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Assessment of the Lower Urinary Tract Microbiota during Symptom Flare in Women with Urologic Chronic Pelvic Pain Syndrome: A MAPP Network Study

J. Curtis Nickel[1], Alisa Stephens[2], J. Richard Landis[2], Chris Mullins[3], Adrie van Bokhoven[4], M. Scott Lucia[4], Garth D. Ehrlich[5], and The MAPP Research Network[6]*

[1]Department of Urology, Queen's University, Kingston, Ontario, Canada

[2]Department of Biostatistics and Epidemiology, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA

[3]National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD

[4]Department of Pathology, University of Colorado Anschutz Medical Campus, Aurora, CO

[5]Departments of Microbiology & Immunology and Otolaryngology-Head and Neck Surgery, Drexel University College of Medicine, Philadelphia, PA

[6]See Appendix 1

Corresponding Author: J. Curtis Nickel MD
Department of Urology, Queen's University
Kingston, Ontario Canada K7L 2V7
Phone: 613-548-2497
Email: jcn@queensu.ca
*MAPP Research Network is described in Appendix 1

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ABSTRACT

Purpose:

We compared culture-independent assessment of microbiota of the lower urinary tract in standard culture negative female UCPPS patients who reported symptom flare to those who did not report a flare.

Materials and Methods:

Initial stream (VB1) and midstream (VB2) urine specimens (n=233 UCPPS patients) were analyzed with Ibis T-5000 Universal Biosensor system technology for comprehensive identification of microorganism species. Differences between flare and non-flare groups for presence or number of different species within a higher level group (richness) were examined by permutational multivariate analysis of variance and logistic regression.

Results:

Overall, 81 species (35 genera) were detected in VB1 and 73 (33) in VB2. Mean (SD) VB1 and VB2 species count per person was 2.6 (1.5) and 2.4 (1.5) for 86 flare patients and 2.8 (1.3) and 2.5 (1.5) for 127 non-flare patients, respectively. Overall, species composition did not significantly differ between flare and non-flare patients at any level (p=0.14 species, p=0.95, genus in VB1 and VB2, respectively) in multivariate analysis for richness. Univariate analysis, both unadjusted and adjusted, confirmed significantly greater prevalence of fungi (Candida and Saccharomyces) in flare group (15.7%) compared to non-flare group in VB2 (3.9%) (p=0.01). When adjusted for antibiotic use and menstrual phase, women who reported a flare remained more likely to have fungi present in VB2 specimens [OR= 8.3, CI=1.7-39.4)].

Conclusion:

Among women with UCPPS the prevalence of fungi (Candida and Saccharomyces sp.) was significantly greater in those who reported a flare compared to those who did not.

INTRODUCTION

Interstitial cystitis/bladder pain syndrome (IC/BPS) is characterized by waxing and waning symptoms of bladder pain and storage urinary symptoms[1]. Symptom exacerbations among patients with IC/BPS, often called "flares", well recognized by both physicians and patients, have not been well characterized. Many patients, as well as physicians, attribute flares to an infection and antibiotics are often prescribed[1,2]. Following the culture status of women with IC/BPS over almost 2 years failed to provide evidence to support the bacterial etiology flare hypothesis[3]. However, standard culture techniques have many limitations, including the fact that 99% of known bacteria cannot be cultivated using standard culture media techniques[4]. Enhanced culture techniques are reportedly better (larger volumes, multiple media, multiple atmospheric conditions, longer incubation times) in capturing more of bacterial and fungal species[5,6], while new culture-independent methods (such as the Ibis T-500 Universal Biosensor technology[7]) allows for detection of up to 1-3% of the total microbiome without a need for an apriori hypothesis of which species are present..

The objective of this study was to utilize a novel, state-of-the-art, culture-independent method to compare the microbiota of the lower urinary tract in standard culture negative (for bacteria) female urologic chronic pelvic pain syndrome (UCPPS) (i.e., IC/BPS) enrolled in the MAPP-EP Study[8,9] who reported a current flare at study entry compared to those women who did not..

METHODS:

The Trans-MAPP EP Study.

