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Search for Microorganisms in Men with Urologic Chronic Pelvic Pain Syndrome: A Culture-Independent Analysis in the MAPP Research Network

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*MAPP Research Network is described in Appendix 1

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Introduction:

We used next-generation, state-of-the-art, culture-independent methodology to survey urine microbiota of UCPPS males and control participants enrolled in the MAPP Network to investigate a possible microbial etiology.

Methods:

Male UCPPS patients and matched controls were asked to provide VB1, VB2 and VB3 urine specimens. Specimens were analyzed with Ibis T-5000 Universal Biosensor technology to provide comprehensive identification of bacterial and select fungal species. Differences between UCPPS and control study participants for presence of species or species variation within a higher taxonomic grouping (genus) were evaluated using permutational multivariate analysis of variance and logistic regression.

Results:

VB1 and VB2 urine specimens were obtained from 110 (VB3 in 67) UCPPS participants and 115 (VB3 in 62) controls. A total of 78, 73 and 54 species (42, 39 and 27 genera) were detected in VB1, VB2 and VB3 respectively. Mean (SD) VB1, VB2 and VB3 species count per person was 1.62 (1.28), 1.38 (1.36) and 1.33(1.24) for cases and 1.75(1.32), 1.23(1.15) and 1.56 (0.97) for controls respectively. Overall species and genus composition differed significantly between UCPPS and control participants in VB1 ($p=0.002$ species level, $p=0.004$ genus level) with *Burkholderia cenocepacia* over represented in UCPPS cases. No significant differences were observed at any level in VB2 or VB3 samples.

Conclusions:

Assessment of baseline culture-independent microbiological data from male subjects enrolled in the MAPP Network has identified over representation of *B cenocepacia* in UCPPS. Future studies are planned to further evaluate microbiota associations with variable and changing UCPPS symptom patterns.

INTRODUCTION 4

Chronic prostatitis was traditionally considered an infectious disease of bacterial origin and for decades treated primarily with antibiotics[1]. In contrast, CP/CPPS and IC/BPS diagnosed in men, collectively referred to as male Urologic Chronic Pelvic Pain Syndrome (UCPPS), have been defined by the absence of identifiable bacterial infection as a cause for the chronic pain and urinary symptoms. The microbiologic diagnosis of infection in the prostate has traditionally been based upon the use of cultivation techniques in which bacteria are isolated from voided urine or EPS on specific nutritive media and under environmental conditions that only support growth for certain species. However, the vast majority of bacteria resist such cultivation and most chronic bacterial infections are nearly universally associated with a biofilm mode of growth[2,3] that is highly recalcitrant to antibiotic therapy and difficult to culture. Bacterial biofilms have been identified in culture-negative patients with a past history of chronic bacterial prostatitis who had become refractory to antibiotics[4.5]. Molecular techniques, that do not rely on bacterial growth *in vitro*, including molecular-phylogenetic approaches based on ribosomal RNA gene sequences (16S RNA PCR techniques)[6] have resulted in conflicting conclusions regarding the contributions of infectious agents in UCPPS[7-14].

The objective of this study was to utilize a novel, state-of-the-art, culture-independent method to characterize the microbiota of male UCPPS and control (i.e. no UCPPS related symptoms) study participants recruited within the MAPP Network EP Study[15,16] and to analyze microbiome differences relative to the extensive associated clinical data collected in that study. We employed the next-generation molecular diagnostic Ibis T-5000 Universal Biosensor technology[17] which

provides universal and comprehensive identification of all bacterial species present at >1-3% of the microbiome. The use of this advanced technology in combination with highly detailed clinical data from a large number of UCPPS patients and matched controls provides the MAPP Research Network an unparalleled opportunity to perform discovery analyses and test hypotheses relating to the contribution of the microbiome as an etiology for male UCPPS.

METHODS:

Participants and Specimens:

The Trans-MAPP EP Study recruited UCPPS participants for baseline phenotyping and longitudinal follow-up of the treated history of UCPPS symptoms, with standardized data acquisition and analysis and biological sample collection across network sites. In addition, the study enrolled positive controls (individuals with non-urologically associated chronic pain syndromes), and age/sex matched healthy controls for the same baseline phenotyping assessments[15,16].

Inclusion criteria for UCPPS study participants for the MAPP EP Study included: 1) a diagnosis of IC/BPS or CP/CPPS, with urologic symptoms present a majority of the time during any 3 of the past 6 months (CP/CPPS) or the most recent 3 months (IC/BPS); 2) at least 18 years old; 3) reporting a non-zero score for bladder/prostate and/or pelvic region pain, pressure or discomfort during the past 2 weeks; and 4) appropriate consent. Exclusion criteria have been described [15]. Healthy controls were recruited to be age- and sex-matched to UCPPS patients, and positive controls were defined as subjects meeting criteria for the non-urological associated syndromes,

primarily fibromyalgia, chronic fatigue syndrome, and/or irritable bowel syndrome. Further details of the study design, including descriptions of the study population enrollment criteria and disease specific questionnaires are available[15].

The current study details data collected from male participants who provided both initial stream urine (VB1) and midstream urine (VB2) specimens. A reduced number of case and control subjects were able to provide a post-prostate massage urine (VB3) specimen. Patients with a positive urine culture (traditional uropathogen isolated in VB2 employing traditional culture technique) **at baseline or within last 6 weeks** were excluded from analysis.

