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A case controlled study examining the bladder microbiome in women with Overactive Bladder (OAB) and healthy controls.

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N Curtiss collected and processed samples and wrote the manuscript

A Balachandran collected the samples and edited the manuscript

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S Wildman developed the concept and edited the manuscript

C Peppiatt-Wildman edited the manuscript

J Duckett developed the concept and edited the manuscript

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Abstract

Objective: To characterise the microbiome in healthy women with no bladder symptoms and to compare this to the bladder microbiome in patients with overactive bladder syndrome (OAB).

Study Design: MSU specimens from 63 women with OAB were compared to urine from 35 controls. Urine was centrifuged and the resulting sediment pellet was re-suspended in supernatant and plated under aerobic conditions for 48 hours and anaerobic conditions for 7 days. Each morphologically distinct colony was purity plated. Bacterial colonies were lysed and polymerase chain reaction undertaken to amplify the 16s ribosomal RNA gene. This DNA was purified and sequenced allowing identification of bacterial genera.

Results: The mean number of different bacterial genera was 5.0 in both controls and OAB patients (p = 0.99). The uropathogenic bacteria *Proteus* (P=0.01) was more commonly isolated from women with OAB. The genus *lactobacillus* was present more commonly in urine taken from controls when compared to urine from OAB patients (p=0.02). Overall the most commonly grown bacteria were *staphylococcus* (grown in 59% of samples), *streptococccus* (51%), *corynebacterium* (37%) and *lactobacillus* (28%). A total of 95 different genera were identified from the urine samples.

Conclusion: The female human bladder has a diverse microbiome with stastistically significant differences between bacterial species present in OAB patients and controls.

Introduction

Historically urine was considered to be sterile but current evidence suggests that this is not the case [1]. The recent development and application of 16s ribosomal RNA (rRNA) gene sequencing technology has revealed that like the skin or gut, the 'healthy' bladder contains bacteria which are not always associated with a disease process [2]. Multiple organisms can be identified in the urine of women with and without overactive bladder (OAB) with a negative mid- stream urine (MSU) culture processed in a hospital microbiology laboratory [1,3]. The way in which these organisms exist and interact with their environment is called the microbiome [4]. Several studies have now identified differences in the bacterial load [5] and species present [6] in urine taken from women with overactive bladder (OAB) compared to healthy controls. The presence of low levels of bacteria in the bladder could be considered as an interesting idiosyncrasy without any consequences but importantly these differences are associated with clinical symptoms and outcomes [7-8]. OAB is a syndromal diagnosis with multiple different pathologies causing similar symptoms. Variations in the microbiome may be important in patients with OAB. In some patients the microbiome may have a pivotal role in the aetiology of OAB symptoms and additional identification and characterisation may allow us to maximise our therapeutic interventions. Known pathogens have been identified more commonly in patients with OAB compared to controls [9]. Conversely,not all organisms are found more commonly in patients with OAB compared to controls. Several studies have suggested an increased prevalence of certain bacteria, such as lactobacillus, in controls compared to patients with OAB [9-11]. Similarly, some studies have found a reduced prevalence of *Lactobacillus* in patients with bladder symptoms [1,6,12].

The study aimed to identify which organisms might be the focus for further research as therapeutic targets; either to promote organisms associated with positive microbiological health or targeting those organisms associated with OAB symptoms. The hypothesis was that lactobacillus would be found more commonly in control patients compared to OAB patients and that other known uropathogens would be found more commonly in OAB.

Materials and Methods

Patients and Sample Collection:

A consecutive cohort of 60 women attending a urogynaecology clinic with symptoms of OAB were identified and consented to participate in the study. All control female subjects (n=35) were recruited from patients attending a general gynaecology clinic and members of staff. Demographic data were recorded for all patients and a pelvic examination was undertaken. Symptoms were assessed by careful clinical questioning for symptoms of OAB and by completion of a validated symptom questionnaire (ICIQ- FLUTS). Control patients were excluded if they reported any bladder symptoms on the ICIQ Short Form. Any patients with prolapse (>Stage 1 POP-Q) or urodynamic evidence of stress incontinence were excluded from the study. Severity of symptoms was assessed using the patient global impression of severity scale (PGI-S). Leaks per week were assessed subjectively on the scale 0-6, 7-14, >14. Urodynamic studies were not required for inclusion but most patients had undergone this test. The use of vaginal, transdermal or oral hormone replacement therapy was a contraindication for this study.

