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CARRIAGE OF BACTERIAL VAGINOSIS-ASSOCIATED SPECIES IN MALE SEXUAL PARTNERS

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ABSTRACT

BACKGROUND: Bacterial vaginosis (BV) is a poorly understood syndrome. The condition seems to represent a disruption of commensal vaginal bacteria in which Lactobacilli are reduced and other genera, particularly anaerobes, are increased. No individual agent has been identified as the cause of BV; however, evidence suggests the condition can be sexually transmitted. Metronidazole is the most commonly prescribed treatment, yet treatment failure and recurrence rates are high. Recent cultivation-independent studies have revealed that bacterial genera, such as *Atopobium vaginae* and uncultivated *Megasphaera* species, are associated with BV. Carriage of these new genera by males has not been investigated. We speculate that high BV recurrence rates might be due to re-infection of females with BV-associated bacteria by male partners. **METHODS**: We developed specific quantitative real time PCR assays targeting the 16S rRNA gene of ten vaginal bacterial species including *Atopobium vaginae*, *Gardnerella vaginalis*, *Megasphaera* type 1 and BVAB1, genera that are highly associated with BV. We investigated their distribution and abundance in vaginal specimens of 20 patients, 14 with and 6 without BV, as well as in urine, urethral swab and coronal sulcus swab specimens of their male partners. **RESULTS**: The results show that BV-associated bacteria are detectable in male partners of BV patients. The highest concentrations of the BV-associated bacteria *G. vaginalis*, *A. vaginae*, *Megasphaera* type 1 and BVAB1 were found in coronal sulcus swab specimens, while the lowest concentrations were found in urine specimens. The concentrations of BV-associated bacteria were low or undetectable in specimens of non-BV patients and their male partners. **CONCLUSION**: Male partners of women with BV carry recently recognized BV-associated bacterial genera and penile surface specimens appear to harbor the highest concentrations of these bacteria.

METHODS

SAMPLE COLLECTION AND CLINICAL MEASUREMENTS
A total of 20 couples were enrolled in this study. Couples had to be 18 years or older, with no antibiotics taken in the past 28 days, presenting to the std clinic together for evaluation. A vaginal swab was collected from each women. Male partners provided a urethral swab, a coronal sulcus swab and a urine specimen. All swabs and urine specimens were transported in GeneLock preservative (Sierra Molecular Inc.). The female specimens were characterized clinically using Amsel criteria and Nugent scores. The samples were designated "normal" (Nugent score= 0-3), "intermediate" (Nugent score= 4-6) or BV (Nugent score= 7-10).

DNA EXTRACTION AND QUANTIFICATION
DNA extraction was performed using a QIAamp DNA mini kit (vaginal swabs) and micro kit (male specimens). Plasmids for standard curves were purified using a QIAprep Spin Miniprep kit (Qiagen). Genomic and plasmid DNA were quantified using a TBS-380 fluorometer (Turner Biosystems) using the Quant-iT PicoGreen dsDNA reagent (Invitrogen Inc.). Plasmid copies were calculated with the aid of the DNA copy number calculator at the URI Genomics and Sequencing Center web site (http://www.uri.edu/research/gsc/resources/cndna.html).

qRT-PCR ASSAYS
PCR primers targeting the 16S rRNA gene of thirteen bacterial species commonly found in both normal women and women with BV were designed using the freeware package Primrose (http://rdp.cme.msu.edu). Specificity of the primers was assessed with the BLAST algorithm on the NCBI web site (http://www.ncbi.nlm.nih.gov/blast). Primers were synthesized by Integrated DNA Technologies. A universal qRT-PCR assay (15) to measure concentrations of total bacterial 16S rRNA genes in all DNA specimens was also applied. qRT-PCR amplifications were performed on an iCycler thermal cycler equipped with a real time detection system (Bio-Rad) using HotStart-IT SYBR Green qPCR master mix (USB Corporation). Plasmids containing 16S rDNA inserts of the bacterial species tested were used to generate ten-fold serial dilutions for standard curves. The amplification products were visualized on a 1.5% agarose gel and their identity was confirmed by sequencing.

STATISTICAL ANALYSIS
Statistical calculations were performed using GraphPad Prism v5.0 for Windows. Differences in the qRT-PCR values obtained for all the bacteria measured across patient groups defined by Nugent scores were evaluated by Mann-Whitney U test. Spearman rank correlations were used to evaluate the associations between the bacteria quantified. Statistical significance was set at α 0.05, and all tests were two-sided.

