

Assessment of the Urinary Microbiota of MSM Using Urine Culturomics Reveals a Diverse Microbial Environment

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BACKGROUND: The urinary tract is not sterile and is populated by microbial communities that influence urinary health. Men who have sex with men (MSM) are understudied yet have increased risk factors for genitourinary infections. Our objective was to interrogate the composition of MSM urinary microbiota.

METHODS: Midstream urine specimens (n = 129) were collected from MSM (n = 63) and men seen for routine care (clinical cohort, n = 30). Demographics and sexual/medical history were documented. Specimens underwent culture using standard-of-care and enhanced methods designed to isolate fastidious and anaerobic microorganisms. Isolates were identified by MALDI-TOF mass spectrometry or 16S rRNA gene sequencing.

RESULTS: The MSM cohort was younger (mean (SD), 35.4 (11.26) years) compared to the clinical cohort (62.7 (15.95) years). Organism recovery was significantly increased using enhanced vs standard culture for the MSM (mean of 9.1 vs 0.6 species/sample [$P < 0.001$]) and clinical (7.8 vs 0.9 species/sample [$P < 0.001$]) cohorts. The microbial composition of MSM urine specimens was dominated by Gram-positive and anaerobic microbes and clustered distinctly from that of clinical urine specimens. Composition of microbial species recovered within the same subject was dynamic between urine specimens but more similar relative to inter-individual comparisons. Principal coordinate analysis showed no correlation between urinary microbiota composition and age, recent antibiotic use, sexually transmitted infection/HIV status, or sexual practices.

CONCLUSIONS: Enhanced culture recovered a large diversity of microbial species from MSM urine specimens, especially taxa typically associated with mucosal surfaces.

These findings may increase understanding of urologic disease in MSM and improve diagnostic methods for detection of genitourinary infections.

Introduction

Long-standing dogma has held that the human urinary tract is sterile (1, 2). However, characterization of urine specimens obtained from human bladders has revealed the existence of unique bacterial communities that make up the urinary microbiota, although the significance of these communities is not well understood (1–3). Studies of other human microbial communities such as the intestinal microbiota suggest that such communities often serve a critical role in protection against development of disease (4, 5).

An analysis of studies of the human urinary tract found that this microenvironment harbors more than 500 bacterial taxa (6). Many of these organisms have been previously associated with the gut, which is thought to be a frequent source of pathogens that cause urinary tract infection (UTI) (7, 8). Most studies of the urinary tract microbiota to date have focused on women, who have a higher incidence of UTI compared to men (7). It has been shown that the urinary microbiota influences risk for development of UTI, urinary incontinence, and chronic lower urinary tract symptoms in women (3, 9–11). However, there is a paucity of data on the urinary microbiota of males, particularly in men who have sex with men (MSM). These men represent a vastly understudied population who are at higher risk of genitourinary diseases such as UTI and sexually transmitted infections (STIs) (12). One recent study used 16S rRNA gene sequencing of first void urine samples to characterize the urethral microbiota of MSM with a

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focus on emerging causes of non-gonococcal urethritis (NGU) (13). This study found associations between the presence and absence of 16S rRNA of specific taxa in MSM with NGU in comparison to a non-NGU cohort. However, organismal viability from urine was not assessed in this study, and more studies are needed to broaden our understanding of the urinary microbiota of asymptomatic MSM in the context of other genitourinary diseases.

While uropathogenic strains of *Escherichia coli* are the cause of the majority (74.4%) of UTIs in the outpatient setting, over 20% of UTI are attributable to other uropathogens (7), and this number is likely higher in males (14). Standard approaches for the isolation of uropathogens from urine specimens include biochemical urinalysis and culture-based methods that are tuned for the optimal isolation of *E. coli* (11). However, standard culture methods are not permissive for isolation of fastidious and anaerobic microorganisms; this represents a major roadblock in recognition of emerging uropathogens (15). Enhanced urine culture methods that combine the use of additional media, inoculation of an increased volume of urine, and extended incubation times to facilitate recovery of fastidious organisms have been developed to expand the repertoire of urine culture (1, 11). In a recent study, standard urine culture recovered only 50% of the number of uropathogens recovered from urine of female patients with symptoms of UTI in comparison to enhanced urine culture (11).

Exposure to unique microenvironments such as gut and oral microbiota through anal and oral intercourse may lead to shifts in urinary microbiota of MSM that increase predilection for genitourinary disease. While 16S rRNA gene sequencing is frequently used to assess microbiota of many habitats (13, 16, 17), this technique does not provide information about organism viability and exhibits lower resolution for identification of some taxa at the species level (18). To overcome these issues, microbial culturomics is a method that uses enhanced culture-based techniques to isolate many organisms that were previously thought to be non-cultivable (19). Enhanced culture techniques on urine specimens can isolate microorganisms that are non-cultivable by standard urine culture methods and have been used to characterize the composition of the urinary microbiota of women (1–3, 20) and men (10, 21) with various disease pathologies. However, this method has not been applied to specifically interrogate the urinary microbiota of MSM. Thus, our objective was to use culturomics to describe the microbial composition of the urinary tract of MSM compared to a clinical cohort of men with urine specimens submitted for culture as part of routine clinical care.

Materials and Methods

MSM COHORT RECRUITMENT AND SAMPLE COLLECTION

Following approval from the Washington University in St. Louis institutional review board, participants were recruited by the Infectious Disease Clinical Trials Unit (IDCTU) at Washington University School of Medicine, which provided research coordinators to assist in recruitment, participant consent, and data and sample collection. The IDCTU is co-located with the infectious diseases clinic, which sees a large population of MSM as part of a pre-exposure prophylaxis program to prevent HIV transmission, for routine clinical care, and to participate in ongoing clinical studies. All subjects recruited for this cohort met the following inclusion criteria: men (sex assigned at birth) who were greater than or equal to 18 years of age and who self-identified as MSM. After giving verbal and written informed consent, all enrolled MSM subjects submitted a urine specimen with a volume of >15 mL and responded to a standardized questionnaire for collection of pertinent demographic data and metadata on recent antibiotics use, presence of urinary symptoms, recent history of STI diagnosis, HIV or other immunosuppression status, and sexual preference and practices (see online Supplemental Fig. 1). Participants were provided with a collection kit and detailed instructions for collection of a mid-stream urine. Briefly, participants were instructed to withdraw foreskin (if applicable), clean and rinse glans beginning at the urethra with a Castile soap towellette (PDI Healthcare), and urinate, passing the first portion into the toilet and the mid-portion into the collection container. All urine specimens were processed within 6 h of collection. Remuneration in the form of a gift card (\$10) was provided to each subject upon completion of the questionnaire and submission of the urine specimen.