A clean catch mid-stream urine (MSU) was taken from all participants. A sample was sent for routine hospital clinical microbiological assessment and patients with a positive MSU (> 1x 10^5 cfu) for UTI were excluded. The urine remaining from the original sample collection was subsequently taken for further study specific laboratory testing within four hours of initial collection.

Bacterial species isolation and amplification:

A 5 ml urine sample was taken and centrifuged at 800rpm for 5 mins, the resulting pellet was re-suspended in 50 µl of the supernatant and 5 µl streaked onto two chocolate agar plates (E and O laboratories). Both plates were incubated at 37^oC, one in 5% CO_2 for 48 hours, the other in an anaerobic glove box isolator for 7 days to ensure anaerobic conditions. Each morphologically distinct

colony was then purity plated and incubated in the same conditions for a further 48 hours. Aseptic techniques were employed throughout.

Bacteria colonies were picked from purity plates and 5 µl inoculations of each isolate were suspended in 100 µl sterile nuclease free water (Thermo Scientific) in a PCR reaction tube. Bacterial lysis was performed using an Eppendorf Mastercycler set to 94°C for 5 minutes to extract DNA. Polymerase chain reaction (PCR) was then performed to amplify a variable region (V9) of the bacterial 16S rRNA gene 5 µl of the lysed bacteria was combined with 12.5 µl Q5 Polymerase Mastermix (NEB), 1.25 µL forward primer (DG74Fwd - 5'-AGG AGC TGA TCC AAC CGC A-3' - Eurofins), 1.25 µl reverse primer (RDR080Rev - 5'-AAC TGG AGG AAG GTG GGG AC-3'-Eurofins) and 5 µl sterile nuclease free water. The reaction was run on an Eppendorf Mastercycler at the following temperatures and times: - 1) 98°C for 30 seconds (denature), 2) 98°C for 10 seconds (denature), 3) 55°C for 20 seconds (anneal), 4) 72°C for 20 seconds (extend) [Steps 2-4 repeated 37 times] 5) 72°C for 2 minutes (final extension). All PCR amplicons were checked for reaction success and purity by loading on a 1% agarose gel containing ethidium bromide (Fisher) and run at 120 V for 90 minutes. PCR reactions were run alongside nuclease free water as a negative control.

The resultant DNA was purified using a PCR Purification Kit (Qiagen) and the concentration of each product was recorded using a Nanodrop 2000 (Thermo Scientific). For each sample, 15 µl of the PCR product, adjusted to 5 ng/µl using purified nuclease free water, was added to 2.5 µl primer in a mix2Seq tube (Eurofins) and sent to Eurofins Genomics, Ebersburg, Germany for sequencing. A microbe genomic BLAST (Basic Local Alignment Search Tool, PubMed) was performed on the returned sequences and the identity of the individual microbes recorded. The results of the sequencing was correlated with the results of gram staining. Sequences that could not be classified to the bacterial genus level were excluded from further analysis.

Using the 16s rRNA gene sequence information a microbiome profile was developed for each woman. The percentage frequencies of genera were then compared between control and women with

OAB and analysed by Fisher Exact test. A minimum sample size of more than 25 in each arm was used as per recommendations by Machin when means and standard deviations are uncertain [13]. Demographic data was compared between controls and women with OAB using the unpaired Student's t test. All terminology and urodynamic definitions conform to IUGA/ICS standardization [14]. The study was performed in accordance with the Declaration of Helsinki. The collection of urine samples in the study were approved by the National Research Ethics Service where required.

