Antimicrobial Susceptibility and Clonality of Vaginally Derived Multidrug-Resistant *Mobiluncus* Isolates in China

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ABSTRACT Here, the antimicrobial susceptibility, resistance mechanisms, and clonality of *Mobiluncus* sp. isolates recovered from gynecological outpatients in China were investigated. Compared to *M. mulieris*, *M. curtisii* exhibited higher antimicrobial resistance to metronidazole, clindamycin, and tetracycline. Whole-genome sequencing indicated that the clindamycin resistance gene *erm(X)* was located on a transposable element, Tn5432, which was composed of two IS1249 sequences. Phylogenetic analysis indicated that *Mobiluncus* spp. had high diversity, with isolates being grouped into several sporadic clades.

KEYWORDS *Mobiluncus* spp., antimicrobial resistance, clindamycin, metronidazole, whole-genome sequencing

Mobiluncus spp. are obligate anaerobic, Gram-labile or Gram-negative curved bacilli of the vaginal flora that are highly correlated with bacterial vaginosis (1, 2). Analysis of the 16S rRNA gene sequences revealed that *Mobiluncus* spp. comprise two distantly related species, namely, *Mobiluncus curtisii* and *Mobiluncus mulieris*. Both species are associated with different types of conditions, such as vaginal abscesses, umbilical abscesses, endometritis, ulcerative colitis, and spontaneous preterm birth (3, 4). The treatment of *Mobiluncus* sp. infections is primarily based on empirical broad-spectrum antimicrobial therapy against anaerobes using metronidazole and clindamycin, which are frequently used first-line antianaerobic agents (5, 6). However, the increase in antimicrobial resistance of anaerobes over the recent decades is worrisome, since such resistance has been described in multidrug-resistant anaerobic bacteria (7). Because of their fastidious nature and strict nutritional requirements, these organisms are difficult to culture and isolate.

To date, there is only one study that has reported the prevalence and antimicrobial resistance rate in *Mobiluncus* isolates, which were recovered from Turkish women with gynecological infections from 1999 to 2002 (8). Data on the antimicrobial susceptibility and, more importantly, the resistance mechanisms and clonality of *Mobiluncus* isolates recovered from China are completely lacking. To address this point, the present study assessed the susceptibility of *Mobiluncus* isolates to penicillin, cefoxitin, imipenem, tetracycline, metronidazole, and clindamycin in China. The phylogenetic relationships among isolates and the mechanisms of resistance were also investigated.

In this study, all vaginal swabs were collected from gynecological outpatients between January 2017 and November 2018 at the Women's Hospital, Zhejiang University School of Medicine, Hangzhou, China. Two specimens of vaginal secretion were

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TABLE 1 The MICs of six antimicrobial agents against 30 *M. curtisii* isolates and 33 *M. mulieris* isolates recovered from patients with bacterial vaginosis

Antimicrobial agent	MICs ($\mu\text{g/ml}$) for: ^a							
	<i>M. curtisii</i> (n = 30)				<i>M. mulieris</i> (n = 33)			
	Range	50%	90%	R (%)	Range	50%	90%	R (%)
Penicillin	0.008–0.064	0.032	0.032	0	<0.008–0.008	<0.008	0.008	0
Cefoxitin	0.5–4	2	2	0	<0.064–1	0.064	0.125	0
Imipenem	0.008–0.032	0.016	0.032	0	<0.008–0.016	0.008	0.008	0
Tetracycline	0.064–16	4	8	30	<0.064–16	4	8	24.2
Clindamycin	<0.064–>256	>256	>256	73.3	<0.064–>256	>256	>256	54.5
Metronidazole	>32–>32	>32	>32	100	0.25–>32	>32	>32	63.6

^aR, resistance.