The MAPP EP Study recruited UCPPS participants for baseline phenotyping and 12 month longitudinal follow-up of the treated history of UCPPS symptoms, with standardized data acquisition and analysis and

biological sample collection across network sites[8]. Inclusion and exclusion criteria have been described[9]. This study evaluated the female participants with a diagnosis of IC/BPS, with urologic symptoms present a majority of the time during any 3 of the past 6 months. Further details of the study design, including descriptions of the study population enrollment criteria and disease specific questionnaires are available[8,9,10] or can be accessed online at http://www.biomedcentral.com/content/pdf/1471-2490-14-58.pdf

Participants and Specimens:

At the time of enrollment (baseline), each participant was asked the following question, "Are you currently experiencing a flare of your urologic or pelvic pain symptoms By this we mean, are you currently experiencing symptoms that are much worse than usual?" (possible responses "yes" or "no"). Participants who responded with "yes" were included in the flare group. Patients with a "flare" at baseline were compared to women who did not report a flare. Each participant was asked to provide an initial stream urine specimen (VB1, ~20mL) and classic midstream clean catch specimen (VB2, ~20mL) at the baseline clinic visit. Participants were asked to clean the genital area using saline wipes before urine collection. A second VB2 urine taken at the same in-clinic visit was cultured (standard culture technique) on-site to identify active infection. Participants with a positive urine culture (traditional uropathogen colony forming units $\geq 10^5$/mL) were excluded from the EP study.

Specimen handling

Urine specimens were collected using standardized collection kits and frozen as soon as possible (85% were frozen within 15 minutes; >95% within 30 minutes). After collection at the MAPP Network Discovery sites[8,9], specimens were transferred from urine cups to 50 mL conical tubes and frozen at -80^{o}C until shipping to the central MAPP Network Tissue Analysis and Technology Core (TATC). Upon receipt at the TATC the specimens were thawed, thoroughly mixed, and aliquoted into 1 and 3 mL aliquots. Specimens were frozen at -80^{o}C until used.

DNA extraction and Ibis eubacterial and fungal domain Ibis T-5000 assays:

We subjected VB1 and **VB2** specimens to next-generation molecular diagnostic Ibis T-5000 Universal Biosensor technology[7]. In brief, total DNA was extracted using 3 mL from VB1 and VB2 urine samples, and the bacterial and fungal DNAs were amplified by polymerase chain reaction (PCR) using the 16 primer pair BAC (Bacterial:Antibiotic resistance genes:Candida)/Fungal detection systems developed by Ibis[7]. The individual amplicons were "weighed" using the Ibis instrumentation for electrospray ionization (ESI) time-of-flight (TOF) mass spectrometer (MS) which reports molecular mass. The species 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, including limitations, are available in[7] and Appendix 2.

Statistical Analysis:

Demographic characteristics and relevant clinical factors were compared between flare and non-flare patients by Chi-Square tests. Differences in the overall microbial composition for flare versus non-flare IC/PBS patients were assessed by permutational multivariate analysis (PERMANOVA)[11]. 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 genus classification such as genus. All testing was conducted on a specific species and genus if it was detected in at least 10 subjects (the number required to detect a difference in a specific species or genus between flare and non-flare patients). Tests for differences in overall microbial composition between participants who reported flares and those who did not at the species and

genus level were adjusted for antibiotic use (previous 2 years) and menstrual cycle phase. Tests of individual taxa were adjusted for multiple comparisons by controlling the false discovery rate (FDR)[12].

RESULTS:

Baseline urine specimens were obtained from 233 UCPPS female participants: We studied 213 participants with reported flare status and full specimen collection. Baseline demographics and data are presented in Table 1. Considering clinical factors, women in the flare group reported significantly more pain and urinary symptoms as measured by the SYMQ-1, ICSI, and GUPI Total scores. There was no significant difference in respect to menstrual cycle phase (p=0.201) and 2-year antibiotic use (p=0.523) between the two groups.

We detected at least one species in 97% of the VB1 specimens (93.0% in flare compared to 98.4% of non-flare patients) and 88.0% of VB2 specimens (86.7% in flare compared to 90.0% of non-flare patients). Overall, 81 species (35 genera) were detected in VB1 and 73 in VB2 (33 genera) (Tables 2a and 2b). Mean (SD) VB1 and VB2 species count per person was 2.6 (1.5) and 2.4 (1.5) for flare patients and 2.8 (1.3) and 2.5 (1.5) for non-flare patients, respectively. Overall species composition did not significantly differ between flare and non-flare patients at any level (p=0.14, 0.75 species level, p=0.95, 0.87 genus level, p=0.42 in VB1 and VB2, respectively in multivariate analysis for richness). Tables 2a and 2b describe differences in the occurrence of species and genus respectively that were represented in at least 10 subjects (tests between flare and non-flare patients using logistic regression). There was also no significant difference in the number (percent) or specific genus composition of traditional uropathogens detected between groups (Table 3). In IC/BPS patients with negative standard plate cultures, uropathogens were detected in 8.1% vs 9.4% of VB1 in flare vs non-flare subjects, respectively; the corresponding values for VB2 specimens were 1.2% vs 3.9%. . Univariate analysis revealed a significantly greater prevalence of