Results

The urine samples of 95 women were analysed (60 from women with OAB and 35 from healthy female controls with over 700 bacterial colonies characterised. Three patients had a confirmed UTI on hospital culture and were excluded from further analysis. The demographics of controls and women with OAB were similar although more control patients had undergone previous urogynaecological surgery (see table 1). The significance of this is uncertain. No patient had a history of recurrent UTIs. 88% of OAB patients were either currently on or had previously been on anticholinergic medication consistent with the referral pattern to a specialist urogynaecological clinic. The majority of our OAB women scored their OAB symptoms as either moderate or severe and included some women who had previously tried 4 or more anticholinergic medications. Most of the patients were OAB wet with leaks daily or more often in 68% of women (see table 2). No subjects were using oral or vaginal lactobacillus preparations

The mean number of different bacterial genera was 5 in urine samples of both the control and OAB group (5.0 control v 5.0 OAB). Of all urine samples tested, 97.8 % grew at least one bacterial species and a total of 66 different bacterial genera were identified resident in the urine of both cohorts at different frequencies (appendix 1). The most frequently grown organisms from all samples expressed as percentage frequency, were *staphylococcus* (59%), *streptococccus* (51%), *corynebacterium* (37%) and *lactobacillus* (28%). Figure 1 shows the frequency in which the 25 most commonly grown bacterial genera were found in healthy female controls and women with OAB.

There were significant differences found in the prevalence of certain organisms in the bladder of women with OAB and healthy controls (Fisher's exact test). *Lactobacillus* was more prevalent in bladders of controls (43% controls v 20% OAB p= 0.02) whilst *Proteus* featured more often in women with OAB (3% controls v 23% OAB p=0.01). 16% of bacterial sequences could not be classified to bacterial genus level.

Comment

This study supports the previous findings of the existance of a bladder microbiome and also shows significant differences between the prevalence of different bacterial genera in healthy controls compared to patients with OAB. *Lactobacillus* is found more commonly in control patients compared to patients with OAB. This is the first study assigning statistical significance to this finding. The urinary pathogen *Proteus* was found statistically more commonly in patients with OAB.

There have been several previous studies focused on characterising the bladder microbiome in healthy individuals and in patients with bladder symptoms [1,6,12]. In common with other studies [1,6,9-12], the most noticeable result from the current study is the significantly greater prevalence of *Lactobacillus* in controls compared to patients with bladder symptoms. Hilt et al found *Lactobacillus* in 29% of control urine samples compared with 12% of urine samples from patients with OAB [1]. Moreover, another study comparing bacteria in the urine from women with urgency urinary incontinence (UUI) to that in the urine of controls found decreased *Lactobacillus* amongst the UUI group [6]. The urine samples in both of these studies were collected by transurethral catheter as opposed to MSU suggesting that lactobacillus is likely to be present in the female bladder and not present simply from contamination from the genital tract during MSU collection [1,6]. *Lactobacillus* has additionally been identified in the urine of men [12,15] and from suprapubic aspirate [2] providing further evidence that *Lactobacillus* colonisation of urine is not solely due to vaginal contamination.

Lactobacilli are able to produce bacteriocins which have antibacterial activity [16] which could possibly help explain its protective role. Diversity has been identified as a factor by other authors but this did not seem to be a factor in this study as both controls and OAB patients grew the same mean number of genera (5 genera).

In this study, unlike preceding studies, large numbers of patient urine samples allowed statistical significance to be determined for differences in the prevalence of some bacterial genera. The data statistically confirmed some trends previously noted in other studies and whilst no formal allowance for multiple analyses has been made, the fact that our findings mirror other smaller studies and are biologically plausible means, the results are likely to be reliable. The methodological approach to this study is culture dependant, which has advantages and disadvantages. There is the possibility of not identifying bacteria which do not grow in the culture conditions used in this study. However, all bacteria identified can be assumed to be viable. Other authors have identified bacteria using culture independent methods amplifiying 16S rRNA gene sequences directly from urine. Work comparing extended culture and culture independent methods have found there to be similar genera identified using both approaches [1,6] . Culture independent methods tends to identify large numbers of bacteria species but not all samples contain DNA in sufficient quantities [17] and cannot confirm the sequences are from bacteria that can survive, reproduce and be metabolically active within the bladder [2,3]. The number of urine samples from which bacteria were successfully cultured was higher in our study than Hilt et al in the extended culture arm of their study who identified bacteria in just 80% of samples [1]. Neither technique allows true quantification as primers will amplify genera with different degrees of efficiency. The study of microbiomes is therefore largely descriptive and comparative.