Specimen handling

After collection of urine specimens (**20 ml volumes collected after initial saline wipe of glans**) using standardized collection kits at MAPP Network Discovery sites, specimens were transferred to 50 mL conical tubes and frozen at -80 ^{o}C until shipping to central MAPP Network Tissue Analysis and Technology Core (TATC). Specimens were then thawed, thoroughly mixed, and aliquoted into 1 and 3 mL aliquots and refrozen at -80 ^{o}C until use. Three mL frozen aliquots were transferred to the Center for Genomic Sciences in Pittsburgh for microbial analyses.

Ibis T-5000 Universal Biosensor Analysis: **DNA extraction and Ibis eubacterial and fungal domain assays:**

In brief, total DNA was extracted from all urine samples, and microbial (i.e., bacterial and fungal) DNAs were amplified by the polymerase chain reaction (PCR) using the 16 primer pair BAC (Bacteria, antibiotic resistance genes and Candida) and Fungal detection systems developed

by Ibis as described[17]. The individual amplicons were "weighed" using the Ibis instrumentation by electrospray ionization (ESI) time-of-flight (TOF) mass spectrometer (MS) which reports out the molecular mass. The amplicon masses are then used to determine their base compositions as a particular mass can only be produced by a single combination of the four nucleotides (A, C, G, & T). The taxonomic identities of the amplicons were then revealed using a database containing base composition data on virtually all bacterial/fungal species sequenced to date. Details of the methodology are available in Appendix 2.

Statistical Analysis:

Demographic characteristics and relevant clinical features were compared between male UCPPS and control participants by Chi-Square tests. Differences in the overall microbial composition for UCPPS males versus control males were assessed by permutational multivariate analysis (PERMANOVA)[18]. This procedure is a nonparametric analogue of multivariate ANOVA that uses resampling for inference. The presence or absence of particular taxa for each subject is converted into a numerical matrix from which distance matrices are calculated and compared between groups according to a selected distance measure. The Euclidean distance was chosen as the basis of this analysis. Differences in the representation of individual taxa were tested using logistic regression for the presence or absence and PERMANOVA for differences in species richness within a higher level classification such as genus or Gram-Stain. All testing was conducted at the species, genus, and Gram-stain level. At the genus and Gram-stain levels, the primary test considered differences in species richness, but inference for differences in the presence or absence of a group within a level are also presented. Tests of individual taxa were

adjusted for multiple comparisons by controlling the false discovery rate (FDR)[19]. Models adjusted for potential confounding by the demographic variables of age, race, and employment.

RESULTS:

VB1 (urethral) and VB2 (bladder) urine specimens were obtained from 110 male UCPPS participants and 115 age and sex matched healthy and positive controls at their MAPP EP Study baseline assessment. VB3 samples (containing variable amount of EPS) were also collected from 67 UCPPS and 62 control participants. Baseline demographic data are shown in Table 1 (VB1/2) and Table 2 (VB3). There was no significant difference in race or ethnicity between UCPPS and control groups, however, minor differences were noted in age, employment and income between UCPPS participants and controls. In men with UCPPS who provided both VB1 and VB2 specimens, 86% reported a previous diagnosis of CP, whereas 20% reported a previous diagnosis of IC (Table 3); similar proportions of CP and IC diagnoses were also reported by UCPPS participants providing all 3 specimens (Table 4). All male UCPPS participants assessed met a clinically defined CP/CPPS diagnostic criteria (i.e., reported pain or discomfort in any of the 8 domains of the Male Genitourinary Pain Index (GUPI)[15] during any 3 months in the previous 6 months) while 69 - 71.6% met a clinically defined IC/BPS diagnostic criteria (i.e., unpleasant sensation of pain, pressure, or discomfort, perceived to be related to the bladder and/or pelvic region, associated with lower urinary tract symptoms in the most recent 3 months). The mean (SD) chronic prostatitis symptom score (CPSI) was 22.1 (8.3) and 2.5 (4.2) for VB1/2 UCPPS and control participants, respectively; 20.9 (7.6) and 1.8 (2.6) for VB3 UCPPS and control participants, respectively. The mean (SD) male GUPI scores were 24.2 (9.0) and 2.5 (4.5)

for VB1/2 UCPPS and control participants, respectively; 23.1 (8.4) and 1.8 (2.6) for VB3 UCPPS and control participants, respectively (Tables 3 and 4).

Analysis of urine samples revealed a total of 78 species (42 genera) in VB1 while VB2 and VB3 samples were shown to contain a total of 73 (39 genera) and 54 species (27 genera), respectively. Mean (SD) VB1 species count per person was 1.62 (1.28) and 1.75 (1.32) for UCPPS and control participants, respectively. Mean VB2 species count per person was 1.38 (1.36) and 1.23 (1.15) among UCPPS and control participants, respectively. Among VB3 samples mean (SD) species count per person was 1.33(1.24) and 1.56(0.97) for UCPPS and control participant, respectively

