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Presence and concentrations of select bacterial vaginosis-associated bacteria are associated with increased risk of pelvic inflammatory disease

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Abstract

In a vaginal 16S ribosomal RNA gene quantitative PCR study of 17 pelvic inflammatory disease (PID) cases and 17 controls who tested positive for *Chlamydia trachomatis*, women who additionally tested positive for *Atopobium vaginae*, *Sneathia* spp., BVAB-TM7, *Megasphaera* spp., *Eggerthella*-like bacterium or *Mobiluncus* spp. were more likely to develop PID.

Short Summary

A vaginal 16S rRNA qPCR study demonstrated that *Chlamydia trachomatis* positive women who also tested positive for *Atopobium vaginae*, *Sneathia* spp., *Megasphaera* spp., *Eggerthella*-like bacterium, or *Prevotella amnii* were at elevated PID risk.

Keywords

Pelvic inflammatory disease; Bacterial vaginosis; Endometritis; Epidemiology

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Conflicts of Interest: None.

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INTRODUCTION

Pelvic inflammatory disease (PID), infection and inflammation of the female upper genital tract, is a common condition among young women that often results in infertility, chronic pelvic pain (CPP), and recurrent PID.[1] Although PID has a polymicrobial etiology, with *Chlamydia trachomatis* and/or *Neisseria gonorrhoeae* accounting for approximately one third to one half of cases,[1] up to 70% of PID cases have an unidentified etiology. Bacterial vaginosis (BV), determined by analysis of Gram stained vaginal smears, and specific cultivable BV-associated species including anaerobic gram-negative rods have also been associated with PID.[1]

BV is a polymicrobial condition characterized by a shift from a lactobacilli predominant vaginal microbiota to one with high concentrations and diversity of facultative and anaerobic bacteria. BV-associated microorganisms have been cultured from upper tract samples from women with PID.[1] However, the associations between individual BV-associated bacteria and risk of PID have not been determined using highly sensitive PCR methods. Cultivation-independent studies using 16S rRNA gene sequence polymerase chain reaction (PCR) amplified from vaginal DNA have revealed previously unrecognized bacterial genera associated with BV.[2] We sought to determine the associations among key BV-associated bacteria and PID incidence among a population of women at high risk for sexually transmitted infection.

MATERIALS AND METHODS

We conducted an ancillary study of 34 women enrolled in the Gynecologic Infections Follow-Through (GIFT) study, which has been described in detail elsewhere.[3] Briefly, 1,199 women 13 to 36 years of age were recruited into the parent GIFT Study from family planning clinics, university health clinics, gynecology clinics, and STD units at five clinical sites in the United States between May 1999 and June 2001 and were followed approximately three years for the development of PID. Women were eligible for the GIFT study if they were not specifically seeking care for an STI but were considered at elevated risk for having chlamydial cervicitis, according to a modification of the Stergachis et al. risk paradigm.[4] Approximately two-thirds of women were aged 19 to 24 years of age and 75% were black. As part of the parent study, participants collected vaginal specimens at baseline, 6, 12, 24, and 36 months after being educated by study staff on a standardized method for self-collection using the BD CultureSwab™ collection and transport system (Becton Dickinson, NJ). DNA amplification for *C. trachomatis* and *N. gonorrhoeae* was performed by using a strand displacement DNA Amplification (SDA) Assay (Becton Dickinson, NJ) from self-obtained vaginal swabs and residual specimens were archived and frozen at −80° C. Women were educated on the signs and symptoms of PID and advised to contact study staff at any point during follow-up to report pelvic pain, abnormal bleeding or urethritis, or a diagnosis of chlamydial or gonococcal cervicitis. To detect PID, women who reported pelvic pain at any point in the study and women who tested positive on *C. trachomatis* and *N. gonorrhoeae* screening were scheduled for an additional, symptomatic visit involving a pelvic examination and an endometrial biopsy.

