Methods and Applications

Development of a Multiplex Real-Time Quantitative PCR Assay for Detecting Vaginal Microbiota in Chinese Women — China, 2021–2022

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ABSTRACT

Introduction: The Nugent score, limited by subjectivity and personnel requirements, lacks accuracy. Establishing a precise and simple molecular test is therefore essential for detecting vaginal microbiota compositions and evaluating vaginal health.

Methods: We evaluated the vaginal health of Chinese women using quantitative polymerase chain reaction (qPCR) to target *Lactobacillus crispatus* (*L. crispatus)*, *L. iners*, *Gardnerella vaginalis* (*G. vaginalis)*, *Atopobium vaginae* (*A. vaginae)*, and *Megasphaera phylotype1*. bacterial vaginosis (BV)-related bacteria shared a fluorescent channel. Using 16S rDNA sequencing as a reference standard, we evaluated and validated the diagnostic accuracy of the qPCR assay.

Results: Both qPCR and 16S rDNA sequencing demonstrated 90.5% concordance in segregating vaginal community state type (CST), as visualized through heatmaps and PCoA. Spearman's correlation analysis revealed strong correlations between the two methods in calculating the RA of *L. crispatus* (CST I), *L. iners* (CST III), and BV-related bacteria (CST IV), with coefficients of 0.865, 0.837, and 0.827, respectively. Receiver operating characteristic analysis showed that qPCR had significant diagnostic accuracy for CST I, CST III, and CST IV (molecular BV), with area under the curve values of 0.967, 0.815, and 0.950, respectively, indicating strong predictive power.

Discussions: Vaginal health can be evaluated using a single qPCR amplification experiment, making the multiplex qPCR assay a highly accurate tool for this purpose.

The human vaginal microbiota is dominated by *Lactobacillus* species, with *L. crispatus* serving as a crucial indicator of reproductive health (*1*). Vaginal dysbiosis, characterized by a shift in microbiota composition from lactobacilli to pathogenic bacteria, commonly leads to bacterial vaginosis (BV) (*2*). BV is associated with an increased risk of negative reproductive health outcomes, including sexually transmitted infections and spontaneous preterm births (*3*–*4*). Traditional BV diagnosis relies on the Nugent score, which involves the examination of Gram-stained smears and a score greater than 7 indicating BV (*5*). However, this method requires experienced personnel and can be subjective, leading to reduced sensitivity and specificity. Recently, 16S ribosomal DNA (16S rDNA) sequencing has emerged as a tool for assessing vaginal health status by calculating community state types (CSTs). Notably, CST IV has been termed "molecular-BV" (*1*,*6*). However, the high cost and complexity of gene sequencing hinder its implementation in developing countries with large populations, such as China. Therefore, a simple and accurate diagnostic method for BV would be highly beneficial for both clinicians and patients.

Real-time quantitative polymerase chain reaction (qPCR) has revealed various dominant BV-related bacteria. *G. vaginalis* and *A. vaginae* were the most common species in BV with high abundance, and *Megasphaera* spp. was associated with BV independently (*7*). In this study, we developed a multiplex qPCR assay targeting the aforementioned BV-related bacteria and two *Lactobacillus* species (*L. crispatus* and *L. iners*), followed by 16S rDNA sequencing for validation. This assay allows vaginal health to be evaluated using a single qPCR amplification experiment, thereby facilitating early identification of patients at high risk of severe vaginal dysbiosis and prevention of future adverse sequelae.

METHODS

Study Participants and Specimen Collection

This study enrolled 84 participants at Beijing Ditan

Hospital, Capital Medical University, between October 2021 and November 2022. All participants were over 18 years old, with a median age of 38 (range 31.5–43), and had not used antimicrobials for two weeks prior to enrollment. Vaginal swabs were collected from the upper third of the vagina by an experienced gynecologist and used to determine Nugent scores via Gram staining. Additionally, swabs were immediately transported to the laboratory and stored at −80 °C for subsequent DNA extraction, qPCR, and 16S rDNA sequencing. This study received ethical approval from the Beijing Ditan Hospital Ethics Committee (No. 2021-22-01).