This study is based on clean catch MSU samples but there is debate as to whether MSUs or CSUs are the best way to sample the bladder microbiome. A review of this subject failed to reach a conclusion as to which technique is preferable [18]. Some research centres favour CSUs whilst others

use MSU samples citing the fact that similar organisms are isolated from MSU and CSU samples [9]. This is supported by the current study where 4 of the 5 most prevalent bacteria were the same as those demonstrated by Hilt using a CSU technique [1]. Other research sites have argued in favour of CSUs but have recognised the necessity to use MSUs in ongoing research [7]. In common with other centres, our current research found serial CSUs unacceptable to patients [9]. This has been overcome in some centres by obtaining urine specimens under general anaesthesia [2] but this was not an option in the current study. CSUs sample urine in the bladder and also any organisms shed from higher in the renal tract. There may be some benefit in obtaining organisms that reside in the urethra as this is deemed to be the origin of urgency in some theories of incontinence. A study comparing CSUs to MSUs and relating this to symptoms is needed to fully address this.

Data trends observed in this study regarding pathogenic organisms, commonly seen in patients with urinary tract infection, indicated that these bacteria were more commonly identified in the urine of patients with OAB than urine of controls. *Proteus* was statistically more commonly grown from the urine of women with OAB. Khasriya also found *proteus* more commonly in patients with lower urinary tract symptoms (LUTS) compared to controls [9]. There were similar but non significant trends for other known urinary pathogens such as E. coli.. It is likely that the study is underpowered to show all associations. The mechanism by which these organisms cause OAB is uncertain but may be due to altered urothelial ATP signalling [19]. Observational non randomised studies using antibiotics have suggested a benefit in alleviating OAB symptoms but they did not include an assessment on the effect on the microbiome [20]. Current studies are in progress to assess the effect of antibiotic therapy on the bladder microbiome. There was no significant difference between the age of cases and controls but OAB patients were 5.5 years older. Although not statistically different this might result in a different microbiome in the vaginal which might affect the bladder microbiome. This can only be tested by therapeutic trials assessing the effect of interventions in the vagina to identify if these affect the bladder microbiome. To date these trails have not been performed.

There are statistically significant differences, in the prevalence of some organisms between controls and women with OAB. Collectively these results suggest that *Lactobacillus* may be protective against OAB but cause and effect needs further study. Future research should focus on antibiotic treatment against pathogenic organisms and probiotics to stimulate a positive bladder microbiome.