fungi (Candida and Saccharomyces sp.) in the flare group (15.7%) compared to the non-flare group in VB2 (3.9%) (Table 2b p=0.01). When adjusted for antibiotic use and menstrual phase, women who reported a flare remained more likely to have fungi present in VB2 specimens than women who did not report a flare [OR= 8.3, CI=(1.7-39.4)]. Representative VB2 heatmap by flare status were developed to illustrate the differences between groups in VB2 (Figure 1).

DISCUSSION:

In our study, symptom exacerbations were referred to as "flares" and were defined as "symptoms that are much worse than usual". This definition was based on expert clinical opinion, as no published data is available to objectively define flares. Almost 40% of female IC/BPS patients evaluated in this Trans-MAPP EP study reported such a symptom exacerbation at baseline evaluation. In a condition in which patient reported pain and voiding symptoms wax and wane over time, it is difficult to develop a generalizable definition for "flare" or understand the relevance of flares to the course of disease. Yet it has been documented that most IC/BPS patients do report flares[13,14] and there is little doubt that flares have a negative impact on several aspects of patients' lives, including their work lives and their relationships[14]. Over a six month period in a longitudinal study of 400 IC patients, symptom severity improved in 15-20% and deteriorated in another 15-20%, often attributed to changes in stress, diet, sexual intercourse, and premenstrual flares [15,16]. The proportion of patients with flares is consistent with the 32% reported in a cohort of more than 100 newly diagnosed female patients with IC/BPS followed for 2 years[17]. Other cohort data suggest, however, flares may be more frequent in the IC/BPS population ranging in duration from 1 day to more than 2 weeks[13,14].

We failed to identify a difference in the "bacterial" microbiota of women with IC/BPS who reported flares at study entry compared to those who did not report a flare. Approximately 2.5 species per patient on average were detected in both groups. In multivariate analysis, there was no

difference in bacterial species or genus composition between flare and non-flare. However, in VB2, univariate analysis did show a significant difference in the prevalence of fungi between flare and non-flare groups. A significantly greater prevalence of fungi (Candida and Saccharomyces sp.) was detected in the flare group (15.7%) compared to the non-flare group in VB2 (3.9%) (p=0.006). It could be argued that this finding which was not observed in the VB1 analyses suggests that the bladder is involved rather than this just being a urethral/introital difference, however this conclusion would require analysis of specific bladder (catheterized) urine specimens and vaginal/introital swab specimens before such a conclusion could be made. It is possible that fungi presence might be influenced by previous antibiotic use or menstrual cycle status, however when controlled for these two variables, women who reported a flare remained 8 times more likely to have fungi present in VB2 specimens than women who did not report a flare. If this discovery is replicated in future studies, the ramifications for patient care are obvious, even if we have not yet proven causality. Our standard cultures did not detect fungi (in this case Candida and Saccharomyces sp.) in patients enrolled in this study implying that non-culture techniques are more sensitive and may be required to detect a clinically significant fungal presence in IC/BPS patients. If detected or suspected, anti-fungal therapy may be appropriate. This suggestion has not been proven in this study.

In a questionnaire study[12] evaluating flare "triggers", patients reported that a urinary tract infection (UTI) episode was associated with a flare in 54% of UCPPS patients (included male and female patients). But there has been a paucity of attempts to employ standard culture techniques to document this association with flares. In one of the few longitudinal studies reported evaluating bacteriuria and UTI in patients with IC/BPS, 106 consecutive women with newly diagnosed IC/BPS were followed up for 24 months[17] . Thirty four women (32%) presented with 54 flares, of which 44 were standard culture-negative and 10 were culture-positive with Gram-negative uropathogens. These investigators suggested that a small proportion of the symptom flares of

IC/PBPS might be associated with recurrent UTI, although data were not provided regarding the status after bacterial eradication. Nickel and colleagues[3] were not able to show an association between significant bacteriuria with uropathogenic bacteria and flares. That study also suggested a minimal response with antibiotic treatment, in respect to ameliorating flare symptoms in patients with positive bacterial cultures.