collected from each patient. One specimen was used for Gram staining to confirm the typical *Mobiluncus* sp. morphology under the microscope (i.e., curved bracket with two tapered ends), and the other specimen was used for culture-based separation. The vaginal discharge samples were inoculated onto Columbia blood agar plates (Oxoid, Basingstoke, United Kingdom) and placed under anaerobic conditions in anaerobic bags (bioMérieux, Marcy-l'Étoile, France) at 35°C for 3 to 5 days. Colonies were Gram stained to obtain organisms with the characteristic curved rod shape and subcultured to obtain pure cultures of each colony. A total of 63 *Mobiluncus* sp. isolates recovered from the vaginal discharge were identified using 16S rRNA gene sequencing. In all, 30 isolates (47.6%) were identified as *M. curtisii*, and 33 isolates (52.4%) were identified as *M. mulieris* (see Table S1 in the supplemental material). The antimicrobial susceptibility of *Mobiluncus* isolates against penicillin, cefoxitin, imipenem, tetracycline, clindamycin, and metronidazole was assessed using the Etest method. Briefly, *Mobiluncus* isolates were suspended in Brucella broth to obtain a density equivalent to the McFarland 1 standard and were further inoculated onto Brucella agar supplemented with 5% lysed sheep blood, 5 $\mu\text{g/ml}$ hemin, and 1 $\mu\text{g/ml}$ vitamin K1. The Etest strip was placed in the center of each 90-mm plate and incubated anaerobically at 35°C for 48 h (9, 10). The MICs of antibacterial agents were interpreted according to the breakpoints of anaerobes provided by the Clinical and Laboratory Standards Institute (CLSI) guidelines (11). *Bacteroides fragilis* strain ATCC 25285 was used for quality control. Ten *M. curtisii* isolates and eleven *M. mulieris* isolates were further selected for whole-genome sequencing using the Illumina NovaSeq platform. *De novo* genome assembly was performed using SPAdes 3.14. The macrolide resistance gene *erm(X)*, tetracycline resistance gene *tet(O)*/*tet(W)*, and lincomycin resistance gene *lnu(C)* were identified using the ResFinder 3.2 database. The reported mechanisms of antimicrobial resistance against metronidazole in other bacterial species were also searched, including the nitroimidazole reductase gene *nim*, the oxygen-insensitive NADPH nitroreductase gene *rdxA*, the NADPH flavin oxidoreductase gene *frxA*, and RND-family efflux pump genes. Genome-wide phylogenetic trees were constructed between the *Mobiluncus* isolates recovered in this study and previously sequenced strains retrieved from the NCBI GenBank database using a core genome single-nucleotide polymorphism (cgSNP) strategy. The phylogenetic relatedness of *M. curtisii* and *M. mulieris* strains was assessed using an online bacterial whole-genome sequence typing and source tracking database (BacWGSTdb) (12, 13). Easyfig was used to analyze the genetic surroundings of antimicrobial resistance genes and homologous regions between different isolates.

The results of antimicrobial susceptibility testing indicated that *M. curtisii* had a higher resistance rate than *M. mulieris* to (i) metronidazole (100% versus 63.6%), (ii) clindamycin (73.3% versus 54.5%), and (iii) tetracycline (30% versus 24.2%). None of the isolates had a reduced susceptibility to penicillin, cefoxitin, or imipenem. Detailed results of MIC distributions, MIC₅₀ and MIC₉₀ values, and the percentages of resistance of *Mobiluncus* isolates are shown in Table 1.

Among the 21 *Mobiluncus* isolates that were subjected to whole-genome sequencing, 16 isolates carried the macrolide resistance gene *erm(X)*, 18 isolates carried the

FIG 1 Comparison of the homologous regions shared by clindamycin-resistant (I and II) and clindamycin-susceptible (III and IV) isolates. Arrows represent coding sequences [red arrows, *erm(X)*; pink arrows, IS1249] and indicate the direction of transcription. The arrow size is proportional to the gene length.