Multiplex qPCR Set-up

Total DNA was extracted using the MagaBio Bacterium DNA Fast Purification Kit (Hangzhou Bori Biological Technical, China) according to the manufacturer's instructions. Using a broad-coverage 16S rDNA gene sequence as an internal reference, PCR was performed for *G. vaginalis*, *A. vaginae*, *Megasphaera phylotype1*, *L. crispatus*, and *L. iners* according to a previously described methodology (*8*). Real-time qPCR was conducted on an ABI QuantStudio 1 Plus instrument (Thermo Fisher Scientific, USA) using multiplex Taq polymerase (Vazyme Biotech, China), with the three BV-associated bacteria sharing a fluorescent channel (Supplementary Table S1, available at https://weekly.chinacdc.cn/). The 25 μL qPCR mix contained 5 μL of DNA. Primer/probe concentrations for broad-coverage 16S rDNA were set at 1.5 μmol/L (forward), 1.0 μmol/L (reverse), and 0.5 μmol/L, and at 0.5 μmol/L and 0.25 μmol/L for other species. Primer and probe specificities were confirmed using PRIMER BLAST. The cycling conditions included a 2 min incubation at 37 °C and initial denaturation for 30 s at 95 °C, followed by 45 cycles of denaturation for 10 s at 95 °C and annealing/extension for 30 s at 60 °C. Samples were run in duplicate, with distilled sterile water as a negative control. Additionally, single-plex PCR was performed for the three BV-related bacteria and compared with the multiplex qPCR.

The cycle threshold (Ct) values obtained for each fluorescent channel (*L. crispatus*, *L. iners*, and BV-related bacteria) and the 16S rDNA gene sequences (internal reference) were designated as Ct1 and Ct2, respectively. The relative abundance (RA) of each species was calculated using $2^{-\Delta(Ct1-Ct2)}$ (*9*). Species with an RA exceeding 20% were considered dominant and used to determine the CST of the vaginal microbiota (*10*).

16S rDNA Sequencing

The V3–V4 regions of the 16S rDNA gene were amplified using primers 341F-805R and Q5 Hot Start High-Fidelity 2X Master Mix (New England Biolabs). Two rounds of PCR were performed before sequencing on the Illumina HiSeq^{TM} platform. QIIME (version 2022.11.1, NAU, Flagstaff, USA) was used to analyze the data and identify operational taxonomic units and species. CSTs were determined based on the RA and distribution of dominant bacteria. CST I is dominated by *L. crispatus* and is usually linked to a healthy vaginal state. CST III is dominated by *L. iners* and can be found in both healthy and pathological states. CST IV is highly diverse with low levels of *lactobacilli* and is typically associated with BV.

Statistical Analysis

Chi-square and Fisher's exact tests were used to compare categorical variables (N/%) across groups. CST segregation by qPCR and 16S ribosomal DNA (16S rDNA) was assessed using principal coordinate analysis (PCoA) with Bray-Curtis distance. Spearman's correlation test was used to analyze the relationship between qPCR and 16S rDNA data. The diagnostic performance of qPCR for vaginal microbiota (CST I, III, and IV), including sensitivity, specificity, area under the curve (AUC), and positive and negative predictive values, was evaluated using receiver operating characteristic (ROC) curves. Statistical analyses were performed using SPSS (version 26.0, IBM, Armonk, NY, USA), GraphPad (version 9.0, GraphPad Software, San Diego, California), MedCalc (version 20.0, MedCalc Software, Mariakerke, Belgium), Easy application (*11*), and ImageGP (*12*). The two-tailed significance level was set at 0.05.

RESULTS

Basic Information of Patients and 16S rDNA-seq

16S rDNA sequencing revealed three CSTs at the species level. Of the 55 participants exhibiting *Lactobacillus* dominance, 28.6% (*n*=24) and 32.1% (*n*=27) exhibited *L. crispatus* (CST I) and *L. iners* (CST III) dominance, respectively. Four women had other *Lactobacillus* species, including two with *L. jensenii* and one each with *L. gasseri* and *L. delbrueckii*. CST IV, characterized by a lack of dominant species and a mix of *G. vaginalis*, *A. vaginae*, and other anaerobes, was

identified in 34.5% (n=29) of the participants (Table 1).

Establishment of A Multiplex qPCR Set

The effectiveness of multiplex qPCR was assessed using the relative abundance (RA) of 16S rDNA amplicon sequencing as the standard. Figure 1A compares the amplification efficiency between single-plex and multiplex qPCR. Each row represents the amplification curve of each sample for *G. vaginalis*, *A. vaginae*, and Megasphaera phylotype 1 obtained using single-plex and multiplex qPCR. Both single-plex and

TABLE 1. Comparison of vaginal flora typing results based on qPCR and 16S rDNA V3V4 sequence analysis [*n* (%)].

