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Higher levels of a cytotoxic protein, vaginolysin, in *Lactobacillus*-deficient community state types at the vaginal mucosa

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Abstract

Vaginolysin (VLY), a cytotoxic protein produced by *Gardnerella vaginalis*, may contribute to bacterial vaginosis (BV). Women with *G. vaginalis*, low levels of lactobacilli, history of vaginal douching, higher Nugent scores, and higher vaginal pH had increased VLY. Inflammatory markers were not highly expressed with increasing VLY. VLY's role in BV warrants further evaluation.

Keywords

Gardnerella vaginalis; vaginolysin; bacterial vaginosis; vaginal microbiota; 16S rRNA gene amplicon sequencing

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Conflicts of Interest

None of the authors have conflicts of interest to declare.

Data availability: The questionnaire, VLY and immunologic marker data are available at the National Center for Biotechnology Information (NCBI) Database of Genotypes and Phenotypes (dbGaP) under Accession number phs001386.v1.p1. Metagenomic sequence data were submitted to the public NCBI Sequence Read Archive (SRA) with the accession number PRJNA391039.

NOTE

Bacterial vaginosis (BV) is a common condition associated with increased risk of sexually transmitted infections, as well as development of adverse outcomes in the female reproductive tract.(1) Although the etiology of BV remains unclear, it is characterized by an overabundance of strict or facultative anaerobic bacteria such as *Gardnerella vaginalis* and a decrease in *Lactobacillus* spp. The latter is known to produce lactic acid which drive an acidic vaginal microenvironment.(2, 3) Laboratory studies have demonstrated that certain strains of *G. vaginalis* can displace some *Lactobacillus* spp. at the mucosa (4) and BV-related isolates of *G. vaginalis* are associated with a 2-fold increase in its cytotoxic protein, vaginolysin (VLY), compared to non-BV isolates of *G. vaginalis* in an *in vitro* biofilm model.(5) VLY acts by creating pores that alter cell integrity and shape and is thought to disrupt local innate immunity and thus may promote persistence of BV.(6, 7) It is hypothesized that BV may not induce clinical signs of inflammation because BV associated virulence factors could alter the expression of IL-1 β and IL-8 to prevent neutrophil recruitment.(8, 9) To date, expression of VLY has been limited to *in vitro* and animal studies.(5–7) The objective of this study was to determine the association between VLY, vaginal microbiota composition and correlates of local immunity *in vivo*.

Forty reproductive-aged women were enrolled as part of a previously described study at the University of Maryland School of Public Health.(10) Briefly, non-pregnant women aged 18 to 45 years who did not report using antibiotic or antimycotic drugs in the prior 30 days were recruited and completed detailed demographic and behavioral surveys. Participants self-collected a vaginal sample by inserting a double headed rayon swab (Starplex Scientific Corp., Cleveland, TN) 1–2 inches and rotated along the vaginal wall for several full circles for at least 20 seconds. Swabs were immediately snap frozen at –20°C and subsequently transferred to –80°C until processing. Participants determined their vaginal pH using a CarePlan® VpH test glove. The study originally characterized the composition of the vaginal microbiota using 454 pyrosequencing of the V1–V3 hypervariable regions of the 16S rRNA genes. Vaginal microbiota were clustered into community state types (CSTs) as previously reported.(10) Thirty-seven types of human papillomavirus (HPV) were detected using the Linear Array HPV Genotyping test (Roche Molecular Diagnostics, Indianapolis, IN), of which 13 were considered high risk: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68. The University of Maryland Baltimore and College Park Institutional Review Boards approved this study.

One head of the double-headed swabs was cut using sterile scissors and vortexed in 1 ml of PBS. Aliquots (200 μ l) were sent to the Ratner Lab for VLY quantification and the University of Maryland Baltimore Cytokine core for the cytokine/chemokine quantification. VLY concentration was determined by ELISA as previously described (11) with the exception that a mouse monoclonal anti-VLY antibody (GenScript; 1:250 dilution) was used as the primary antibody. Total absolute bacterial abundance was estimated using a pan-bacterial 16S rRNA gene TaqMan quantitative PCR (qPCR) assay.(12) Total number of copies of 16S rRNA genes from 1 μ l of sample in a total reaction volume of 20 μ l were normalized to a standard curve (ranging from 10⁸ to 10¹ copies/ μ l in 10-fold serial dilutions) of cloned *E.*

