Antibiotic resistance and pathogenicity assessment of various Gardnerella sp. strains in local China

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Gardnerella overgrowth is the primary cause of bacterial vaginosis (BV), a common vaginal infection with incidences as high as 23–29% worldwide. Here, we studied the pathogenicity, drug resistance, and prevalence of varying Gardnerella spp. We isolated 20 Gardnerella strains from vaginal samples of 31 women in local China. Ten strains were then selected via phylogenetic analysis of cpn60 and vly gene sequences to carry out genome sequencing and comparative genomic analysis. Biofilm-formation, sialidase, and antibiotic resistance activities of the strains were characterized. All strains showed striking heterogeneity in genomic structure, biofilm formation and drug resistance. Two of the ten strains, JNFY3 and JNFY15, were classified as Gardnerella swidsinskii and Gardnerella piotii, respectively, according to their phenotypic characteristics and genome sequences. In particular, seven out of the ten strains exhibited super resistance (≥ 128 µg/mL) to metronidazole, which is the first line of treatment for BV in China. Based on the biochemical and genomic results of the strains, we proposed a treatment protocol of prevalent Gardnerella strains in local China, which provides the basis for accurate diagnosis and therapy.

KEYWORDS
Gardnerella vaginalis, prevalent strains, comparative genomics, antibiotic resistance, accurate diagnosis and therapy

Introduction

Gardnerella vaginalis is a facultatively anaerobic bacterium of the Bifidobacteriaceae family and part of the normal vaginal microbiome. Often described as a Gram variable organism with a Gram-positive wall type, the genome size of the type strain ATCC 14018 is remarkably small compared to other facultative anaerobes (Greenwood and Pickett, 1980). At only 1.6 M, it is one-third and one-fifth that of E. coli and
G. vaginalis diagnostic basis of BV (Swidsinski et al., 2005). Additionally, adhere to the vaginal epithelium, forming clue cells for the (Anahtar et al., 2018; Rosca et al., 2020). This biofilm can formation of a stubborn biofilm that is indicative of BV overgrowth in particular being implicated in the vaginal microbiome relative to Lactobacilli. This involves the overgrowth of various anaerobic bacteria microflora in the vagina, the host, and the environment. disrupt the dynamic balance between the bacterial vaginal tract provides only partial information on the physiological potential of the organism (Yeoman et al., 2010). Comparative genomic analyses of strains 5-1 (from healthy hosts) and AMD (from bacterial vaginosis) showed that the copy number and amino acid sequence of vaginolysin in these two strains were almost identical, with only one amino acid difference. Strain AMD contains more toxin-antitoxin (TA) systems; Strain 5-1 lacks two key adhesion proteins, Rib, significantly reduced ability to adhere to epithelial cells, and is more sensitive to erythromycin, leading to weakened pathogenicity (Harwich et al., 2010). All these studies have provided a solid basis for the development of novel diagnostics and treatments against Gardnerella infections. However, given the high heterogeneity of Gardnerella, and the large population with high BV infection rates in China, the lack of in-depth research on varying Gardnerella strains poses a concern for the treatment of patients in China.

Therefore, this study investigates ten epidemic strains of Gardnerella in local China. First, we used molecular biology and biochemistry techniques to classify and determine the epidemic percentage of each subtype. Then, we analyzed both common and unique pathogenic genes of different Gardnerella strains through comparative genomics. Based on these results, we constructed a database of epidemic Gardnerella strains in local China, which may act as a foundation for developing accurate diagnostics and therapeutics for Gardnerella-induced BV.
Materials and methods

Selection of patients

Samples were obtained from 31 women who attended private gynecology clinics in Jinan Maternal and Child Health Care Hospital, China. The study was approved by the Jinan Health Committee (approval no. 2019-1-25). Written informed consent was obtained from all study participants prior to enrollment. All were Han Chinese > 18 years of age (range, 21–55 years; mean, 31.2 years). All had come to the clinic for a routine gynecological examination, with self-reported complaints of vaginal itching/burning sensations, or with increased and/or malodorous vaginal discharge. All participants were asked to complete a questionnaire on the current use of hormonal contraceptives, menstrual cycle, and frequency of vaginal infections. Exclusion criteria included menstruation for a routine gynecological examination, with self-reported complaints of vaginal itching/burning sensations, or with increased and/or malodorous vaginal discharge. All participants were asked to complete a questionnaire on the current use of hormonal contraceptives, menstrual cycle, and frequency of vaginal infections. Exclusion criteria included menstruation at the time of enrollment, human immunodeficiency virus (HIV) infection, and antibiotic/antimicrobial treatment within 14 days of sampling.

