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CASE REPORT

Identification of *Burkholderia fungorum* in the urine of an individual with spinal cord injury and augmentation cystoplasty using 16S sequencing: copathogen or innocent bystander?

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Abstract

Introduction People with neuropathic bladder (NB) secondary to spinal cord injury (SCI) are at risk for multiple genitourinary complications, the most frequent of which is urinary tract infection (UTI). Despite the high frequency with which UTI occurs, our understanding of the role of urinary microbes in health and disease is limited. In this paper, we present the first prospective case study integrating symptom reporting, urinalysis, urine cultivation, and 16S ribosomal ribonucleic acid (rRNA) sequencing of the urine microbiome.

Case presentation A 55-year-old male with NB secondary to SCI contributed 12 urine samples over an 8-month period during asymptomatic, symptomatic, and postantibiotic periods. All bacteria identified on culture were present on 16S rRNA sequencing, however, 16S rRNA sequencing revealed the presence of bacteria not isolated on culture. In particular, *Burkholderia fungorum* was present in three samples during both asymptomatic and symptomatic periods. White blood cells of ≥5–10/high power field and leukocyte esterase ≥2 on urinalysis was associated with the presence of symptoms.

Discussion In this patient, there was a predominance of pathogenic bacteria and a lack of putative probiotic bacteria during both symptomatic and asymptomatic states. Urinalysis-defined inflammatory markers were present to a greater extent during symptomatic periods compared to the asymptomatic state, which may underscore a role for urinalysis or other inflammatory markers in differentiating asymptomatic bacteriuria from UTI in patients with NB. The finding of potentially pathogenic bacteria identified by sequencing but not cultivation, suggests a need for greater understanding of the relationships amongst bacterial species in the bacteriuric neuropathic bladder.

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Introduction

People with spinal cord injury (SCI) are at high risk of genitourinary complications, including bladder and kidney infections [1, 2], calculi [3, 4], and bladder cancer [5, 6], among others, due to the neuropathic bladder (NB) that accompanies the neurologic injury. Urinary tract infections (UTI) have been and remain the most common genitourinary complication, one of the most common and costly secondary health conditions and are the most common cause of rehospitalization in people with SCI [7–9].

Accurate diagnosis and treatment of UTI in patients with NB secondary to SCI is challenging. First, the almost universal presence of bacteriuria in people with SCI presents a major diagnostic and therapeutic challenge. Clinical dogma has been that "healthy" urine is sterile, and as such, bacteriuria (defined as "the presence of bacteria in the urine not due to contaminated specimen collection") is considered

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"abnormal" and perhaps a precursor to UTI. However, in most patient populations asymptomatic bacteriuria (ABU) is not treated, as frequent antibiotic use promotes the development of multidrug-resistant organisms, and because of data demonstrating that rarely do children with NB and ABU sustain renal injury [10, 11].

Contradicting clinical dogma, our team has shown using 16S ribosomal ribonucleic acid (rRNA) next-generation sequencing (NGS) that healthy urine is not sterile and that a urine microbiome does exist in the absence of symptoms [12]. This urine microbiome varies by gender, bladder function, and possibly other factors. When compared with the urine microbiomes of asymptomatic individuals with normally functioning bladders, the urine microbiomes of asymptomatic people with NB due to SCI are more heavily populated by potential uropathogens and notably lack a predominance of commensals and putative probiotic bacteria [12, 13]. This recent discovery has the potential to advance our understanding of urinary health and disease, as well as change our approach to the diagnosis and management of urinary symptoms, the presence of uropathogens, and ultimately, UTI.

In this paper, we report the first prospective description of the urinary microbiome of a patient with NB and augmentation cystoplasty (in 2000) secondary to SCI by 16S rRNA NGS during differing phases of urinary health and disease. In addition, we describe the first case in the literature of *B. fungorum* occurring in the urine of a person with NB and augmentation cystoplasty due to SCI.

Case presentation

The patient is a 55-year-old male with T4 ASIA Impairment Scale A SCI secondary to a skiing accident when he was 19 years old. He has NB with bladder augmentation managed by intermittent urethral catheterization using single-use sterile catheters every 4–6 h. Routine medications include: Oxybutynin 5 mg three times daily (bladder spasticity); Baclofen 40 mg three times daily, Diazepam 5 mg daily and Tizanidine 2 mg nightly (spasticity); and Docusate daily (neurogenic bowel). Twelve urine samples were collected prospectively over a 8-month period and stratified by patient's reported state of health: asymptomatic (no urinary symptoms, one sample), symptomatic (symptoms present and perceived by subject to be due to UTI, nine samples), and postantibiotic treatment (two samples). The most common reported symptoms that he attributed to UTI included: increased spasticity, malodorous urine, cloudy urine, worsening urine leakage, and sweating. All urine samples were collected by sterile technique using betadine and a single-use intermittent catheter (via urethra) and immediately placed on ice. Within 1 h, the samples were separated into two aliquots: one, for standard urine analysis and culture (Quest Diagnostics) and another for metagenomic analysis. Quest diagnostics performed analysis of urine samples for nitrites, leukocyte esterase, and leukocytes. Bacterial cultures were performed by inoculation of blood agar plates and incubation at 37 °C for 48 h.