Overall genus and species composition significantly differed between UCPPS and control participants in VB1 samples (p=0.002 species level, p=0.004 genus level, p=0.027 Gram-stain level). Examining individual species, the overall difference was driven by *Burkholderia cenocepacia*, *Propionibacterium acnes*, and *Staphylococcus capitis/caprae*. Only *B. cenocepacia* was overrepresented in UCPPS (Adjusted OR=1.9, p=0.0159), (Table 5a). Prompted by the finding that comparison of species in VB1 appeared to show the most robust differences between groups, a **representative VB1 species cluster analysis was conducting based on Euclidean distances and hierarchical clustering with complete linkage (Figure 1).** Similar trends were observed at the genus level with significance retained after FDR adjustment. At the Gram-stain level, overall difference in composition was also detected in VB1, driven by differences in the prevalence of Gram-positive and Gram-negative species. No significant differences in overall composition or prevalence of individual species at the Gram-stain level were observed in VB2 or VB3 (Table 5b,c). Fifty-one species (26 genera) were present in

individual participants' VB3 but not in their respective VB1 or 2 samples. However, only one species and two genera were noted in more than 10 subjects (no fungal species or genera were noted in more than 10 subjects) and the difference between UCPPS and control participants were not significant (Table 5d), suggesting these microbes may not have a major contribution to UCPPS pathophysiology. Uropathogenic bacteria were identified in 8.7% of control vs 5.5% of UCPPS participants in VB1; 5.2% of controls vs 11.8% of UCPPS participants in VB2; 6.5% of controls vs 4.5% of UCPPS participants in VB3 (Table 6). However, only five uropathogens were localized to VB3 (i.e., VB3 but not VB1 and/or VB2) in subjects who provided all three specimens; three (4.8%) control group and two (3%) UCPPS participants (Table 6).

DISCUSSION:

Earlier generation molecular diagnostic techniques employed to search for the presence of causative organisms in patients with negative urine cultures and a diagnosis of CP/CPPS, have produced contradictory results[7-14]. The Ibis T-5000 Universal Biosensor technology[17] (see appendix 2 for details) employs a PCR-ESI-TOF MS coupled to a sophisticated dynamic relational database that is able to generate a definitive species-level diagnostic for all known bacterial species. In addition, the system provides a "most-closely-related match" for unknown organisms. This technology allows for a powerful discovery-based approach that is not subject to restrictions based on a priori assumptions of microbial profiles[20,21].

The Trans-MAPP EP Study enrolled male UCPPS study participants who met the basic criteria of a defined CP/CPPS diagnosis, although only 86% self-reported a diagnosis of chronic

prostatitis. Approximately 22% also self-reported a diagnosis of interstitial cystitis while 69% **11** met the pre-defined criteria for IC/BPS. In this cohort of male UCPPS patients, we were not able to show a clear clinically significant difference in the microbiome (either individual microorganisms or groups of microorganisms) between UCPPS participants and control participants without UCPPS symptoms. **We noted specific microbiome differences for *Burkholderia cenocepacia* (more prevalent in VB1 in UCPPS participants compared to controls) and others have described this organism as a pathogen[22], possibly involved in the etiology of CP/CPPS[23,24].** The minor differences observed in VB1 for *Propionibacterium acnes* and *Staphylococcus capitis/capare (*both under represented in UCPPS participants compared to controls) may be clinically insignificant, but they could also indicate a change in the overall species balance.

No differences at the species, genus or gram stain level were detected between UCPPS and control participants for VB2 or VB3 samples. When we further analyzed those organisms presumed to be localized to VB3 through deletion of those also detected in VB1 and/or VB2 (similar to culture localization using the 3 glass test technique), we did not note any difference between UCPPS and control participants. Furthermore, examination of UCPPS participants identified with known uropathogenic bacteria in any specimen (as well as in VB3 localization), failed to show any clinically significant difference. A similar observation was previously noted in a traditional culture-based case/control cohort study[25]. Studies within the MAPP Network are currently in progress to correlate the presence of uropathogenic bacteria with inflammatory biomarker patterns (eg IL-6).

A number of limitations of our study should be noted. The fact that we did not identify any significant alterations in the microbiome of patients with a chronic urologic pain condition can not address the possibility that chronic inflammation and pain my persist after an offending organism has been cleared [26,27]. We have also not ruled out the possibility that various organisms, particularly those localized to VB3, identified in UCPPS participants might influence various symptom changes (e.g., flare status) or are associated with select UCPPS patient subgroups (e.g., patients with differing symptom profiles, natural history, and/or underlying biological characteristics). Furthermore, this approach did not provide a comprehensive assessment of all fungal species or viruses. An additional limitation was that our specimens collected using standardized lower urinary tract collection methodology to enrich anatomical areas (urethra, bladder, prostate) would include mixed populations of microbes from all these areas as well as the kidney. The final caveat that remains with our approach is that we tested urine specimens which may or may not contain biofilm bacteria which in theory may only be detected if there is dispersion from the biofilm or mechanical disruption of the biofilm (in our study was only attempted by prostate massage). **Our data is not sufficient to recommend empiric antimicrobial therapy for similar patients with CPPS.** The strengths of our analyses lie in our use of the next generation Ibis T-5000 Universal Biosensor technology to accurately assess segmented urine microbiota in combination with comprehensive phenotyping of patients conducted in the MAPP EP Study. This allows for comparisons of microbial profiles to varied, complex clinical measures. The ambitious NIH funded Human Microbiome Project (HMP) recognizes the need to characterize microbial communities found at multiple human body sites and to look for correlations between changes in the microbiome in human health and disease[28]. Unfortunately there is a paucity of data for the urinary tract microbiome in health and disease

[http://www.hmpdacc.org/]. Further analyses are planned in the MAPP Network to identify potential sub-groupings of UCPPS patients that may show significant difference in their urologic microbiome when stratified based on differing phenotypic characteristics, as well studies to correlate microbial profiles (including more comprehensive fungal survey) with symptom progression and change over time.