CLINICAL COHORT RECRUITMENT AND SAMPLE PROCESSING

Following approval from the Washington University in St. Louis institutional review board, remnant urine specimens submitted to the Barnes-Jewish Hospital microbiology laboratory as part of routine clinical care were enrolled in the clinical cohort arm of the study. Samples were enrolled for the study if the following inclusion criteria were met: a midstream urine specimen was collected from a male patient (as noted by the electronic medical record) who was greater than or equal to 18 years of age. The specimen was required to be submitted in a C&S preservative vacutainer tube (BD) and had to be < 24 h from time of collection to be included. Specimens were excluded if they were unpreserved urine specimens, were > 24 h from time of collection, or were collected from female patients. Demographic and clinical data were obtained through review of the laboratory

information system and electronic medical record using a standardized case report form for all individual subjects included in the study (see online [Supplemental Fig. 2](#)).

URINE ANALYSIS, MICROSCOPY, AND GRAM STAIN

An aliquot of each urine specimen was analyzed via urine dipstick, microscopy, and Gram stain. For dipstick analysis, urine was gently mixed and the urine dipstick (Multistix 10 SG, Siemens) was lowered into the urine until all test pads were covered. After removal of excess, the test strip was placed on a paper towel and read visually by comparison of the colors on the test strip to the color blocks on the bottle label. For urine microscopy, 10 μ L of urine were transferred to a disposable hemocytometer (C-Chip, INCYTO). Urine was examined for red blood cells (RBCs), white blood cells (WBCs), epithelial cells, and bacteria at 400 \times magnification and quantified by the number of cells/high power field (hpf). For Gram stain, one drop of uncentrifuged urine was added to a clean glass slide and allowed to air dry. The slide was fixed with methanol and stained according to standard procedure.

STANDARD AND ENHANCED URINE CULTURE PROTOCOL

Urine was subjected to both standard and enhanced culture. For the standard culture method, urine was plated to media using a 1 μ L calibrated loop in a cross-streak pattern. For the enhanced urine culture method, 100 μ L of urine was inoculated with a pipet to media using a cross-streak pattern. See online [Supplemental Table 1](#) for additional details regarding media, incubation conditions, and incubation interval for standard and enhanced methods. All microorganism growth was quantified as <10 000, 10 000 to 100 000, or >100 000 colony forming units (CFU)/mL and identified by MALDI-TOF mass spectrometry (VITEK MS MALDI-TOF MS, bioMerieux) or 16S rRNA gene sequencing (performed by ID Genomics).

COMPUTATIONAL ANALYSIS

Presence-absence matrices were generated at the species and group level and hierarchically clustered using the *pheatmap* function (R *pheatmap* v1.0.12, <https://cran.r-project.org/web/packages/pheatmap/index.html>), while barplots and boxplots were visualized using the *ggplot* function [R *ggplot2* package (22)]. The Fisher exact test with false discovery rate (FDR) correction was used to compare frequency of recovery of microbial taxa between cohorts. Jaccard dissimilarity indexes and principal coordinate decomposition with Cailliez correction were calculated using the *vegdist* and *pcoa* functions [R *vegan* v2.5-5 and *ape* packages (23)] and visualized using *ggplot2*. Significant differences in Jaccard distance

between temporal and cross-sectional urine specimens of repeat MSM donors were determined by the Kruskal–Wallis test with FDR correction, while significant clustering following principal coordinate analysis (PCoA) was determined by permutational multivariate analysis of variance (PERMANOVA) using the *adonis* function (R *vegan* package v2.5-5, <https://cran.r-project.org/web/packages/vegan/vegan.pdf>). Alpha diversity was defined as the number of different taxa recovered in each urine sample. Beta diversity was defined as the difference in microbial composition between samples.

STATISTICAL TESTING

The Student *t*-test was used to compare differences in the number of organisms recovered/specimen between each sub-population and in age among cohorts. The Fisher exact test was used to compare remaining demographic data and patient-associated metadata. All tests were performed using GraphPad Prism V8 (GraphPad Software, San Diego, California USA, www.graphpad.com) or the *fisher.test* function (R *STATS* package, R Core Team (2013), R Foundation for Statistical Computing,), with *P* values and *q* values of <0.05 considered significant.

Results

CHARACTERISTICS OF THE MSM AND CLINICAL COHORTS

In the MSM cohort, 63 individual men were enrolled and a total of 99 urine specimens were collected and cultured using standard and enhanced methods ([Fig. 1](#)). From the clinical cohort, 30 urine specimens were

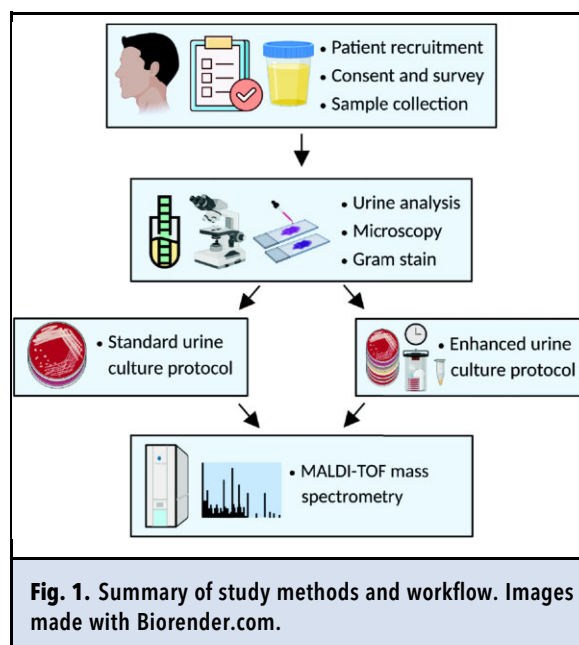


Table 1. Demographics and characteristics of study populations.