Within 6 h of collection, the samples for metagenomic analysis were processed by serial centrifugation; the pellet was isolated and stored at −80 °C[12]. Thawed urine samples were clarified by low-speed centrifugation and bacterial genomic DNA was extracted from urine pellets using the Qiagen DNeasy blood and tissue kit. The DNA obtained from urinary pellets were amplified and checked for purity by measuring the A_{260}/A_{280} ratio prior to amplification of 16S rRNA genes using universal primers 27f (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′). The amplification products were sequenced by single molecule real-time (SMRT) sequencing using the Pacific Biosciences sequencer (PacBio). All-Species Living Tree Project LTP115 and Silva 119 Ref NR 99 (curated) were used for species identification. Number of reads, which is proportional to the number of copies of the 16S rRNA genes, and is a measure of the abundance/fraction of each bacterial species in the sample, were presented.

All bacteria identified by traditional cultivation were also detected by 16S rRNA PacBio sequencing; however, 16S rRNA sequencing revealed the presence of potentially pathogenic bacteria during both symptomatic and asymptomatic states that were not identified on culture (Table 1). There was one sample where the DNA concentration was too low to perform 16S rRNA sequencing (sample T10, Table 1). The amount of bacterial DNA isolated was higher during the symptomatic state than that during the asymptomatic state (Table 2). Urinalysis results demonstrated that white blood cell (WBC) ≥ 5–10 and leukocyte esterase (LE) ≥ 2 were associated with the presence of symptoms in this patient, while an absence of LE, nitrite, and WBC < 5 did not differentiate asymptomatic from symptomatic state.

Discussion

This case report describes changes in urine inflammatory biomarkers and the urine microbiome over an 8-month period in a patient with NB with augmentation cystoplasty secondary to SCI. Urinalysis findings of ≥5 WBC and ≥2 + LE were associated with urinary symptoms in this individual. This may or may not be true for others with NB and SCI, but warrants consideration of more investigation as the Infectious Diseases Society of America currently recommends against use of WBC on urinalysis to differentiate ABU from UTI [14]. There is evidence demonstrating an

Table 1 Urine samples summary in chronological order by symptom

Timepoint and Symptom Status	Culture Isolate(s) Urinalysis Inflammatory and Bacterial Markers	Colonies (CFU/mL)	Bacterial species Identified by 16S rRNA Gene Sequencing (number of copies)
T1. Baseline Asymptomatic (10/1/2013)			*Granulicatella adiacens* (17,951)
	Staphylococcus	50,000-10,0000	*Staphylococcus epidermidis* (8925)
	Low High		*Burkholderia fungorum* (7716)
	WBC 1-2		*Pseudomonas brenneri* (109)
	LE 1+		*Klebsiella pneumoniae* (12)
	Nitrite +		*Pseudomonas trivalis* (1)
T2. Symptomatic (odor, cloudy) (10/15/2013)	*Proteus mirabilis*	1,000-10,000	*P. mirabilis* (14,592)
	Low High		*B. fungorum* (12307)
	WBC 5-9		*S. epidermidis* (2604)
	LE 2+		*P. brenneri* (236)
	Nitrite -		*S. flexneri* (52)
			Serratia proteamaculens (1)
			Propionbacter acnes (1)
T3. Asymptomatic (On antibiotics) (10/30/2014)	Low High	0	
	WBC 0		
	LE -		
	Nitrite -		
T4. Symptomatic (odor) (11/13/2013)	*K. pneumoniae*	>100,000	*K. pneumoniae* (15163)
			K. pneumoniae (15)[I]
	Low High		*Veillonella parvula* (37)
	WBC 0-1		
	LE 2+		
	Nitrite +		
T5. Symptomatic (odor) (12/4/2013)			*B. fungorum* (27183)
			P. brenneri (1147)
	Staphylococcus	1000-10,000	*S. epidermidis* (1112)
	Low High		*K. pneumoniae* (477)
	WBC 0-1		*E. faecalis* (24)
	LE -		*Pseudomonas psychotolerans* (4)
	Nitrite -		*Enterobacter cancerogenus* (3)
			P. aeruginosa (2)
T6. Symptomatic (spasticity, sweating, odor, cloudy) (12/18/2013)	*K. pneumonia*	>100,000	*K. pneumoniae* (28709)
	Low High		*E. cloacae* (2)
	WBC 15-19		
	LE 2+		
	Nitrite +		
T7. Asymptomatic (On antibiotics) (3/13/2014)	*P. aeruginosa*	10,000-50,000	*P. aeruginosa* (28859)
	Low High		*B. fungorum* (3092)
	WBC 3-4		*P. brenneri* (34)
	LE 1+		*K. pneumoniae* (12)
	Nitrite +		*Staphylococcus caprae* (1)
			K. pneumoniae (1)
T8. Symptomatic (spasticity, odor) (3/25/2014)	*K. pneumoniae*	>100,000	*K. pneumoniae* (33250)
	Low High		*E. cloacae* (1)
	WBC 1-2		
	LE 1+		
	Nitrite +		
T9. Symptomatic (spasticity, odor) (4/3/2014)	*P. mirabilis*	>100,000	*P. mirabilis* (16380)
	Low High		*V. parvula* (2)
	WBC 10-14		*P. aeruginosa* (1)
	LE 2+		
	Nitrite +		