Dominant species	CST	qPCR	16S rDNA	χ^2	*P*	Common cases
Lactobacillus crispatus	CST I	24 (28.6)	24 (28.6)			24 (32.9)
Lactobacillus iners	CST III	31 (36.9)	27 (32.1)			27 (32.9)
BV-related bacteria	CST IV	21 (25.0)	29 (34.5)	2.889	0.409	21 (28.8)
Other species	Other types	8 (9.5)	4 (4.8)			4 (5.5)
Total cases		84	84			76

Abbreviation: CST=community state type; qPCR=quantitative polymerase chain reaction.

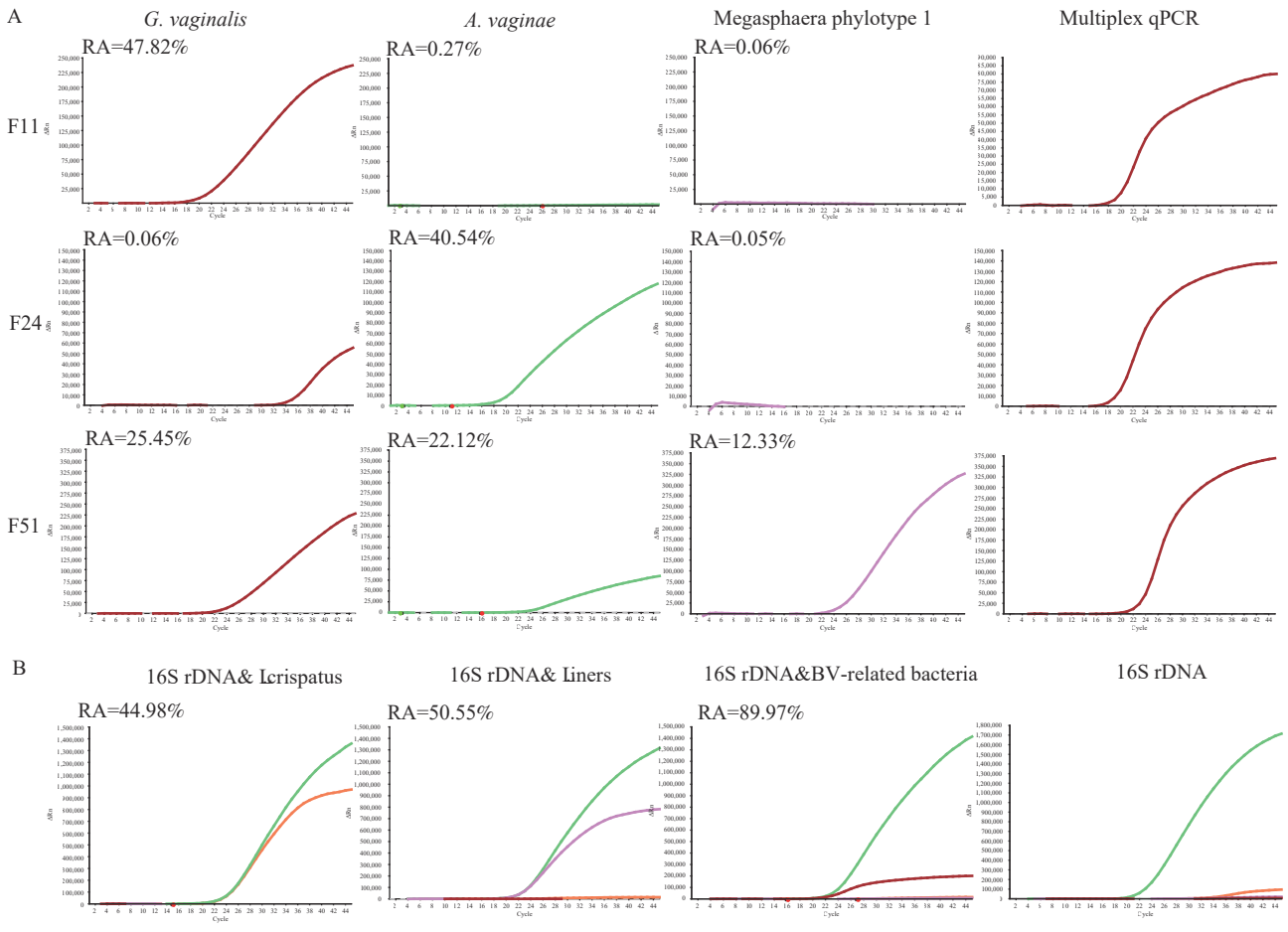

FIGURE 1. Comparison of amplification curves from multiplex qPCR and single-plex qPCR using 16S rDNA sequencing as the standard. (A) Three samples (F11, F24, F51) with absolutely higher RA of single BV-related species (*G. vaginalis*, *A. vaginae* and *Megasphaera phylotype1*) were selected to perform by single-plex qPCR with the same primer and probe of the multiplex qPCR. (B) Using RA calculated by 16S rDNA-seq, three samples with higher RA of single species and less abundance of the remaining species and one sample without these species were detected by the multiplex qPCR.
Note: For (A), the amplification curves were compared with those of the same sample detected by the multiplex qPCR.
Abbreviation: CST=community state type; RA=relative abundance; qPCR=quantitative polymerase chain reaction.

multiplex qPCR showed sigmoidal curves, reaching a plateau, indicating consistency with 16S rDNA sequencing data and validating the accuracy of multiplex qPCR in detecting these targets.

Figure 1B highlights the specificity of multiplex qPCR using the RA of 16S rDNA sequencing as the standard. In the first sample, *L. crispatus* (44.98% RA, CST I) was accurately amplified using multiplex qPCR (Ct=20.41), while the other four species were not detected. In the second sample, qPCR amplification (Ct=20.35) revealed *L. iners* dominance (50.55% RA, CST III), and other species were not detected. The third sample demonstrated *G. vaginalis* dominance (89.97% RA, CST IV) with amplification of both BV-related bacteria and universal primers (Ct=19.49), while *L. crispatus* and *L. iners* were not detected. In the fourth sample, characterized by a low RA for all targets, only the universal primer was amplified (Ct=18.84), aligning with the sequencing data.