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For the current ancillary nested pilot study utilizing a case to control ratio of 1:1, we randomly selected 17 women who experienced histologically confirmed PID over follow-up and 17 controls selected randomly among all study participants who did not experience PID signs or symptoms, matched by follow-up visit and race. DNA was extracted from 200 µL of the archived vaginal material using the MoBio BiOstic Bacteremia DNA Isolation Kit (MoBio Laboratories- Carlsbad, CA). Mycoplasmal bacteria previously associated with PID in cross-sectional studies[1] and key BV-associated bacteria[5–8] were selected for analysis. Species-specific 16S ribosomal RNA (rRNA) gene quantitative PCR (qPCR) assays targeting *Atopobium vaginae*,[5] *Gardnerella vaginalis*,[5] *Sneathia spp.*,[5] bacterial vaginosis associated bacterium 1 (BVAB1),[5] BVAB2,[5] *Mageeibacillus indolicus*,[5] *Megasphaera spp.*,[5] *Eggerthella*-like bacterium,[8] *Mobiluncus* spp.,[6] *Prevotella timonensis*,[8] *Prevotella amnii*, [8] *Ureaplasma urealyticum*,[7] *Ureaplasma parvum*,[7] and *Mycoplasma genitalium*[9] were applied to DNA from vaginal samples from visits occurring immediately preceding and within 3 months of PID using methods which have been previously described.[5–9] All these assays used a StepOne Plus thermal cycler, with 45 cycles of amplification, input DNA in 3 uL in a 30 uL reaction. In addition, an assay was developed targeting BVAB-TM7 for this study. For that assay, each 30 µL qPCR assay contained 1X Buffer A (Life Technologies- Carlsbad, CA), 3 mM magnesium chloride, 0.8 µM forward primer (TM7–992F; 5' TGACATCCCTAGAATTTCTCC-3'), 0.8 µM reverse primer (TM7–1051R; 5'-GGATCTGTCACCTAGTTCT-3'), 150 nM probe (TM7_1015; 5'–6FAM-AAGGAGAGAGTGCTTTTTA-MGBNFQ-3'), 0.05 units uracil-N-glycosylase (UNG), 1.0 unit AmpliTaq Gold DNA polymerase (Life Technologies-Carlsbad, CA) and 3 µL sample DNA. Assays were run on the StepOne Plus qPCR instrument (Life Technologies) using the following amplification conditions: 50°C 2 min (UNG activation), 95°C 10 min (pre-melt), 45 cycles of 95°C 15 sec (melt), 59°C 39 sec (annealing), 72°C 30 sec (extension). Relationships between the presence and concentrations of individual bacteria and PID were determined using conditional logistic regression models. As all cases and no controls tested positive for *C. trachomatis*, we were unable to include chlamydia as a covariate in this pilot study. Variables measuring 16S rRNA gene copies were log transformed (base 10) and negative samples were assigned a value equal to half the lower limit of detection for each respective bacterium. Analyses were performed using R version 3.6.0.

RESULTS

Cases were more likely to have BV determined by Gram stain as compared to controls, although the differences in proportions were not statistically significant (45.5% vs. 29.4%, p=0.119). Generally, the bacteria we assayed were frequently identified in the lower genital tract prior to the development of PID (See Table 1). *M. genitalium* was the least prevalent and *P. timonensis* was the most prevalent, identified in 8% and 94% respectively of vaginal samples collected among cases prior to PID diagnosis. In unadjusted analyses, several BV-associated bacteria were significantly associated with subsequent PID (Table 1). Women who tested positive for *A. vaginae* (OR_{adj} 13.7 (2.7, 108.5), *Sneathia* spp. (OR_{adj} 5.8 (1.4, 27.7), *Megasphaera* spp. (OR_{adj} 6.0 (1.4, 29.7), *Eggerthella*-like bacterium (OR_{adj} 10.6 (2.4, 59.0) and *Prevotella amnii* (OR_{adj} 4.6 (1.1, 22.5) were significantly more likely to develop PID. There was a trend that women who tested positive for *G. vaginalis*, BVAB1, BVAB2,

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M. indolicus, BVAB-TM7, *P. timonensis,* and *Mobiluncus* spp. were also more likely to develop PID. Women who tested positive for *M. genitalium*, *U. urealyticum* or *U. parvum* did not have an elevated risk of subsequent PID.

Cases had significantly higher mean 16S rRNA gene copies/mL as compared to controls for the following bacteria: *A. vaginae* (2.6 E6 ± 8.1E6 vs. 1.9E6 ± 5.7E6, p=0.01), *Megasphaera* spp. (9.8E5 ± 2.0E6 vs. 1.1E5 ±3.2E5, p=0.02), *Eggerthella*-like bacterium (1.6E6 ± 3.0E6 vs. 1.3E6 ± 1.6E6, p=0.009), and *P. timonensis* (2.2E6 ± 4.4E6 vs. 4.0E5 ± 1.4E6, p=0.02). Mean 16S rRNA gene copies/mL of *Sneathia* spp. (1.6E6 ± 4.6 E6 vs. 5.3E5 ± 1.7E6, p=0.05) and BVAB2 (9.7E4 ± 2.2E5 vs. 5.2E4 ± 1.3E5, p=0.07) were also higher among cases as compared to controls, although differences were of borderline statistical significance. Higher bacterial concentrations of *G. vaginalis*, BVAB1, *M. indolicus*, BVAB-TM7, *U. urealyticum*, *U. parvum*, *M. genitalium, Mobiluncus* spp.*, and P. amnii* were not predictive of PID.

