies, which pointed out that *erm(X)* was the most crucial gene that contributed to MLS resistance. However, neither the *erm(B)* gene encoding the ribosomal RNA methylase Erm(B) nor the *mef(A-E)* gene encoded a membrane efflux pump was detected in all isolates in this study.

Aminoglycosides are commonly used as complementary antibiotics to treat serious infections. In this study, gentamicin showed relatively good activity against non-diphtheriae *Corynebacteria* with a resistance rate of 22.2% on average. Aminoglycoside resistance occurs through several mechanisms that could coexist simultaneously, but enzymatic inactivation of the antibiotic molecule is the most prevalent mechanism in clinical situations.<sup>40</sup> The *aac(3)-XI* gene, encoding an

aminoglycoside 3-N acetyl transferase conferring resistance to gentamicin, could be detected in all gentamicin-resistant isolates excluding *C. jeikeium* and *C. amycolatum* species. Other mechanisms might contribute to the aminoglycoside resistance among these two species. Other aminoglycosides not listed in CLSI M45 were not tested in this study.

Fluoroquinolones have been extensively used in the empirical treatment of UTIs because of their accumulation in the body's organ.<sup>41,42</sup> On average, however, a high rate of 93.3% against ciprofloxacin was detected. Moreover, in this study, ciprofloxacin was completely resistant to *C. glucuronolyticum*, *C. jeikeium*, and *C. amycolatum* isolates. The fluoroquinolone-resistance-related mutations in *gyrA* gene were determined. There was a single mutation at position 87 as well as a double mutation at position 87 and 91. It was noticed that two non-conservative mutations at position 87 and position 91 might significantly increase the MIC of ciprofloxacin to values greater than 32 µg/mL. However, a single mutation at position 87 was more common among those isolates resistant to ciprofloxacin but with lower MICs (in the range of 4–16 µg/mL). The high resistance rates of fluoroquinolones in almost all non-diphtheriae *Corynebacterium* species would restrict their use as empirical treatment options.

As previously reported, rifampicin has been used as a complementary agent for managing *C. striatum* infections.<sup>43,44</sup> In this study, rifampicin showed excellent in vitro activity against non-diphtheriae *Corynebacterium* isolates, second only to vancomycin and linezolid. Of note, isolates of *C. glucuronolyticum* and *C. amycolatum* were fully susceptible to rifampicin. There were only six out of 45 isolates resistant to rifampicin among all isolates. Moreover, MICs of five of the resistant isolates ranged from 1 to 4 µg/mL, which indicated a low level of resistance against rifampicin. Resistance to this compound typically results from substituting some highly conserved residues in the RNA polymerase  $\beta$  subunit (*ropB*). We compared the *ropB* gene sequences of the six rifampicin-resistant isolates with those of rifampicin-susceptible isolates. However, no mutations within the 81-bp rifampicin resistance-determining region (RRDR) of the *rpoB* gene were detected, which might be explained by the low-level resistance.

Tetracyclines are also a class of broad-spectrum antibiotics that inhibit the growth of bacteria mainly through interfering with protein synthesis. In this study, the average rate of tetracycline resistance among all isolates of non-diphtheriae *Corynebacterium* was 37.8%. Isolates of *C. glucuronolyticum* exhibited the highest resistance rate (77.8%), while isolates of *C. tuberculostearicum* were susceptible to this compound. The *tetA* and *tetB* genes were detected for the tetracycline-resistant mechanisms. The results showed that *tetB* gene was more prevalent than the *tetA* gene, which indicated that the *tetB* gene played a crucial role in tetracycline resistance.

In this study, biofilm formation capability among different species of non-diphtheriae *Corynebacterium* was another important area of concern, contributing to the potential pathogenesis, drug resistance and infection recurrence to some extent. From the data listed in Figure 1, it was clear that both *C. striatum* and *C. glucuronolyticum* isolates exhibited the strongest biofilm formation ability on abiotic surfaces, nearly the same as the positive control ATCC 43300. However, isolates of *C. urealyticum*, *C. tuberculostearicum*, and *C. jeikeium*, like the negative control, were unable to form biofilm. Moreover, the biofilm formation ability of *C. amycolatum* isolates was distributed moderately to strongly among different isolates. The in vitro results of biofilm formation abilities reflected the colonization probability on indwelling devices in vivo to some extent, which needed further investigation in the future.

This study has some limitations too. It was a retrospective study, so we could not clearly distinguish between the actual infection and contamination because of the absence of established clinical and bacteriological markers of infection for *Corynebacterium* spp. In addition, the number of non-diphtheriae *Corynebacterium* isolates in this research was small, especially for some species, including only four to five isolates.

## Conclusions

This study illustrated the high prevalence of MDR in non-diphtheriae *Corynebacterium* isolates and related molecular mechanisms, highlighting the importance of focusing on non-diphtheriae *Corynebacterium* related infections. All species of isolates showed 100% susceptibility to vancomycin and linezolid but high resistance rates to compounds of the MLSB group, fluoroquinolones, tetracyclines, and  $\beta$ -lactams. The definite antibiotic profiles varied a lot among different isolates. All isolates could form biofilm on abiotic surfaces except those of *C. urealyticum*, *C. tuberculostearicum*, and *C. jeikeium*. Furthermore, among the non-diphtheriae *Corynebacterium* species, *C. striatum* was the most common and resistant, as well as having the strongest biofilm formation ability. Therefore, surveillance of MDR *C. striatum* should be given more attention. *C. urealyticum* and *C. jeikeium* were also important pathogens related to UTIs.<sup>45–47</sup> Although they could not form biofilm on

abiotic surfaces, the MDR characteristics should be paid more attention to in clinical therapy. Reliable laboratory diagnosis and appropriate actions to control MDR isolates of non-diphtheriae *Corynebacterium* dispersion are required. Clinicians are advised to make an effort to classify suspicious isolates to genus, preferably to the species level. In order to offer laboratory support for effective therapy, additional research is still required to investigate the clinical, epidemiological, and microbiological features of more prevalent species of non-diphtheriae *Corynebacterium*.

## Data Sharing Statement

The datasets generated for this study are available from the corresponding author Pro Jianrong Su on request.

## Ethics Approval and Informed Consent

This retrospective study was approved by the Ethics Committee of Beijing Friendship Hospital, Capital Medical University (20210208). Adult patients wrote the informed consent and a parent or legal guardian of patients under 18 years of age provided informed consent prior to the experiment described below. This study was conducted in accordance with the Declaration of Helsinki.

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## Disclosure

The authors declare no conflicts of interest for this study.

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