Urinary Microbiota; Which Non-Invasive Urine Collection Method Should We Use?

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Urinary Microbiota; Which Non-Invasive Urine Collection Method Should We Use?

Objective: The aim of this study is establish the optimal non-invasive urine sample collection method for the microbiota studies.

Methodology: 12 men with bladder carcinoma underwent first voided and midstream urine collection. Urine samples were analyzed by using V3-V4 regions of bacterial 16s ribosomal RNAs. Bacterial groups with relative abundance above 1% were analyzed in first voided urine and midstream urine samples at phylum, class, order, and family level. At the genus level, all of the identified bacterial groups' relative abundances were analyzed. The statistical significance ($p<0.05$) of differences between first voided and midstream urine sample microbiota were evaluated using the Wilcoxon test.

Results: According to analysis, 8 phyla, 14 class, 23 orders, 39 families, and 29 different genera were identified in the first voided and the midstream urine samples. Statistical differences were not identified between first voided and mid-stream urine samples of all bacteria groups except the Clostridiales at order level (p:0.04) and Clostridia at class level (p:0.04).

Conclusions: Either first voided or midstream urine samples can be used in urinary microbiota studies as we determined that there is no statistically significant difference between them regarding the results of 16s ribosomal RNA analysis.

What's known?

According to widespread acceptance, first voided urine and midstream urine should be collected separately for standard microbiologic evaluation.

What's new?

We found that there is no statistically significant difference between two collection methods even on microbiota analysis. We believe that either first voided or midstream urine samples can be used in urinary microbiota studies.

Introduction:

Urine culture is the gold standard for the diagnosis of urinary tract infections (UTI). The specimen for the urine culture is mostly obtained from midstream.[1] To prevent urethral contamination midstream urine collection is recommended, especially for men.[1] The first 10 ml and the following 50 ml of urine represent the urethra and the bladder, respectively.[2] According to this hypothesis, two-step urine collection is recommended for the evaluation of the urinary tract in men.

Although urine culture is the most specific method to evaluate UTI, there are some concerns about its sensitivity.[2] Moreover there's no complete consensus on the urine sample collection method as many variations have been suggested in different studies.[3–9] Recently, it was shown that 16S ribosomal RNA sequencing can detect bacteria residing in the urinary tract from the urinary samples with a high sensitivity and specifity, even if the patient has UTI symptoms or not. [2,10] Therefore, we have decided to investigate the reliability of a two-step urine collection method and the validity of the hypothesis which argues that the first voided urine reflects the urethra and the midstream urine reflects the bladder. Our goal was to establish the optimal non-invasive urine sample collection method for the microbiota studies, preferably without collecting two different samples. To answer these questions we analyzed

and compared the first voided and the midstream urine samples with 16S ribosomal RNA sequencing.

Methods:

Subject Recruitment and Specimen Collection:

The first voided urine and the midstream urine specimens were collected from 12 male patients with bladder carcinoma who admitted to Dokuz Eylul University Hospital in Turkey between March-July 2019. Subjects who had a recent history of UTI, who used antibiotics within a month, who had previously underwent an urological surgery, who had confirmed sexually transmitted infections were excluded from the study. All subjects were asked to sign a written informed consent. Ethics committee approval was obtained from Dokuz Eylul University Clinical Trials Ethics Committee (Date: 28.03.2019 and No: 2019/ 06-28). Study was supported by The Scientific and Technological Research Council of Turkey (TUBITAK) Project number was 217S075.

According to the classical knowledge[2], we collected and examined first voided and midstream urine samples. The glans and external meatus were disinfected by an urology specialist with 10 % povidone iodine, to minimize contamination. After disinfection, the first voided urine (10 ml) and the midstream urine specimens (50 ml) were collected. The specimens were brought to the laboratory within 60 minutes (fresh urine) upon collection. The samples were centrifuged at 4000 revolutions per minute (rpm) for 10 minutes and stored at -80 ° C until all subjects' samples are collected. A 5 ml ribosaver was added to each sample to prevent deterioration.