We hypothesized that flares might be due to unculturable micro-organisms, micro-organisms that were either overlooked or not identified as an uropathogen or a change (or imbalance) in the microbial ecology of an individual's lower urinary tract microbiota (dysbiosis). We proposed that the application of next generation molecular diagnostics (in this case, the Ibis T-5000 Universal Biosensor technology[9] – see appendix 2 for an explanation and description of this technique, its comparison to other nonculture microbial identification techniques and limitations of the technique) to analyze MAPP Network specimens of patients with flare and no flare status would more definitively address questions regarding the presence of unique micro-organisms or ecological communities of micro-organisms than previous approaches, and thus potentially identify one possible etiology for flares in IC/BPS.

Our study has some important weaknesses and limitations. We excluded patients with positive standard cultures at baseline and therefore cannot speculate that significant bacteriuria at baseline might have been associated with flares in some excluded study candidates. The specimens were voided urine specimens and therefore do no necessarily represent the microbiome of the bladder, but rather the lower urinary tract and introitus/vagina. We have only reported on the detection of microorganisms and therefore we could only assess the presence rather than the dominance of any species. The available antibiotic usage data was not comprehensive (yes or no only) and we could not account for the scope of antibiotic use over the previous 2 years. We did not comprehensively identify all fungal species (a full Ibis fungal panel would be required to identify all fungal

organisms). There is a risk of type II error in analyzing our results, with the possibility that we did not have enough subjects to detect minor differences for some bacterial species. The strengths of our analyses lie in the comprehensive clinical assessment of patients with IC/BPS in the Trans-MAPP EP study and the fact that we *a priori* collected reported flare status at initial evaluation. The analysis also adjusted and/or corrected for demographic differences including menstrual status and antibiotic use, although antibiotic use was measured fairly coarsely as any use within the past 2 years. The next generation technology that we have used to analyze IC/BPS specimens in this Trans-MAPP EP flare study was the Ibis T-5000 Universal Biosensor technology which has demonstrated an ability to provide extremely accurate and comprehensive data[18,19] in clinical studies involving complex infectious processes.

Our finding that the prevalence of fungi was greater in participants who reported flares needs to be replicated. We are planning a hypotheses driven studies to examine flares in this same cohort (MAPP 1 EP study participants) at 6 and 12 months as well as the upcoming MAPP-2 Microbiome Project employing similar methodology with the addition of including a full fungal screen.

CONCLUSION:

Assessment of baseline culture independent microbiological data from female subjects enrolled in the MAPP Research Network has identified a higher prevalence of certain fungi (Candida and Saccharomyces sp.) in the urine microbiota that may be involved in etiology or pathogenesis of a subset of patients with IC/BPS related flares. This preliminary discovery is currently being further tested in order to validate findings.

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FIGURE LEGENDS

Figure 1. Presence of species by flare status, VB2
The pink bar denotes non flare participants, and the red bar denotes flare participants. Yellow lines within the heatmap indicate the presence of the species listed alphabetically in the column along the bottom of the heatmap.

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Table 1: Baseline demographics for subjects (No = no reported flare at baseline; yes – flare reported at baseline).

Factor	Level	No	Yes	Total	p-value
Number of Participants	N (%)/ Mean (SD)	127	86	213	
Age	Mean years	41.7 (15.0)	41.2 (14.0)		0.190
Race	Caucasian	107 (84.2)	78 (90.6)%		0.022
Employment	Employed	81 (63.8)	46 (53.5)		0.01
SYMQ – 1* (0-10)		4.6 (2.0)	6.4 (1.8)	5.3 (2.1)	< 0.001
ICSI score (0-20)		10.1 (4.1)	12.2 (4.8)	10.9 (4.5)	0.001
GUPI score (0-45)		24.7 (8.1)	30.8 (8.4)	27 (8.7)	< 0.001
Menstrual Cycle Phase	No Cycle	46 (36.2%)	38 (44.2%)	84 (39.4%)	0.201
	Follicular (0-12)	23 (18.1%)	13 (15.1%)	36 (16.9%)	
	Ovulation (13-14)	3 (2.4%)	2 (2.3%)	5 (2.3%)	
	Luteal (15-40)	40 (31.5%)	19 (22.1%)	59 (27.7%)	
	>40 days	7 (5.5%)	11 (12.8%)	18 (8.5%)	
	Missing	8 (6.3%)	3 (3.5%)	11 (5.2%)	
Antibiotic Use (in last 2 years)	Yes	71 (82.61%)	61 (85.92)	132	0.523
	No	16 (18.39%)	10 (14.08)	26	
	Missing			55	

*Pain, pressure and discomfort associated with the bladder/pelvis rated 0-10 (with 10 being the most severe discomfort imagined).

