Original Research

Vaginal microbiota composition as a diagnostic tool for bacterial vaginosis in pregnant Korean women

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Summary

Objectives: To analyze the relative abundance of five *Lactobacillus* species, *Gardnerella vaginalis*, and *Atopobium vaginae* in the vaginal microbiota of pregnant Korean women to establish diagnostic criteria for bacterial vaginosis (BV). *Materials and Methods:* Pregnant Korean women under antenatal care at Eulji University Hospital were enrolled at 10–14 weeks gestation. Twelve women were diagnosed with BV, nine had intermediate flora, and 93 had normal flora determined by Nugent score. Vaginal samples were collected to determine the quantification of seven microorganisms by qPCR and compared among the three groups. *Results:* This study indicated that *G. vaginalis, A. vaginae,* and *L. iners* were significantly increased specifically in the BV group ($p < 0.001$). While *L. crispatus* was significantly decreased in the BV group ($p < 0.001$). The area under curve (AUC) values for receiver operator characteristic (ROC) analysis were 0.934, 0.918, 0.763, and 0.742 for *G. vaginalis, A. vaginae, L. iners,* and *L. crispatus*, respectively, suggesting that patients with a higher quantification of *G. vaginalis* and *A. vaginae* were more likely to be diagnosed with BV (91.7% and 83.3%, respectively). *Conclusions:* The quantification of *G. vaginalis* and *A. vaginae* may be more useful diagnostic marker for BV in pregnant Korean women than those of *Lactobacillus species*.

Key words: Bacterial vaginosis; Intermediate flora; *Lactobacillus crispatus*; *Lactobacillus iners*; *Gardnerella vaginalis*; *Atopobium vaginae*.

Introduction

Bacterial vaginosis (BV) results from an imbalance of vaginal flora characterized by a decrease in *Lactobacillus* species and overgrowth of anaerobes, such as *Gardnerella vaginalis* and *Atopobium vaginae* [1-3]. An efficient means to diagnose BV in pregnant women is critical because of its association with adverse perinatal outcomes, such as preterm labor and abortion [1-5]. Most clinicians diagnose BV based on the Nugent score [2]; however, this method is limited in that it requires an experienced microbiologist who is well practiced in BV diagnosis because of the diversity of the vaginal flora [6-8]. In addition, it is unable to detect some BV-associated bacteria, particularly *Atopobium vaginae* [6-9]. For these reasons, some researchers have sought to develop molecular-based methods to accurately diagnose BV. Menard *et al.* [6, 7] reported that the quantification of *A. vaginae* and *G. vaginalis* could be a more sensitive and specific diagnostic tool, and asserted that *A. vaginae* quantification could differentiate BV from intermediate flora (IF). In addition, Bretelle *et al.* [8, 9] suggested that it may predict preterm birth. Molecular analysis of *Lactobacillus* species could also be used to diagnose BV since its quantitative reduction is an important aspect of BV development. For instance, Jesper, et al. reported that the relative prevalence of *Lactobacillus* species-including *L. crispatus* and *L. iners*-was significantly less in BV patients than of those in the normal flora (NF) group [10]; however, *L. iners* abundance varies across reports [11-14]. Moreover, the relative presence of *Lactobacillus* species can differ depending on race, ethnicity, residential area, food, and some physical conditions-such as infection and pregnancy [13-16].

Despite the number of published quantification methods, an effective tool to diagnose BV in pregnant Korean women has yet to be developed. Therefore, the authors aimed to address this gap by developing a diagnostic system based on the relative abundance of five *Lactobacillus* species, *Gardnerella vaginalis,* and *Atopobium vaginae* in the vaginal microbiota for this population

Materials and Methods

Pregnant women under antenatal care at Eulji University Hospital were enrolled in the study at 10-14 weeks gestation. After providing written consent, each subject recorded her history of preterm birth, abortion, obstetrics, and the presence or absence of an existing genital infection via a questionnaire. Women who received antibiotics and/or vaginal suppositories within the last two weeks and those with a major medical condition were excluded from the study.

