Non-gonococcal urethritis and its relationship to three novel bacterial vaginosis (BV) associated bacteria

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A thesis
submitted in partial fulfillment of the
requirements for the degree of

Master of Public Health

University of Washington
2020

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Program Authorized to Offer Degree:
Epidemiology

University of Washington

Abstract

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Objective: Non-gonococcal urethritis (NGU) is the most common urethral syndrome in the United States, yet up to 50% of cases are of unknown etiology. Bacterial-vaginosis associated bacteria (BVAB), highly predictive of BV in cisgender women, have been detected in cisgender men and may explain some cases of NGU. We sought to determine the frequency and correlates of three BVAB in cisgender men and evaluate whether they were associated with NGU.

Methods: We enrolled cisgender men who have sex with women (MSW) from a sexually transmitted disease (STD) clinic in Seattle, WA. Participants completed a computer-assisted self-interview (CASI), underwent a clinical interview and examination, and provided a urethral swab and urine specimen. We quantitated polymorphonuclear leukocytes (PMNs) on a Gram stained urethral smear and defined NGU as ≥5 PMNs per high power field plus either urethral symptoms or visible discharge in the absence of *Neisseria gonorrhoeae.* First void urine specimens were tested for *Chlamydia trachomatis* (CT) and *Mycoplasma genitalium* (MG), using Aptima assays (Hologic, Inc. San Diego, CA) and for BVAB2, *Mageeibacillus indolicus*, and *Sneathia spp.* using laboratory-developed quantitative polymerase chain reaction (qPCR) assays. We also tested a subset of men for *Trichomonas vaginalis* (TV), *Ureaplasma urealyticum* (UU), herpes simplex virus, and adenovirus using qPCR. We used logistic regression to evaluate potential associations between BVAB and NGU.

Results: A total of 317 MSW met inclusion criteria and were included in these analyses. Of these, thirty-six (11.4%) had BVAB2, 17 (5.4%) had *M. indolicus* and 67 (21.1%) had *Sneathia spp.* No sociodemographic characteristics were associated with any of the BVAB. However, men with each of these three BVAB were significantly more likely to report ≥3 sex partners in the past two months (BVAB2: 47.2% vs. 23.0%, p=0.01; *M. indolicus*: 52.9% vs. 24.2%, p=0.03; *Sneathia spp.*: 42.2% vs. 21.5%, p<0.01). In the subset of 174 men with UU test results, UU was significantly more common among men with BVAB2 (68.4% vs. 21.3%, p <0.001), *M. indolicus* (66.7% vs. 25.0%, p =0.04), and *Sneathia spp.* (54.1% vs. 19.0%, p<0.001). There was no association of NGU with BVAB2 or with *M. indolicus* in bivariate or multivariable analyses. Men with *Sneathia spp.* were significantly less likely to have NGU (25.4% vs. 40.0%, AOR=0.36, 95% CI=0.13-0.97). Results were similar when restricting the analysis to men without other known pathogens, although the association with *Sneathia spp.* was no longer significant. In a subset of men with longitudinal data, all three BVAB were frequently detected over multiple visits and often detected together.

Conclusions: This analysis did not confirm prior observations of increased risk of NGU associated with any of these three BVAB. More research investigating how the number of female partners influences BVAB colonization in MSW may inform our understanding of acquisition and clearance in women.

INTRODUCTION

While urethritis is the most common urethral syndrome in the United States, up to 50% of cases are not due to *Neisseria gonorrhoeae* (GC), *Chlamydia trachomatis* (CT), or *Mycoplasma genitalium* (MG), the three primary known causes [1]. These cases of unknown etiology are often referred to as idiopathic nongonococcal urethritis (NGU). In previous studies, other pathogens that have been associated with NGU include *Trichomonas vaginalis* (TV), herpes simplex virus (HSV) and adenovirus [2]. *Ureaplasma urealyticum* (UU) has also been associated with NGU in some, but not all studies [3,4]. Demographic factors such as younger age, racial minority status, lower education and income as well as a prior history of NGU have also been previously associated with NGU [5]. High risk sexual behaviors such as having multiple sex partners and lack of condom use have been commonly associated with STIs more generally [6,7]. More recently, bacteria known to cause inflammation in the female genital tract have been explored as potential causes of NGU.

Bacterial vaginosis associated bacteria (BVAB) have been highly predictive of and specific for bacterial vaginosis (BV), one of the most common genital tract syndromes in women [8]. In addition to the association with BV, three specific novel species, BVAB2, *Mageeibacillus indolicus*, and *Sneathia spp.*, were more common in women with cervicitis than in those without cervicitis in one study [9]. This finding, along with evidence that BVAB may be sexually transmitted [10,11], suggests that these bacteria may result in similar inflammatory conditions in the male genital tract. Although several prior studies have detected various BVAB in the male urogenital tract [12–14], results have been conflicting. A case-control study of idiopathic NGU among men in Seattle detected a significant association of *Sneathia spp.* with idiopathic NGU, and BVAB2 and *M. indolicus* were more common among men with NGU than those without NGU [2]. In contrast, studies among Swedish men have not detected associations with these

BVAB and NGU. In a case-control study using broad-range 16S rRNA gene PCR and sequencing, Frølund et al detected *Sneathia spp.* in the male urogenital tract but there was no association between *Sneathia spp.* and idiopathic NGU [14]. A separate cross-sectional study using qPCR also found no association between NGU and either *Sneathia sanguinegens* or *Sneathia amnii* [12].