Table 2A. Prevalence of Species in women with and without flares in VB1 (only taxa present in at least 10 subjects were tested individually)

VB1	No Flare N (%)	Flare N (%)	p unadjusted (richness)	p unadjusted (presence/ absence)	p adjusted * (richness)	P adjusted* (presence/ absence)
Overall	127	86				
Species				0.128		0.142
Bifidobacterium subtile	17 (13.4%)	8 (9.3%)		0.366		0.6091
Candida albicans	3 (2.4%)	7 (8.1%)		0.0655		0.0664
Escherichia coli	6 (4.7%)	4 (4.7%)		0.9802		0.8632
Finegoldia magna	10 (7.9%)	7 (8.1%)		0.9441		0.9263
Lactobacillus acidophilus	18 (14.2%)	7 (8.1%)		0.1847		0.2996
Lactobacillus crispatus	44 (34.6%)	32 (37.2%)		0.7016		0.6713
Lactobacillus gasseri	12 (9.4%)	5 (5.8%)		0.3413		0.5272
Lactobacillus johnsonii	37 (29.1%)	24 (27.9%)		0.8459		0.9183
Lactobacillus sp.	5 (3.9%)	6 (7%)		0.3316		0.227
Staphylococcus epidermidis/haemolyticus	45 (35.4%)	42 (48.8%)		0.0517		0.0265
Staphylococcus haemolyticus	16 (12.6%)	2 (2.3%)		0.0184		0.0212
Staphylococcus hominis	29 (22.8%)	16 (18.6%)		0.4587		0.4719
Streptococcus agalactiae	7 (5.5%)	5 (5.8%)		0.9252		0.9817
Genus			0.923	0.938	0.954	0.931
Bifidobacterium	23 (18.1%)	11 (12.8%)	0.277	0.3005	0.262	0.3868
Candida	9 (7.1%)	11 (12.8%)	0.291	0.1669	0.324	0.1917
Corynebacterium	8 (6.3%)	3 (3.5%)	0.550	0.3698	0.662	0.7284
Escherichia	6 (4.7%)	4 (4.7%)	1.000	0.9802	0.87	0.8632
Finegoldia	10 (7.9%)	7 (8.1%)	1.000	0.9441	0.926	0.9263
Lactobacillus	83 (65.4%)	56 (65.1%)	0.788	0.9714	0.882	0.7414
Staphylococcus	83 (65.4%)	56 (65.1%)	0.929	0.9714	0.924	0.7465
Streptococcus	19 (15%)	13 (15.1%)	0.854	0.9751	0.866	0.8971
Fungi[+]	14 (11%)	14 (16.3%)	0.334	0.268	0.38	0.3646

*adjusted for employment (employed, disabled, or other and race)
[+] Ibis BAC Screen only detects Candida and Saccharomyces sp.

Table 2B. Prevalence of Species in women with and without flares in VB2 (only taxa present in at least 10 subjects were tested individually)

VB2	No Flare N (%)	Flare N (%)	p unadjusted (richness)	p unadjusted (presence/ absence)	p adjusted * (richness)	P adjusted* (presence/ absence)
Overall	127	83				
Species				0.697		0.752
Bifidobacterium subtile	15 (11.8%)	10 (12%)		0.9586		0.8616
Finegoldia magna	8 (6.3%)	5 (6%)		0.9355		0.7899
Lactobacillus acidophilus	13 (10.2%)	8 (9.6%)		0.8878		0.6615
Lactobacillus crispatus	34 (26.8%)	28 (33.7%)		0.2803		0.3235
Lactobacillus gasseri	7 (5.5%)	7 (8.4%)		0.4099		0.477
Lactobacillus johnsonii	36 (28.3%)	22 (26.5%)		0.7706		0.784
Lactobacillus sp.	10 (7.9%)	5 (6%)		0.6118		0.7961
Propionibacterium acnes	8 (6.3%)	4 (4.8%)		0.6524		0.9557
Staphylococcus epidermidis/haemolyticus	41 (32.3%)	32 (38.6%)		0.3514		0.2587
Staphylococcus haemolyticus	9 (7.1%)	4 (4.8%)		0.5075		0.8359
Staphylococcus hominis	20 (15.7%)	17 (20.5%)		0.3798		0.4221
Streptococcus agalactiae	8 (6.3%)	4 (4.8%)		0.6524		0.5768
Genus			0.846	0.513	0.871	0.407
Bifidobacterium	22 (17.3%)	12 (14.5%)	0.757	0.5821	0.73	0.5122
Candida	5 (3.9%)	8 (9.6%)	0.130	0.1042	0.121	0.1071
Finegoldia	8 (6.3%)	5 (6%)	1.000	0.9355	0.803	0.7899
Lactobacillus	74 (58.3%)	55 (66.3%)	0.601	0.2452	0.533	0.1251
Propionibacterium	8 (6.3%)	4 (4.8%)	0.759	0.6524	0.976	0.9557
Staphylococcus	70 (55.1%)	48 (57.8%)	0.927	0.6985	0.998	0.5355
Streptococcus	18 (14.2%)	11 (13.3%)	0.716	0.8501	0.567	0.7927
Fungi[+]	5 (3.9%)	13 (15.7%)	0.003	0.0058	0.007	0.0103