INTRODUCTION

Bacterial vaginosis (BV) is a common cause of vaginal irritation that is characterized by a shift from a normal Lactobacilli-predominant vaginal flora to a flora with higher concentrations of other bacterial species. No single organism has been implicated as the cause of BV; in fact, BV is now considered a polymicrobial disease. The onset mechansim of BV has not been elucidated. Previous work using cultivation-independent molecular methods (1-7), revealed associations between BV and several cultivated and uncultivated bacterial species that were not previously recognized. BV is associated with preterm birth, miscarriage, pelvic inflammatory disease (PID), urinary tract infections, and postoperative infections. BV has also been associated with increased susceptibility to HIV (8) and infection with *C. trachomatis* and *N. gonorrhoea* (9). It is unclear whether BV is a sexually transmitted infection (STI), but it is clearly associated with sexual activity (recent change of sexual partner, multiple partners, lesbian couples). BV has also been found in 12% virginal women, suggesting that factors other than sexual activity may be also important in the development of the condition.
Women with BV are treated with metronidazole or clindamycin. This treatment, however, is not very effective: cure rate is 70-80%; recurrences 50-70%. **There is controversy as to whether recurrences are due to relapse or reinfection by the male partners.** Recently, BV bacteria have been cultured from male partners of BV women (10), and one molecular study has been conducted to assess the presence of uncultivated BV-associated bacteria in males (11). Concurrent male treatment with oral metronidazole (12), oral clindamycin (13) or topical 62% EtOH in gel (14) didn't reduce the risk of recurrence in their female partners. However, in this studies BV cure/recurrence was diagnosed only clinically by Nugent scores/Amsel criteria. We have been developing specific quantitative real time PCR (qRT-PCR) assays targeting prominent vaginal bacteria to better understand the etiology of the BV syndrome. **In this study our goals were 1) to use our specific qRT-PCR assays for the identification of microbial communities associated with the BV syndrome in both the female cases and their male partners; and 2) to determine the best specimen or combination of specimens (urethral swab, urine and coronal sulcus/foreskin swab) to describe genital bacterial communities carried by men.**

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DNA Yield

Vaginal swab	300 ng – 15 mg	($7x10^6$ to $1x10^8$ *)
Penile Skin swab	<1 ng – 1 mg	($2x10^4$ to $1x10^7$)
Urethral swab	1 ng – 5 mg	($3x10^4$ to $1x10^7$)
Urine (10 ml)	40 ng – 3 mg	($1x10^4$ to $8x10^5$)

* Total bacteria concentration in 5ng of DNA

--There was no correlation between circumcised /uncircumcised and DNA yield--

RESULTS

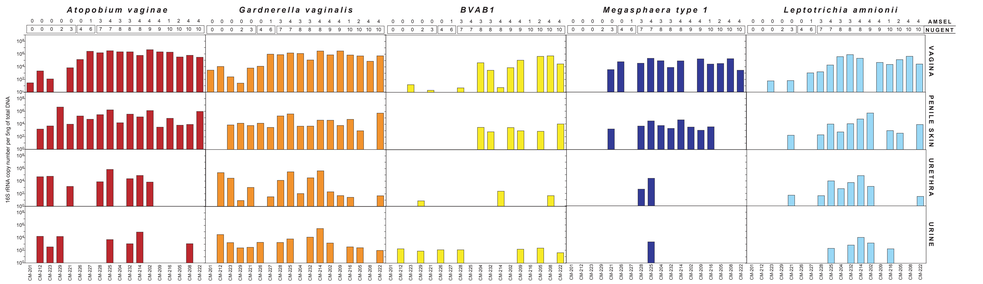

FIGURE 1. Quantitative analysis of 16S rDNA from BV-associated bacteria in vaginal samples, and in coronal sulcus (penile skin), urethra and urine specimens from their male partners. Specimens are arranged according to increasing Nugent score (Normal 0-3, intermediate 4-6, BV 7-10). Bacteria associated with BV in women were detected in specimens of their corresponding male partners. The highest concentrations of BV-associated bacteria were found in penile skin specimens, while the lowest concentrations were found in urine specimens. The concentrations of BVAB1, *Megasphaera* type 1 and *L. amnionii* were low or undetectable in specimens of non-BV patients and their male partners. *G. vaginalis* and *A. vaginae* were the most prevalent bacteria in all specimens regardless of clinical diagnosis.

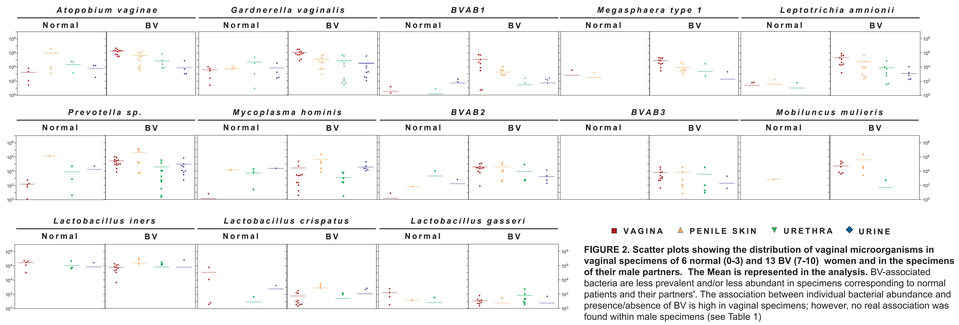

FIGURE 2. Scatter plots showing the distribution of vaginal microorganisms in vaginal specimens of 6 normal (0-3) and 13 BV (7-10) women and in the specimens of their male partners. The Mean is represented in the analysis. BV-associated bacteria are less prevalent and/or less abundant in specimens corresponding to normal patients and their partners'. The association between individual bacterial abundance and presence/absence of BV is high in vaginal specimens; however, no real association was found within male specimens (see Table 1)