To further explore BVAB in men, we estimated the prevalence of and characteristics associated with BVAB2, *M. indolicus*, and *Sneathia spp.* in a cross-sectional study of cisgender men who have sex with women (MSW). We also evaluated their association with NGU. Additionally, we described the natural history of these bacteria in a subset of men.

METHODS

Study Design and Procedures

Cisgender men attending the Public Health – Seattle & King County (PHSKC) STD Clinic in Seattle, Washington were enrolled in a longitudinal study of the male urethral microbiota. The detailed study recruitment procedures have been previously described [15]. Briefly, persons assigned male sex at birth who have sex with women (MSW) attending a sexually transmitted disease (STD) clinic who were 16 years or older were eligible. To be included, men were required to have had exclusively female partners in the past year, sexual activity during the past 60 days, no antibiotics in the past 30 days, no evidence of gonorrhea on Gram stain and no known contact to a partner with *Neisseria gonorrhoeae* (GC) diagnosis. After providing informed consent, participants underwent a clinical interview and examination, during which a urethral swab and urine specimen were collected. Urethral swabs were utilized for Gram staining for GC as well as quantification of polymorphonuclear leukocytes (PMNs). All patients attending the clinic routinely completed a clinic computer-assisted self-interview (CASI) which collects basic socio-behavioral information ("Kiosk CASI"). Study participants also completed a study CASI that collected more detailed behavioral data. Clinical data on urethral symptoms, examination findings and medical history were abstracted from an electronic medical record.

NGU was defined as ≥5 PMNs per high power field (hpf) plus either urethral symptoms or visible discharge in the absence of *N. gonorrhoeae*. Men who were diagnosed with NGU received either azithromycin (1g single dose) or doxycycline (100 mg/day x 7 days). Participants were followed for 6 months, with study visits at one-month intervals throughout the study period; men with NGU at enrollment had one additional study visit after initial treatment. Study procedures at follow-up visits were identical to those at enrollment.

Urine specimens from patients were routinely tested for GC and CT using Aptima Combo2 assays while *Mycoplasma genitalium* (MG) was detected using Aptima with analyte-specific reagents (Hologic, Inc. San Diego, CA). Species-specific quantitative polymerase chain reaction (qPCR) assays developed in the Fredricks Laboratory at the Fred Hutchinson Cancer Research Center (FHCRC) were used to detect BVAB2, *M. indolicus*, and *Sneathia spp.* in all men and *Trichomonas vaginalis* (TV) and *Ureaplasma urealyticum* (biovar 2 or UU) in urine in a subset of 174 men. Previously established qPCR assays developed by the Jerome laboratory at FHCRC were utilized to identify herpes simplex virus (HSV) and adenovirus in the subset of individuals that participated in a separate case-control study (n=175) [16].

Statistical Analysis

We compared characteristics of participants with and without NGU, as well as those with and without each of the three BVAB (BVAB2, *M. indolicus, Sneathia spp.*) using Fisher's exact tests and two-sample t-tests with unequal variances. Specifically, we focused on characteristics for which there was prior evidence of potential association with NGU and STI's in general, including sociodemographic characteristics (age, race/ethnicity, education), sexual behaviors (condom use, number of sex partners), clinical characteristics (urethral symptoms, presence of urethral discharge, prior STI and NGU diagnoses) and known etiologies of NGU.

We utilized multivariable logistic regression to evaluate the association between presence of each BVAB and NGU, adjusting for organisms that have been associated with NGU (*C. trachomatis, M. genitalium*, and *U. urealyticum*), the other BVAB, and number of sex partners and having a new sex partner in the past two months. Given that only a subset of men were tested for UU, we explicitly included a category for those missing UU test results to retain them in the multivariable analysis, but do not report estimates for this category.

Additionally, we conducted two secondary analyses. In the first, we assessed the relationship between these bacteria and idiopathic NGU by restricting analyses to those without CT, MG, and known infections with TV and UU. In the second, to evaluate the influence of how NGU is defined, we compared associations with NGU and with idiopathic NGU using our study definition (Inflammation-Based) to two alternative definitions of NGU. The first was that used in our previous study that demonstrated an association of *Sneathia spp.* with NGU [2] (Objective Signs-Based, defined as clinician-observed discharge OR ≥5 PMNs/hpf on a Gram stain). The second definition required the presence of urethral symptoms and at least one objective clinical sign (Symptom-Based, defined as patient reported urethral symptoms AND clinical observed discharge OR ≥5 PMNs/hpf).

Finally, to describe the natural history of infection with each of these three novel BVAB over a period of 6 months, we constructed a heatmap in the subset of individuals who had follow-up specimens tested for these three BVAB. We included the presence and quantity of each BVAB, NGU status and its components (elevated PMNs, patient-reported urethral symptoms, clinician-observed discharge), MG and CT infections, and antibiotic use by visit.