Demographics/genitourinary history	MSM cohort (n = 63)	Clinical cohort (n = 30)	P
Age: mean (SD), years	35.4 (11.26)	62.7 (15.95)	$P < 0.0001$
Race			
White (non-Hispanic)	46 (73%)	NA ^a	
Black	15 (24%)	NA	
Multi-racial	1 (2%)	NA	
Unknown	1 (2%)	NA	
HIV positive	12 (19.0%)	1 (3.3%)	$P = 0.0545$
Other immunosuppression	1 (1.6%)	5 (16.7%)	$P = 0.0126$
Recent diagnosis of STI (any)	14 (22.2%)	0 (0%)	$P = 0.0039$
Gonorrhea	8 (12.7%)	0 (0%)	
Chlamydia	7 (11.1%)	0 (0%)	
<i>Trichomonas</i>	1 (1.6%)	0 (0%)	
Syphilis	7 (11.1%)	0 (0%)	
Other (HPV ^b and HSV ^c)	2 (3.2%)	0 (0%)	
Recent history of urinary symptoms	1 (1.6%)	7 (23.3%)	$P = 0.0013$
Recent antibiotic use for any reason	12 (19.0%)	8 (26.7%)	$P = 0.4273$

^aNot available
^bHuman papillomavirus
^cHerpes simplex virus.

enrolled from 30 different men (Table 1). The mean age of the clinical cohort was significantly higher than that of the MSM cohort (62.7 vs 35.4 years, $P < 0.0001$). A higher proportion of the MSM cohort, as compared to the clinical cohort, were HIV positive (19.0 vs 3.3%, $P = 0.0545$) and had a recent diagnosis of at least one STI (22% vs 0%, $P = 0.0039$). Of the clinical cohort, 23.3% had experienced recent urinary symptoms compared to 1.6% of MSM ($P = 0.0013$), though there was no difference in reported recent antibiotic usage in either cohort (19% for MSM vs 27% for clinical, $P = 0.4273$).

MICROBIAL COMPOSITION OF URINE SAMPLES USING ENHANCED CULTURE METHODS

A comparison of the number of different species recovered per urine sample showed an average recovery of 0.6 and 0.9 organisms/specimen in the MSM and clinical cohort samples, respectively, using standard culture methods, with a maximum of 3 organisms recovered in a specimen (see online Supplemental Table 2). The average number of organisms recovered using enhanced culture methods was significantly higher in both the MSM (9.1 organisms/specimen; $P < 0.0001$) and clinical (7.8 organisms/specimen, $P < 0.0001$) cohorts

compared to standard culture, with a range of 0 to 21 different species recovered in a specimen.

A total of 60 organisms representing 18 unique species (9 Gram-positive cocci, 6 Gram-positive bacilli, 2 Gram-negative bacilli, and 1 anaerobe) were recovered from MSM cohort urine samples using standard culture. In comparison, 901 organisms representing 143 unique species (37 Gram-positive cocci, 39 Gram-positive bacilli, 17 Gram-negative bacilli, 5 Gram-negative coccobacilli, 1 yeast, and 44 anaerobes) were recovered from the same samples using enhanced culture. In the clinical cohort, 17 unique microbial species were recovered using standard culture, in comparison to 89 species recovered by enhanced culture. Species that were frequently recovered from urine specimens using only enhanced culture methods included *Peptoniphilus asaccharolyticus* (n = 33), *Cutibacterium acnes* (n = 31), *Streptococcus anginosus* (n = 31), *Gardnerella vaginalis* (n = 28), *Veillonella atypica* (n = 28), *Haemophilus parainfluenzae* (n = 26), and *Dermabacter hominis* (n = 26) (see online Supplemental Fig. 3). Notably, *Escherichia coli* was recovered at a similar frequency between standard and enhanced culture methods. Emerging uropathogens such as *Alloscardovia omnicoles* (n = 15), *Haemophilus influenzae* (n = 7), and *Actinotignum schaalii* (n = 8) were

more frequently recovered with enhanced culture in comparison to standard methods (only one instance of recovery of *A. omnicolens* with standard culture) (24–26).

Among the MSM cohort, several subjects provided a urine specimen multiple times over the course of the study. In total, 33 participants gave multiple urine samples (31 subjects gave 2, one gave 3, and one gave 4 samples) throughout the study (mean 126 days between sampling, range 7 to 245 days). The number of organisms recovered per specimen remained relatively stable over time within the same subject but was varied between subjects (Fig. 2, A). Overall, the composition of microbial species recovered within the same individual was more dynamic between urine specimens collected over 30 days apart but remained significantly similar within individuals over time relative to between-subject comparisons ($P < 0.05$) (Fig. 2, B and C). Specimens collected less than 30 days apart were more similar in composition in comparison to urine specimens collected more than 30 days apart (Fig. 2, C).

COMPARISON OF URINARY MICROBIOTA USING ENHANCED CULTURE OF MSM AND CLINICAL COHORTS

Analysis of urine specimens for indicators of UTI showed that urine specimens from MSM, in comparison to clinical specimens, were more often negative for blood (73% of MSM vs 37% of clinical), leukocyte esterase (94% vs 33%), nitrites (100% vs 63%), epithelial cells (92% vs 33%), and bacteria (82% vs 43%). MSM urine specimens less frequently had high numbers (>10 /hpf) of white (1% vs 47%) and red (1% vs 30%) blood cells, especially among samples collected from symptomatic males in the clinical cohort (see online Supplemental Table 3). Gram stain analysis of urine specimens showed that samples collected from the clinical cohort were more likely to have bacteria seen on Gram stain (see online Supplemental Table 4), and the presence of high numbers of bacteria in symptomatic males correlated with a positive culture result by standard urine culture methods.