DNA Isolation:

200 mg of the both urine samples were transferred to seperate tubes containing 0.1 mm diameter glass beads and 300 µL of lysis buffer (200 mM Tris-HCl, pH 8.0; 20 mM

EDTA; 10% TritonX-100) and homogenized for 1 minute at 6000 rpm. 10 µL Lysozyme solution (200 µg / µL) was added to the samples and the mix was transferred to a new tube to separate them from the glass beads. The mix was incubated for 15 minutes at 37°C. After incubation, 250 µL of lysis buffer (0.5 µg / µL Proteinase K, 5% Tween® 20, 3M Guanidine thiocyanate, 20 mM Tris-HCl, pH 8.0) was added to the sample, and the mix was re-incubated for 15 minutes at 70°C and then for 5 minutes at 95°C. After re-incubation, 250 µL of 2-propanol was added to the tube and the sample was loaded onto the silica column. The DNAs in the sample were passed through the silica column by centrifugation at 13000 rpm for 1 minute and kept by the silica column, then were washed twice with wash solution (20 mM NaCl, 2 mM Tris-HCl, pH 8; 80% v / v Ethanol). The silica column was dried by centrifugation. DNAs retained in the silica column were taken from the column with 50 µL of 100 mM Tris-HCl prepared with nuclease-free, sterile, deionized water (pH 7) and stored at -20 ° C until analysis.

The amount and quality of DNAs were measured by spectrophotometric methods and their suitability for the next steps was tested. Other molecular processes were performed using DNAs with an OD260 / OD280 ratio of 1.8-2.0, an OD260 / OD230 ratio of 2.0-2.2 and a concentration of at least 10 ng / µL (preferably 50-300 ng / µL).

Next-Generation Sequencing (NGS):

The primer pair to be used to construct the amplicon libraries targeted a region of approximately 460 bp, covering the V3-V4 region of the 16S rRNA gene (Klindworth et al. 2013). Connector DNA sequences were added to the 5 ’end of the target-specific primer pairs for compatibility with the Illumina index and sequence adapters of the generated library. The forward primer sequence of the primer-connector oligos specific for 16S rRNA is 5’TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-CCTACGGGNGGCWGCAG-3’,

and the reverse primer sequence is 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-ACTACHVGGGTATCTAATCC-3'. The first PCR step was performed using "Bio-Speedy® 2X qPCR Mix" and 200 nm from each primer. The following thermal cycling program was monitored on the Biorad CFX Connect (Bio-Rad, USA): 3 minutes at 95°C; 25 cycles of 30 seconds at 95°C, 30 seconds at 55°C and 30 seconds at 72°C; 5 minutes at 72°C. By carrying out agarose gel electrophoresis of PCR product size (~ 550 bp) was confirmed and "Bio-Speedy® PCR Product Cleaning Kit" (Bioeksen, Turkey) is used as eluent.

By performing the second PCR step, binary index and Illumina sequencing adapters were added to the first PCR amplicons by using the Nextera XT Index Kit (Illumina, USA), and the following thermal cycling program was used: 95°C for 3 minutes; 8 cycles of 30 seconds at 95°C, 30 seconds at 55°C and 30 seconds at 72°C; 5 minutes at 72°C. PCR products were purified with a "Bio-Speedy® PCR Product Cleaning Kit" (Bioeksen, Turkey). The final library was verified for size (~ 630 bp) by using the "Bioanalyzer DNA 1000 chip. The final library was diluted to 4 nM using 10 mM Tris pH 8.5 and 5 μL aliquots were mixed to form a library pool. For batch formation and sequencing preparation, the pooled libraries were denatured with NaOH, diluted with hybridization buffer (HT1), and denatured with temperature before MiSeq sequencing. Illumina MiSeq v3 reaction kits were used in the studies. A minimum of 5% PhiX was added to each reaction as an internal control.

Unprocessed sequence data (forward and reverse reads merged) were analyzed by using Mothur version 1.39.1. First, the index and primary sequences were trimmed, and then the specific sequences were identified. The trimmed unique sequences were aligned using the silva database sequences (https://www.arb-silva.de/) and the BLAST (Basic local alignment search tool) algorithm. The unaligned sequences at both ends of the sequences were removed by filtering and error checking was performed. Pollution was prevented by pre-clustering. The

UCHIME code was used for chimera removal.[11] The sequences were classified using the Bayesian classifier built into Mothur. Reference and taxonomy files were obtained from the Ribosomal Database Project (RDP) database. After the operational taxonomic unit (OTU) was selected and taxonomic determination was made according to the RDP database, OTUs were grouped according to their phylotypes. Obtained microbial community profiles with each other using Minitab 17 software (Minitab, UK) they will be compared and dendrograms created. Calculation of PCA (Principal Component Analysis) ordinations and aftermath Minitab 17 software will be used for correlation analysis. $P \leq 0.05$ obtained the results will be considered statistically significant.