Examination of vaginal samples, *Gardnerella vaginalis* isolation

All samples were subjected to Gram-staining and microscopy to assess their Nugent score (NS) (Nugent et al., 1991). BV diagnosis was also defined by the clinician. BV diagnosis included the mandatory satisfaction of three out of the four Amsel criteria (elevated pH, clue cells, fishy odor, and characteristic vaginal discharge); this was supplemented by chemical analysis results (H₂O₂ concentration, activity of leucocyte esterase, sialidase, coagulase and beta-glucosidase) (Amsel et al., 1983; Workowski et al., 2010). A sample was considered as BV-positive if the NS ranged from 7 to 10 and at least three Amsel criteria were present (Workowski et al., 2010). In the case of any inconsistency between the results of chemical analysis and morphology, it should be subjected to morphology.

For *G. vaginalis* isolation, a swab taken near mid-vagina was placed in a BHI liquid medium and then the 10 × gradient dilution was spread on a Chocolate Agar Medium (Haibo, Qingdao, China). Chocolate agar plates were incubated at 37°C in 5% CO₂ for 48 h. Colonies of *G. vaginalis* were identified as described previously (Pleckaityte et al., 2012).

Growth condition

Planktonic cells were grown in sBHI [Brain-heart infusion supplemented with 2% (wt/wt) gelatin (Aladdin, Shanghai, China), 0.5% (wt/wt) yeast extract (Oxford, UK), 0.1% (wt/wt) glucose and 0.1% (wt/wt) soluble starch (Aladdin, Shanghai, China)] for 24 h at 37°C with 5% CO₂. For biofilm formation, the glucose was replaced by maltose, and 5% (v/v) goat blood was added. 2% mid-log phase seed was inoculated to a fresh medium for the following tests.

Gene-specific PCR assays and phylogeny construction

*Gardnerella vaginalis* identification was confirmed by PCR amplification of the 16S rRNA gene (Rainey et al., 1996) and sequencing of the obtained PCR product. *G. vaginalis* subtypes were classified through phylogenetic tree construction using *cpn60* and *vly* gene sequences according to previous studies (Janulaitiene et al., 2017, 2018). The primers are listed in Supplementary Table 1.

Sialidase assay

To further characterize the virulence factors of *Gardnerella* clinical isolates, we investigated the presence and expression of the sialidase gene. The presence of the sialidase gene in clinical isolates of *Gardnerella* was identified by PCR using specific primers (Supplementary Table 1). In addition, The *Gardnerella* cultures were diluted to an OD₆₀₀ value of 0.8 with 50 mM MES buffer (pH 5.5). The reaction system contained 2.2 mM NBT (Nitrotetrazolium Blue chloride, Sigma-Aldrich, St. Louis, MO), 146 mM sucrose, 10.5 mM MgCl₂, 6.3 mM BCIN (5-Bromo-4-chloro-3-indolyl α-D-N-acetyleneuraminic acid sodium salt, Sigma-Aldrich, St. Louis, MO), with 0.5% sulfonyl 440 added to avoid bubbles. The mixture was then incubated at 37°C for 20 min and 100 µL of the incubated mixture was added to the wells of black polystyrene microplates (Nunc, Thermo Fisher Scientific). The plates were sealed with an optically clear seal, and BCIN hydrolysis was monitored by measuring the fluorescence at a wavelength of 616 nm using a SynergyH4 hybrid multi-mode microplate reader (Biotek, Winooski, VT, USA) (Zhang and Rochefort, 2013; Janulaitiene et al., 2018). The fluorescence of each supernatant was analyzed in triplicate.

Biofilm assay

For biofilm formation, the cell concentration of 24 h-old cultures was assessed by measuring the optical density of the cultures at 600 nm (Model Sunrise, Tecan, Switzerland). The cultures were further diluted to obtain a final concentration of approximately 10⁶ CFU/mL. After homogenization, 200 µL of *G. vaginalis* suspensions were dispensed into each well of three 96-well flat-bottom tissue culture plates (Orange Scientific, Braine L’Alleud, Belgium). The tissue culture plates were incubated at 37°C in 10% CO₂. After 24 h, the culture medium covering the biofilm was removed, then replaced by
Phylogenetic analysis of Gardnerella strains based on cpn60 and vly sequences. (A) Phylogenetic tree of Gardnerella cpn60 sequences comprising four distinct clades: (A–D). Bootstrap values for each node are indicated. ATCC 14018, ATCC 14019, 41V, 101, 1500E, AMD, 409-05, JCP8066, and JCP7719 are G. vaginalis isolates with whole genome sequence information available in Genbank (Accession numbers ADNB00000000, CP002104, AEJE00000000, AEJD00000000, GCA_000263595, ADAM00000000, CP001849, GCA_000414565, and GCA_000414625, respectively). (B) Phylogenetic tree of Gardnerella vly sequences comprising four distinct clades: 1, 2, 3, and 4 (no vly gene).

Fresh sBHI and allowed to grow under the same conditions for an additional 24 h. The biofilm produced was quantified by crystal violet staining and then scanned at OD 570 (Jayaprakash et al., 2012; Janulaitiene et al., 2018).