CONCLUSION:

Assessment of baseline culture-independent microbiological data from male subjects enrolled in the MAPP Network study has identified *B. cenocepacia* as significantly increased in VB1 urine samples of UCPPS. Further work is needed to explore the microbial signature from mid stream urine and prostatic massage specimens in UCPPS men with variable and changing symptom patterns.

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Table 1: Baseline demographics for subjects who provided VB1 and VB2 urine specimens.

	Category	UCPPS	Controls	Total	p
Number of Participants	N (%)	110	115	225	
Age Group	<35 years	23 (20.9%)	39 (33.9%)	62 (27.6%)	0.038
	35-50 Years	37 (33.6%)	40 (34.8%)	77 (34.2%)	
	50+ Years	50 (45.5%)	36 (31.3%)	86 (38.2%)	
Race	White	102 (92.7%)	97 (84.3%)	199 (88.4%)	0.147
	Black	4 (3.6%)	5 (4.3%)	9 (4.0%)	
	Asian	3 (2.7%)	4 (3.5%)	7 (3.1%)	
	Multi Race		4 (3.5%)	4 (1.8%)	
	Other	1 (0.9%)	5 (4.3%)	6 (2.7%)	
Ethnicity	Hispanic	6 (5.5%)	6 (5.2%)	12 (5.3%)	1.000
	Non-Hispanic	103 (93.6%)	109 (94.8%)	212 (94.2%)	
	Unknown	1 (0.9%)		1 (0.4%)	
Employment	Employed	74 (67.3%)	73 (63.5%)	147 (65.3%)	0.007
	Unemployed	8 (7.3%)	24 (20.9%)	32 (14.2%)	
	Retired	21 (19.1%)	13 (11.3%)	34 (15.1%)	
	Full-time homemaker		2 (1.7%)	2 (0.9%)	
	Disabled	7 (6.4%)	3 (2.6%)	10 (4.4%)	
Income (Annual)	$10,000 or less	6 (5.5%)	10 (8.7%)	16 (7.1%)	0.002
	$10,001 to $25,000	6 (5.5%)	20 (17.4%)	26 (11.6%)	
	$25,001 to $50,000	17 (15.5%)	22 (19.1%)	39 (17.3%)	
	$50,001 to $100,000	34 (30.9%)	36 (31.3%)	70 (31.1%)	
	More than $100,000	39 (35.5%)	18 (15.7%)	57 (25.3%)	
	Prefer not to Answer	7 (6.4%)	9 (7.8%)	16 (7.1%)	
	Missing	1 (0.9%)		1 (0.4%)	

Table 2: Baseline data for subjects who were able to provide all three urine specimens (VB1, VB2, and VB3).

	Category	UCPPS	Controls	Total	p
Number of Participants	N (%)	67	62	129	
Age Group	<35 years	12 (17.9%)	17 (27.4%)	29 (22.5%)	0.452
	35-50 Years	20 (29.9%)	17 (27.4%)	37 (28.7%)	
	50+ Years	35 (52.2%)	28 (45.2%)	63 (48.8%)	
Race	White	65 (97.0%)	55 (88.7%)	120 (93.0%)	0.081
	Black		3 (4.8%)	3 (2.3%)	
	Asian	2 (3.0%)	1 (1.6%)	3 (2.3%)	
	Multi Race		2 (3.2%)	2 (1.6%)	
	Other		1 (1.6%)	1 (0.8%)	
Ethnicity	Hispanic		3 (4.8%)	3 (2.3%)	0.111
	Non-Hispanic	66 (98.5%)	59 (95.2%)	125 (96.9%)	
	Unknown	1 (1.5%)		1 (0.8%)	
Employment	Employed	43 (64.2%)	37 (59.7%)	80 (62.0%)	0.275
	Unemployed	4 (6.0%)	10 (16.1%)	14 (10.9%)	
	Retired	15 (22.4%)	11 (17.7%)	26 (20.2%)	
	Full-time homemaker		1 (1.6%)	1 (0.8%)	
	Disabled	5 (7.5%)	3 (4.8%)	8 (6.2%)	
Income	$10,000 or less	3 (4.5%)	7 (11.3%)	10 (7.8%)	0.002
	$10,001 to $25,000	3 (4.5%)	12 (19.4%)	15 (11.6%)	
	$25,001 to $50,000	11 (16.4%)	10 (16.1%)	21 (16.3%)	
	$50,001 to $100,000	19 (28.4%)	22 (35.5%)	41 (31.8%)	
	More than $100,000	27 (40.3%)	9 (14.5%)	36 (27.9%)	
	Prefer not to Answer	3 (4.5%)	2 (3.2%)	5 (3.9%)	
	Missing	1 (1.5%)		1 (0.8%)	

Table 3: Disease specific characteristics for subjects who provided VB1 and VB2 urine specimens