Statistical Analysis:

Data were presented as a median for continuous variables or number of cases (%) for counted data. Bacterial groups with relative abundance above 1% were analyzed in first voided urine and midstream urine samples at phylum, class, order, and family level. At the genus level, all of the identified bacterial groups' relative abundances were analyzed. The statistical significance of differences between first voided and mid-stream urine sample microbiota were evaluated using the Wilcoxon test for percentages data through SPSS software (Version 24.0, SSPS Inc, Armonk, NY). $P < 0.05$ were considered statistically significant.

Results:

Subjects:

The first voided and the midstream urine samples of 12 male patients were analyzed. The mean age of patients was 67.0 ±8.2 and the average cigarette usage was 35.0 ±13.9 packs/year. While 6 patients were diagnosed with pTa low-grade urothelial carcinoma, 2 of

them were with pTa high-grade urothelial carcinoma. There were only 1 patient for each pTa low-grade urothelial carcinoma, carcinoma in situ, pT1 high-grade urothelial carcinoma, pT1 high-grade urothelial carcinoma and carcinoma in situ diagnoses.

Relative abundance of urinary bacteria in urine samples:

Eight phyla, 14 class, 23 orders, 39 families, and 29 different genera were identified in the first voided and the midstream urine samples. A cladogram is a graphical description of the hypothetical evolutionary relationship between taxonomic levels in phylogenetic analyses.[12] One of the patients' bacterial taxa were shown as a cladogram according to relative abundances. Starting from the outermost circle, genus (purple), family (blue), order (green) and class (yellow) to innermost circle phylum (red) bacterial hypothetical relationship were demonstrated. Each different color of lines in the cladogram represents different bacterial taxa (Figure 1).

At the phylum level, *Firmicutes* were the most common bacteria in the both urine samples. It was followed by and *Proteobacteria, Bacteroidetes and Actinobacteria*. Based on the relative abundance of bacteria, at the phylum level statistical differences were not identified between the first voided and the midstream urine samples (Table 1).

At the class level, *Gammaproteobacteria* were the most common bacteria. Based on the relative abundance of bacteria, at class level statistical differences were not identified between first voided and midstream urine samples of all bacteria groups except the *Clostridia* (p=0.04, Table 1).

At the order level, *Enterobacteriaceae* were the most common bacteria. Based on the relative abundance of bacteria, at the order level, statistical differences were not identified between first voided and midstream urine samples of all bacteria groups except the *Clostridiales* (p=0.04, Table 1).

At the family level, *Enterobacteriaceae* were the most common bacteria. Based on the relative abundance of bacteria, at the family level, statistical differences were not identified between first voided and midstream urine samples (Table 1).

At the genus level, *Escherichia* and *Shigella* were the most common bacteria. Based on the relative abundance of bacteria, at the genus level, statistical differences were not identified between first voided and midstream urine samples (Table 2).

Discussion:

In the present study, we analyzed the first voided and the midstream urine samples with 16S ribosomal RNA sequencing to establish the optimal non-invasive urine sample collection method for the microbiota studies.

Most of the bacteria that resides in the urinary tract can be detected with the standard urinary culture. However, only the fast-growing bacteria which are above the culture threshold level are identified which may cause underdiagnosis.[1,6,13] The fact that bacteria that are too few to be grown in the culture that can be revealed with 16 sRNA sequencing, suggests that the sensitivity of the microbiota analysis is higher than the bacteria culture. Next-generation sequencing in UTI diagnosis is a new and highly sensitive method that helps to detect clinically important species.[10,13] Even replacing urine culture with microbiota analysis in UTI diagnosis may be considered in the future. Traditionally it was thought that all of the identified bacteria in the urinary tract are pathological. However recent studies revealed that there are non-pathological bacteria residing in the human urinary tract. Some of them may have mutualistic relationship with the host and prevent transient pathogenic organisms from colonizing. Moreover, the microbiome is not static, it's characteristics can change in different conditions. [1,13] Therefore, we decided to use microbiota analysis in our study.

Microbiome studies may be holding the key for the novel diagnosis or treatment options, just like the one we proposed in this study.