For these analyses we used Stata V.16 and two-sided tests with a significance level of alpha = 0.05. The parent study procedures were approved by the University of Washington Institutional Review Board; these analyses were restricted to anonymized data.

RESULTS

A total of 322 MSW enrolled, of whom 249 were included in an earlier report describing characteristics associated with NGU in MSW and in men who have sex with men (MSM) [15]. We excluded five of the 322 enrolled men: one subsequently reported male sexual contact; one had a positive GC NAAT test; one reported antibiotic use prior to enrollment; one reported no female sexual partners in the last 12 months and one did not sign the HIPAA release form. The remaining 317 MSW were tested for both the presence and quantity of BVAB2, *M. indolicus*, and *Sneathia spp.* and were included in these analyses.

Among the 317 participants, the average age was 33.8 years (SD ± 10.2 years; range = 17-71 years). Most were non-Hispanic white (46.1%) or non-Hispanic black (22.4%), while 36 individuals reported Hispanic/Latino ethnicity (11.4%) and 64 reported other/multiple races or did not report their race (20.2%). Of the 317 men, 117 had NGU at enrollment (36.9%). Thirty-six men (11.4%) had BVAB2, 17 men (5.4%) had *M. indolicus,* and 67 men (21.1%) had *Sneathia spp.*

Characteristics associated with NGU

As previously reported [15], non-Hispanic Black MSW, those with less than high school education, those who reported inconsistent condom use or a history of CT, and those with MG or CT at enrollment were significantly more likely to have NGU than those without these characteristics ($p<0.05$ for all). Participants with and without NGU did not differ significantly by age, sexual history in the past two months, detection of adenovirus, HSV, TV, or UU, or history of NGU or GC infection.

BVAB2

Sociodemographic and sexual behavior characteristics. Men with BVAB2 were significantly more likely to have ≥3 sexual partners in the two months before enrollment than men without BVAB2 (47.2% vs 23.0%, p=0.01; **Table 1**). There were no significant differences in age, race, education, consistent condom use, having a new partner in the past two months, or type of sexual behavior in the past two months (vaginal, anal, or oral) between men with and without BVAB2.

Clinical characteristics. Urethral symptoms were significantly less common among men with BVAB2 than they were among men without BVAB2 (22.2% vs. 45.2%, p=0.01; **Table 2**). Clinician-observed discharge was also significantly less common in men with than without BVAB2 (22.2% vs. 42.0% p=0.03). In contrast, there was no significant association with the quantity of PMNs/hpf. There was also no association between BVAB2 and detection of CT, MG, HSV, adenovirus, and TV at the clinic visit, nor was there any association with reporting a history of NGU, CT, or GC. However, UU infection was significantly more common among men with BVAB2 than among men without BVAB2 (68.4% vs. 21.3%, p<0.001) in the subset of men who were tested for UU.

Association with NGU. Men with BVAB2 were less likely to have NGU than men without BVAB2 although this difference was not statistically significant (22.2% vs. 38.8%, p=0.07; **Table 5)**. In multivariable analyses, adjusting for *M. indolicus, Sneathia spp.*, MG, CT, UU, number of recent sex partners, and having a new partner, this remained relatively unchanged (AOR 0.62; 95% CI: 0.15-2.55; **Table 3**). Among those in whom BVAB2 was detected, quantity of BVAB2 was not associated with NGU (2.17 log copies/ul vs. 2.34 log copies/ul, p=0.62; **Table 4**). When restricting these analyses to men without CT, MG, TV, and UU, the association between BVAB and idiopathic NGU remained relatively unchanged in bivariate analyses (OR=0.38; 95% CI: 0.08-1.71, **Table 4**). Given the small number of men with BVAB2 in this subset, we were unable to develop a multivariable model.

M. indolicus

Sociodemographic and sexual behavior characteristics. Reporting ≥3 sex partners in the past two months was significantly more common in men with than without *M. indolicus* (52.9% vs. 24.2%, p=0.03; **Table 1**). Men who had *M. indolicus* were somewhat more likely to be non-Hispanic Black (35.3% vs. 21.7%) and Hispanic (23.5% vs. 10.7%) than men without *M. indolicus* (p=0.10), although this was not statistically significant. No other sociodemographic and sexual behavior characteristics, including age, race, education, consistent condom use, having a new sexual partner in the past two months, and type of sexual behavior were significantly different between groups.

Clinical characteristics. In contrast to BVAB2, *M.* indolicus was not associated with any patient reported symptoms (**Table 2**). Additionally, there was no association between *M. indolicus* and either clinician observed discharge or quantity of PMNs/hpf. However, among the subset of men with UU test results, UU was significantly more common in participants with than without *M. indolicus* (66.7% vs. 25.0%, p=0.04). History of CT infection was also more common in men with *M. indolicus* than in men without (47.1% vs. 27.2%, p=0.10) but this difference was not statistically significant. There was no association of *M. indolicus* with detection of any other organisms at enrollment (CT, MG, HSV, adenovirus, and TV) or with history of NGU and GC.