Evaluation and Validation of Multiplex qPCR Assay's Accuracy

Table 1 presents a comparison of CSTs identified using qPCR and 16S rDNA, showing no significant difference between the methods (P=0.409). Among the 84 participants, 76 shared CSTs were identified, yielding a concordance rate of 90.5%. Heatmaps depicted the consistency of CST I and CST III across both methods and visually highlighted the 8 mismatches (Figure 2A–2B). Among the 8 samples with mismatched CSTs, F01, F07, and F11 exhibited opposite conclusions regarding CST classification by dominant microbiota, with CST III identified by multiplex qPCR and CST IV by 16S rDNA sequencing. In samples F36, F31, F02, F03, and F33, the dominant microbiota were *Shuttleworthia*, *Peptostreptococcus*, *Bifidobacterium breve*, *Streptococcus gallolyticus*, and *Bifidobacterium animalis*, respectively (Supplementary Table S2, available at https://weekly.chinacdc.cn/). These bacterial species were not within the detection range of the multiplex qPCR assay and were therefore not detected. PCoA demonstrated a clear segregation of the three CSTs using both qPCR and 16S rDNA (adonis P<0.001) (Figure 2C–2D). Additionally, a strong correlation was observed in the RA of *L. crispatus*, *L. iners*, and BV-related bacteria between the two methods, with Spearman's coefficients of 0.865, 0.837, and 0.827, respectively (Figure 2E–2G).

Table 2 presents the diagnostic performance of multiplex qPCR for *L. crispatus* (CST I), *L. iners* (CST III), and BV-related bacteria (CST IV), with optimal Ct cutoffs of 21.4, 22.9, and 22.8, respectively. ROC analysis demonstrated AUCs of 0.967, 0.815, and 0.950 for CST I, CST III, and CST IV, respectively (all P<0.001), indicating strong predictive capabilities (Supplementary Figure S1, available at https://weekly.chinacdc.cn/).

DISCUSSION

This study developed and validated a multiplex qPCR assay to evaluate vaginal health using a single amplification experiment. This assay addresses critical gaps in existing diagnostic approaches while enhancing accuracy and offering a more cost-effective solution for evaluating vaginal health.