tetracycline resistance gene *tet(O)/tet(W)*, 1 isolate carried the lincomycin resistance gene *lnu(C)*, and 15 isolates carried both the *erm(X)* and *tet(O)/tet(W)* genes. Sixteen *Mobiluncus* sp. strains (eight *M. curtisii* strains and eight *M. mulieris* strains) carrying *erm(X)* exhibited a high level of resistance to clindamycin, with an MIC of $>256 \mu\text{g/ml}$, and five clindamycin-susceptible strains did not carry the *erm(X)* gene, which is consistent with the phenotypic data. The *erm(X)* gene was allocated to a specific region in the genome with a similarity of 100% to the composite transposable element Tn5432. Tn5432 comprises two IS1249 sequences, the *erm(X)* gene, and the transposase gene *tnpCX*, which may assist in the horizontal transfer of the *erm(X)* gene. Analysis of upstream and downstream sequences flanking Tn5432 revealed that the transposon was located between an ATP-binding protein and a hypothetical protein of unknown function. However, the *erm(X)* and *tnpCX* genes were missing in the clindamycin-susceptible isolates or carried only one copy of IS1249 in this region (Fig. 1). The *tet(O)* and *tet(W)* genes that encode ribosomal protection proteins were identified in 18 *Mobiluncus* sp. strains (8 *M. curtisii* strains and 10 *M. mulieris* strains). No specific transposon or insertion sequence was detected in the genetic surroundings of the *tet(O)/tet(W)* genes. The MICs of tetracyclines for the *tet(O)/tet(W)*-positive isolates were higher than those for the three *tet(O)/tet(W)*-negative isolates. Moreover, all the *M. curtisii* isolates and 63.6% of the *M. mulieris* isolates were resistant to metronidazole. Therefore, we screened all known metronidazole resistance genes from the whole-genome sequencing data, but none of the antimicrobial resistance determinants previously reported in other bacterial species were identified. However, we characterized an underlying gene that encodes an oxygen-insensitive NADPH nitroreductase, which has not been reported before. Multiple sequence alignments of the amino acid sequence of the nitroreductase among the *M. mulieris* isolates that are resistant and susceptible to metronidazole revealed two unique nonsynonymous mutations in the

FIG 2 Recombination-filtered core genome phylogeny and the distribution of antimicrobial resistance genes for *M. curtisii* isolates. The cell in a different color indicates the presence of antimicrobial resistance genes, and the blank cell indicates the absence of the gene. Isolates sequenced in this study are in red.

metronidazole-resistant isolates (R186Q and T192I), which can be responsible for the metronidazole resistance. In addition, almost all of the *M. curtisii* isolates shared the identical sequence of the nitroreductase gene (Fig. S1). The phylogenetic tree of *Mobiluncus* isolates had high diversity, with isolates being grouped into several clades, which indicated that these isolates are not epidemiologically related (they differed by >1,000 SNPs) (Fig. 2 and 3).

Mobiluncus species are increasingly recognized as clinically significant anaerobic bacteria because of their potential pathogenicity linked to bacterial vaginosis and their ability to affect the normal vaginal flora (1, 14). An *in vitro* study indicated that *Mobiluncus* spp. significantly increased the proinflammatory cytokine production of vaginal epithelial cells, including the production of interleukin-1 α (IL-1 α), IL-1 β , tumor necrosis factor- α (TNF- α), and IL-8 (15), and may promote the occurrence of bacterial vaginosis and accelerate its progression. The emergence of multidrug-resistant anaerobic bacterial strains that cause vaginosis, particularly those that are resistant to metronidazole and clindamycin, represents an increasing threat to public health (16–18). Oral or vaginal administration of metronidazole or clindamycin produced an initial response in 80% to 90% of patients, but 15% to 30% of these patients relapsed within 3 months due to the possible selection of potential antimicrobial-resistant strains (19). The limited therapeutic options available to combat infections caused by *Mobiluncus* spp. indicates the importance of studying the prevalence and mechanisms of resistance to first-line antimicrobial agents such as metronidazole and clindamycin.

Currently, limited data are available on the antimicrobial susceptibility of *Mobiluncus* spp., especially for individual species. One of the reasons for this limitation in data availability on *Mobiluncus* susceptibility relates to the known difficulty in cultivating these pathogens and in the performance of the agar dilution method. The antimicrobial resistance rate in *Mobiluncus* spp. varies widely among different countries and studies.