Antibiotic resistance

All antibiotic reserve fluids were prepared at a concentration of 5,120 µg/mL, filtered by a filter membrane to ensure sterility, and stored separately at –20°C. The final antimicrobial concentration was obtained by double dilution with sBHI broth, which was diluted to 512, 256, 128, 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25, and 0.125 µg/mL, respectively. The microdilution plate was made of a 96-well plate with 100 µL of prepared working fluid added to each well. At least one well containing only 100 µL of the antimicrobial-free broth was used as a growth control for the test strain. At the same time, at least one well containing only 100 µL of prepared working fluid added to each well. At least one well containing only 100 µL of the antimicrobial-free broth was used as a growth control for the test strain. At the same time, at least one well containing only 100 µL of prepared working fluid added to each well. At least one well containing only 100 µL of the antimicrobial-free broth was used as a growth control for the test strain. At the same time, at least one well containing only 100 µL of prepared working fluid added to each well. At least one well containing only 100 µL of the antimicrobial-free broth was used as a growth control for the test strain. At the same time, at least one well containing only 100 µL of prepared working fluid added to each well. At least one well containing only 100 µL of the antimicrobial-free broth was used as a growth control for the test strain. At the same time, at least one well containing only 100 µL of prepared working fluid added to each well. At least one well containing only 100 µL of the antimicrobial-free broth was used as a growth control for the test strain. At the same time, at least one well containing only 100 µL of prepared working fluid added to each well. At least one well containing only 100 µL of the antimicrobial-free broth was used as a growth control for the test strain. At the same time, at least one well containing only 100 µL of prepared working fluid added to each well. At least one well containing only 100 µL of the antimicrobial-free broth was used as a growth control for the test strain. At the same time, at least one well containing only 100 µL of prepared working fluid added to each well. At least one well containing only 100 µL of the antimicrobial-free broth was used as a growth control for the test strain.

Path, absorption rate 0.08–0.13). To maintain the stability of the cell concentration in the inoculation suspension, the microdilution plate must be inoculated within 30 min after preparation of the inoculation suspension. In the microdilution plate containing 100 µL of diluted antimicrobial agents, 5 µL of cell suspension was added to each well so that the number of cells in each well was approximately 5 × 10^5 CFU/mL. The microdilution plate was placed in an incubator at 37°C and 5% CO2 for 18 ± 2 h. When the bacteria in the growth control hole had sufficient growth and the negative control hole without inoculation did not grow, MIC was determined as the minimum drug concentration that could significantly inhibit bacterial growth (Clinical and Laboratory Standards Institute, 2020a,b).

Genome sequencing

The strains were cultured to the middle and late logarithmic stage, and cells were collected. The QIAGEN Genomic DNA extraction kit (QIAGEN, Dusseldorf, Germany) was used for Genomic DNA extraction of the samples according to the standard operating procedure provided by the manufacturer. The extracted genomic DNA was determined with the NanoDrop One UV-vis spectrophotometer (Thermo Fisher Scientific, Wilmington, Delaware, USA).
Genome analysis and phylogenomic tree construction of Gardnerella taxon

After quality control, the data was assembled with CANU\(^1\) and corrected with PILON (Walker et al., 2014)\(^2\) combined with second-generation sequencing data. The corrected genome uses its own script to detect whether it is ringed or not. After the redundant parts are removed, the origin of the ringed sequence is moved to the replication starting site of the genome with Circuitor (parameter: Fixstart) to obtain the final genome sequence.

The coding gene was predicted by Prodigal\(^2\) and the complete CDS was retained. The tRNA gene was predicted by Transcan-SE, and the rRNA gene was predicted by RNAmmer. Other ncRNA searched the RFAM database for predictions using Infernal, retaining the predicted length (Kalvari et al., 2020). CRISPR was predicted with MinCED (Bland et al., 2007) and Islander was predicted with IslandViewer.\(^3\)

Genome-encoded proteins were extracted and annotated with InterPro\(^4\) to extract annotation information from TIGRFAMs, Pfam and GO databases. Blastp was used to compare the coded proteins to KEGG and Refseq databases, and the best result of the comparison coverage > 30% was kept as the annotation result. The encoding protein was compared with the COG database for COG annotation by RPSBLAST. ABRicate could obtain resistance genes from contig, which correlated with databases such as NCBI, CARD [The Comprehensive Antibiotic Resistance Database (mcmaster.ca)] and ARG-ANNOT (Gupta et al., 2014). ABRicate software was also used to predict the resistance genes in the genome. Sequencing strain genes were compared with the Pathogenic Bacteria Virulence factor database (VFDB).\(^5\) The VFDB database, developed by the Chinese Academy of Medical Sciences, is widely used in the identification of virulence factor genes. VFDB collected the sequence information of bacterial virulence genes from 30 genera (74 pathogens). The bacterial genes were predicted using AntiSMASH.\(^6\) AntiSMASH used a rules-based clustering method to identify 45 different types of secondary metabolite biosynthesis pathways through its core biosynthesis enzyme.