	Category	UCPPS	Controls	Total	p
Number of Participants	N (%)	110	115	225	
Self-reported IC diagnosis	No	88 (80.0%)	109 (94.8%)	197 (87.6%)	<.001
	Yes	22 (20.0%)		22 (9.8%)	
	Missing		6 (5.2%)	6 (2.7%)	
Self-reported CP diagnosis	No	24 (21.8%)	109 (94.8%)	133 (59.1%)	<.001
	Yes	86 (78.2%)		86 (38.2%)	
	Missing		6 (5.2%)	6 (2.7%)	
Meet IC/PBS Criteria (see text)	No	34 (30.9%)		34 (15.1%)	
	Yes	76 (69.1%)		76 (33.8%)	
	Missing		115 (100.0%)	115 (51.1%)	
Meets CP/CPPS Criteria (see text)	Yes	110 (100.0%)		110 (48.9%)	
	Missing		115 (100.0%)	115 (51.1%)	
Mean CPSI (SD)		22.1 (8.3)	2.5 (4.2)	12.0 (11.8)	<.001
Mean male GUPI (SD)		24.2 (9.0)	2.5 (4.5)	13.1 (13.0)	<.001
Meds for urologic or pelvic pain symptoms	No	36 (32.7%)	113 (98.3%)	149 (66.2%)	<.001
	Yes	74 (67.3%)	2 (1.7%)	76 (33.8%)	
Pain medication class	None	39 (35.5%)	89 (77.4%)	128 (56.9%)	<.001
	Peripheral	12 (10.9%)	15 (13.0%)	27 (12.0%)	
	Central	49 (44.5%)	11 (9.6%)	60 (26.7%)	
	Opioid	10 (9.1%)		10 (4.4%)	

Table 4: Disease specific characteristics for subjects who were able to provide all three urine specimens (VB1, VB2, and VB3). **1**

	Category	UCPPS	Controls	Total	p
Number of Participants	N (%)	67	62	129	
Self-reported IC diagnosis	No	54 (80.6%)	56 (90.3%)	110 (85.3%)	<.001
	Yes	13 (19.4%)		13 (10.1%)	
	Missing		6 (9.7%)	6 (4.7%)	
Self-reported CP diagnosis	No	18 (26.9%)	56 (90.3%)	74 (57.4%)	<.001
	Yes	49 (73.1%)		49 (38.0%)	
	Missing		6 (9.7%)	6 (4.7%)	
Meet IC/PBS Criteria (see text)	No	19 (28.4%)		19 (14.7%)	
	Yes	48 (71.6%)		48 (37.2%)	
	Missing		62 (100.0%)	62 (48.1%)	
Meets CP/CPPS Criteria (see text)	Yes	67 (100.0%)		67 (51.9%)	
	Missing		62 (100.0%)	62 (48.1%)	
Mean CPSI (SD) score		20.9 (7.6)	1.8 (2.6)	11.7 (11.2)	<.001
Mean male GUPI Total (SD) score		23.1 (8.4)	1.8 (2.6)	12.9 (12.4)	<.001
Meds for urologic or pelvic pain symptoms	No	17 (25.4%)	61 (98.4%)	78 (60.5%)	<.001
	Yes	50 (74.6%)	1 (1.6%)	51 (39.5%)	
Pain medication class	None	21 (31.3%)	45 (72.6%)	66 (51.2%)	<.001
	Peripheral	6 (9.0%)	10 (16.1%)	16 (12.4%)	
	Central	33 (49.3%)	7 (11.3%)	40 (31.0%)	
	Opioid	7 (10.4%)		7 (5.4%)	

Table 5A. Differences in Species Composition in VB1 (only taxa present in at least 10 subjects **1** were tested individually)

VB1	Controls N (%)	UCPPS N (%)	p unadjusted (richness)	p unadjusted (presence/ absence)	p adjusted (richness)	p adjusted (presence/ absence)
Number of Participants	115	110				
Species[+]				0.004		0.002
Bifidobacterium subtile	9 (7.8%)	6 (5.5%)		0.4782		0.6718
Burkholderia cenocepacia[+]	5 (4.3%)	16 (14.5%)		0.0129		0.0159
Finegoldia magna	11 (9.6%)	9 (8.2%)		0.7157		0.4784
Listeria innocua/monocytogenes	5 (4.3%)	5 (4.5%)		0.9427		0.9473
Propionibacterium acnes[+]	19 (16.5%)	7 (6.4%)		0.0213		0.0097
Staphylococcus capitis/caprae[+]	12 (10.4%)	2 (1.8%)		0.0178		0.0107
Staphylococcus epidermidis/haemolyticus	38 (33%)	28 (25.5%)		0.2124		0.0781
Staphylococcus hominis	23 (20%)	14 (12.7%)		0.1441		0.1074
Streptococcus agalactiae	9 (7.8%)	5 (4.5%)		0.3141		0.2483
Streptococcus parasanguinis/pneumoniae	7 (6.1%)	13 (11.8%)		0.1376		0.1199
Genus[+]			0.013	0.02	0.004	0.006
Bifidobacterium	9 (7.8%)	7 (6.4%)	0.628	0.6701	0.74	0.8496
Burkholderia[+*]	5 (4.3%)	16 (14.5%)	0.015	0.0129	0.007	0.0159
Finegoldia	11 (9.6%)	9 (8.2%)	0.814	0.7157	0.476	0.4784
Lactobacillus	6 (5.2%)	11 (10%)	0.171	0.1819	0.116	0.1028
Listeria	5 (4.3%)	6 (5.5%)	0.787	0.7009	0.82	0.816
Propionibacterium[+*]	19 (16.5%)	7 (6.4%)	0.024	0.0213	0.006	0.0097
Staphylococcus[+*]	60 (52.2%)	44 (40%)	0.02	0.0678	0.005	0.0164
Streptococcus	21 (18.3%)	24 (21.8%)	0.904	0.5053	0.943	0.5008
Gram Stain[+]			0.087	0.12	0.027	0.083
Gram negative[+*]	22 (19.1%)	34 (30.9%)	0.066	0.0426	0.041	0.0267
Gram positive[+*]	85 (73.9%)	78 (70.9%)	0.095	0.6143	0.039	0.5671