*adjusted for employment (employed, disabled, or other and race)
[+] Ibis BAC Screen only detects Candida and Saccharomyces sp.

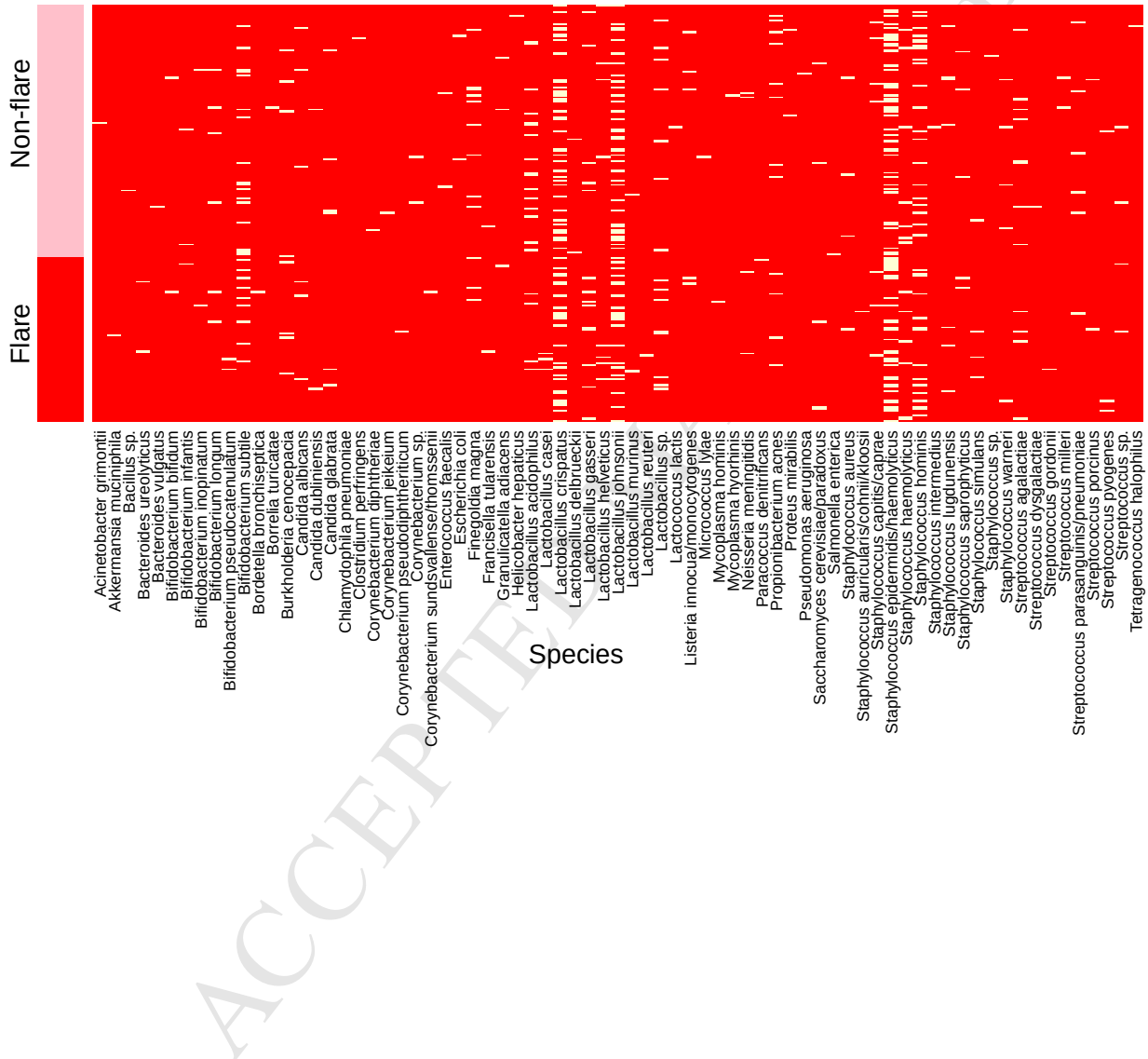
Table 3. Prevalence of uropathogens in UCPPS women with and without flares in VB1 and VB2 (note that this data refers to nonculture identification technique since all subjects had standard culture sterile VB2 urine sample)