To assess genome differences between Gardnerella strains, a phylogenetic analysis involving 22 genome sequences retrieved from NCBI and our data was performed. For this purpose, the genome sequences were aligned using MAFFT, and the phylogenetic tree was constructed using the neighbor-joining method in Clustal W v2.1; the image was produced using FigTree software.\(^7\)

Results

Collection and phylogenetic analysis of Gardnerella isolates

Twenty Gardnerella clinical strains were isolated from characterized vaginal samples of 31 women in local China. Each plate was inoculated from a single vaginal swab. Gardnerella strains were identified as described in Methods. Isolates from individual colonies were then subtyped by clade-specific PCR. Based on cpn60 gene sequences, 13 isolates were defined as belonging to clade C, six isolates belonged to clade B, and one isolate belonged to clade A. We found no Gardnerella strains belonging to clade D (Figure 1A). Two isolates (JNFY3 and JNFY13) originated from vaginal samples with normal vaginal microflora (NS = 1 and 0, respectively), whereas the other 29 vaginal swabs with NS values ranging from 4-10 (Table 1). The 20 Gardnerella clinical strains were divided into four clades based on their vly gene sequences (Figure 1B), and strains without the vly gene (JNFY15 and JNFY21) were classified as subtype 4.

Biofilm, biomass, and sialidase activity in Gardnerella strains

The measurement of biofilm formation capacity of 10 strains showed that, except for JNFY3, JNFY15, and JNFY17, other prevalent strains could form biofilm with varied thickness. JNFY14 produced the largest amount of biofilm, and cpn60 subtype C prevalent strains generally formed a certain amount

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1 https://github.com/marbl/canu
2 https://github.com/hyattpd/Prodigal/wiki/introduction
3 http://www.pathogenomics.sfu.ca/islandviewer/upload/
4 http://www.ebi.ac.uk/interpro/search/sequence/
5 http://www.mgc.ac.cn/VFs/
6 http://antismash.secondarymetabolites.org/
7 http://tree.bio.ed.ac.uk/software/figtree/
of biofilm (Table 1, Figure 2, and Supplementary Figure 2). Sialidase activity was detected in strains JNFY3, JNFY4, JNFY11, and JNFY17. Moreover, strain JNFY14 exhibited weak sialidase activity (Table 1).

General genome features and phylogenomic tree of *Gardnerella* strains

The genome size of *Gardnerella* was between 1.5 and 1.8 Mb, which was smaller than other members of the *Bifidobacteriaceae* family, one-third the size of *E. coli* and one-fifth the size of *P. aeruginosa*. The genomes of these ten strains contained about 1,300 genes, with a GC content of about 41–43%. The basic features of the ten prevalent GV strains were similar to those of the American strain ATCC 14019 isolated from BV patients (genome size 1,667,350 bp, GC content 41%). No obvious plasmid sequence was found in any of the ten strains. One genomic island was predicted in JNFY1, JNFY11, and JNFY17, and two genomic islands were predicted in JNFY3. No genomic island was predicted in other strains. A CRISPR sequence was predicted in JNFY1, JNFY3, JNFY4, JNFY13, and JNFY28, but not in other prevalent strains (Table 2). The phylogenomic tree showed that the *Gardnerella* strains were generally divided into four groups, which was similar to the *cpn60* phylogenetic tree (Figure 1A). Strains JNFY1, 4, 9, 11, 13, 28, and ATCC 14019 were located in the same group in both trees; likewise, strains 409-05, JNFY3, 5-1, and JNFY15, 17, UMB0830 located to the same groups. Strains JNFY3 and JNFY15 were classified as *Gardnerella swidsinskii* and *Gardnerella piotii*, respectively, according to their genome sequences (Supplementary Figure 1). On the basis of the distance from the position of each strain to the root of the phylogenomic tree (Supplementary Figure 1), we calculated that the evolutionary order of each strain is as follows: ATCC 14018, JNFY3, 409-05, 5-1, JNFY14, JNFY15, JNFY17, JNFY4, JNFY11, JNFY9, JNFY13, JNFY1, and ATCC 14019.

Carbohydrate transport and metabolism

In general, biofilm formation is tightly related to carbohydrate transport and metabolism. Compared with the biofilm-rich strain JNFY14, the biofilm-lacking strains JNFY3, JNFY15, JNFY17, and JNFY28 lacked many genes involved in polysaccharides synthesis and sugar transport. Absent genes included *afuC*, *araD*, *fucP*, *galM*, *galT*, *lacZ*, *mglA*, *xylB*, and *xylF*. In addition, there were fewer copies of *nagC*, *ugpA*, *ugpB*, and *ugpE* in biofilm-lacking strains, compared to the biofilm-rich strain JNFY14 (Supplementary Table 2). The
Biofilm formation by *Gardnerella* strains. Isolates were cultured in 96-well plate in BHI medium, stained at 48 h, after removal of planktonic cells.