Although there are many studies on the urinary microbiome, there's no complete consensus on the method of urine sample collection.[3–9] In comparison between the first and the midstream urine samples of our study, there is no statistically significant difference in almost all taxonomic levels. Only one statistically significant difference was found in the midstream urine contained higher levels of *Clostridia* in contrast to the first voided urine at the class and the order level comparisons. Although there is no data about the significance of *Clostridia* in the midstream urine, this finding may be due to the its obligatory aerobic characteristics.

A study by Pohl et al compared the microbiome of midstream urine and catheterized urine samples and found a difference between the two groups.[8] Conversly, another study by Hourigan et al. compared the microbiome of midstream urine samples and urine samples obtained with cystoscopy and found no differences between groups except beta diversity differing only in males.[5] In the present study, we did not prefer to collect catheterized urine samples due to its invasive nature, contamination risk, and the textbook knowledge that the first 10 ml of urine represents the urethra.[2] Additionally, study by Bundgaard-Nielsen et al. supports our method as it recommends the storage of samples at -80 ˚C where urinary microbiota remains stable over time.[9]

Limitations:

Our study has some limitations. Firstly, urine samples of only male patients with a bladder cancer diagnosis were analyzed without a control group for comparison. It is known that bladder cancer patients have rich in bacterial variation in their urine when compared with healthy subjects.[14,15] Moreover, no female patients were enrolled in the study. Hence gender

differences could not be analyzed in this study. A comparison with suprapubic aspiration and other collection methods was not performed in this study. Small sample size is another limitation. On the other hand, 16S rRNA sequencing has its limitations. Contrary to the culture, the rRNA sequencing may be detecting genetic material from the inactive bacteria in the urine. Our current knowledge on the clinical importance of microbiota and 16s rRNA sequencing is limited.

Conclusions:

In conclusion, either first voided or midstream urine samples can be used in urinary microbiota studies as we determined that there is no statistically significant difference between them regarding the results of 16s rRNA analysis, which is considered to be more sensitive than the bacteria culture in terms of evaluating UTI. The results of our study are consistent with the results of studies performed by Wu et al and Liu et al.[15,16]

To the best of our knowledge, this is the first study that undertakes the hypothesis of the first voided urine reflects the urethra and midstream urine reflects the bladder. The current opinion stating that bacterial content of the urine samples differs with the collection method needs to be revised.

The present study brings forth a novel proposal and discusses that any urine sample collected by non-invasive methods could reflect the bacterial contents of the entire urinary system regardless of the collection method. Even the data of our modestly sampled study establishes that the bacterial contents of the first voided and the mid-stream urine samples are almost identical. Further larger sample-size studies are needed to support our results.

Conflict of interest: None declared.

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Table 1. Comparison of relative abundance (median %) of urinary microbiome between first voided urine group and midstream urine group at all taxonomic levels.				
Taxa		**First Voided Urine**	**Midstream Urine**	**P**
Phylum				
	Firmicutes	32.54	33.79	Ns
	Proteobacteria	32.44	31.14	Ns
	Bacteriodetes	13.88	16.59	Ns
	Actinobacteria	11.28	11.87	Ns
	Tenericutes	4.8	4.56	Ns
Class				
	Gammaproteobacteria	22.34	20.31	Ns
	Bacilli	19.18	17.46	Ns
	Bacteroidia	12.20	14.79	Ns
	Actinobacteria	10.77	11.55	Ns
	Clostridia	9.46	10.72	0.041

	Mollicutes	4.89	4.34	Ns
	Alphanobacteria	5.34	5.49	Ns
	Betaproteobacteria	4.40	3.46	Ns
	Negativicutes	1.38	1.65	Ns
	Flavobacteriia	1.33	1.02	Ns
Order				
	Enterobacteriales	15.61	13.27	Ns
	Lactobacillales	14.03	13.07	Ns
	Bacteriodales	12.20	14.79	Ns
	Actinomycetales	9.84	10.12	Ns
	Clostridiales	9.46	10.72	0,041
	Mycoplasmatales	4.86	4.34	Ns
	Bacillales	4.12	3.57	Ns
	Xanthomonadales	3.17	3.49	Ns
	Burkholderiales	2.78	2.26	Ns
	Rhizobiales	2.11	2.34	Ns
	Pseudomonadales	1.95	1.70	Ns
	Selenomonadales	1.38	1.65	Ns