Association with NGU. There was no association between *M. indolicus* and NGU in bivariate analyses. There was also no significant association in multivariable analyses, although given the small number of men with *M. indolicus* the estimates were unstable (**Table 3**). Among men with

M. indolicus, there was no association with quantity and NGU (2.55 log copies/μl vs. 2.40 log copies/μl, p=0.83; **Table 4**), nor was there any association with idiopathic NGU in bivariate analyses (OR=0.86; 95% CI: 0.18-4.19; **Table 5**). Multivariable models for idiopathic NGU could not be developed.

Sneathia spp.

Sociodemographic and sexual behavior characteristics. Only the number of sex partners in the past 2 months was significantly associated with *Sneathia spp.* Men with *Sneathia spp.* were more likely to report ≥3 sex partners in the past two months than men without *Sneathia spp.* (42.2% vs. 21.5%, p<0.01, **Table 1**). Neither age, nor race, education, consistent condom use, having a new sex partner, or type of sexual behavior were significantly different between groups.

Clinical characteristics. Men with *Sneathia spp.* were significantly less likely to have any urethral symptoms than men without *Sneathia spp.* (29.9% vs. 46%, p=0.02; **Table 2**). Clinician-observed discharge was significantly less common among men with *Sneathia spp.* than men without (26.9% vs. 43.2%, p=0.02). Similarly, men with *Sneathia spp.* were less likely to report discharge as a symptom than men without *Leptrotrichia/Sneathia spp*., although this difference was not statistically significant (35% vs. 56.5%, p=0.09). Detection of UU among men tested for this organism was significantly more common in men with *Leptrotrichia/Sneathia spp.* than in men without *Sneathia spp.* (54.1% vs. 19%, p<0.001). Men with *Sneathia spp.* were less likely to have CT at enrollment than those without *Sneathia spp.,* although this was not statistically significant (4.5% vs. 12.8%, p=0.08). There was no association between *Sneathia spp.* and PMNs/hpf, detection of other organisms at enrollment (MG, HSV, adenovirus, TV), or history of NGU, GC and CT.

Association with NGU. Men with *Sneathia spp.* were significantly less likely to have NGU than those without *Sneathia spp.* (25.4% vs. 40.0%, p=0.03; **Table 5**). In multivariable analyses, adjusting for BVAB2, *M. indolicus*, MG, CT, number of recent sex partners, and having a new partner, this association was similar (AOR 0.36; 95% CI=0.13-0.97; **Table 3**). However, there was no association between quantity and NGU among those with *Sneathia spp.* (3.21 log copies/µl vs. 3.47 log copies/µl, p=0.52; **Table 4**). When restricting these analyses to men without CT, MG, TV, and UU, the association between *Sneathia spp.* and idiopathic NGU was no longer statistically significant (OR=0.48; 95% CI: 0.18-1.32; **Table 3**).

Sub-analyses investigating the influence of NGU definitions

The prevalence of NGU was higher when the definition of NGU required either ≥5 PMNs/hpf or clinician-observed discharge (Objective Signs-Based definition) than under our Inflammation-Based study definition (50.8% vs. 36.9%; **Table 5**). There remained no association between BVAB2 and *M. indolicus* and NGU under this definition and the association between *Sneathia spp.* and NGU was no longer statistically significant. The prevalence of idiopathic NGU was also higher when one or more objective signs comprised the definition of NGU (40.5% vs. 22.4%) but there remained no associations with the three BVAB (data not shown).

In contrast, the prevalence of NGU was slightly lower when urethral symptoms were required in the definition of NGU (Symptoms-Based definition) than under our study definition (35.7% vs. 36.9%). Although this difference was small, the inverse association of NGU with BVAB2 was statistically significant (19.4% vs. 37.7%, p=0.04) under this definition. There was no change to the associations of *Sneathia spp.* and *M. indolicus* with NGU; the former remained inversely associated with NGU (p=0.03) while the latter remained not significantly associated. The prevalence of idiopathic NGU was also slightly lower with the Symptoms-Based definition than with our study definition (21.5% vs 22.4%). However, there were no substantive changes in the associations between any of the BVAB and idiopathic NGU (data not shown).

Exploratory Analysis of Follow-Up Visits

Of the 317 men included in this study, qPCR results for BVAB2, *M. indolicus* and *Sneathia spp.* were available for almost all follow-up visits from 109 men. Thirty-four of these men (31.2%) had at least one visit with a positive qPCR test for BVAB2, *M. indolicus,* or *Sneathia spp.* (**Figure S1**).

Overall, men with BVAB frequently had the same bacterium over multiple visits, and the bacteria were often detected together. Twenty-two (64.7%) of the 34 men had more than one of the BVAB present at one or more visits. BVAB2 was rarely detected in the absence of *Sneathia spp.* All 21 men with BVAB2 at any time during follow-up also had *Sneathia spp.* detected at least once. Only four of these men (19%) had a time-point where BVAB2 was present alone. *M. indolicus* was infrequently detected and almost exclusively present together with *Sneathia spp.* and BVAB2. *Sneathia spp.* was detected alone more often that the other BVAB, but this was still relatively rare. Consistent with the cross-sectional analyses, we observed no consistent patterns with NGU, PMNs and antibiotic use for any of the three bacteria.