Table 1 (continued)

Timepoint and Symptom Status	Culture Isolate(s)	Colonies (CFU/mL)	Bacterial species Identified by 16S rRNA Gene Sequencing (number of copies)
	Urinalysis Inflammatory and Bacterial Markers		
T10. Symptomatic (spasticity, odor) (5/19/2014)	*E. faecalis* Low High WBC 3-4 LE 1+ Nitrite -	1000-10,000	RNA concentration too low to sequence
T11. Symptomatic (spasticity) (6/3/2014)	*P. mirabilis* Low High WBC 3-4 LE 2+ Nitrite +	>100,000	*P. mirabilis* (37472) *V. parvula* (25) *S. intermedius* (16) *B. fungorum* (1) *S. flexneri* (1)
T12. Symptomatic (spasticity, odor, cloudy) (6/17/2014)	*K. pneumonia* Low High WBC 1-2 LE 2+ Nitrite +	>100,000	*K. pneumoniae* (29924) *B. fungorum* (1)

[I]Different strain of same species

Table 2 Comparing asymptomatic and symptomatic urine samples

	Asymptomatic ($N = 3$)	Symptomatic ($N = 9$)
Average DNA (ng/μL[I])	10.4	73.3
WBC 0–4	3	5
WBC 5–9	0	2
WBC 10+	0	2
LE negative	1	1
LE 1+	2	2
LE 2+	0	6
Nitrite NEG	1	3
Nitrite POS	2	
	6	

[I]Nanograms per microliter

association between positive nitrites and ≥6 WBC on urinalysis results and positive urine culture in NB [15], however, this was not supported by our data in this individual. In addition, the same review found that the presence of symptoms associated with UTI in NB was more reliable when patients were reporting symptoms of spasticity, autonomic dysreflexia, and subjective feelings of unease [15]. Inflammatory marker findings in this patient were not consistent with this as the presence of increased spasticity was associated with variable white cells (WBC ranged from 1–2/hpf to 15–19/hpf; LE ranged from 1+ to 2+); however, spasticity was consistently associated with nitrite + urine. In 5 of 6 samples collected during periods of urinary symptoms that included spasticity, culture confirmed >100,000 cfu of a uropathogen.

SMRT sequencing of 16S rRNA genes demonstrated a bacterial ecosystem that, regardless of state of health and presence of symptoms, was largely composed of pathogenic or potentially pathogenic bacteria. Bacterial species in greatest abundance on sequencing correlated with identification by cultivation in 8 of 12 samples. In one instance, while the subject was taking antibiotics, bacteria were not isolated on cultivation or identified by 16S rRNA sequencing. In another sample when the subject was symptomatic, *Enterococcus faecalis* grew on culture, however, there was not enough total bacterial DNA for amplification. Nonetheless, subsequent samples indicated reinfection with a similar bacterial milieu as previously identified. Further, the urine microbiome on all occasions lacked *Lactobacillus* species, which we have previously identified in the urine microbiome of healthy subjects without NB [12], and also determined to be present to a lesser extent in subjects with NB due to SCI. In this same work we have determined that *Lactobacillus* identified in people with SCI differs at the species level from that found in people with normally functioning bladders [13]. Similar and confirmatory absence of *Lactobacillus* in this subject with NB with augmentation cystoplasty due to SCI suggests that further examination of this microbiome change warrants further attention, which is the subject of an ongoing project.

At five time points, *Burkholderia fungorum* was identified by 16S rRNA gene sequencing only and not by cultivation. *B. fungorum* is ubiquitous in our environment and has been identified in soil and plant based samples [16], respiratory secretions of patients with cystic fibrosis [16, 17], patients with infectious endocarditis [18], in the vaginal

microbiota of females with bacterial vaginosis [19], the proximal gut of HIV patients with low CD4+ T cell counts [20], and now in the urine of an individual with SCI and NB with augmentation cystoplasty. More recently, pathogenic *B. fungorum* has been described in case reports of *B. fungorum* causing septic arthritis [21], synovial tissue infection [22], and infectious granuloma [23], however, there is still not much known regarding its pathogenicity.