The total patient population consisted of 124 women. Of these, ten were excluded because of medical illness or lack of follow-up, leaving 114 study participants. The study was approved by the

Table 1. — *PCR primers for Lactobacillus species, Gardnerella vaginalis, and Atopobium vaginae.*

PCR	Primers	Cycling conditions
Lactobacillus species	F-LBF: 5′-ATGGAAGAACACCAGTGGCG-3′ R- LBR: 5′-CAGCACTGAGAGGCGGAAAC-3′	15 min, 95°C, (15 sec, 95°C; 45 sec, 50°C; 45 sec, 72°C) ×37
L. crispatus	LcrisF: 5′-AGCGAGCGGAACTAACAGATTTAC-3′ LcrisR : 5′-AGCTGATCATGCGATCTGCTT-3′	15 min, 95°C, (15 sec, 95°C; 60 sec, 60°C; 20, sec 72°C) ×40
L. gasseri	LgassF: 5′-AGCGAGCTTGCCTAGATGAATTTG-3′ LgassR: 5′-TCTTTTAAACTCTAGACATGCGTC-3′	15 min, 95°C, (15 sec, 95°C; 60 sec, 57°C; 60 sec, 65°C) ×40
L. iners	InersFw: 5′-GTCTGCCTTGAAGATCGG-3′ InersRev: 5′-ACAGTTGATAGGCATCATC-3′	15 min, 95°C, (15 sec, 95°C; 55 sec, 60°C; 60 sec, 65°C) ×35
L. jensenii	LjensF: 5′-AAGTCGAGCGAGCTTGCCTATAGA-3′ LjensR: 5′-CTTCTTTCATGCGAAAGTAGC-3′	15 min, 95°C, (15 sec, 95 °C; 55 sec, 60°C; 60 sec, 72°C) ×40
L. vaginalis	LV16s_23s_F: 5′-GCCTAACCATTTGGAGGG-3′ LV16s_23s_R3: 5′-CGATGTGTAGGTTTCCG-3′	15 min, 95 °C, (15 sec, 95 °C; 30 sec, 56°C; 30 sec, 72°C) ×37
G. vaginalis	R-GV3: 5′-CCGTCACAGGCTGAACAGT-3′ F-GV1: 5′-TTACTGGTGTATCACTGTAAGG-3′	15 min, 95 °C, (45 sec, 95 °C; 45 sec, 55°C; 45 sec, 72°C) ×50
A. vaginae	ATOVAGRT3Fw: 5′-GGTGAAGCAGTGGAAACACT-3′ ATOVAGRT3Rev: 5′-ATTCGCTTCTGCTCGCGCA-3′	15 min, 95°C, (20 sec, 95°C; 45 sec, 60°C; 45 sec, 72°C) ×45

Table 2. — *The demographic characteristics of the patient population.*

	NF (n = 93)	IF (n = 9)	BV (n = 12)	P-value
Age (years)	33.68 ± 4.16	35.11 ± 2.42	32.92 ± 3.99	0.270
Parity	1.37 ± 0.80	1.44 ± 0.52	1.42 ± 0.79	0.953
Gestational age (weeks)	38.55 ± 2.16	37.69 ± 2.19	39.38 ± 1.39	0.099
Preterm delivery history	0.24 ± 0.62	0.33 ± 0.71	0.08 ± 0.29	0.642
Abortion history	0.41 ± 0.77	0.11 ± 0.33	0.08 ± 0.29	0.214

NF: normal flora; IF: intermediate flora; BV: bacterial vaginosis. Patient demographics among the three groups were analyzed by Kruskal-Wallis (statistical significance, p < 0.05). Mann-Whitney tests were used to make comparisons between groups (statistical significance, p < 0.05/3).