The Nugent score has traditionally been used to assess the degree of clinical BV (*5*,*13*). However, its limitation to genus-level bacterial identification makes precise BV assessment challenging, hindering clinical diagnosis and treatment. In contrast, multiplex qPCR using 16S rDNA as a reference demonstrated 90.5% consistency, surpassing the Nugent score in accuracy. Excluding five cases (6.0%) beyond the detection scope, three discrepancies were observed for CST III and IV. This discrepancy may be attributed to the genomic variability of *L. iners*, which allows it to exist as either a symbiont or parasite in healthy, imbalanced, or diseased vaginal environments (*14*).

Previous studies have shown that single-plex qPCR to detect *G. vaginalis* or *A. vaginae* for predicting BV yielded limited diagnostic accuracy, with a sensitivity and specificity of 83.6% and 84.1%, respectively, for *G. vaginalis* and 87.1% and 90.7%, respectively, for *A. vaginae* (*15*). Conversely, a combined approach incorporating *G. vaginalis*, *A. vaginae*, and *Megasphaera phylotype1* significantly improved diagnostic performance, achieving a sensitivity of 92% and a specificity of 95% in diagnosing BV (*15*). Consistent with these results, we found that multiplex qPCR had strong diagnostic performance in predicting molecular BV, with a sensitivity of 89.7% and specificity of 94.5%. Notably, an innovative aspect of this multiplex qPCR method is its ability to detect three BV-related bacteria using a common fluorescent channel. Additionally, it incorporates two primary *Lactobacillus* species: *L. crispatus*, which is used to assess vaginal health, and *L. iners*, which is employed to evaluate the intermediate state of BV. Multiplex qPCR focuses on analyzing the composition of the vaginal microbiota as a whole rather than targeting individual BV marker organisms, thereby reducing both the time and costs associated with diagnosis.