	Flavobacteriales	1.33	1.02	Ns
	Sphingomonadales	1.26	1.31	Ns
Family				
	Enterobacteriaceae	15.62	13.27	Ns
	Porphyromonadaceae	10.42	11.82	Ns
	Corynebacteriaceae	4.79	4.29	Ns
	Mycoplasmataceae	4.79	4.34	Ns
	Lachnospiraceae	4.23	4.77	Ns
	Xanthomonadaceae	3.17	3.51	Ns
	Lactobacillaceae	2.77	3.46	Ns
	Clostridiales Incertae Sedis XI	1.57	2.45	Ns
	Actinomycetaceae	1.54	1.71	Ns
	Streptococcaceae	1.48	3.33	Ns
	Moraxellaceae	1.45	1.04	Ns
	Veillonellaceae	1.39	1.74	Ns
	Flavobacteriaceae	1.33	1.02	Ns
	Prevotellaceae	1.33	1.51	Ns
	Sphingomonadaceae	1.09	1.26	Ns

	Rhodobacteraceae	0.60	1.52	Ns
	Ruminococceae	0.52	107	Ns

Bacterial taxa with a relative abundance >%1 were included. P-value; Ns, not significant (based on P <0.05).

Table 2. Wilcoxon tests comparing first voided urine and midstream urine at genus level

Genus	**First Voide Urine**			**Mid-stream Urine**			
	25th percentile	**Median**	**75th percentile**	**25th percentile**	**Median**	**75th percentile**	**p**
EscherichiaShigella	7.20	8.537	11.96	5.88	6.90	8.48	Ns
Streptococcus	1.19	4.44	5.93	1.03	3.21	3.65	Ns
Ureaplasma	3.31	4.06	4.87	2.45	3.98	4.74	Ns
Corynebacterium	2.22	2.96	3.66	2.04	2.88	3.56	Ns
Stenotrophomonas	1.97	2.66	3.96	2.54	3.04	3.72	Ns
Lactobacillus	0.75	2.65	3.17	2.94	3.18	4.51	Ns
Staphylococcus	0.91	1.42	1.68	0.67	1.13	1.68	Ns
Prevotella	0.84	1.19	1.70	1.02	1.27	1.60	Ns
Peptoniphilus	0.60	0.84	1.06	0.60	0.81	1.00	Ns
Acinetobacter	0.59	0.81	1.12	0.59	0.71	0.85	Ns
Turicella	0.21	0.74	1.02	0.30	0.50	0.88	Ns
Brevundimonas	0.52	0.70	1.00	0.43	0.63	0.85	Ns

Mycoplasma	0.42	0.65	0.95	0.33	0.71	1.01	Ns
Bosea	0.37	0.64	0.87	0.49	0.76	0.94	Ns
Hydrogenophilus	0.40	0.64	1.16	0.47	0.59	0.92	Ns
Dialister	0.08	0.57	1.09	0.57	0.85	1.28	Ns
Actinomyces	0.40	0.56	0.82	0.55	0.74	1.27	Ns
Finegoldia	0.40	0.47	0.71	0.36	0.48	0.86	Ns
Barnesiella	0.11	0.41	0.57	0.25	0.45	0.72	Ns
Methylobacterium	0.29	0.41	0.52	0.36	0.57	0.81	Ns
Mobiluncus	0.00	0.38	0.51	0.13	0.47	0.64	Ns
Bifidobacterium	0.19	0.31	0.41	0.29	0.33	0.46	Ns
Massilia	0.17	0.30	0.41	0.27	0.32	0.40	Ns
Peptostreptococcus	0.00	0.25	0.51	0.12	0.22	0.39	Ns
Mucispirillum	0.10	0.20	0.34	0.00	0.29	0.33	Ns
Pseudocitrobacter	0.03	0.16	0.20	0.02	0.16	0.28	Ns
Propionimicrobium	0.00	0.12	0.25	0.10	0.22	0.39	Ns
Blautia	0.00	0.00	0.05	0.00	0.00	0.10	Ns
Haloferula	0.00	0.00	0.21	0.00	0.04	0.16	Ns

All identified bacterial genera were included. P-value; NS, not significant (based on $P < 0.05$).

Figure 1: Cladogram representation of the urinary microbial taxa.