Recovery of organisms from the first urine sample provided by unique subjects in the MSM cohort ($n = 63$) and all specimens from the clinical cohort ($n = 30$) were compared using enhanced culture. Organisms that were recovered at a significantly higher frequency from the MSM cohort included *Streptococcus mitis/oralis* ($P < 0.001$), *G. vaginalis* ($P < 0.01$), *H. parainfluenzae* ($P < 0.01$), *V. atypica* ($P < 0.01$), and *Streptococcus agalactiae* ($P < 0.05$) (Fig. 3, A). In contrast, organisms considered typical pathogens when recovered from human clinical specimens, such as *Enterococcus faecalis* ($P < 0.05$), *E. coli* ($P < 0.05$), and *Staphylococcus aureus* ($P < 0.05$), were recovered at

significantly higher rates in specimens in the clinical cohort. Genus and group level analysis showed similar trends (Fig. 3, B and C).

ORDINATION METHODS REVEAL DISTINCT CLUSTERING BETWEEN MICROBIAL CONTENT OF MSM URINE AND THE CLINICAL COHORT

To further interrogate the global relationships between the urinary microbial burden of the 2 cohorts, Jaccard distances were calculated from a presence/absence matrix of microbial groups for all urine specimens of the clinical cohort ($n = 30$) and the first specimen collected from each MSM subject ($n = 63$). PERMANOVA testing revealed significant separation of microbial content by cohort ($P < 0.0010$), further evidenced by distinct clustering through principal coordinate analysis (Fig. 4, A). The clustering of specimens was impacted by the absence of viridans group Streptococci, anaerobic Gram-positive cocci, anaerobic Gram-negative organisms, and coryneforms, and the presence of *Enterococcus* spp. and Enterobacterales in the clinical cohort (Fig. 4, B).

ANALYSIS OF IMPACT OF SEXUAL AND GENITOURINARY FACTORS ON URINARY MICROBIOTA OF MSM

For the MSM cohort, information about recent sexual and genitourinary history, including circumcision status, history of UTI, and oral and anal sex practices, was collected (Table 1 and online Supplemental Table 5). Alpha-diversity was increased in MSM with a recent history of gonorrhea/chlamydia diagnosis in comparison to MSM with no recent diagnosis of these STIs (median of 12 vs 8 organisms recovered/specimen, $P = 0.026$) (Fig. 5, A). Similarly, organism recovery was increased in MSM with recent antibiotic exposure compared to no exposure (15 vs 8 organisms recovered/specimen, $P = 0.007$) (Fig. 5, B). While there were an increased median number of species recovered from MSM who reported recent anal sex (9.5 organisms recovered/specimen), oral sex (9 species recovered/specimen), or who were circumcised (9 organisms recovered/specimen) compared to MSM who did not report these factors (median 8 organisms recovered/specimen for each of the three factors), this increase was not statistically significant (Fig. 5, C, D, and E). A comparison of the alpha diversity of specimens from MSM with no reported urinary symptoms to specimens from symptomatic men in the clinical cohort showed that there were significantly fewer organisms recovered from symptomatic men (4 vs 9 organisms recovered/specimen; $p = 0.009$) (Fig. 5, F). To independently assess associations between microbial composition of urine and each of these factors, the data were analyzed by Jaccard distance and principal coordinate analyses. There was no association between microbial composition of urine at the group level and any one