N (%)		**Entero-bacter**	**Entero-coccus**	**Esche-richia**	**Kleb-siella**	**Proteus**	**Pseudo monas**	**Any Uro-pathogen**
VB1	No Flare (n=127)	0 (0%)	4 (3.1%)	6 (4.7%)	2 (1.6%)	0 (0%)	1 (0.8%)	12 (9.4%)
	Flare (n=86)	0 (0%)	4 (4.7%)	4 (4.7%)	1 (1.2%)	0 (0%)	1 (1.2%)	7 (8.1%)
	p unadjusted	-	0.574	0.9802	0.803	-	0.7818	0.7424
	p adjusted*	-	0.4996	0.8632	0.6449	-	0.7253	0.5677
VB2	No Flare (n=127)	0 (0%)	1 (0.8%)	2 (1.6%)	0 (0%)	2 (1.6%)	0 (0%)	5 (3.9%)
	Flare (n=83)	1 (1.2%)	0 (0%)	0 (0%)	0 (0%)	1 (1.2%)	2 (2.4%)	1 (1.2%)
	p unadjusted	1.000	0.7625	0.9957	-	0.9957	0.9966	0.5505
	p adjusted*	1.000	0.6369	0.9971	-	0.9971	0.9978	0.9059

*adjusted for employment (employed, disabled, or other and race)

Figure 1. Presence of species by flare status, VB2

The pink bar denotes non flare participants, and the red bar denotes flare participants. Yellow lines within the heatmap indicate the presence of the species listed alphabetically in the column along the bottom of the heatmap.



Abbreviations:

EP	Epidemiological/Phenotyping
FDR	false discovery rate
GUPI	Genitourinary Pain Index
IC	Interstitial Cystitis
IC/BPS	Interstitial Cystitis/Bladder Pain Syndrome
ICSI	Intersitial Cystitis Symptom Index
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
SYMQ – 1	Pain, pressure, discomfort scale
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

Appendix 1 MAPP Research Network

MAPP Research Network Study Group

MAPP Network Executive Committee
J. Quentin Clemens, MD, FACS, MSci, Network Chair, 2013-
Philip Hanno, MD
Ziya Kirkali, MD
John W. Kusek, PhD
J. Richard Landis, PhD
M. Scott Lucia, MD
Chris Mullins, PhD
Michel A. Pontari, MD

Northwestern University Discovery Site
David J. Klumpp, PhD, Co-Director
Anthony J. Schaeffer, MD, Co-Director
Apkar (Vania) Apkarian, PhD
David Cella, PhD
Melissa A. Farmer, PhD
Colleen Fitzgerald, MD
Richard Gershon, PhD
James W. Griffith, PhD
Charles J. Heckman II, PhD
Mingchen Jiang, PhD
Laurie Keefer, PhD
Darlene S. Marko, RN, BSN, CCRC
Jean Michniewicz
Todd Parrish, PhD
Frank Tu, MD, MPH

University of California, Los Angeles Discovery Site and PAIN Neuroimaging Core
Emeran A. Mayer, MD, Co-Director
Larissa V. Rodríguez, MD, Co-Director
Jeffry Alger, PhD
Cody P. Ashe-McNalley
Ben Ellingson, PhD
Nuwanthi Heendeniya
Lisa Kilpatrick, PhD
Jason Kutch, PhD
Jennifer S. Labus, PhD
Bruce D. Naliboff, PhD
Fornessa Randal
Suzanne R. Smith, RN, NP

University of Iowa Discovery Site
Karl J. Kreder, MD, MBA, Director
Catherine S. Bradley, MD, MSCE
Mary Eno, RN, RA II
Kris Greiner, BA
Yi Luo, PhD, MD
Susan K. Lutgendorf, PhD
Michael A. O'Donnell, MD
Barbara Ziegler, BA