**Table 2** The general features of the 10 *Gardnerella* genomes.

| Strains | Size (bp) | CDS number | Plasmid | Island | GC % | tRNA | rRNA | CRISPR | Accession No. |
|---------|-----------|------------|---------|--------|------|------|------|--------|---------------|
| JNFY1   | 1,711,437 | 1,316      | NO      | 1      | 41.61| 45   | 6    | 1      | CP083177      |
| JNFY3   | 1,602,355 | 1,245      | NO      | 2      | 42.02| 45   | 6    | 1      | CP083176      |
| JNFY4   | 1,742,450 | 1,359      | NO      | NO     | 41.56| 45   | 6    | 1      | CP083175      |
| JNFY9   | 1,640,523 | 1,243      | NO      | NO     | 41.41| 45   | 6    | NO     | CP083174      |
| JNFY11  | 1,743,456 | 1,357      | NO      | 1      | 41.82| 45   | 6    | NO     | CP083173      |
| JNFY13  | 1,682,566 | 1,290      | NO      | NO     | 41.64| 45   | 6    | 1      | CP083172      |
| JNFY14  | 1,680,963 | 1,290      | NO      | NO     | 41.67| 45   | 6    | NO     | CP083171      |
| JNFY15  | 1,541,442 | 1,164      | NO      | NO     | 42.55| 45   | 6    | NO     | CP083170      |
| JNFY17  | 1,595,814 | 1,236      | NO      | 1      | 42.74| 45   | 6    | NO     | CP083169      |
| JNFY28  | 1,542,082 | 1,392      | NO      | NO     | 42.23| 45   | 6    | 1      | CP083168      |

Absence and reduced copy numbers of these genes may explain why the strains of this group cannot form normal biofilm.

**Antibiotic resistance**

According to the genomic data, all of the ten *Gardnerella* isolates contained antibiotic resistance genes, with a total of four detected antibiotic resistance genes. JNFY1, JNFY3, JNFY4, JNFY9, JNFY11, and JNFY15 contained the macrolide erythromycin resistance gene *ermX*. Strains JNFY1, JNFY4, JNFY9, JNFY11, JNFY13, JNFY14, JNFY15, JNFY17, and JNFY28 contained the lincosamide antibiotic resistance gene *lsaC*, while strains JNFY4, JNFY9, and JNFY13 contained the tetracycline resistance genes *tetL* and *tetM*. The comparative genomics data also showed that all strains contain the daunorubicin resistance protein (Table 3).

Resistance tests showed that *Gardnerella* strains containing erythromycin resistance genes were resistant to macrolide azithromycin. Similarly, prevalent strains containing tetracycline resistance genes were resistant to tetracycline. Strains JNFY13 and JNFY28 showed weak resistance to erythromycin, despite their lack of *ermX*. Alternatively,
Table 3 Antibiotic-resistance genes predicted in genome of the Gardnerella strains.

| Strains     | Macrolides-resistant | Tetracyclines-resistant |
|-------------|-----------------------|-------------------------|
|             | ermX    | IsaC    | tetL    | tetM    |
| ATCC 14019  | 0       | 1       | 0       | 0       |
| JNFY1       | 1       | 1       | 0       | 0       |
| JNFY3       | 1       | 0       | 0       | 0       |
| JNFY4       | 1       | 1       | 1       | 1       |
| JNFY9       | 1       | 1       | 1       | 1       |
| JNFY11      | 1       | 1       | 0       | 0       |
| JNFY13      | 0       | 1       | 1       | 1       |
| JNFY14      | 0       | 1       | 0       | 0       |
| JNFY15      | 1       | 1       | 0       | 0       |
| JNFY17      | 0       | 0       | 0       | 0       |
| JNFY28      | 0       | 1       | 0       | 0       |

Strain JNFY3 showed strong resistance to lincosamide, despite its lack of IsaC. YadH (ABC-type multidrug transport system) and McrA (5-methylcytosine-specific restriction endonuclease) existed only in metronidazole-resistant strains JNFY3, JNFY4, JNFY14, JNFY17, and JNFY28 (Table 4), thus these genes are likely involved in metronidazole resistance.