[+] Significant at the 0.05 level in adjusted analysis

* Significant under FDR of 0.05 for multiple comparisons in adjusted analysis

Table 5B. Differences in Species Composition in VB2 (only taxa present in at least 10 subjects **2** were tested individually)

VB2	Controls N (%)	UCPPS N (%)	p unadjusted (richness)	p unadjusted (presence/ absence)	p adjusted (richness)	p adjusted (presence/ absence)
Overall	115	110				
Species				0.354		0.477
Enterococcus faecalis	5 (4.3%)	5 (4.5%)		0.9427		0.9838
Propionibacterium acnes	14 (12.2%)	6 (5.5%)		0.0842		0.0533
Staphylococcus capitis/caprae	4 (3.5%)	6 (5.5%)		0.4757		0.7136
Staphylococcus epidermidis/haemolyticus	28 (24.3%)	30 (27.3%)		0.6162		0.927
Staphylococcus hominis	12 (10.4%)	11 (10%)		0.9143		0.9269
Streptococcus parasanguinis/pneumoniae	4 (3.5%)	6 (5.5%)		0.4757		0.8238
Genus			0.238	0.227	0.380	0.370
Bifidobacterium	7 (6.1%)	3 (2.7%)	0.282	0.2335	0.472	0.4757
Enterococcus	5 (4.3%)	5 (4.5%)	1	0.9427	0.988	0.9838
Lactobacillus	9 (7.8%)	10 (9.1%)	0.451	0.7333	0.249	0.4739
Listeria	3 (2.6%)	7 (6.4%)	0.207	0.1856	0.263	0.2562
Propionibacterium[+]	14 (12.2%)	6 (5.5%)	0.108	0.0842	0.046	0.0533
Staphylococcus	47 (40.9%)	52 (47.3%)	0.307	0.3338	0.539	0.7397
Streptococcus	11 (9.6%)	8 (7.3%)	1	0.5376	0.62	0.2818
Gram Stain			0.426	0.762	0.724	0.935
Gram negative	19 (16.5%)	21 (19.1%)	0.644	0.6146	0.554	0.6911
Gram positive	67 (58.3%)	68 (61.8%)	0.405	0.5862	0.673	0.9603

[+] Significant at the 0.05 level in adjusted analysis
* Significant under FDR of 0.05 for multiple comparisons in adjusted analysis

Table 5C. Differences in Species Composition in VB3 ((only taxa present in at least 10 subjects were tested individually) **3**

VB3	Controls N (%)	UCPPS N (%)	p unadjusted (richness)	p unadjusted (presence/ absence)	p adjusted (richness)	p adjusted (presence/ absence)
Overall	62	67				
Species				0.82		0.804
Listeria innocua/monocytogenes	6 (9.7%)	4 (6%)		0.4356		0.698
Propionibacterium acnes	5 (8.1%)	5 (7.5%)		0.8984		0.6228
Staphylococcus epidermidis/haemolyticus	20 (32.3%)	18 (26.9%)		0.5025		0.4159
Staphylococcus hominis	6 (9.7%)	8 (11.9%)		0.6802		0.5491
Genus			0.433	0.613	0.332	0.592
Lactobacillus	7 (11.3%)	7 (10.4%)	0.843	0.8779	0.878	0.8185
Listeria	6 (9.7%)	4 (6%)	0.52	0.4356	0.731	0.698
Propionibacterium	5 (8.1%)	5 (7.5%)	1	0.8984	0.628	0.6228
Staphylococcus	36 (58.1%)	32 (47.8%)	0.196	0.2424	0.11	0.194
Streptococcus	8 (12.9%)	8 (11.9%)	0.822	0.8684	0.894	0.7573
Gram Stain			0.523	0.099	0.448	0.078
Gram negative	13 (21%)	7 (10.4%)	0.191	0.105	0.269	0.1513
Gram positive	49 (79%)	45 (67.2%)	0.603	0.1324	0.446	0.0881

[+] Significant at the 0.05 level in adjusted analysis
* Significant under FDR of 0.05 for multiple comparisons in adjusted analysis

Table 5D. Differences in Species Composition in VB3 not VB1 or VB2 (only taxa present in at **4** least 10 subjects were tested individually)