DISCUSSION

In this population of cisgender MSW, *Sneathia spp.* was detected in 21.1% of men, whereas BVAB2 (11.4%) and *M. indolicus* (5.4%) were less common. Aside from detection of *U. urealyticum* and reporting a higher number of sex partners in the past two months, few characteristics were associated with these BVAB. Although all three of the BVAB were somewhat less common among men infected with CT, this was not statistically significant. Overall, NGU was inversely associated with *Sneathia spp.* and not significantly associated with either BVAB2 or *M. indolicus*. In the subset of men with longitudinal data, all three of these BVAB commonly co-occurred and often persisted in those in whom they were detected.

Given that BV in women is associated with race [8,17], and these 3 BVAB are highly predictive of BV [9], it was somewhat surprising that neither BVAB2, *M. indolicus,* nor *Sneathia spp.* were significantly associated with race. The exception was *M. indolicus,* which was somewhat more common in individuals reporting non-Hispanic Black race or Hispanic ethnicity and the lack of statistical significance may be due to small sample size. However, the lack of association with other sociodemographic characteristics in men is consistent with other studies. In their case-control study using broad-range PCR and sequencing on first-void urine among Swedish men, Frølund et al did not observe any association between age and *Sneathia spp.*, although the sample size was small [14]. In our previous observations of men in Seattle, there were no associations between any of the 3 BVAB and age or race, although BVAB2 was significantly associated with lower education [2].

While demographic characteristics were generally not associated with these BVAB, having ≥3 sex partners in the past two months was significantly more common in men with all three of the BVAB that we evaluated. Frølund et al, in comparison, did not find any association between number of sex partners in the last 6 months with *Sneathia spp.* in first-void urine of Swedish men [14]. The difference between their results and ours may be due to the longer period over which they measured the number of sex partners. In our prior study, none of these BVAB were associated with either total or new number of sex partners in the past two months. However, our prior study only considered a continuous measure of number of sex partners [2]. Despite this association with the number of different sex partners in the past two months, there was no association of having a new partner and any of the three BVAB, a pattern which was consistent in multivariable analyses. This suggests that the overall number and variety of recent female partners is more relevant to the carriage of these bacteria than new exposures from a new partner. This may reflect that BV in women is often persistent [18,19]. While antibiotic treatment for symptomatic BV is recommended for women, one randomized controlled trial in women who have sex with women (WSW) found that treatment did not result in a return to normal flora for all women and recurrences of BV after one month were relatively common [20]. The sustained detection of BVAB that we observed in these men parallels the persistent BVAB infection that occurs in women. However, a better understanding of the relationship between BVAB in women and their male partners in necessary. While prior studies have failed to find a significant decrease in recurrence when treating male partners of women with BV [21–24], both not using condoms [10] and having a recent new sex partner [25] have been associated with BV in women. In addition, one study found a significant association between BV in women and NGU in their male partners, although the sample size was small [26]. More research is needed on the transmission dynamics of BVAB between male and female sex partners.

BV is polymicrobial in nature and is characterized by a decrease in lactic acid-producing lactobacilli and an increase in facultative and anaerobic bacteria [27]. Additionally, it is associated with a significant increase in bacterial richness and diversity and a larger number of anaerobic bacteria, which includes species such as BVAB2*, M. indolicus,* and *Sneathia spp.,* among others [28,29]. One study that followed thirty-three women longitudinally observed that multiple BVAB (including BVAB2, *M. indolicus,* and *Sneathia spp.*) were detected in women with BV simultaneously [17]. Although the microbiota in the female vagina are more diverse [30], this co-occurrence of BVAB in women is somewhat analogous to the co-occurrence and persistence of BVAB2, *M. indolicus*, and *Sneathia spp.* in men that we observed.

In the subset of men tested for *U. urealyticum*, each of the BVAB was strongly associated with *U. urealyticum*. Although the data regarding the relationship between UU and NGU are conflicting (one meta-analysis identified an association between UU and NGU [31], several other studies did not [3,32–36]), Ureaplasmas have been previously associated with BV in women, although there is disagreement regarding this relationship [37–39]. The strong association between UU and BVAB may be due to the hydrolysis of urea by Ureaplasmas that results in increased ammonia and higher pH, which may encourage colonization with BVAB [12].

Few clinical characteristics were significantly associated with any of the three BVAB, and the relationships that we did detect were in the opposite direction from what we had originally hypothesized (e.g., signs and symptoms were less common in men with any of these three BVAB). This lack of association is consistent with observations in prior studies [2,14]. Notably BVAB2 and *M. indolicus* were not significantly associated with NGU, and MSW with *Sneathia spp.* were significantly less likely to have NGU. None of these BVAB were significantly associated with idiopathic NGU. This was the opposite of what we observed in our prior study of men in Seattle [2], Our current result is consistent with two prior studies that found no association between *Sneathia spp.* and NGU [12,14]. Specifically, a cross-sectional study among Swedish men utilized species-specific qPCR to detect *Sneathia amnii*, *Sneathia sanguinegens*, and BVAB2, and observed no association with idiopathic NGU (≥5 PMNs/hpf and discharge and/or dysuria) [12], despite a stricter definition for their controls (<5 PMNs/hpf and no urethritis symptoms). A case-control study among Swedish men by the same investigators that utilized broad-range qPCR and sequencing with first-void urine to characterize urethral microbiota [14] also did not identify any relationship between *Sneathia spp.* and idiopathic urethritis, defined as ≥10 PMNL/hpf and urethritis symptoms lasting <30 days).