**Virulence**

Typically, epithelial cell adhesion is mediated by pili. All ten strains of Gardnerella contained ppK, a gene encoding for type IV pili. Some Gardnerella strains share virulence factors involved in pathogenic mechanisms like epithelial cell adhesion, iron absorption, secretion, acting as toxins and endotoxins, and immune escape; virulence factors can also have regulatory functions (Supplementary Table 3). Strains lacking these virulence factors such as JNFY3, JNFY15, and JNFY17 have the common feature of impaired biofilm formation. Gardnerella produces vaginolysin, which plays a critical role in BV pathogenesis. The gene encoding vaginolysin was detected in nine strains, with the exception of JNFY15 (Supplementary Table 4). Strains JNFY3 and JNFY4 exhibited nine and one unique virulence factor(s), respectively, with virulence factors having roles in allantoin utilization, adhesion, secretion and iron uptake (Supplementary Table 5). The TA components and other competitive exclusion genes of the Gardnerella strains are listed in Table 5. There are mainly four TA systems in Gardnerella genomes including HicAB family, PHD-RelE family, ParE family, and RelB-Txe family, with various distribution in the 11 strains. However, the other genes related to competitive exclusion showed a similar pattern in each strain.

**Secondary metabolites biosynthesis**

Analysis of secondary metabolite gene clusters in ten isolates of Gardnerella demonstrated the presence of polyketosynthesase (PKS)-related secondary metabolite gene clusters in the Gardnerella genome. PKS genes encode an enzyme, or enzyme complex with multiple domains, capable of synthesizing polyketo compounds, including common antibiotics such as erythromycin, tetracycline and so on. Type I, type II, and type III PKS synthesize T1PKS, T2PKS, and T3PKS, respectively. The T3PKS gene cluster was predicted in all strains except JNFY3.

**Discussion**

Bacterial vaginosis (BV) is a common vaginal infection in women of child-bearing age, with Gardnerella spp. being the main pathogen of BV. Alongside BV, Gardnerella spp. has also been associated with vertebral osteomyelitis and discitis (Graham et al., 2009), retinal vasculitis (Neri et al., 2009), acute hip arthritis (Sivadon-Tardy et al., 2009), and bacteremia (Chen et al., 2018). While the vaginal overgrowth of several anaerobic bacteria has been associated with BV, Gardnerella spp. has a stronger adhesion to vaginal epithelium and a stronger tendency to form biofilm compared to other BV-related microorganisms. It can be used as a scaffold to attach other anaerobic bacteria and is associated with the onset and recurrence of BV (Anahit et al., 2018). The treatment of recurrent BV is difficult, and existing treatment measures include the prolongation of an antibiotic course and consolidation treatment. However, due to biofilm formation and strain differences in Gardnerella, antibiotics cannot eliminate the bacteria, making the current treatment of BV unable to achieve ideal therapeutic effect (Cohen et al., 2020; Laniewski et al., 2020). Therefore, it is of great significance to construct a database detailing prevalent Gardnerella strains in local China, which can provide the basis for the accurate diagnosis and therapy of BV and allow for the development of more effective antibacterial drugs targeting prevalent strains.

In this study, 20 prevalent strains of Gardnerella from local China were collected from clinical specimens to study the differences in their pathogenicity and drug resistance. This would provide a theoretical basis for the accurate diagnosis and treatment of BV. First, based on cpn60 sequence genotyping, the 20 prevalent strains were divided into subtypes A, B, and C. Based on vly sequence genotyping, prevalent strains were divided into subtypes 1, 2, 3, and 4. Ten strains of subtypes C1 (JNFY9, 13 and 28), C2 (JNFY14 and 11), B1 (JNFY14 and 17), A1 (JNFY3), and B4 (JNFY15) were selected for comparative genomics. The genome size of the 10 strains ranged from 1.54 to 1.74 Mb, and the GC content was about 41–43%. No plasmid was observed, and 1–2 gene islands were predicted in 4 strains. The phylogenomic tree of the Gardnerella strains showed that strains JNFY3, JNFY15,17, and JNFY1,4,9,11,13,14,28 were
TABLE 4  MIC (minimum inhibitory concentration) of the Gardnerella strains on common antibiotics in clinic.

| Antibiotics-resistant genes | MIC (µg/mL) |
|-----------------------------|-------------|
|                            | First-line  | Second-line | Third-line |
|                            | Beta-lactam | Macrolide   | Quinolone  | Nitroimidazole | Aminoglycoside | Macrolide | Aminoglycoside | Beta-lactam | Glycopeptide |
|                            | AMP | TE | AZM | ERM | CLI | CIP | MTZ | GM | DNR | TO | CMR | VA |
| ATCC 14019                 | <0.25 | <0.25 | <0.25 | 0 | 0 | 2 | 8 | 16 | 2 | 64 | 0.25 | 1 |
| JNFY1                      | 0.5 | 0.25 | 4 | 0.5 | 0 | 16 | 128 | 8 | 0 | 16 | 0.25 | 1 |
| JNFY3                      | 0.5 | 0.25 | 16 | 0.125 | 0.0625 | 16 | >128 | 16 | 8 | 32 | 0.5 | 1 |
| JNFY4                      | 0.5 | 16 | 16 | 32 | 128 | 16 | >128 | 16 | 2 | 32 | 0.5 | 1 |
| JNFY9                      | 0.25 | 16 | 8 | 0 | 0 | 16 | 4 | 4 | 0.125 | 16 | 0.5 | 1 |
| JNFY11                     | 1 | 0.25 | 64 | 0.125 | 16 | 16 | 2 | 8 | 1 | 32 | 1 | 1 |
| JNFY13                     | 0.25 | 32 | 0.25 | 0.125 | 0 | 1 | >128 | 16 | 2 | 128 | 1 | 1 |
| JNFY14                     | 4 | 0.125 | 0.5 | 0 | 0.0625 | 32 | >128 | 32 | 0.5 | 32 | 1 | 1 |
| JNFY15                     | 0.5 | <0.25 | 8 | 128 | 128 | 16 | 16 | 32 | 1 | 128 | 0.5 | 0.5 |
| JNFY17                     | 4 | 0.5 | 0.5 | 0 | 0 | 32 | >128 | 32 | 0 | 64 | 2 | 1 |
| JNFY28                     | 0.5 | 0.25 | 0.25 | 0.25 | 0 | 8 | >128 | 16 | 32 | 128 | 0.5 | 1 |