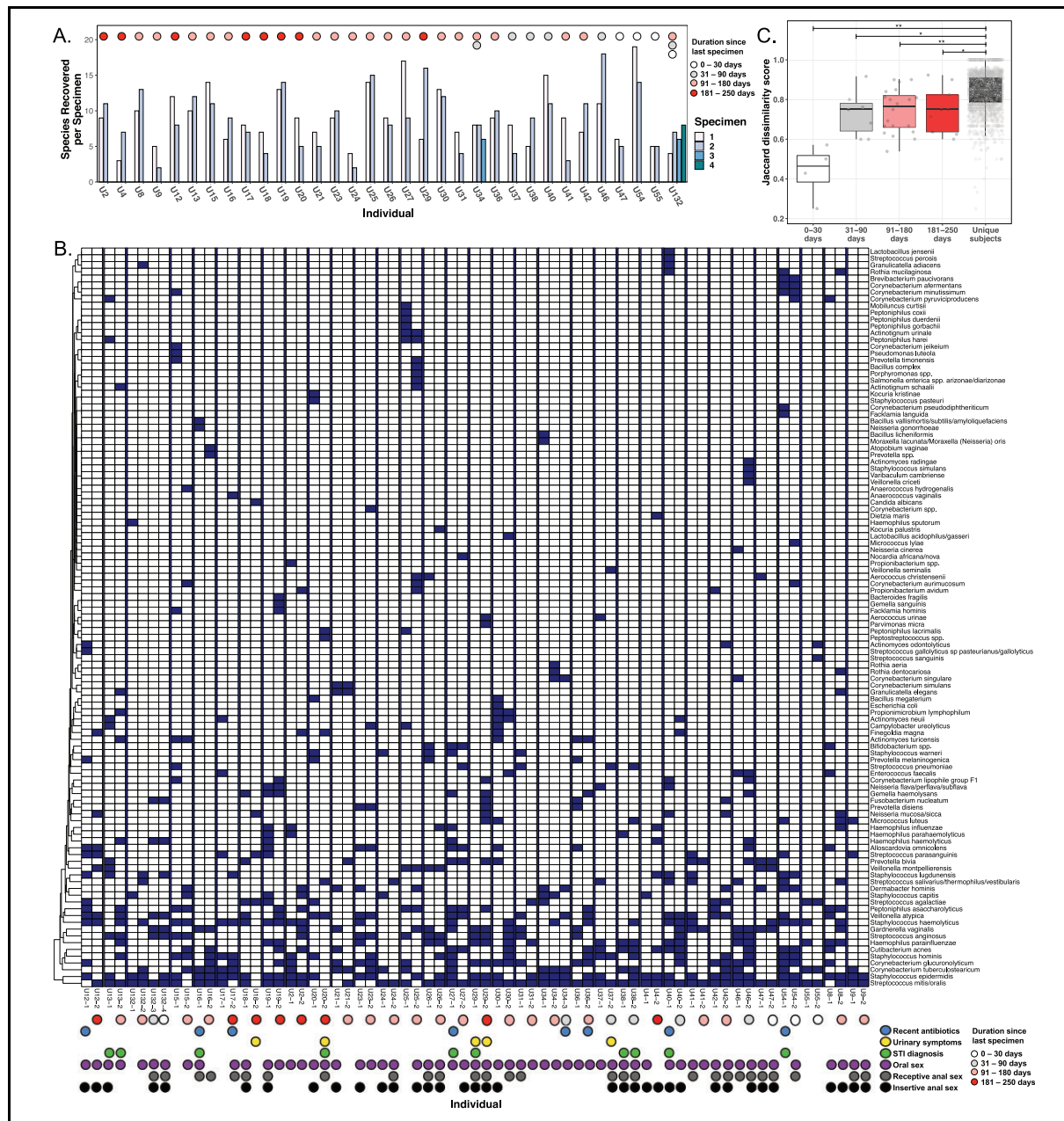
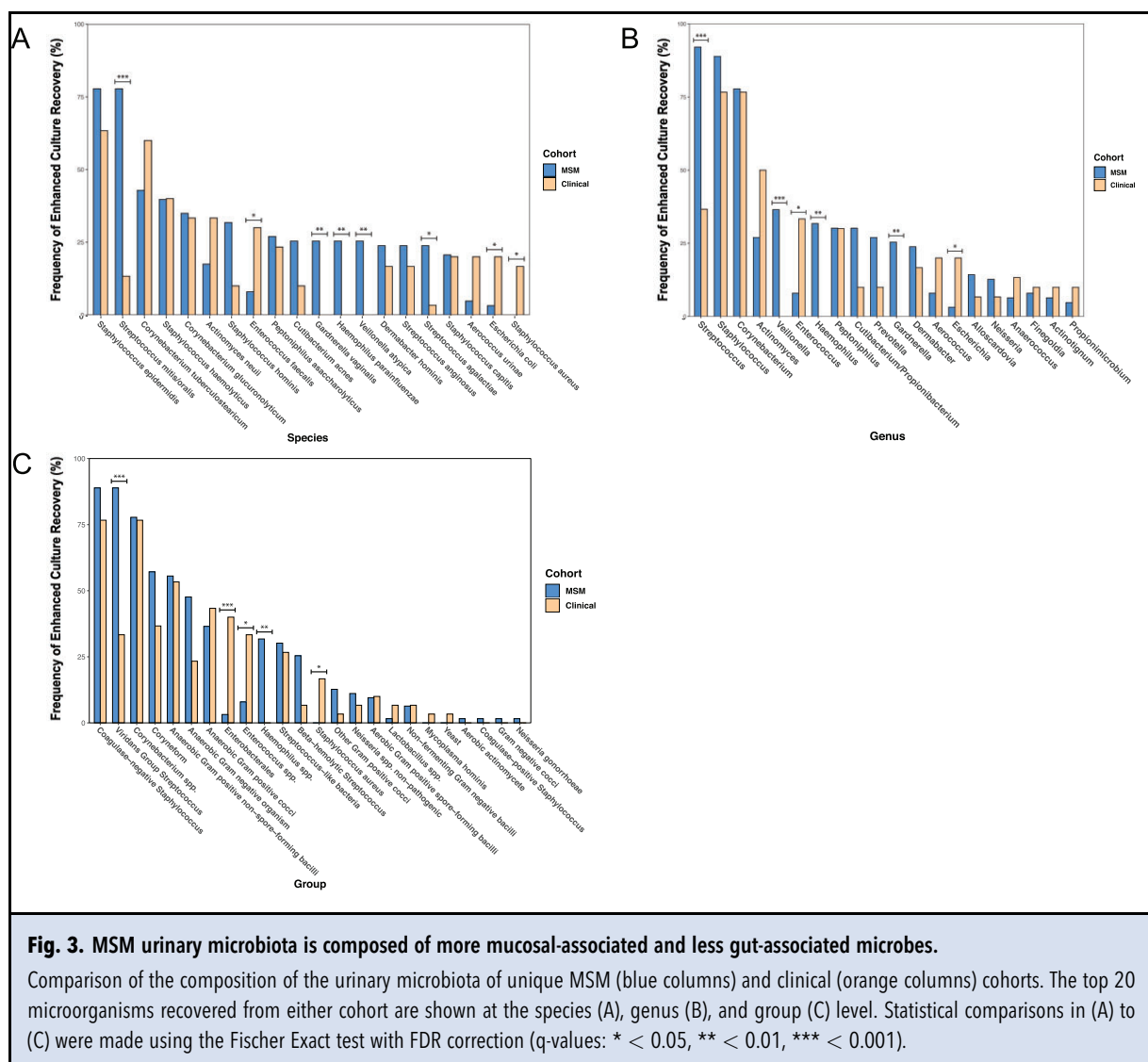


Fig. 2. The composition of microbial species recovered within the same individual is dynamic between urine specimens yet less divergent relative to inter-individual comparisons.