coli 16S rRNA gene. Absolute abundance of *G. vaginalis* was calculated by multiplying the relative abundance of *G. vaginalis* obtained by 16S rRNA gene sequencing data by the total bacterial 16S rRNA gene copy number generated by qPCR. A panel of 21 cytokines and chemokines were quantified from 25µl of sample using Milliplex Multiplex Magnetic assays (Merck KGaA, Darmstadt, Germany) and the Luminex 100 system (Luminex Corp, Austin, TX). Quality control samples were included during all steps. One sample was excluded because of insufficient volume for VLY quantification. The final analytic sample size was 39.

The primary outcome variable, VLY concentration (ng/ml), was transformed to meet the normality assumption by taking the cube root. The main independent variable was one of three CST categories previously assigned. (10) Two CSTs were dominated by *Lactobacillus* spp. (*L. iners* (CST-III) or *L. crispatus* (CST-I), and one lacked *Lactobacillus* spp. and was dominated by BV-associated bacteria including *G. vaginalis* and *Atopobium vaginae* (CST-IV). Other independent variables were any HPV, any high-risk HPV (HR-HPV), log₁₀-transformed absolute abundance of *G. vaginalis*, Nugent's Gram's stain score (0–3, 4–6, and 7–10) (13), vaginal pH (4.0, 4.1–4.5, 4.6–5.0, and 5.1), douching history, and immune markers (IL-1β, TNF-α, TGF-β, and IL-8). Because all transformations of the immune markers did not meet the normality assumption, IL-1β and TNF-α were dichotomized at the lower limit of detection (3.2 pg/ml), and TGF-β and IL-8 were dichotomized above and below the median. Thirty-four samples were below the level of detection for IL-1β and/or TNF-α. The baseline demographics of the participants by CST were compared using Fisher's exact tests for categorical variables and analysis of variance (ANOVA) for continuous variables. Univariate linear regression models were used to estimate regression coefficients and 95% confidence intervals (CI) for the association between independent variables and VLY. Because some of the independent predictors were highly correlated with one another, 10 separate models were built (Table 1). Race, age, lifetime number of sex partners, and smoking status were evaluated as potential confounders, but none were significantly associated with CSTs and VLY. The univariate models were the most parsimonious and are presented in the table. Analyses were performed using Stata Statistical Software: Release 13 (College Station, TX: StataCorp LP).

Among the thirteen women assigned to the low-*Lactobacillus* CST-IV, the median age was 40 years (interquartile range 29–43). Nine (69.2%) were African-American, ten (76.9%) were smokers, and six (54.6%) had ten or more lifetime sex partners. The highest concentrations of VLY were among women in CST-IV and the lowest in CST-I, a *L. crispatus*-dominated state ($p<0.001$) (Figure 1). The *L. iners*-dominated CST-III had an intermediate concentration of VLY which may suggest that *L. iners* can co-exist with *G. vaginalis* and its cytotoxin, and in turn, why some strains of *L. iners* have been associated with frequency and duration of BV in prior studies. (14–18)

In the univariate analyses, *Lactobacillus*-deficient CST-IV ($p<0.01$), *G. vaginalis* absolute abundance ($p=0.01$), increasing vaginal pH ($p_{\text{trend}}<0.01$), increasing Nugent score ($p_{\text{trend}}<0.01$) and douching history ($p<0.01$) were all associated with increasing VLY concentrations. Collectively, these data suggest that *G. vaginalis* cytotoxin may represent a local marker of BV. Pro-inflammatory cytokines (IL-1 β, TNF- α, and IL-8), as well as a

regulator of inflammation (TGF- β), were not highly expressed with increasing VLY concentrations and none reached statistical significance ($p>0.05$).

As VLY causes breakdown of tissue, we had hypothesized that VLY might allow HPV to flourish as the mucosal surface is repaired from the basal epithelial cell layers. Detection of any genotype of HPV ($p=0.50$, data not shown) and any HR-HPV were not associated with VLY concentration ($p=0.73$) (Table 1).