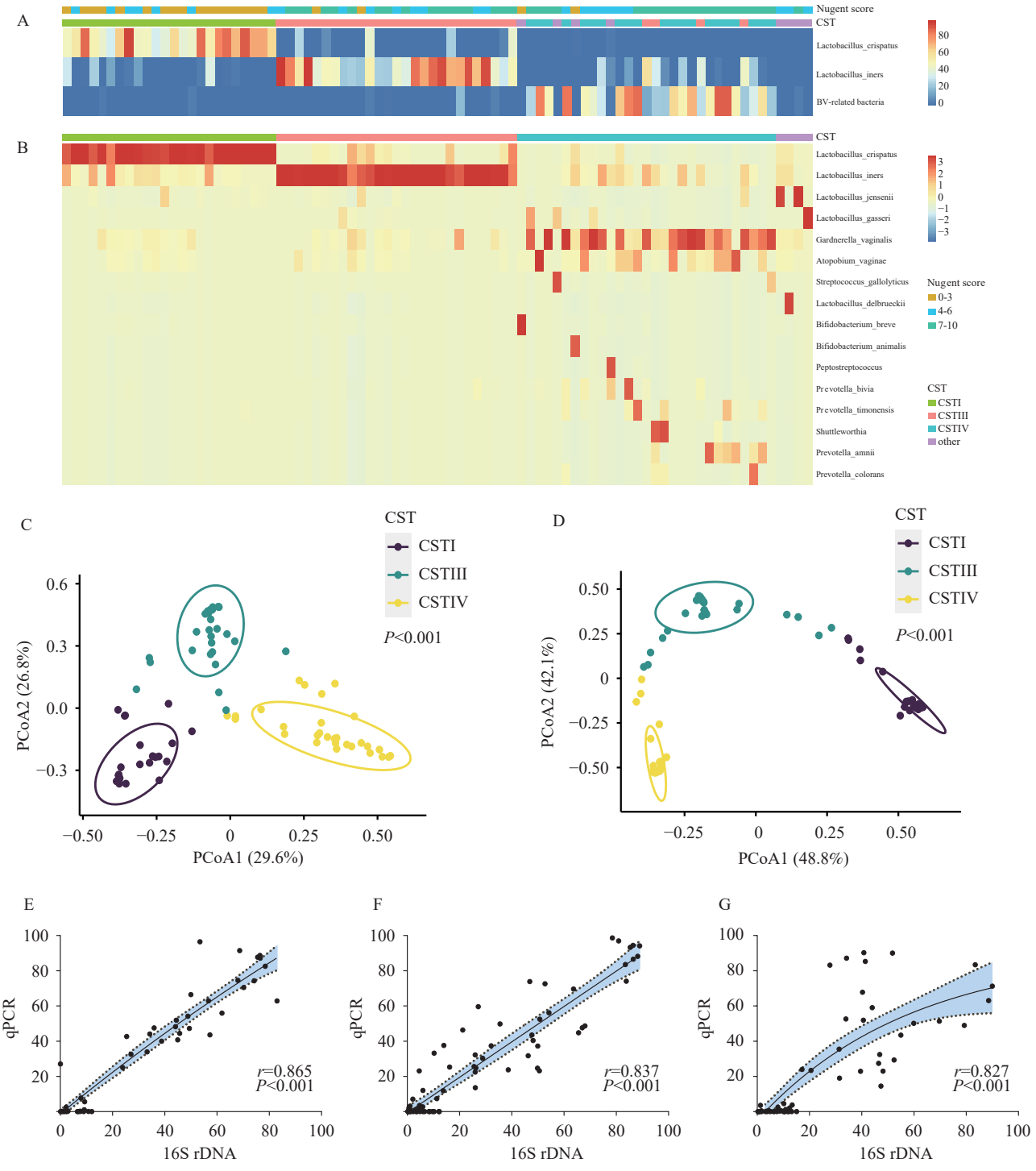

FIGURE 2. Comparison of CST categorized by multiplex qPCR and 16S rDNA sequencing. (A) Heatmap categorizing CSTs by dominant species using multiplex qPCR. (B) Heatmap categorizing CSTs by dominant species based on 16S rDNA sequencing. (C) PCoA based on the Bray-Curtis dissimilarity index from 16S rDNA sequencing. (D) PCoA based on the Bray-Curtis dissimilarity index from multiplex qPCR. (E) Correlation between multiplex qPCR and 16S rDNA sequencing in the RA of *Lactobacillus crispatus*. (F) Correlation between multiplex qPCR and 16S rDNA sequencing in the RA of *Lactobacillus iners*. (G) Correlation between multiplex qPCR and 16S rDNA sequencing in the RA of BV-related bacteria.
Note: *Lactobacillus crispatus* (CST I), *Lactobacillus iners* (CST III), and BV-related bacteria (CST IV).
Abbreviation: CST=community state type; RA=relative abundance; qPCR=quantitative polymerase chain reaction.

TABLE 2. Sensitivity, specificity and predictive values of bacterial signatures distinguishing the vaginal health condition and molecular BV by the multiplex qPCR.

AUC (95% *CI*)	Sensitivity (%) (95% *CI*)	Specificity (%) (95% *CI*)	PPV (%) (95% CI)	NPV (%) (95% *CI*)
Lactobacillus crispatus predicts CST I (21.4)*				
0.967 (0.904, 0.994)	100.0 (85.8, 100.0)	90.0 (79.5, 96.2)	80.0 (65.2, 89.5)	100.0
Lactobacillus iners predicts CST III (22.9)*				
0.815 (0.716, 0.892)	96.3 (81.0, 99.9)	59.7 (45.8, 72.4)	53.1 (45.0, 61.0)	97.1 (83.1, 99.6)
BV-related microbiota predicts CST IV (22.8)*				
0.950 (0.880, 0.986)	89.7 (72.6, 97.8)	94.6 (84.9, 98.9)	89.7 (74.1, 96.3)	94.5 (85.6, 98.1)

Abbreviation: AUC=area under the cure; BV=bacterial vaginosis; CST=community state type; *CI*=confidence interval; NPV=negative predictive value; PPV=positive predictive value; qPCR=quantitative polymerase chain reaction.
* Cutoff value.

This study provides valuable insights for accurately assessing vaginal health, but it has some limitations. First, this study is a single-center study and lacks multicenter validation, validation across multiple centers is necessary. Additionally, the small sample size of this cross-sectional study may have introduced bias. Therefore, future research should include larger longitudinal cohort studies to better understand causality.

Conflicts of interest: No conflicts of interest.

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SUPPLEMENTARY MATERIAL

SUPPLEMENTARY TABLE S1. The multiplex qPCR target and primer/probe sequences.