Table 3. — *Analysis of vaginal flora species in pregnant Korean women. (Log $2^{-\Delta}$).*

	NF (n = 93) Mean ± SD	IF (n = 9) Mean ± SD	BV (n = 12) Mean ± SD	*p*-value
L. crispatus	4.08 ± 2.37	1.06 ± 0.43	1.49 ± 1.17	<0.001*+
L. iners	3.90 ± 1.95	5.04 ± 2.41	5.81 ± 2.26	0.003 +
L. gasseri	0.77 ± 1.26	1.27 ± 1.77	0.75 ± 1.05	0.812
L. jensenii	1.55 ± 1.77	1.47 ± 2.34	0.67 ± 0.43	0.316
L. vaginalis	2.25 ± 0.69	2.19 ± 0.87	2.00 ± 0.61	0.502
G. vaginalis	1.75 ± 0.96	4.50 ± 2.12	5.31 ± 1.60	<0.001*+
A. vaginae	3.05 ± 0.52	3.73 ± 1.46	5.13 ± 1.16	<0.001+#

*NF: normal flora; IF: intermediate flora; BV: bacterial vaginosis. * p < 0.05 between NF and IF groups. + p < 0.05 between NF and BV groups. # p < 0.05 between IF and BV groups*

Institutional Review Board (IRB) of Eulji University Hospital (IRB #2014-10-002).

Vaginal samples were collected from the post fornix of the vaginal wall with a swab for the qPCR and a cotton swab for the Gram stain. The Gram stain swab was smeared on a slide and dried to fix, while the qPCR swab was stored at -80ºC until analysis.

The Nugent scoring system with Gram staining was used to diagnose BV and intermediate flora [3]. From this, 93 women showed normal flora, nine had intermediate flora, and 12 were diagnosed BV.

Vaginal swabs were thawed at room temperature for 30 minutes and diluted in a 1:9 solution of PBS/saline (pH 7.4). Bacterial DNA was isolated with a specific kit and stored in a -80ºC freezer.

Vaginal flora qPCR for *L. crispatus, L. iners, L. jensenii, L. gasseri, L. vaginalis, G. vaginalis*, and *A. vaginae* was performed using SYBR Green Real Time PCR Master Mix and the primers shown in Table 1. Reactions were performed with a real-time system and the amplification conditions shown in Table 1. Experiments were run in 96-well plates with triplicate samples and normal saline as a negative control. Data was analyzed using the $2^{-\Delta Ct}$ method and then log-transformed to yield final values.

Statistical analysis was performed SPSS, version 18.0. Patient demographics and differences in the presence microorganisms among the three diagnostic groups were analyzed by Kruskal-Wallis (statistical significance, $p < 0.05$). Mann-Whitney tests were used to make comparisons between groups (statistical significance, $p < 0.05/3$). Significant factors were used for Receiver
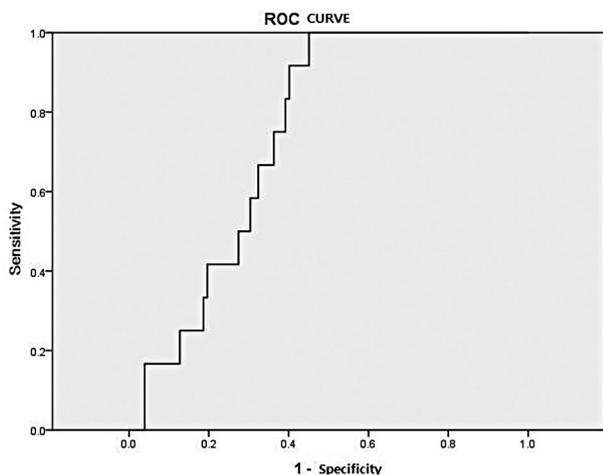
Figure 1. — Receiver operating characteristic (ROC) curve of *L. crispatus* for bacterial vaginosis diagnosis. Area under curve (AUC), 0.742.