Observations of the local immune response during BV have been inconsistent across studies. Some publications report a pro-inflammatory response with high levels of IL-1 β , TNF- α , and IL-8 (19–22). Other studies suggest a dampened immune response that lack IL-8 and neutrophil infiltration.(8, 9) In a 2002 study which defined BV by Amsel's clinical criteria, Cauci *et al.* found the pro-inflammatory immune marker IL-8 was positively correlated with leukocyte counts and an IgA response to a hemolysin produced by *G. vaginalis*.(23) It may be that women who have higher VLY concentration could have insufficient IL-8 concentrations to mount a humoral immune response. Certainly other bacterial species and bacterial toxins also alter local immunity but the heterogeneity in immune responses to BV merits further evaluation.

In this study, the small sample size may have limited our power to detect significant associations between the cytokines and VLY concentration. However, many of our hypothesized associations between markers of BV and VLY were detected. Second, proteins in the samples may have adhered to the swabs and could not be completely eluted during the extraction process, resulting in low or undetectable measurements of immune markers. Third, detection of VLY may vary depending on the presence of genomic variants of *G. vaginalis* and whether *G. vaginalis* is planktonic or nested in a biofilm.(24) These data were not available in the current dataset and should be considered in future studies to further elucidate the relationship between *G. vaginalis*, VLY and BV. Lastly, this cross-sectional study could not assess temporality. Despite these limitations, this study provides information on the associations between vaginal microbiota, *G. vaginalis* absolute abundance, and its cytotoxin. Further studies of VLY and BV in a larger study of women are ongoing.(25)

In summary, this study adds to available *in vitro* findings by reporting that higher concentrations of VLY *in vivo* were associated with women who had a vaginal microbiota lacking lactobacilli, as well as other clinical indicators of BV. We also found that a community state type dominated by *L. iners* was often associated with VLY-producing *G. vaginalis*. Longitudinal studies are needed to assess the role of VLY in the development and persistence of BV.

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Short summary

Women with indicators of bacterial vaginosis, including a vaginal microbiota lacking *Lactobacillus* spp., had higher *in vivo* concentrations of vaginolysin, a cytotoxic protein.

Figure 1.

Vaginolysin (ng/ml) by community state type

Note: Analysis of variance (ANOVA) was used to test differences in vaginolysin by community state type.

Table 1

Univariate analysis of risk factors associated with vaginolysin

Model	Variable	Regression coefficient*	
		Unadjusted (95% CI)**	P
1	Vaginal community state type (CST)		
	CST-I, <i>L. crispatus</i> -dominated	Ref.	
	CST-III, <i>L. iners</i> -dominated	0.48 (0.06, 0.90)	0.03
	CST-IV, <i>Loctobocillus</i> -deficient	0.75 (0.39, 1.11)	<0.01
2	<i>Gardnerella vaginalis</i> (log ₁₀ counts)	0.19 (0.05, 0.33)	0.01
3	HR-HPV		
	No	Ref.	
	Yes	0.07 (−0.33–0.47)	0.73
4	Nugent Gram stain score		<0.01
	0–3	Ref.	
	4–6	0.43 (−0.13, 1.00)	
	7–10	0.65 (0.24, 1.06)	
5	Vaginal pH		<0.01
	4.0	Ref.	
	4.1–4.5	0.05 (−0.60, 0.70)	
	4.6–5.0	0.45 (−0.04, 0.95)	
	5.1	0.70 (0.32, 1.07)	
6	History of douching		
	No	Ref.	
	Yes	0.53 (0.15–0.91)	<0.01
7	IL-1 β [†]		
	3.20 (LLOD)	Ref.	
	3.20	0.10 (−0.28, 0.48)	0.61
8	TNF- α [‡]		
	<3.20 (LLOD)	Ref.	
	>3.20	−0.20 (−0.67, 0.27)	0.39
9	TGF- β [‡]		
	<47.39	Ref.	
	47.39	−0.30 (−0.67, 0.07)	0.10
10	IL-8 [‡]		
	<340.88	Ref.	
	340.88	−0.19 (−0.56, 0.19)	0.32

Note: HR-HPV, high risk human papillomavirus; α , alpha; β , Beta; P, p-value; LLOD, lower limit of detection; CI, confidence interval; Ref., reference.

* Vaginolysin concentration transformed with cube root to satisfy normality assumption

** Unadjusted values are reported because race, age, lifetime number of sex partners, and smoking status did not confound the main associations and the more parsimonious models were reported.

γ
pg/ml

γ
latent TGF- β 1