DISCUSSION

In our targeted study of women considered at high risk for sexually transmitted infection, women who tested positive for BV-associated bacteria including *A. vaginae*, *Sneathia* spp., *Megasphaera* spp., *Eggerthella*-like bacterium, *Mobiluncus* spp., BVAB-TM7, and *P. timonensis* in the vagina were at significant risk for developing subsequent PID within 3 months. Further, greater bacterial load of *A. vaginae*, *Megasphaera* spp., *Eggerthella*-like bacterium, *P. timonensis*, *Sneathia* spp., and BVAB2 was predictive of subsequent PID. All women with PID were co-infected with *C. trachomatis*, thus the listed bacteria could modulate PID risk among *C. trachomatis* infected women but we do not have evidence from this study that BV-bacteria alone are associated with PID risk.

To our knowledge, this is the first qPCR study to prospectively demonstrate that the presence and quantity of fastidious BV-associated bacteria are associated with subsequent PID in women with Chlamydial infection. Results are consistent with a prior GIFT Study finding, where we demonstrated that a cluster of cultured vaginal BV-associated organisms (absence of hydrogen peroxide-producing lactobacilli, presence of *G. vaginalis*, *Mycoplasma hominis*, anaerobic Gram-negative rods, and undifferentiated ureaplasmas) was associated with a two-fold risk of incident PID.[10]

Our study has a number of strengths, including quantitative measurements of specific vaginal bacterial concentrations and prospectively assessed histologically confirmed incident PID. The selection of vaginal specimens within a critical exposure window of 3 months preceding PID diagnosis supports a temporal association between the bacteria and PID. Our pilot study was limited by its sample size, which resulted in large confidence intervals and may have biased some of our models toward the null. As women without signs and symptoms of PID over follow-up did not receive pelvic examinations, it is possible that some women in the control group may have had subclinical PID, potentially biasing models toward the null. Although *M. genitalium* has been cross-sectionally associated with endometritis and clinically suspected PID in prior studies,[1] it was infrequently detected among samples from women in our study. As no controls tested positive for *M. genitalium*,

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we were unable to model the relationship between this bacterium and PID. Additional well powered studies are needed to confirm these preliminary findings, improve precision, examine a broader range of BV-associated bacteria, and allow for additional confounder adjustment and examination of bacterial interactions. All cases in our study tested positive for *C. trachomatis*, highlighting the need for future studies with repeated vaginal sampling to explore the potential for BV-associated bacteria to increase the risk of chlamydial infection and ascension to the upper genital tract. Our findings raise the question of whether screening and treatment of women harboring bacteria associated with high risk of PID has the potential to reduce the incidence of PID.

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Table 1.

The risk of pelvic inflammatory disease for women testing positive for vaginal bacteria preceding and within 3 months of diagnosis by species-specific quantitative 16S ribosomal RNA gene polymerase chain reaction assays

Bacterial Species	Case N=17 n (%)	Control N=17 n (%)	Crude OR (95%CI)
Atopobium vaginae	15 (88%)	6 (35%)	13.7 (2.7, 108.5)
Gardnerella vaginalis	15 (88%)	11 (65%)	4.1 (0.8, 31.7)
Sneathia spp.	12 (71%)	5 (29%)	5.8 (1.4, 27.7)
[a]BVAB1	9 (53%)	4 (24%)	3.7 (0.9, 17.5)
BVAB2	9 (53%)	4 (24%)	3.7 (0.9, 17.5)
Mageeibacillus indolicus	9 (53%)	4 (24%)	3.7 (0.9, 17.5)
BVAB-TM7	5 (29%)	2 (12%)	3.1 (0.6, 24.6)
Megasphaera spp.	11 (65%)	4 (24%)	6.0 (1.4, 29.7)
Eggerthella-like bacterium	13 (76%)	4 (24%)	10.6 (2.4, 59.0)
Mobiluncus spp.	4 (24%)	1 (6%)	4.9 (0.6, 102.8)
Prevotella timonensis	16 (94%)	12 (71%)	6.7 (0.9, 136.8)
Prevotella amnii	10 (59%)	4 (24%)	4.6 (1.1, 22.5)
Ureaplasma urealyticum	3 (18%)	3 (18%)	1.0 (0.2, 6.2)
Ureaplasma parvum	9 (53%)	12 (71%)	0.5 (0.1, 1.9)
Mycoplasma genitalium	1 (6%)	0 (0%)	-

[a]BVAB denotes bacterial vaginosis-associated bacterium