AMP, ampicillin; TE, tetracycline; AZM, azithromycin; ERM, erythromycin; CLI, clindamycin; CIP, ciprofloxacin; MTZ, metronidazole; GM, gentamicin; DNR, daunorubicin; TO, tobramycin; CMR, chloramphenicol; VA, vancomycin.
### TABLE 5 TA system components and other competitive exclusion genes.

| TA system                      | ATCC 14019 | JNFY1 | JNFY3 | JNFY4 | JNFY9 | JNFY11 | JNFY13 | JNFY14 | JNFY15 | JNFY17 | JNFY28 |
|-------------------------------|------------|-------|-------|-------|-------|--------|--------|--------|--------|--------|--------|
| HicA-family TA system         |            | 1     |       |       |       | 2      |        |        |        |        |        |
| toxin                         |            |       |       |       |       |        |        |        |        |        |        |
| HicB-family TA system         |            |       |       |       |       |        |        |        |        |        |        |
| antitoxin                     |            | 1     |       |       |       | 2      |        |        |        |        |        |
| PHD/YefM family TA system     |            | 1     |       |       |       |        |        |        |        |        |        |
| antitoxin                     |            |       |       |       |       |        |        |        |        |        |        |
| RelE/StbE family TA system    |            | 3     |       |       |       |        |        |        |        |        |        |
| toxin                         |            |       |       |       |       |        |        |        |        |        |        |
| PHD only                      |            |       |       |       |       |        |        |        |        |        |        |
| PHD only                      |            |       |       |       |       |        |        |        |        |        |        |
| PHD only                      |            |       |       |       |       |        |        |        |        |        |        |
| RelB only                     |            |       |       |       |       |        |        |        |        |        |        |
| RelB only                     |            |       |       |       |       |        |        |        |        |        |        |
| RelB only                     |            |       |       |       |       |        |        |        |        |        |        |
| Other genes with potential roles in competitive exclusion |
| Abi-like protein              | n/a        | 3     |       |       |       | n/a    |        |        |        |        |        |
| CHAP domain protein           | 2          | 2     |       |       |       | 2      |        |        |        |        |        |
| GH25 enzyme                   | 1          | 1     |       |       |       | 1      |        |        |        |        |        |
| SalY-family antimicrobial      | 4          | 5     |       |       |       | 5      |        |        |        |        |        |
| peptide ABC transport system  |             |       |       |       |       |        |        |        |        |        |        |
| ATP-binding protein           |             |       |       |       |       |        |        |        |        |        |        |

n/a indicates protein was not identified within the genome.
located in three different branches. This was almost identical to the \textit{cpn60} phylogenetic tree, except for the position of strain JNFY14. Therefore, the \textit{cpn60} distribution could represent genomic classification to some extent. The \textit{G. vaginalis} strains 5-1, 409-05 and JNFY3 were grouped in the same branch in all three trees, further indicating the weak pathogenicity of these strains in healthy hosts.

Virulence factors associated with microbial pathogenesis were found in the genome of prevalent strains, including factors for adhesion, secretion, iron and magnesium absorption, immune escape, and toxins. Common virulence factors were found in the ten prevalent strains, although differences also exist amongst the strains. It is worth noting that JNFY3 had nine specific virulence factors while JNFY4 had one. In addition, JNFY3, JNFY15, and JNFY17 had severely hindered abilities in biofilm formation. These three strains not only lacked certain carbohydrate metabolism genes (Supplementary Table 2), but also lacked virulence factors related to adhesion, iron absorption and toxins, which might be conductive to biofilm formation in the comparative genomic analysis.