Recovery of organisms from enhanced culture of repeat urine specimens collected from MSM (n = 33). (A) The number of individual isolates recovered/specimen are shown for each subject in which >1 urine specimen was collected over the course of the study. The specimen collection number is denoted by bar color (n = 31 subjects with 2 specimens, 1 subject with 3 specimens, and 1 subject with 4 specimens) and the duration between specimen collections is denoted by circles. (B) The presence (blue squares) or absence (white squares) of unique microbial species recovered from MSM subjects shown in A. Colored circles denote reported recent antibiotic use, urinary symptoms, STI diagnosis, and oral and anal sex. (C) Jaccard dissimilarity scores of urinary microbiota composition were calculated for repeat urine specimens within individuals (temporal distances of 0 to 30 days, 31 to 90 days, 91 to 180 days, 180 to 250 days) and for urine specimens collected across unique subjects. Statistical comparisons in (C) were made using the Kruskal-Wallis test with FDR correction (q-values: * < 0.05, ** < 0.01).

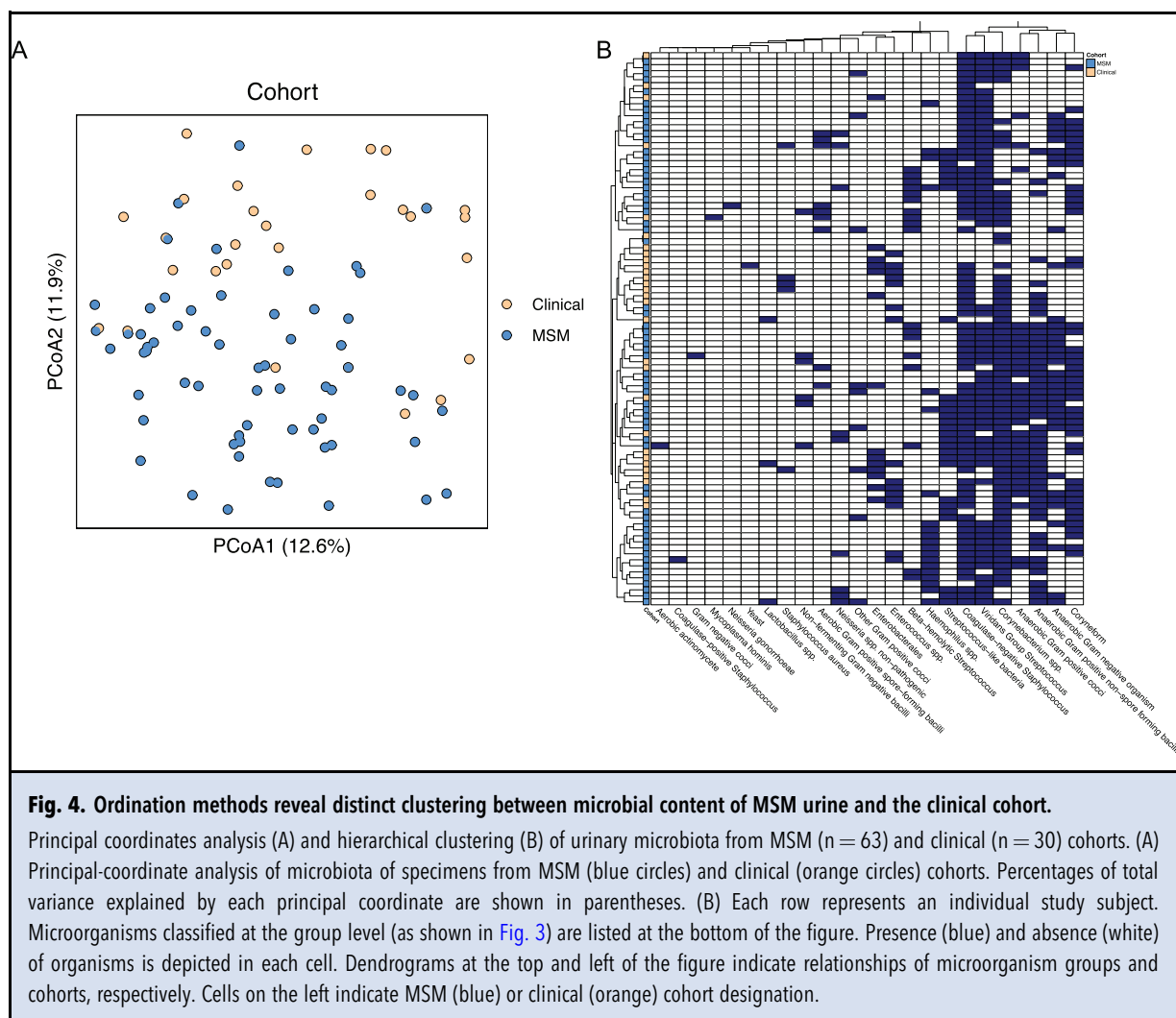


factor analyzed, including age, HIV status, recent oral or anal sex, urinary symptoms, or recent history of STI diagnosis (see online [Supplemental Fig. 4](#)).

Discussion

Our results show that enhanced urine culture is a robust method for identification of a large diversity of microbial species present in the urinary microbiota of MSM. The large number of fastidious and anaerobic organisms recovered from urine samples of MSM supports prior studies showing that the male urinary tract harbors a complex bacterial community that is distinct from other body microenvironments (6, 17, 20). Prior studies of

the urinary microbiota using enhanced culture techniques have shown that shifts in the urinary microbiota occur in many genitourinary diseases, such as UTI, overactive bladder, and STIs (1, 3, 10, 27). The majority of the subjects included in these studies were women, which is not surprising given their overall higher risk for UTI and other urogenital diseases (7, 15). This male undersampling effect may have contributed to a biased understanding of microbial contributions to urinary health and disease in men. To our knowledge, this is the first study to use a culturomics approach to characterize the urinary microbiota of MSM, an understudied population at higher risk of genitourinary diseases such as STIs due to risk factors



such as an increased number of sexual partners and exposures to additional microenvironments through oral and anal sex practices (12).