Word count 2500

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Legend figure 1

This graph depicts the 25 most commonly, grown bacterial genera and compares the frequency in which they were grown from the bladder microbiome of women with OAB (orange) and Control women (blue). P values are shown.
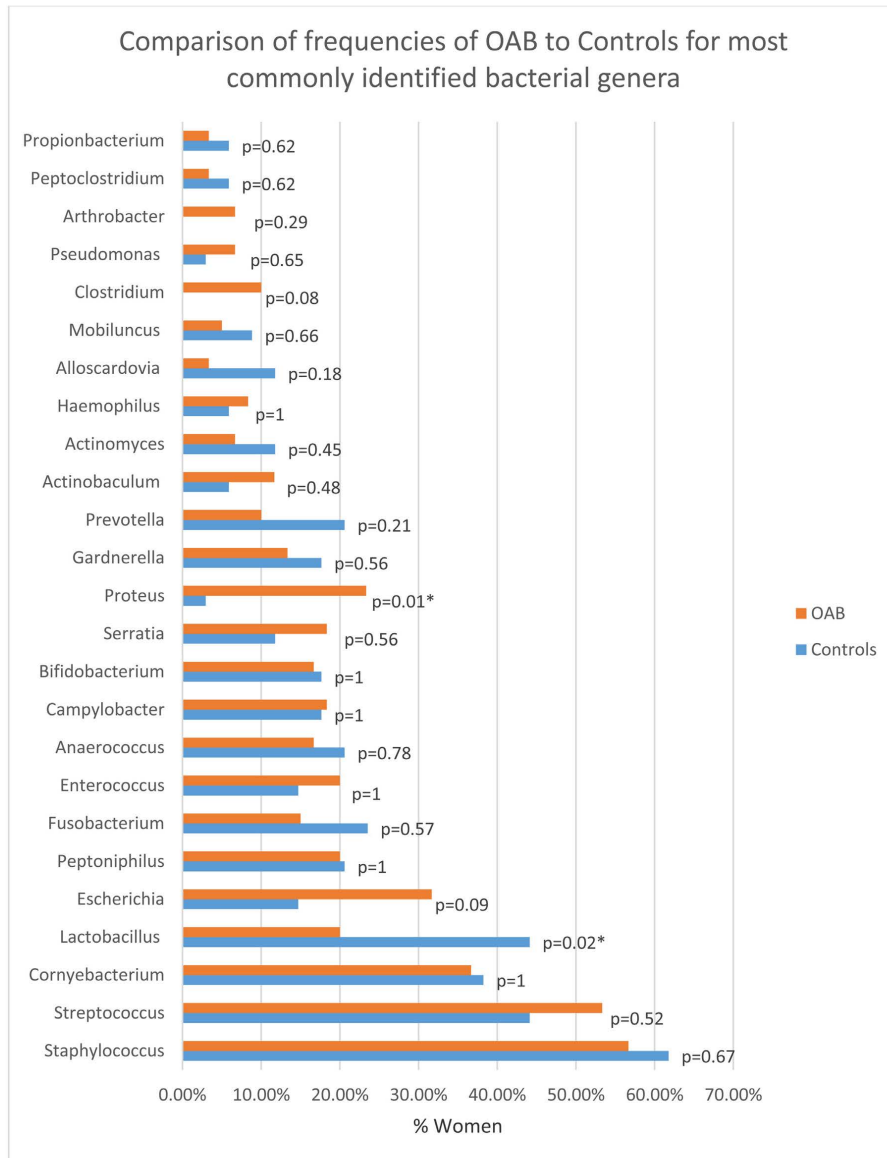
*= statistically significant

Table 1
Demographics details of patient and controls

	Controls (n=35)	OAB (n=60)	P value
Age (range)	53.7 (41-83)	59.2 (14-87)	0.07
Diabetes	9%	7%	1
BMI	27.2	29.4	0.11
Median Parity	2	2	1
Anticholinergics	0%	87.7%	<0.01[a]
Previous urogynae surgery	10/35	32/57	0.02[a]

[a] Statistically significant difference. Urogynae=urogynaecological; prolapse or incontinence surgery.

Table 2
Characteristics of patients with OAB

Percentage of OAB women		
Subjective Severity	Mild	12.3 % (7/57)
	Moderate	19.3 % (11/57)
	Severe	68.4% % (39/57)
Number of Anticholinergics Previously used	0	12.3 % (7/57)
	1	36.8 % (22/57)
	2	7.0 % (4/57)
	3	14.0 % (8/57)
	4	22.8 % (13/57)
	>4	5.3 % (34/57)
Leaks per week	0-6	32.1 % (18/57)
	7-14	7.1 % (4/57)
	>14	59.6 % (34/57)
Previous intravesical botox	0%	(0/57)

Comparison of frequencies of OAB to Controls for most commonly identified bacterial genera

*statistically significant.

Fig. 1. This graph depicts the 25 most commonly, grown bacterial genera and compares the frequency in which they were grown from the bladder microbiome of women with OAB (orange) and Control women (blue). P values are shown. * = statistically significant. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)