Our study had several limitations. While our study definition for NGU required ≥5 PMNs/hpf plus either symptoms or signs of urethritis, other studies have used less stringent definitions. Because there is no gold standard definition of NGU, it is not possible to determine the extent of misclassification of NGU status. Therefore, it is not possible to determine if our definition overestimated or underestimate the odds ratio for the association between any of the three BVAB and NGU. However, our sub-analyses using two alternative definitions of NGU did not appreciably change our overall results, suggesting that the definition of NGU is not highly influential. Additionally, in contrast to our prior study, we included men with either clinician observed discharge or urethral symptoms in the absence of elevated PMNs in the group without NGU, as well as men with elevated PMNs alone. This may have attenuated any relationship of the BVAB with NGU. Another limitation is that the qPCR assay that we used to detect *Sneathia spp.* did not differentiate between the several subtypes of *Sneathia spp.* It is possible that one of the subspecies is associated with NGU and other species in the men obscured this. Finally, we only had test results for UU, TV, HSV and adenovirus in approximately 55% of our study population. This is particularly relevant to our observed associations of UU with each of the three BVAB, and future studies should explore this in more depth with larger study populations.

Despite these limitations, this study also had several strengths. We used highly sensitive and specific assays that had been previously validated. In addition, all participants were assessed for NGU by the same two physicians, which enhanced the consistency of the diagnosis. Finally, while this is a cross-sectional study, the availability of longitudinal data on a subset of men allowed us to observe some of the natural history of these three BVAB in men and yielded unique insights.

In summary, we observed a significant inverse association between *Sneathia spp.* and NGU, but BVAB2 and *M. indolicus* were not associated with either NGU overall or with idiopathic

NGU. The intriguing association of *U. urealyticum* with each of the three BVAB merits more investigation. The consistent association of multiple partners and these BVAB should motivate research investigating how the number and variety of female partners influences BVAB infection in MSW and subsequent transmission to women. Regardless of whether these BVAB lead to adverse health outcomes in MSW, BVAB are important risk factors for female sexual health. Given the potential for BVAB to be transferred between sex partners [10,11], understanding these relationships in MSW may inform our understanding of acquisition and clearance in women, and provide new information to design more effective prevention interventions.

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Table 1 –Sociodemographic and Behavioral Characteristics by BVAB2, *M. indolicus*, and *Sneathia spp.* Status Among Cisgender Men who have Sex with Women Attending the PHSKC STD Clinic (n=317)

	BVAB2+ (n=36) *n (%)*	BVAB2- (n=281) *n (%)*	*p*	*M. indolicus+* *(n=17)* *n (%)*	*M. indolicus-* *(n=300)* *n (%)*	*p*	*Sneathia+* *(n=67)* *n (%)*	*Sneathia-* *(n=250)* *n (%)*	*p*
Age, ***mean (±SD) years***									
	34.25 (±10.46)	33.70 (±10.19)	0.76	34.47 (±10.43)	33.72 (±10.21)	0.78	33.49 (±10.12)	33.83 (±10.25)	0.81
Race/Ethnicity									
NH[a] White	14 (38.89)	132 (46.98)	0.219	4 (23.53)	142 (47.33)	0.096	26 (38.81)	120 (48.00)	0.558
NH[a] Black	7 (19.44)	64 (22.78)		6 (35.29)	65 (21.67)		17 (25.37)	54 (21.60)	
Hispanic	8 (22.22)	28 (9.96)		4 (23.53)	32 (10.67)		8 (11.94)	28 (11.20)	
Other[b]	7 (19.44)	57 (20.28)		3 (17.65)	61 (20.33)		16 (23.88)	48 (19.20)	
Education*									
≤ High School/GED	14 (41.18)	97 (35.02)	0.570	7 (43.75)	104 (35.25)	0.593	26 (40.00)	85 (34.55)	0.467
>High School/GED	20 (58.82)	180 (64.98)		9 (56.25)	191 (64.75)		39 (60.00)	161 (65.45)	
Condom Use*									
Not Always	27 (75.00)	212 (78.23)	0.671	12 (70.59)	227 (78.28)	0.546	51 (79.69)	188 (77.37)	0.738
Always	9 (25.00)	59 (21.77)		5 (29.41)	63 (21.72)		13 (20.31)	55 (22.63)	
Sexual History, Past Two Months *									
Number Sex Partners									
1	10 (27.78)	117 (42.70)	**0.011**	3 (17.65)	124 (42.32)	**0.034**	22 (34.38)	105 (42.68)	**0.004**
2	9 (25.00)	94 (34.31)		5 (29.41)	98 (33.45)		15 (23.44)	88 (35.77)	