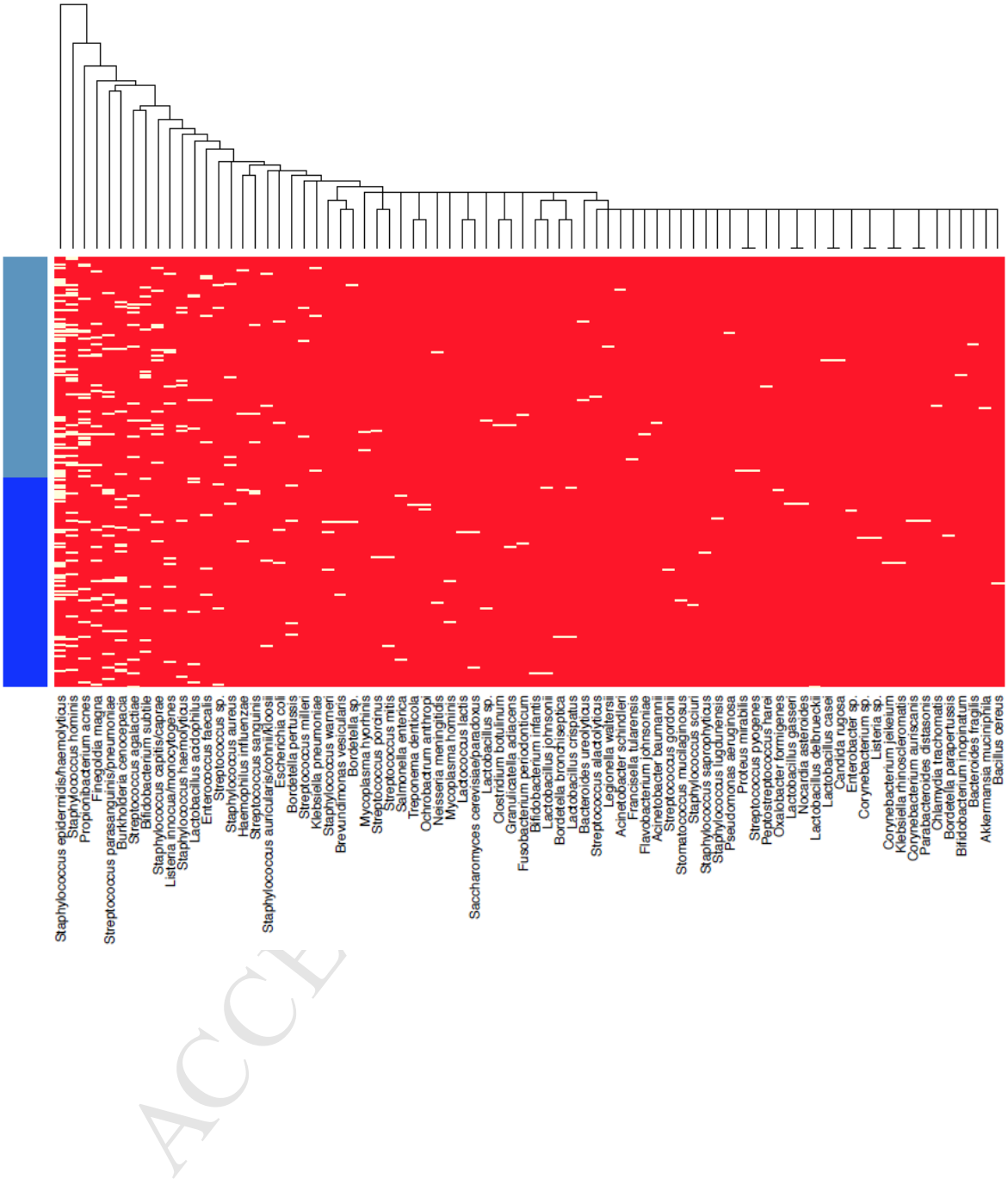
VB3 not VB1 or VB2	Controls N (%)	UCPPS N (%)	p unadjusted (richness)	p unadjusted (presence/ absence)	p adjusted (richness)	p adjusted (presence/ absence)
Overall	62	67				
Species				0.376		0.510
Staphylococcus epidermidis/haemolyticus	7 (11.3%)	6 (9%)		0.6604		0.7298
Genus			0.223	0.24	0.224	0.245
Staphylococcus	23 (37.1%)	18 (26.9%)	0.152	0.2139	0.154	0.1878
Streptococcus	7 (11.3%)	3 (4.5%)	0.381	0.1619	0.274	0.1303
Gram Stain			0.28	0.187	0.325	0.292
Gram negative	12 (19.4%)	5 (7.5%)	0.128	0.0537	0.18	0.0883
Gram positive	35 (56.5%)	33 (49.3%)	0.463	0.4137	0.369	0.4241

[+] Significant at the 0.05 level in adjusted analysis
* Significant under FDR of 0.05 for multiple comparisons in adjusted analysis

Table 6. Differences in Uropathogen Presence by cohort in VB1-VB3 **1**

N (%)		**Entero-bacter**	**Entero-coccus**	**Esche-richia**	**Kleb-siella**	**Proteus**	**Pseudo monas**	**Any Uro-pathogen**
VB1	Controls (n=115)	0 (0%)	5 (4.3%)	2 (1.7%)	3 (2.6%)	1 (0.9%)	1 (0.9%)	10 (8.7%)
	UCPPS (n=110)	1 (0.9%)	3 (2.7%)	2 (1.8%)	1 (0.9%)	0 (0%)	0 (0%)	6 (5.5%)
	p unadjusted	0.9968	0.5154	0.9642	0.3567	0.9969	0.9969	0.3484
	p adjusted	0.9984	0.7121	0.9207	0.3238	0.9978	0.9985	0.5475
VB2	Controls (n=115)	0 (0%)	5 (4.3%)	0 (0%)	1 (0.9%)	0 (0%)	0 (0%)	6 (5.2%)
	UCPPS (n=110)	0 (0%)	5 (4.5%)	2 (1.8%)	0 (0%)	3 (2.7%)	3 (2.7%)	13 (11.8%)
	p unadjusted	1	0.9427	0.9949	0.9969	0.9947	0.9947	0.0828
	p adjusted	1	0.9838	0.9976	0.9993	0.9965	0.9964	0.1045
VB3	Controls (n=62)	0 (0%)	0 (0%)	1 (1.6%)	2 (3.2%)	1 (1.6%)	0 (0%)	4 (6.5%)
	UCPPS (n=67)	0 (0%)	1 (1.5%)	1 (1.5%)	0 (0%)	0 (0%)	1 (1.5%)	3 (4.5%)
	p unadjusted	1	0.9963	0.9559	0.9959	0.9961	0.9963	0.6228
	p adjusted	1	0.9974	0.8787	0.9981	0.9981	0.9982	0.7106
VB3 not 1 or 2	Controls (n=62)	0 (0%)	0 (0%)	0 (0%)	2 (3.2%)	1 (1.6%)	0 (0%)	3 (4.8%)
	UCPPS (n=67)	0 (0%)	1 (1.5%)	0 (0%)	0 (0%)	0 (0%)	1 (1.5%)	2 (3%)
	p unadjusted	1	0.9963	1	0.9959	0.9961	0.9963	0.5893
	p adjusted	1	0.9974	1	0.9981	0.9981	0.9982	0.761