NOTE: *A. vaginae, G. vaginalis, Megasphaera type 1, L. amnionii and BVAB1 assays were run on all penile skin specimens. DNA yield from 10 specimens was very low, therefore, the remaining assays include only 8 penile skin specimens (1 normal, 7 BV).*

	A. vaginae	*G. vaginalis*	*BVAB1*	*Megas 1*	*Prevotella*	*L. amnionii*	*BVAB2*	*BVAB3*	*M. mulieris*	*M. hominis*	*L. crispatus*	*L. iners*	*L. gasseri*
VAGINA ■	0.0007***	0.0007***	0.0143*	0.0061**	0.0007***	0.0009***	0.0018**	0.0018**	0.0464*	0.0025**	0.3798	0.0178*	0.5069
PENILE SKIN ▲	0.1393	0.1667	0.3242	0.0332*	0.6381	0.0195*	*0.0649*	*0.0649*	0.2249	0.2397	0.6076	0.6368	1.0000
URETHRA ▼	0.5536	0.6048	1.0000	0.6669	0.5652	*0.0716*	0.5329	0.1763	0.6809	0.4446	0.8417	0.3550	0.0988
URINE ◆	0.1641	0.6858	0.7357	0.6426	0.2693	0.1893	0.5117	0.9216	n/a	0.9250	0.9415	0.2935	0.5697

TABLE 1. Mann-Whitney U test comparing the levels of each individual bacteria in normal vs BV specimens and the specimens of the corresponding males. Bold type indicates a significance level of P<0.05. There is a significant difference in the amount of BV-associated bacteria between normal and BV vaginal specimens. The numbers of *A. vaginae, G. vaginalis, Prevotella* sp. and *L. amnionii* were significantly higher (P=0.0007) in the BV group (median 8.8 e^4 to 1.9 e^6) than in the normal group (median 10 to 2.8 e^3). On the other hand, the amount of bacteria in male specimens corresponding to either normal or BV women was not significantly different, with the exception of *L. amnionii* and *Megasphaera* type1 in penile skin specimens.

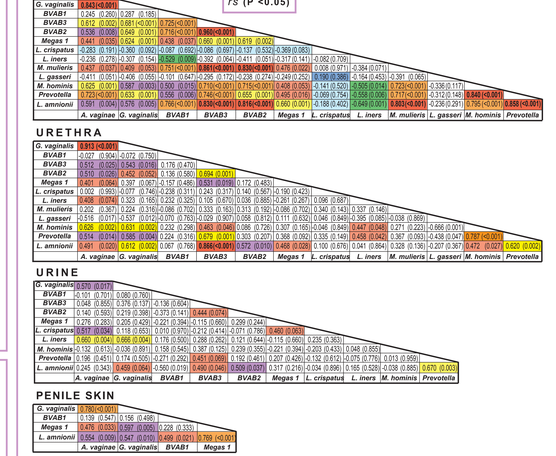

FIGURE 3. Spearman Rank Correlation between vaginal microorganisms in vaginal specimens and in specimens of their male partners. *Spearman Correlation Coefficient: rs > 0.9, very high correlation; rs < 0.9, high correlation; rs < 0.7, moderate correlation; rs < 0.5, low correlation; rs < 0.2, very low correlation; rs = 0, no correlation; rs 0 to -1, negative or inverse correlation.* In vaginal and urethral specimens there is a very high correlation between *A. vaginae* and *G. vaginalis*, while in penile skin and urine there is a moderate and low correlation, respectively, between these organisms. In vaginal specimens, the highest correlation was found between BVAB2/BVAB3, *L. amnionii* and BVAB3, BVAB2, *M. mulieris* and *Prevotella* sp. There is a negative correlation between all the *Lactobacillus* and BV-associated bacteria. BVAB1's correlation to other bacteria ranged from low to moderate in vaginal and penile skin samples. In general, bacteria in male specimens (with a few exceptions) showed no correlation or low correlation between each other.

CONCLUSIONS

- Male specimens harbor recently recognized BV-associated bacteria, such as the uncultivated BVAB1, *A. vaginae, Megasphaera* type 1, etc.

- Penile skin appears to be the best specimen to detect vaginal microorganisms in males; however, the DNA yield is insufficient to perform numerous qRT-PCR analysis. Therefore, penile skin assessment combined with urethral and urine assessments may provide a more accurate description of the male flora.

- Vaginal specimens of patients clinically diagnosed with BV show higher numbers of BV-associated bacteria but lower numbers of Lactobacilli than specimens of normal patients.

- Quantitative real-time PCR is useful to detect and assess the relative abundance of bacterial species in both vaginal and male specimens.