Standard urine cultures are tuned to detect a defined set of urinary pathogens causing UTI that are commonly associated with the human gut (8, 15). Our data support this, as the species with detection rates most similar between standard and enhanced culture is the common uropathogen, *E. coli*. MSM are potentially at higher risk of exposures to gut-associated organisms through insertive anal sex. Our study revealed an increased number of organisms recovered from MSM who reported recent anal sex. However, gut-associated microbes such as *Enterococcus* spp. and Enterobacterales were infrequently recovered in the MSM urinary microbiota in our study (8). This is in contrast to findings

from a previous study, which reported that the majority of organisms recovered using culturomics on urine specimens were gut-associated, although many of these organisms have also been detected among oral and vaginal microbiota (28). Variations in culture techniques and study populations, including analysis of samples from children, renal transplant patients, use of probiotics, and bladder cancer patients, may explain these differences. Our findings support that the urogenital microbiota overlapped to a larger degree with vaginal and other mucosal-associated organisms such as *S. mitis/oralis*, *Haemophilus* spp., and *G. vaginalis* in addition to skin-associated microorganisms such as coagulase-negative *Staphylococcus*, *Corynebacterium* spp., and *C. acnes* (29, 30). Similar to our findings, a study using enhanced culture methods in specimens collected from

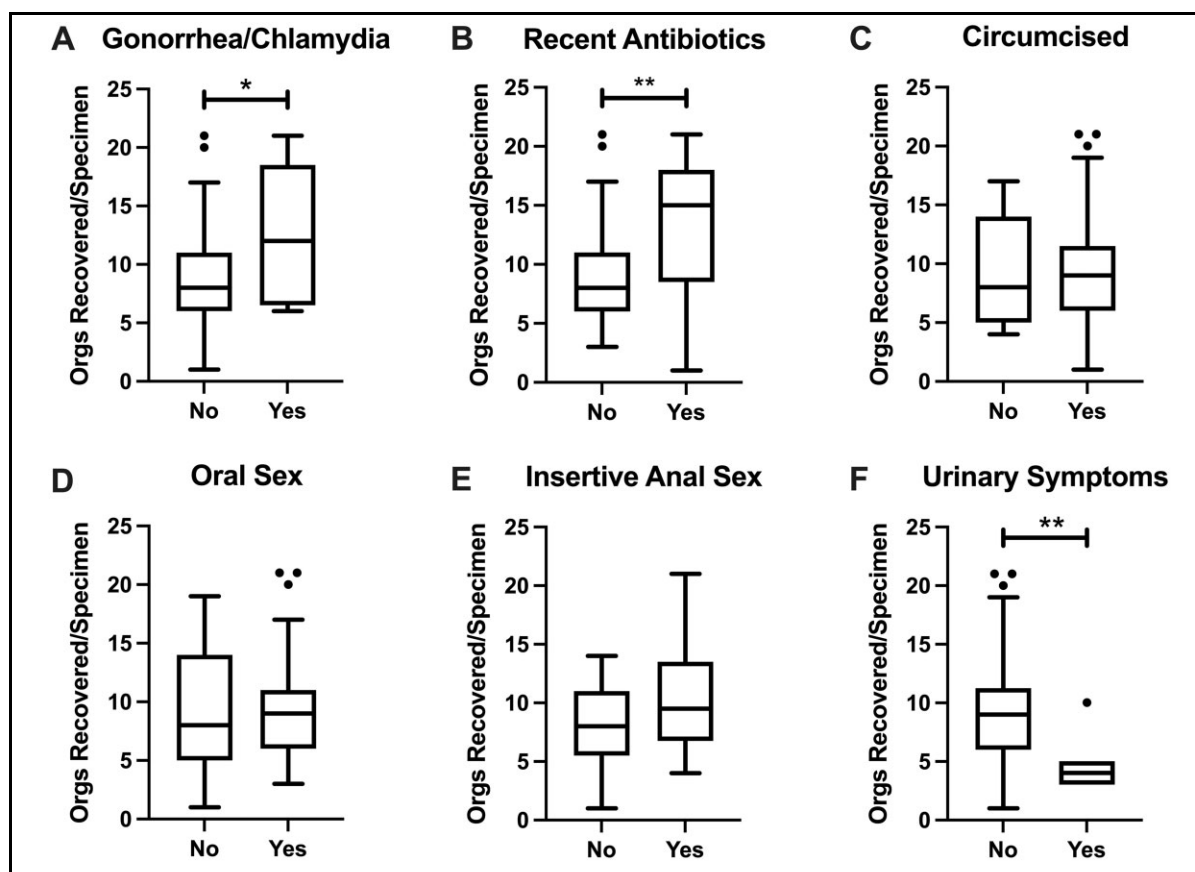


Fig. 5. Diversity of microbial species recovered from urine is not significantly affected by sexual practices among MSM.

Comparison of microbial alpha diversity recovered from MSM associated with (A) recent diagnosis of gonorrhoea or chlamydia ($n = 55$, no; $n = 8$, yes), (B) recent use of antibiotics ($n = 54$, no; $n = 9$, yes), (C) circumcision status ($n = 7$, no; $n = 49$, yes), (D) recent history of oral sex ($n = 11$, no; $n = 52$, yes) or (E) insertive anal sex ($n = 13$, no; $n = 14$, yes), and (F) presence or absence of urinary symptoms. For panel F, symptomatic males from the clinical cohort ($n = 7$) were used for comparison to asymptomatic males from the MSM cohort ($n = 62$). The number of species recovered per specimen for each condition are shown as the range (whiskers), interquartile range (boxes), and median (line within boxes). Statistical comparisons were made using the Student t -test (P values: * < 0.05 and ** < 0.01).

women showed that the female urinary microbiota is distinct from gut-associated microbiota and more closely resembles organisms recovered from vaginal samples (20). Commonly recovered genera from asymptomatic women overlapped with organisms recovered in the asymptomatic MSM cohort in our study, including *Gardnerella*, *Streptococcus*, *Staphylococcus*, and *Corynebacterium*, suggesting that these organisms are broadly represented in the urinary tract of healthy men and women. One major difference between male and female urinary tract microbiota consistently reported in previous comparisons is that *Lactobacillus* spp. are infrequently recovered in male urinary samples but are one of the most commonly recovered bacteria from the

female urinary tract (13, 17, 20). Our results support this finding, as *Lactobacillus* spp. were recovered in only two of 129 specimens in our study.