B. fungorum is an aerobic, nitrate reducing, Gram-negative rod that grows at 37 °C and was previously referred to as "*B. cepacia*-like strains" [14]. Due to its recent discovery, *B. fungorum* is not in commercial databases used for identification by clinical microbiology laboratories and has previously been mistaken as *B. cepacia* and *Brucella melitensis* on culture [17, 21, 22]. The diverse clinical microbiomes that *B. fungorum* has been isolated from contributes to the theory that its interactions with other bacterium are important in the disease process. Additionally, *B. fungorum* has been identified as an isolated pathogen [21–23]. Better understanding of the pathogenicity and bacterial inter-relationships are required to unveil the functional role(s) of *B. fungorum* (and potentially other uropathogens), which will aid in advancing diagnosis and targeted treatment.

Consistent with our previous work, this data demonstrate that there is a urine ecosystem during various states of health and disease, as opposed to healthy urine being sterile. While our understanding of UTI has been the simplistic concept of growth and/or overgrowth of a single (or multiple) organism(s) identified by cultivation and from a previously sterile environment, we consider that a *change in the healthy balance of the urine ecosystem*, leading to a relative overgrowth (which may or may not correlate with >100,000 cfu on cultivation), may in fact, be more representative of UTI when accompanied by urinary symptoms. Furthermore, the healthy urine ecosystem may be unique from person to person. This case report suggests that some degree and combination of uropathogens may be tolerated, and the degree of overpopulation detectable on traditional culture methods may vary by bacterial species, as may associated symptoms. Further, promotion of a better understanding of urinary eubiosis and dysbiosis [12, 13] may lead to preventive and therapeutic options in which manipulation of the ecosystem toward eubiosis to promote health may be possible.

Manipulation of microbiomes in other body systems is currently in practice, with the gastrointestinal system being the most studied to date. Fecal microbiota transplants have been used to treat *Clostridium difficile* colitis and have also been shown to induce remission and decreased symptoms in a variety of other gastrointestinal disease states, including inflammatory bowel disease and functional bowel syndromes [24]. In addition, it has been demonstrated that altering the gut microbiome with fecal transplants in mouse models improves efficiency of cancer immunotherapies [25]. Goldenberg et al. [26] published a recent Cochrane review that found moderate evidence that probiotics are effective in preventing *C. difficile*-associated diarrhea. The success of treating diseases of the gastrointestinal system by manipulation of the microbiome is an indicator that treating other body systems, such as the urinary system in patients with NB, may be an effective approach to other disease states.

16S rRNA gene sequencing has been shown to offer the potential for improved understanding of urinary health and disease through the isolation of organisms not detected through cultivation-based techniques [12]. It provides greater sensitivity regarding the composition of commensal and pathogenic microbes in urine over time. Potential disadvantages of 16s rRNA gene sequencing are that it does not differentiate between living and dead or active and present microbes, neither does it describe the *function* of microbes within the urine ecosystem, including the significance of species detected in low amounts [12, 16]. Further, the sequencing approach used in this report (SMRT sequencing) may not be able to provide results in a timely manner required for clinical practice. However, sequencing technology is advancing rapidly. For example, as of this writing Oxford Nanopore Technologies (Oxford Science Park, UK) offers a portable device called MinION for sequencing of long reads and data analysis in real time. They are currently seeking an Food and Drug Administration approval for a device that can be used for point-of-care diagnostics. Therefore, it is anticipated that in the near future, such assessments may be possible for clinical use.

Limitations of this work include the single case approach, lack of ability to generalize microbiome findings as the patient had augmentation cystoplasty, and lack of information on antimicrobials taken during the study period.

In conclusion, this case supports our previous work that a healthy urine microbiome exists in the absence of urinary symptoms, and in this individual with NB with augmentation cystoplasty due to SCI, the healthy urine microbiome is represented largely by pathogenic or potentially pathogenic bacterial species. Further, this subject had a notable lack of *Lactobacillus* during all states of health, which differs from findings in people with normally functioning bladders, indicating that this may be a target for future work and clinical advancement. Findings from the case are suggestive that monitoring changes in urine inflammatory biomarkers (as opposed to absolute values) in conjunction with symptoms may be more valuable than currently considered in differentiating UTI from ABU. Further, better understanding of SCI-specific urinary symptoms, such as increased spasticity, may be valuable in differentiating ABU from UTI. Lastly, this case represents the first report of *B.*

fungorum in the urine of an individual with SCI, suggesting the role of bacterial inter-relationships and functions in addition to magnitude of growth on traditional cultivation.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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