	BVAB2+ (n=36) *n (%)*	BVAB2- (n=281) *n (%)*	*p*	*M. indolicus+ (n=17) n (%)*	*M. indolicus- (n=300) n (%)*	*p*	*Sneathia+ (n=67) n (%)*	*Sneathia- (n=250) n (%)*	*p*
≥3	17 (47.22)	63 (22.99)		9 (52.94)	71 (24.23)		27 (42.19)	53 (21.54)	
New sex partner									
0	7 (20.00)	85 (30.58)	0.240	3 (17.65)	89 (30.07)	0.412	22 (33.33)	70 (28.34)	0.449
≥1	28 (80.00)	193 (69.42)		14 (82.35)	207 (69.93)		44 (66.67)	177 (71.66)	
Vaginal sex with SP	32 (94.12)	264 (97.42)	0.264	14 (87.50)	282 (97.58)	0.075	59 (96.72)	237 (97.13)	1.00
Anal sex with SP	5 (13.89)	50 (18.18)	0.646	3 (17.65)	52 (17.69)	1.00	12 (18.46)	43 (17.48)	0.856
Oral sex with SP	30 (96.77)	248 (91.85)	0.488	13 (92.86)	265 (92.33)	1.00	57 (95.00)	221 (91.70)	0.587

% represent percentages excluding missing values

P-values are based on Fisher's exact tests, while continuous variables were compared using t-tests assuming unequal variance.

Abbreviations: SP = sex partner

**Missing n =6 for education, n =11 lifetime number of partners, n=10 for condom use, n=7 for 2 month sex history, n=4 for 2 month new partners, n=6 for anal sex 2 mo., n = 12 for vaginal sex 2 mo., n= 16 for oral sex 2 mo.*

[a] *Non-Hispanic*

[b] *Includes other, multiple or unknown race/ethnicity*

Table 2 –Clinical Characteristics by BVAB2, *M. indolicus*, and Sneathia *spp.* status Among Cisgender Men who have Sex with Women Attending the PHSKC STD Clinic (n=317)

Characteristics	BVAB2+ (n=36) n (%)	BVAB2- (n=281) n (%)	*p*	*M. indolicus+ (n=17) n (%)*	*M. indolicus- (n=300) n (%)*	*p*	*Sneathia+ (n=67)* n (%)	*Sneathia- (n=250)* n (%)	*p*
Patient Reported Symptoms									
Any Urethral Sx	8 (22.22)	127 (45.20)	**0.011**	7 (41.18)	128 (42.67)	1.00	20 (29.85)	115 (46.00)	**0.018**
Discharge	3 (37.50)	69 (54.33)	0.472	4 (57.14)	68 (53.13)	1.00	7 (35.00)	65 (56.52)	0.092
Dysuria	3 (37.50)	79 (62.20)	0.262	3 (42.86)	79 (61.72)	0.432	9 (45.00)	73 (63.48)	0.140
Other	5 (62.50)	59 (46.46)	0.476	4 (57.14)	60 (46.88)	0.708	10 (50.00)	54 (46.96)	0.813
Clinician Observed Discharge									
	8 (22.22)	118 (41.99)	**0.029**	4 (23.53)	122 (40.67)	0.206	18 (26.87)	108 (43.20)	**0.017**
PMNs									
0-4	24 (66.67)	155 (55.16)	0.228	7 (41.18)	172 (57.33)	0.067	45 (67.16)	134 (53.60)	0.076
5-9	5 (13.89)	32 (11.39)		5 (29.41)	32 (10.67)		8 (11.94)	29 (11.60)	
≥10	7 (19.44)	94 (33.45)		5 (29.41)	96 (32.00)		14 (20.90)	87 (34.80)	
Organisms Detected									
C. trachomatis	1 (2.78)	34 (12.10)	0.152	1 (5.88)	34 (11.33)	0.705	3 (4.48)	32 (12.8)	0.076
M. genitalium	4 (11.11)	31 (11.03)	1.00	3 (17.65)	32 (10.67)	0.415	8 (11.94)	27 (10.80)	0.827
HSV[a] (n=175)	1/19 (5.26)	1/156 (0.64)	0.206	0/6 (0.00)	2/169 (1.18)	1.00	1/37 (2.70)	1/138 (0.72)	0.379
Adenovirus[a] (n=175)	1/19 (5.26)	0/156 (0.00)	0.109	0/6 (0.00)	1/169 (0.59)	1.00	1/37 (2.70)	0/138 (0.00)	0.211
T. vaginalis [b] (n=174)	0/19 (0.00)	11/155 (7.10)	0.612	1/6 (16.67)	10/168 (5.95)	0.328	3/37 (8.11)	8/137 (5.84)	0.703

Characteristics	BVAB2+ (n=36) n (%)	BVAB2- (n=281) n (%)	*p*	*M. indolicus+* *(n=17)* *n (%)*	*M. indolicus-* *(n=300)* *n (%)*	*p*	*Sneathia+* *(n=67)* n (%)	*Sneathia-* *(n=250)* n (%)	*p*
U. urealyticum[b](n=174)	13/19 (68.42)	33/155 (21.29)	**<0.001**	4/6 (66.67)	42/168 (25.00)	**0.043**	20/37 (54.05)	26/137 (18.98)	**<0.001**
History of STD									
History of NGU*	3 (8.57)	34 (12.14)	0.781	3 (17.65)	34 (11.41)	0.434	10 (15.15)	27 (10.84)	0.389
History of GC*	4 (11.43)	40 (14.29)	0.799	4 (23.53)	40 (13.42)	0.273	8 (12.12)	36 (14.46)	0.695
History of CT*	9 (25.71)	80 (28.57)	0.843	8 (47.06)	81 (27.18)	0.096	21 (31.82)	68 (27.31)	0.539