Subsequent biochemical experiments were conducted to preliminarily investigate the pathogenic mechanism of \textit{Gardnerella}. Experiments on the biofilm-forming capabilities of \textit{Gardnerella} showed variable abilities to form biofilm amongst different prevalent strains with \textit{cpn60} subtype C displaying weak to moderate biofilm-forming abilities. Sialidases are enzymes associated with bacterial invasion of the host and are implicated as virulence factors in diseases such as meningitis, glomerulonephritis, and periodontal disease (Corfield, 1992). Previous studies have shown that BV-associated bacteria produce sialidase, and its activity is inversely related to vaginal IgA response against vaginolysin produced by \textit{Gardnerella} (Cauci et al., 2003). The sialidase assay showed that most strains lacked sialidase activity despite some strains having a higher NS; thus, there is no association between sialidase and pathogenicity.

Resistance tests showed that \textit{Gardnerella} was sensitive to antibiotics, including tetracycline, nitroimidazoles, macrolide and aminoglycosides. Meanwhile, the macrolide erythromycin resistance genes \textit{ermX} and \textit{lauC}, and the tetracycline resistance genes \textit{tetL} and \textit{tetM} were also predicted in the ten strains. It is worth noting that seven out of the ten strains exhibited strong resistance ($\geq 128$ µg/mL) to metronidazole, which is the first line of treatment for BV in China. In addition to nitroreductase, YadH and McrA may also have roles in metronidazole resistance, with YadH involved in metronidazole excretion and McrA hydrolyzing the methyl-nitryl group from metronidazole (Lorca et al., 2007; Lubelski et al., 2007).

Additionally, we compared pathogenic genes of the prevalent strain in local China and the American type strain ATCC 14019. According to the phylogenetic analysis, strain ATCC 14019 belonged to the subtype C1, and possessed fairly thick biofilm and tobramycin resistance. Compared to strain ATCC 14019, the Chinese prevalent strains contained poor and incomplete TA systems with only antitoxin genes included, suggesting that the antitoxins may be essential to neutralize the toxins secreted by other microbes in the vaginal biome. The strains JNFY4, JNFY9, JNFY11, JNFY13, and JNFY14 without Abi-like proteins lack means of defense to a broad-range of bacteriocins produced by opponents, which probably is complemented by the single antitoxins (Kjos et al., 2010). Although there was no intact secretion system in \textit{Gardnerella}, it was speculated that \textit{Gardnerella} secreted toxic proteins to injure other bacteria, such as \textit{Lactobacillus}, to enhance their survivability and displace the balance of the normal vaginal microflora. Strain ATCC 14019 encodes a methicillin resistance protein not found in the ten Chinese prevalent strains. Carbohydrate transport and...
metabolism genes of the 11 Gardnerella strains, including the genes associated with biofilm formation, were also analyzed and combined with biofilm thickness data (Figure 2 and Supplementary Figure 2). AraD, GalM, and GalT may be responsible for constructing biofilm, while FucP, MalA, and XylF are involved in monosaccharide transport. The additional monosaccharide units in the biofilm of strain JNFY14, arabinose and galactose hydrochloride, may play the role of connective elements which help to build a thicker biofilm.

Previous treatment standards involving clindamycin and metronidazole cannot account for all prevalent strains with sufficient efficacy to achieve complete recovery. Therefore, we need targeted treatments for diseases caused by different prevalent strains of Gardnerella to obtain the best results. Although the pathogenic genes of Gardnerella were previously characterized, the pathogenesis of BV is not yet understood. Through genomic and biochemical data analysis, we found that strains of subtype C could generally form biofilm, with varying degrees of antibiotic resistance (apart from invariable tobramycin resistance). Strain G. swidsinskii JNFY3 had a NS of 1, impaired ability to form biofilm, and lacked the sld and lsaC genes. G. vaginalis JNFY14 had the thickest biofilm, whereas strains JNFY17 and G. pioitii JNFY15 rarely produced biofilm. On the whole, with the exception of strain JNFY14, the evolutionary trend appears to be the development of a progressively thicker biofilm from JNFY3 to ATCC 14019. All three strains (JNFY14, JNFY17, and JNFY15) showed some resistance to ciprofloxacin, gentamicin and tobramycin. Additionally, G. pioitii JNFY15 lacked the vly gene and grew slower than other Gardnerella strains (Table 1). Based on this study, we constructed an initial database of Gardnerella prevalent strains in local China, which can be used as a reference to elucidate more accurate diagnostic pathways and treatments for BV (Table 1 and Figure 3).

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary material.

Ethics statement

The studies involving human participants were reviewed and approved by the Medical Ethics Committee of Jinan Maternity and Child Care Hospital. The patients/participants provided their written informed consent to participate in this study.

Author contributions

LG, KZ, and RL conceptualized and designed the study. KZ and XZ analyzed the data. ML, KZ, KW, and HD accessed and verified the data. XJ collected samples from participants. KZ, FZ, and TL wrote the first draft of the manuscript. All authors contributed to drafting of the manuscript and read and approved the final version of the manuscript for submission, had full access to all data in the study, and had final responsibility for the decision to submit for publication.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.1009798/full#supplementary-material
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