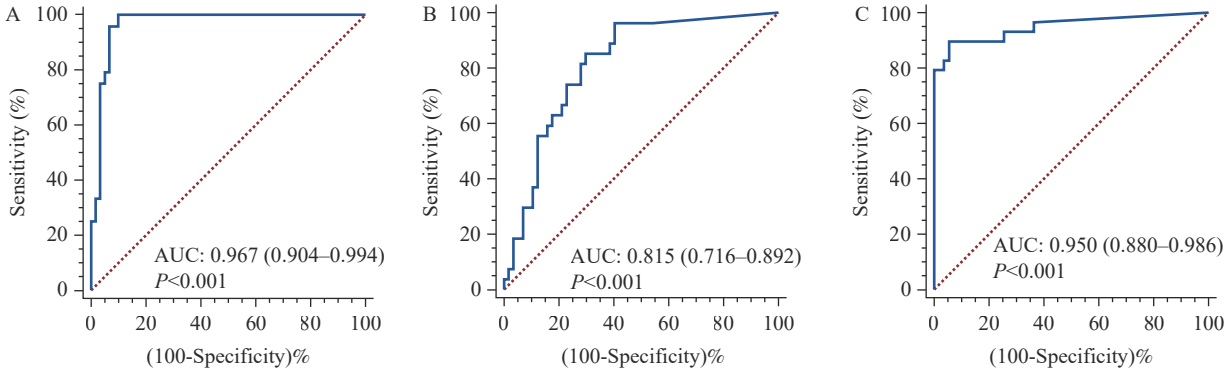
PCR target	Primer name	5′–3′ sequence*
16S rDNA	Forward	CGGTGAATACGTTCYCGG
	Probe	[VIC]CTTGTACACACCGCCCG
	Reverse	GGWTACCTTGTTACGACTT
Lactobacillus_iners	Forward	AGTCTGCCTTGAAGATCGG
	Probe	[FAM]CCAAGAGATCGGGATAACACCT
	Reverse	CTTTTAAACAGTTGATAGGCATCATC
Lactobacillus_crispatus	Forward	AACTAACAGATTTACTTCGGTAATGA
	Probe	[ROX]CCCATAGTCTGGGATACCACTT
	Reverse	AGCTGATCATGCGATCTGC
Atopobium vaginae	Forward	TAGGTCAGGAGTTAAATCTG
	Probe	[CY5]CTACCAGACTCAAGCCTGCC
	Reverse	TCATGGCCCAGAAGACCGCC
Gardnerella vaginalis	Forward	GCGGGCTAGAGTGCA
	Probe	[CY5]CTTCTCAGCGTCAGTAACAGC
	Reverse	ACCCGTGGAATGGGCC
Megasphaera phylotype1	Forward	GATGCCAACAGTATCCGTCCG
	Probe	[CY5]ACAGACTTACCGAACCGCCT
	Reverse	CCTCTCCGACACTCAAGTTCGA

Abbreviation: qPCR=quantitative polymerase chain reaction.
* Primers and probes were obtained from Beijing Tianyi Huiyuan Bioscience & Technology, Inc. (Beijing, China). Cyanine 5 (Cy5), Fluorescein (FAM), X-Rhodamin (ROX), and Victoria (VIC) were used as the 5′-coupled reporter fluorophores of the hydrolysis probes used in the multiplex PCR reaction, and the 3′-coupled with Tetramethylrhodamine (TAMRA) or Black Hole Quenchers (BHQ2) as quenchers.

SUPPLEMENTARY TABLE S2. The eight mismatch cases detected by qPCR and 16S rDNA V3V4 sequence analysis.

Sample ID	qPCR		16S rDNA	
	Dominant species	RA (%)	Dominant species	RA (%)
F01	*L. iners*	59.62	*G. vaginalis*	46.85
F07	*L. iners*	37.61	*G. vaginalis*	39.72
F11	*L. iners*	46.34	*G. vaginalis*	47.82
F36	*L. iners*	33.23	*Shuttleworthia*	27.16
F31	–	–	*Peptostreptococcus*	36.10
F02	–	–	*Bifidobacterium breve*	22.07
F03	–	–	*Streptococcus gallolyticus*	27.74
F33	–	–	*Bifidobacterium animalis*	24.75

Note: "–" means without the detection range of the multiplex qPCR assay.
Abbreviation: RA=relative abundance; qPCR=quantitative polymerase chain reaction.



SUPPLEMENTARY FIGURE S1. Using 16S rDNA sequencing as the gold standard, this figure assesses the predictive capability of multiplex qPCR for vaginal health in predicting CST I, CST III, and CST IV, respectively. (A) ROC curves for *Lactobacillus crispatus*; (B) ROC curves for *Lactobacillus iners*; (C) ROC curves for BV-related bacteria.
Abbreviation: CST=community state type; AUC=area under the curve; ROC=receiver operating characteristic; qPCR=quantitative polymerase chain reaction.