University of Michigan Discovery Site
Daniel J. Clauw, MD, Co-Director; Network Chair, 2008-2013
J. Quentin Clemens, MD, FACS, MSci, Co-Director; Network Chair, 2013-
Suzie As-Sanie, MD
Sandra Berry, MA
Megan E. Halvorson, BS, CCRP
Richard Harris, PhD
Steve Harte, PhD
Eric Ichesco, BS
Ann Oldendorf, MD
Katherine A. Scott, RN, BSN
David A. Williams, PhD

University of Washington, Seattle Discovery Site
Dedra Buchwald, MD, Director
Niloofar Afari, PhD, Univ. Of California, San Diego
John Krieger, MD
Jane Miller, MD
Stephanie Richey, BS
Susan O. Ross, RN, MN
Roberta Spiro, MS
TJ Sundsvold, MPH
Eric Strachan, PhD
Claire C. Yang, MD

Washington University, St. Louis Discovery Site
Gerald L. Andriole, MD, Co-Director
H. Henry Lai, MD, Co-Director
Rebecca L. Bristol, BA, BS, Coordinator
Graham Colditz, MD, DrPH
Georg Deutsch, PhD, Univ. of Alabama at Birmingham
Vivien C. Gardner, RN, BSN, Coordinator
Robert W. Gereau IV, PhD
Jeffrey P Henderson, MD, PhD
Barry A. Hong, PhD, FAACP
Thomas M. Hooton, MD, Univ of Miami
Timothy J. Ness, MD, PhD, Univ. of Alabama at Birmingham
Carol S. North, MD, MPE, Univ. Texas Southwestern
Theresa M. Spitznagle, PT, DPT, WCS
Siobhan Sutcliffe, PhD, ScM, MHS

University of Pennsylvania Data Coordinating Core (DCC)
J. Richard Landis, PhD, Core Director
Ted Barrell, BA
Theressa Creighton, BA
Laura Fluharty, MPH
Philip Hanno, MD
Xiaoling Hou, MS
Michel A. Pontari, MD
Nancy Robinson, PhD
Alisa Stephens, PhD
Yanli Wang, MS

University of Colorado Denver Tissue Analysis & Technology Core (TATC)
M. Scott Lucia, MD, Core Director
Adrie van Bokhoven, PhD
Andrea A. Osypuk, BS
Robert Dayton, Jr
Karen R. Jonscher, PhD
Holly T. Sullivan, BS
R. Storey Wilson, MS

Additional Sites: Harvard Medical School/ Boston Children's Hospital
Marsha A. Moses, PhD, Director
Andrew C. Briscoe
David Briscoe, MD
Adam Curatolo, BA
John Froehlich, PhD
Richard S. Lee, MD
Monisha Sachdev, BS
Keith R. Solomon, PhD
Hanno Steen, PhD

Stanford University
Sean Mackey, MD, PhD, Director
Epifanio Bagarinao, PhD
Lauren C. Foster, BA
Emily Hubbard, BA
Kevin A. Johnson, PhD, RN
Katherine T. Martucci, PhD
Rebecca L. McCue, BA
Rachel R. Moericke, MA
Aneesha Nilakantan, BA
Noorulain Noor, BS

Queens University
J. Curtis Nickel, MD, FRCSC, Director

Drexel University College of Medicine
Garth D.Ehrlich, PhD

National Institutes of Diabetes and Digestive and Kidney Diseases (NIDDK), National Institutes of Health (NIH)
Chris Mullins, PhD
John W. Kusek, PhD
Ziya Kirkali, MD
Tamara G. Bavendam, MD

Appendix 2

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

Introduction:
The Ibis T-5000 Universal Biosensor technology[7] uses a PCR-ESI-TOF MS approach which 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 considered together to arrive at a definitive species-level diagnostic for all known bacteria. In addition, the system provides a most-closely-related match for unknown organisms. The concordance for non-rare taxa between the Ibis analyses and deep 16S sequencing is extremely high between the two methods[18]. The Ibis system, however, is not effective at picking up rare taxa, present at less than 1-3% of the total microbial burden, with the exception of *Staphylococcus aureus* which has its own specific primer. The advantage of this technology was that it freed us from having to choose what organism to test for as there is no need for an a priori hypothesis as to what species are present.

Summary of Methodology:
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[7]. 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[7] 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). 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.