Figure 1. VB1 Species Cluster Analysis by Cohort. light blue – Controls; dark; blue - UCPPS



Abbreviations: **1**

CP	Chronic Prostatitis
CP/CPPS	Chronic Prostatitis/Chronic Pelvic Pain Syndrome
CPSI	Chronic Prostatitis Symptom Index
EP	Epidemiological/Phenotyping
EPS	Expressed Prostatic Secretion
FDR	false discovery rate
GUPI	Genitourinary Pain Index
IC	Interstitial Cystitis
IC/BPS	Interstitial Cystitis/Bladder Pain Syndrome
MAPP	Multidisciplinary Approach to the study of Pelvic Pain
MAPP-EP	Multidisciplinary Approach to the study of Pelvic Pain Epidemiological/Phenotyping study
MS	mass spectroscopic
PCR	polymerase chain reaction
PCR-ESI-TOF MS	polymerase chain reaction –electron spray ionization – time-of-flight – mass spectroscopic
TATC	Tissue Analysis and Technology Core
UCPPS	Urologic Chronic Pelvic Pain Syndrome
VB1	Voided Bladder 1 or initial stream urine (urethral) specimen
VB2	Voided Bladder 2 or midstream urine (bladder) specimen
VB3	Voided Bladder 3 or post prostatic massage urine (prostate) specimen

Appendix 1 MAPP Research Network 1

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Details of Methodology in regard to Ibis T-5000 Universal Biosensor Analysis

Summary:
Total DNA was extracted from all urine samples, and microbial (i.e., bacterial and fungal) DNAs were amplified by the polymerase chain reaction (PCR) using the 16 primer pair BAC (Bacteria, antibiotic resistance genes and Candida) and Fungal detection systems developed by Ibis as described[17]. The individual amplicons were "weighed" using the Ibis instrumentation by electrospray ionization (ESI) time-of-flight (TOF) mass spectrometer (MS) which reports out the molecular mass. The amplicon masses are then used to determine their base compositions as a particular mass can only be produced by a single combination of the four nucleotides (A, C, G, & T). The taxonomic identities of the amplicons were then revealed using a database[217] containing base composition data on virtually all bacterial/fungal species sequenced to date. Details of the methodology is available in Appendix.

Details:
For all samples, 3 ml of urine were centrifuged at 10,000 rpm x 3 min, then all but 100 μL of supernatant was removed and remaining pellet added to 270 μL ATL lysis buffer and proteinase K. Nucleic acid from the lysed sample was extracted using the Qiagen DNeasy Tissue kit (Qiagen cat# 69506). **The amount of DNA recovered varied from specimen to specimen, but the range was 50-400 ng.** 10 μL (total elution volume of 200 μL) of each sample was then loaded per well onto the Ibis BAC/Fungal detection PCR plates (Abbott Molecular; Abbott Park, IL). Each detection plate is comprised of 96 wells containing 16 primer pairs per assay that survey all bacterial (as well as Candida and related yeast species) organisms through a combination of: (1) omnipresent loci (e.g. 16S/18S rDNA sequences); (2) phylum/class/order specific loci; and (3) target loci for specific pathogens of interest (e.g. the *Staphylococcus*-specific *tuf*B gene). PCR amplification was carried out as recommended by the manufacturer followed by desalting of the PCR products, which were then sequentially electrosprayed into the TOF MS. The spectral signals were then processed to determine the masses of each of the PCR products present with sufficient accuracy that the base composition of each amplicon could be unambiguously deduced. Using combined base compositions from multiple PCRs, the identities of the pathogens and a semi-quantitative determination of their relative concentrations in the starting samples was established using a proprietary algorithm to interface with the Ibis database of known organisms[26]. The BAC system also detects the presence of several key antibiotic resistance markers, including van A and van B (vancomycin resistance) in *Enterococcus* species, KPC (carbapenem resistance) in Gram-negative bacteria, and mec A (methicillin resistance) in *Staphylococcus* species. An internal calibrant consisting of a known amount of a synthetic nucleic acid template is also included for each primer pair in each assay, controlling for false negatives (e.g. from PCR inhibitors) and enabling the semi-quantitative analysis of the amount of template DNA present.

Identification of Microbial Species:
This PCR-ESI-TOF MS approach gives the exact base composition for each amplimer generated. Since multiple (8-16) amplimers are generated for each assay it is possible using a comprehensive database to provide a list of possible species matches for each amplimer. A

triangulation approach is used in which all of the amplimers are assessed in combination to generate a definitive species-level diagnostic for all known bacterial species. In addition, the system provides a “most-closely-related match” for unknown organisms. This technology allowed for a powerful discovery-based approach that was not subject to restrictions based on a priori assumptions of microbial profiles.