[a] *Only 175 of the 317 participants were tested for adenovirus and HSV at enrollment.*

[b] *Only 174 of the 317 participants were tested for UU and TV infection*

**Missing n=2 for report of GC hx, n=2 for CT hx, n=2 for NGU hx*

Abbreviations: Sx = symptoms, PMNs = polymorphonuclear leukocytes per high power field, GC = Neisseria gonorrhoeae, CT = Chlamydia trachomatis, HSV = Herpes simplex virus

Table 3 – Association of BVAB2, *M. indolicus*, and *Sneathia spp.* with NGU and Idiopathic NGU Among Cisgender Men who have Sex with Women Attending the PHSKC STD Clinic (N=317)

	NGU						Idiopathic NGU[a]		
BVAB	**OR**	**95% CI**	***p***	***aOR***	***95% CI***	***p***	***OR***	***95% CI***	***p***
BVAB2	0.45	0.20-1.03	0.06	0.62	0.15-2.55	0.51	0.38	0.08-1.71	0.21
M. indolicus	0.93	0.33-2.58	0.89	3.12	0.63-15.54	0.16	0.86	0.18-4.19	0.85
Sneathia spp.	0.51	0.28-0.93	**0.03**	0.36	0.13-0.97	**0.04**	0.48	0.18-1.32	0.16
U. urealyticum	--	--	--	1.39	0.60-3.18	0.44	--	--	--
C. trachomatis	--	--	--	21.49	6.87-67.23	**<0.001**	--	--	--
M. genitalium	--	--	--	8.56	3.46-21.15	**<0.001**	--	--	--
>1 SP (Past 2 months)	--	--	--	1.77	0.95-3.27	0.07	--	--	--
Any New SP (Past 2 months)	--	--	--	0.83	0.43-1.61	0.59	--	--	--

[a] *Restricting to men negative for CT, MG, TV, and UU. Only bivariate odds ratios are shown as the number of men with each bacterium was small and resulted in unstable multivariable models*

Abbreviations: SP = sex partner

Table 4 – Mean Quantity in Log Copies / mL of BVAB2, *M. indolicus*, and *Sneathia spp.* by NGU Status Among Cisgender Men who have Sex with Women Positive for BVAB2, *M. indolicus,* and *Sneathia spp.* at Enrollment (n=71[a])

Characteristics (n)	NGU+ *Mean (SD)*	NGU- *Mean (SD)*	*p-value*
BVAB2 (n=36) [b]			
	2.17 (±0.84)	2.34 (±0.99)	0.62
***M. indolicus* (n=17)** [c]			
	2.55 (±1.50)	2.40 (±1.20)	0.83
***Sneathia spp.* (n=67)** [d]			
	3.21 (±1.38)	3.47 (±1.42)	0.52

P-values were calculated using t-tests assuming unequal variance.

[a] 71 men tested positive for at least one BVAB out of the total 317 men in the study population

[b] Among those positive for BVAB2, 8 had NGU and 28 did not.

[c] Among those positive for M. indolicus, 6 had NGU and 11 did not.

[d] Among those positive for Sneathia spp., 17 had NGU and 50 did not

Table 5: Comparison of NGU Definitions at Enrollment and the Association with BVAB2, *M. indolicus* and *Sneathia spp.* Among Cisgender Men who have Sex with Women Attending PHSKC STD Clinic and the Subset of Men Without *CT, GC, TV, MG and UU* (n=317 for overall NGU)

NGU Status (n)	BVAB2+ *n (%)*	BVAB2- *n (%)*	*p*	*M. indolicus+ n (%)*	*M. indolicus- n (%)*	*p*	*Sneathia + n (%)*	*Sneathia- n (%)*	*p*
Inflammation-Based (PMNs PLUS Clinical Signs OR Sx)									
NGU+ (117)	8 (22.22)	109 (38.79)	0.07	6 (35.29)	111 (37.00)	1.00	17 (25.37)	100 (40.00)	**0.03**
NGU-	28 (77.78)	172 (61.21)		11 (64.71)	189 (63.00)		50 (74.63)	150 (60.00)	
Objective Signs (Clinical Signs OR PMNs)									
NGU+ (161)	14 (38.89)	147 (52.31)	0.16	10 (58.82)	151 (50.33)	0.62	27 (40.30)	134 (53.60)	0.06
NGU-	22 (61.11)	134 (47.69)		7 (41.18)	149 (49.67)		40 (59.70)	116 (46.40)	
Symptoms-Based (Symptoms PLUS Clinical signs or PMNs)									
NGU+ (113)	7 (19.44)	106 (37.72)	**0.04**	6 (35.29)	107 (35.67)	1.00	16 (23.88)	97 (38.80)	**0.03**
NGU-	29 (80.56)	175 (62.28)		11 (64.71)	193 (64.33)		51 (76.12)	153 (61.20)	

P-values are based on Fisher's exact tests, while continuous variables were compared using t-tests assuming unequal variance.