Operating Characteristic (ROC) curve analysis to identify a diagnostic threshold for BV. Statistical significance was defined as *p* < 0.05.

Figure 2. — ROC curve of *L. iners, G. vaginalis,* and *A. vaginae* for bacterial vaginosis diagnosis. AUC for *G, vaginalis, A. vaginae,* and *L. iners* were 0.934, 0.918, and 0.763, respectively. *G. vaginalis*: sensitivity, 0.917; specificity, 0.902; cut-off value, 3.724. *A. vaginae*: sensitivity, 0.833; specificity, 0.833; cut-off value, 3.442.

Results

Twelve women were diagnosed as having BV, nine had intermediate flora, and 93 had normal flora. No significant differences in patient demographics including age, parity, abortion history, and delivery date were found among three groups (Table 2). Table 3 shows the quantification of the seven microorganisms analyzed in the study. Interestingly, *L. crispatus* was more prevalent in NF women than in BV and IF counterparts (*p*-value < 0.001), whereas less *L. iners* was found in the NF group compared to BV patients (*p*-value < 0.001). However, the differences between NF and IF, and between IF and BV were not remarkable. Moreover, no significant differences were observed in the relative abundance of *L. gasseri, L. Jensenii,* or *L. vaginalis*. As expected, *G. vaginalis* and *A. vaginae* were far more prevalent in BV patients than in NF counterparts; and while IF women exhibited more *G. vaginalis* than the NF group, that *A. vaginae* was insignificant.

Four microorganisms—*L. crisp, L. iners, G vaginalis,* and *A. vaginae*—showed significant differences among the three groups. These four factors were analyzed by ROC curve analysis to determine a threshold for BV diagnosis. In addition, *L. crispatus* was less prevalent in BV than in NF patients, while the other species were increased in BV when compared to NF. Therefore, ROC curve was analyzed with two curves (Figures 1 and 2).

The area under curve values were 0.934, 0.918, 0.763, and 0.742 for *G. vaginalis*, *A. vaginae*, *L. iners*, and *L. crispatus*, respectively (Figures 1 and 2). ROC thresholds
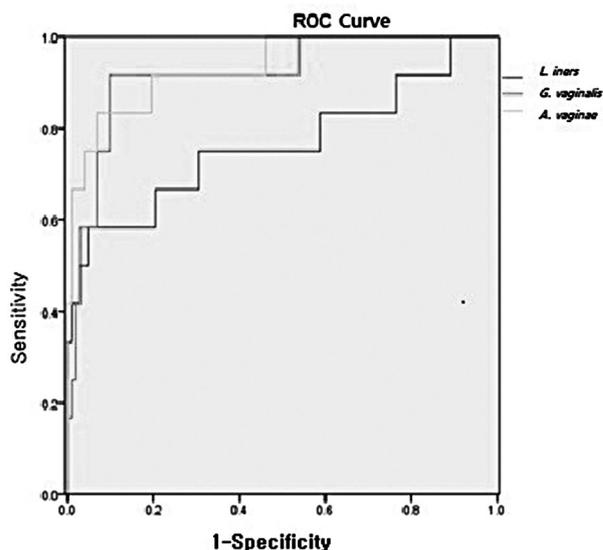
(Log $2^{-\Delta Ct}$) for *G. vaginalis* and *A. vaginae* were 3.724 and 3.442, respectively (*G. vaginalis*: sensitivity, 0.917; specificity, 0.902; *A. vaginae*: sensitivity, 0.833; specificity, 0.833).

The *G. vaginalis* and *A. vaginae* binaries for BV diagnosis were set based on the ROC thresholds and analyzed by Chi-square testing (Table 4). Notably, both measures were statistically accurate diagnostic modalities with *G. vaginalis* and *A. vaginae* predicting BV in 11/12 (91.7%, *p* < 0.001) and 10/12 (83.3%, *p* < 0.001) of cases, respectively.