Direct comparisons of urinary microbiota from healthy men and women have shown that *Streptococcus* and *Gardnerella*, in addition to *Veillonella* and *Haemophilus* spp., are more commonly found in men in comparison to women (17). In support of this, these genera were among the top microorganisms recovered in the healthy MSM cohort in our study. Interestingly, *Veillonella*, *Gardnerella*, and *Haemophilus* were not recovered at all from urine samples of the clinical cohort, suggesting a link between the presence of these organisms and a non-diseased state in the male urinary tract.

This is supported by a recent study using 16S rRNA gene sequencing to characterize the urethral microbiota of MSM and men who have sex with women (MSW), which found an inverse association between the presence of *Veillonella* and NGU in MSM (13). In contrast, a positive correlation was shown for detection of *H. influenzae* and NGU in MSM and MSW (13). While this species was recovered from 7 MSM in our study, none reported current urinary symptoms or had abnormal urinalysis. As our study did not evaluate MSM specifically for NGU, further studies are needed to characterize this association.

Most microbiome studies of MSM to date have focused on the gut microenvironment. These studies have reported a *Prevotella*-enriched gut microbiome for MSM compared to non-MSM populations (31–33). The presence of *Prevotella* spp. in urine specimens has also been positively correlated with STI in men (34) and bacterial vaginosis in women (35). In our study, *Prevotella* spp. was one of the most frequently recovered genera from urine specimens in both the MSM (27%) and clinical (10%) cohorts. Interestingly, among the MSM cohort, *Prevotella* was never recovered from MSM who reported no recent anal sex but was recovered in 59% of MSM who reported recent insertive anal sex, suggesting a potential gut reservoir as the source of *Prevotella* in the urinary microbiota. Additional studies are needed in men to better characterize the role of *Prevotella* in predisposition to STI and other urogenital infections.

Similar to studies that describe a protective role for intestinal microbial communities against disease development (4, 5), members of the urinary tract microbiota likely influence the development of genitourinary disease such as UTI (36). While uropathogenic *E. coli* strains are a major cause of uncomplicated UTI, other uropathogens are emerging as major contributors to disease in women and men (7, 14). Several emerging uropathogens that contribute to pathogenesis and morbidity have recently been characterized, including *Aerococcus urinae*, *Corynebacterium urealyticum*, *A. schaalii*, and *H. influenzae* (2, 24–26). These organisms have fastidious growth requirements that preclude their recovery using standard urine culture methods. Notably, they were frequently recovered from asymptomatic males with normal urinalysis results in the MSM cohort in our study, although *A. urinae* was recovered at a higher rate in the clinical cohort compared to MSM. Ultimately, the role of these organisms as commensals versus pathogens of the urinary tract remains undefined, and a dysbiosis rather than presence or absence may be a major contributing factor in the pathogenesis of UTI (15). In symptomatic males in the clinical cohort of our study, we found a significant decrease in alpha diversity compared to healthy MSM, which is supported by a small longitudinal study that found decreased microbial

diversity in catheterized patients that developed UTI (37). Among MSM from whom more than one specimen was collected, we found that the diversity of the urinary microbiota was both relatively stable yet dynamic within the same subject over time. A prior study analyzing temporal effects on the urinary microbiota of female subjects postulated that shifts may be due to specific lifestyle factors such as vaginal intercourse or receipt of oral sex (38). Independent assessment of similar personal factors in the MSM cohort of our study found no association between urinary microbial diversity and any one factor, including age, HIV infection, recent STI, use of antibiotics, or oral and anal sex practices. While circumcision has been linked to a lower risk of STIs and shifts in the microbiota surrounding the coronal sulcus of males (16, 39, 40), our study did not find any major differences in recovery or diversity in urine samples of circumcised vs uncircumcised males.

Our study has several potential limitations. Midstream urine specimens were collected to approximate the urinary microbiota. Although detailed collection instructions were provided to study subjects, there is potential for urethral contamination during collection that may have affected our results. The lack of epithelial cells noted upon urinalysis in the vast majority of specimens collected from MSM supports that appropriate collection techniques were adhered to during collection. Although several subjects included in our study were living with HIV, the participants were on managed HIV drug regimens and viral loads were well controlled. Future studies on subjects with higher viral loads or lower CD4 counts are necessary to assess the effects of HIV on the urinary microbiota. Lastly, although we noted differences in the number of taxa associated with certain population factors, our study did not collect data on condom usage, number of sexual partners, or incidence of sexual intercourse with women and was underpowered to determine particular microbial signatures characteristic of certain metadata.

In conclusion, enhanced culture recovered a large diversity of microbial species from MSM urine specimens. The utility of enhanced urine culture as a clinical diagnostic in men with genitourinary symptoms is unclear. The techniques and media utilized for this method are familiar to clinical microbiology laboratories and could be incorporated into urine culturing procedures on a selective basis. Our study found that there was a lower diversity of organisms among symptomatic males but did not find any particular microbial signature associated with health over disease. The wide breadth of microbial species recovered by this method may lead to difficulties in interpretation by laboratorians and clinicians, without further knowledge of the role of the urinary microbiota in genitourinary health, and the increased sensitivity provided by this method may lead

to diagnostic confusion and overtreatment for UTI. It is clear that unique populations of microbes are present in MSM that may be cultured from urine specimens, but further studies are needed to determine if the urinary microbiota are stable over time and the positive or negative contributions these organisms make to genitourinary health.

Supplemental Material

Supplemental material is available at *Clinical Chemistry* online.

Nonstandard Abbreviations: MSM, men who have sex with men; MSW, men who have sex with women; STI, sexually transmitted infection; UTI, urinary tract infection; NGU, non-gonococcal urethritis

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