FIG 3 Recombination-filtered core genome phylogeny and the distribution of antimicrobial resistance genes for *M. mulieris* isolates. The cell in a different color indicates the presence of antimicrobial resistance genes, and the blank cell indicates the absence of the gene. Isolates sequenced in this study are in red.

The rates of resistance to clindamycin and metronidazole in our study were much higher than those in previously reported studies. For example, a Turkish study reported a metronidazole resistance rate of 24% in *M. mulieris*, while in a recent study conducted in the United States, a rate of 42% was reported (8, 20). The antimicrobial susceptibility rates of *M. curtisii* and *M. mulieris* to clindamycin were also higher than the rates reported by Bahar et al. (0% and 0%, respectively) and Petrina et al. (18% and 0%, respectively) (8, 20). The empirical overuse of first-line antibiotics in the community is a key driving force for the evolution of antimicrobial resistance in *Mobiluncus* spp. However, the possible inconsistencies in resistance rates between the aforementioned studies may also be due to the use of different methods/techniques for antimicrobial susceptibility testing (e.g., Etest and agar dilution).

Understanding the underlying mechanism of resistance is imperative. The *erm(X)* gene is only part of the nonconjugative composite transposon Tn5432, composed of two IS1249 elements, which is predominantly integrated into the chromosome (21). Whole-genome sequencing analysis of *Mobiluncus* spp. indicated that the horizontal transfer of the *erm(X)* gene via Tn5432 in *Mobiluncus* strains has resulted in an increased proportion of clindamycin-resistant *Mobiluncus* strains in China, which is consistent with the mechanism of resistance to macrolides in other bacterial species, e.g., *Cutibacterium acnes* and *Bifidobacterium* spp. (22, 23). In addition, the *Mobiluncus* strains carried a high proportion of *tet(O)/tet(W)* resistance genes, which encode ribosomal protection proteins that confer increased resistance to tetracycline. Our results indicated that the MIC of tetracycline was higher in *tet(O)/tet(W)*-carrying isolates than in noncarrying isolates, which is consistent with previous studies (24, 25). The mechanisms of metronidazole resistance in anaerobes are complex and multifactorial and have not been fully elucidated (26–28). The *nim* genes, which encode nitroimidazole reductase primarily in *Bacteroides* spp., are reported to be associated with metronidazole resistance (5, 29, 30). Mutations in the nitroreductase-encoding gene *rdxA* and oxidoreductase-encoding gene *frxA* in *Helicobacter pylori* can confer high-level resistance to metronidazole (5, 28). Other mechanisms, including overexpression of the RND-family efflux pump gene *bme* or the DNA repair effector recombinase A gene *recA*, are also involved in metronidazole resistance in *Bacteroides fragilis* (31, 32). In this study, we characterized several unique missense mutations in an underlying oxygen-insensitive NADPH nitroreductase gene in

metronidazole-resistant *Mobiluncus* isolates by direct comparison of whole-genome sequencing data. These mutations identified in the nitroreductase-encoding gene can be associated with the low expression or inactivation of nitroreductases, which are needed to convert metronidazole to its active metabolites and resulting cytotoxicity, and can therefore mediate metronidazole resistance in *Mobiluncus* species. However, these preliminary findings warrant further investigation of the origin, transmission, and control of antimicrobial resistance using a large collection of samples.

In conclusion, our findings increase the knowledge of the antimicrobial susceptibility, resistance mechanisms, and clonality of multidrug-resistant *Mobiluncus* species in China. Continuous epidemiological surveillance is essential to monitor and prevent antimicrobial resistance from progressing to a clinically significant level.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 1 MB.

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Y.Z. and Z.R. designed the study. Z.R. and X.Z. analyzed the data. Z.R., M.S.D., and X.Z. drafted the manuscript. Y.B. and L.Z. contributed to the isolation and phenotypic characterization of bacterial strains. All authors approved the final version of the manuscript.

We declare that there are no conflicts of interest.

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