Discussion

BV is a critical disease to diagnose in pregnant women because of its association with preterm birth [2]. However, the current methods for BV diagnosis are limited and could be improved by the development of a molecular test [5-12]. For instance, the quantification of *A. vaginae* in the vaginal microflora is reported to predict preterm birth [6-9]. Since the vaginal microbiome varies based on race, ethnicity, living area, or medical conditions, such as infection or pregnancy [13-15]; therefore, the present authors attempted to analyze the bacterial distribution in pregnant Korean women to identify useful markers to diagnose BV.

In this study, the authors showed that the quantification of *L. crispatus, L. iners, G. vaginalis,* and *A. vaginae* were significantly different in the BV group versus the NF group. A ROC curve was used to generate a threshold for BV di-

Table 4. — *The relationship between BV and the quantification groups of G. vaginalis and A. vaginae.*

	G. vaginalis quantification group			
	Low group#	high group$	Total	*p*-value
NF+IF group (n (%))	92 (90.1)	10 (9.9)	102 (100)	< 0.001*
BV group (n (%))	1 (8.3)	11 (91.7)	12 (100)	< 0.001*
	A. Vaginae quantification group			
	Low group+	High group&		
NF+IF group (n (%))	85 (83.3)	17 (16.7)	102 (100)	< 0.001*
BV group (n (%))	2 (16.7)	10 (83.3)	12 (100)	< 0.001*

NF: normal flora group, IF: intermediate flora group, BV: bacterial vaginosis group. Cut-off value of quantification of G.vaginalis= 3.724, sensitivity = 0.917, specificity = 0.902 (AUC= 0.918). # Low group ; < 3.724, $ High group ≥ 3.724, *Statistical significance by Chi-square test ; P value < 0.05. Cut-off value of quantification of A. vaginae for BV = 3.442, sensitivity= 0.833, specificity = 0.833 (AUC= 0.934), + Low group < 3.442, & High group ≥ 3.442, *Statistical significance by Chi-square test ; p < 0.05.

agnosis, which was set at 0.918 and 0.934 for *G. vaginalis* and *A. vaginae*, respectively. Notably, these values led to a detection rate of 91.7%, and 83.3% in the present patient population. Menard *et al.* [6, 7] asserted that the quantification of *A. vaginae* and *G. vaginalis* could serve as more sensitive and specific diagnostic tool for BV diagnosis in France, and also asserted that the prevalence of *A. vaginae* could differentiate BV in IF [7]. However, despite several published quantification methods, an effective tool to diagnose BV in pregnant Korean women has yet to be developed. This is particularly important as vaginal microbiota composition can vary according to ethnicity, residence, and other conditions, such as pregnancy and vaginal infection [13-15]. Therefore, the present authors aimed to develop a diagnostic system based on the relative abundance of five *Lactobacillus* species*, Gardnerella vaginalis,* and *Atopobium vaginae* in the vaginal microbiota of this population.

The   present results show that the levels of *L. crispatus* and *L. iners* were relatively unchanged when compared to those of *A. vaginae* and *G. vaginalis;* however, the significance of *L. iners* in BV is controversial. Jesper *et al.* [10] previously reported that *L. iners* is much more prevalent in NF women than BV counterparts, yet the present data showed the opposite (AUC = 0.76). Moreover, *L. crispatus* prevalence was decreased in BV patients (AUC = 0.74) and has been reported to have stronger bacterial inhibitory activity than *L. iners* [17]. However, the present authors have known that it had less diagnostic value than two anaerobes in spite of its significant decrease in BV; nevertheless, these results should be verified with additional studies.

In conclusion, this study demonstrated that *G. vaginalis* and *A. vaginae* quantification in vaginal secretions could be a useful measure to diagnose BV in pregnant Korean women and may facilitate the development of a more accurate microbiome-based molecular diagnostic for BV in the future.

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