RESEARCH ARTICLE

Optimizing bacterial DNA extraction in urine

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Abstract

Urine is an acceptable, non-invasive sample for investigating the human urogenital microbiota and for the diagnosis of sexually transmitted infections. However, low quantities of bacterial DNA and PCR inhibitors in urine may prevent efficient PCR amplification for molecular detection of bacteria. Furthermore, cold temperatures used to preserve DNA and bacteria in urine can promote precipitation of crystals that interfere with DNA extraction. Saline, Dulbecco's Phosphate Buffered Saline, or Tris-EDTA buffer were added to urine from adult men to determine if crystal precipitation could be reversed without heating samples beyond ambient temperature. Total bacterial DNA concentrations and PCR inhibition were measured using quantitative PCR assays to compare DNA yields with and without buffer addition. Dissolution of crystals with Tris-EDTA prior to urine centrifugation was most effective in increasing bacterial DNA recovery and reducing PCR inhibition. DNA recovery using Tris-EDTA was further tested by spiking urine with DNA from bacterial isolates and median concentrations of *Lactobacillus jensenii* and *Escherichia coli* 16S rRNA gene copies were found to be higher in urine processed with Tris-EDTA. Maximizing bacterial DNA yield from urine may facilitate more accurate assessment of bacterial populations and increase detection of specific bacteria in the genital tract.

Introduction

Cultivation and molecular approaches have demonstrated that human urine commonly harbors genital tract bacterial commensals and pathogens [1–8]. Urine samples are frequently used to detect sexually transmitted microbes such as *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, *Mycoplasma genitalium* and *Trichomonas vaginalis* using nucleic acid amplification methods [9–13]. Emerging molecular techniques such as nanopore-based metagenomic sequencing are being considered for clinical applications, including testing for bacterial pathogens in urine samples [14]. The role of the urinary tract microbiota in relation to outcomes of clinical importance, such as urinary tract infections [15–21], urinary incontinence [21–27], interstitial cystitis [28,29], bacterial vaginosis [30], urethritis [31], cancer [32–36], type 2

diabetes [17,37,38], kidney disease [39] and kidney transplants [40,41], is an area of active
investigation. Collection of non-invasive urine samples is more acceptable to men than collection of urethral swabs [42] and is a convenient alternative to urethral swabs in studies of the
male urethral microbiota [43]. However, optimal methods for processing urine samples for
PCR-based studies have not been defined [44].

Concentrations of bacteria in urine are typically low compared to other human body sites,
and urine contains PCR inhibitors such as urea, beta-human chorionic gonadotropin, and
crystals that can impede bacterial DNA detection [45,46]. One approach to circumvent low
DNA concentrations is to perform DNA extraction from large volumes of urine (> 30 mL) to
increase DNA yield. However, many types of crystals such as calcium oxalate, uric acid, and
amorphous phosphate or urate crystals can precipitate out of solution at the low temperatures
commonly used to store and preserve urine [47–49]. On centrifugation of urine to concentrate
bacteria, these crystals form large pellets that can interfere with DNA extraction using commercially available kits and using larger urine volumes can exacerbate this problem further
[49]. We have noted lower DNA yields and higher rates of PCR inhibition from urine samples
with large pellets, and others have noted decreased DNA yields and lower DNA quality when
crystals are present in pellets [49]. Urine temperature, pH, and concentrations of solutes all
affect crystal formation [47,48,50]. Urine pH, which ranges from 4.5–8, has a large impact on
crystal formation and can determine which crystals form [51]. For example, uric acid crystals
form in acidic urine (typically pH <5.8), whereas calcium phosphate and amorphous phosphate crystals form in neutral or alkaline urine [47,48,50]. Conversely, calcium oxalate crystals
form in a wide pH range, and are more dependent on urine calcium and oxalate concentrations [50]. Many of these crystals can be dissolved by heating urine specimens [47], but there is
potential for DNA degradation or growth of bacteria with this approach, possibly biasing
results. We sought to develop a method for reversing crystal precipitation in urine while preserving bacterial DNA and minimizing opportunities for bacterial growth, resulting in higher
yield DNA extractions for molecular applications. A secondary goal was to develop a method
that was cheap, efficient, and easily adaptable for high-throughput applications.

Methods

Sample collection

Urine specimens were collected from men with and without nongonococcal urethritis (NGU)
attending the Public Health—Seattle & King County Sexually Transmitted Diseases Clinic in
Seattle, Washington from February 2015 to September 2017 for a study investigating NGU in
men [52]. The Institutional Review Boards at the University of Washington and Fred Hutchinson Cancer Research Center approved the protocols used in the study and the research was
conducted according to all relevant guidelines and regulations. All men enrolled in the study
provided written informed consent. Participants were ≥16 years of age, had exclusive male or
female partners in the last year and had sex in the past two months. Men who had both male
and female partners, no sex in the past 60 days, antibiotics in the past 30 days, known urethral
contact to *Neisseria gonorrhoeae* (NG) or were diagnosed with NG by Gram stain or nucleic
acid amplification testing were not eligible for the study. Men were enrolled into either a
cross-sectional or cohort study and men in the cohort study provided more than one urine
specimen at different time points. At all study visits, men underwent a standard genital examination and collected 30–45 mL first void urine and a urethral swab specimen for Gram staining. All men also completed a computer-assisted self-interview that collected sociobehavioral
and clinical data. The urethral exudates were Gram stained to enumerate polymorphonuclear
neutrophils (PMNs) and examine for the presence of Gram-negative intracellular diplococci

that are indicative of NG. Urine was tested for NG and *Chlamydia trachomatis* (CT) using the Aptima Combo 2 Assay and *Mycoplasma genitalium* (MG) using the Aptima Assay with analyte-specific reagents (Hologic, Marlborough, MA). In this study, we defined NGU as having either urethral symptoms (urethral discharge, dysuria, or other urethral symptoms) or visible discharge on examination with ≥5 PMNs per high power field in the absence of NG. Men without NGU did not have urethral symptoms or urethral discharge and had <5 PMNs/HPF. Urine samples were stored at 4˚C for 1–3 days prior to processing in the laboratory.

Optimization of crystal dissolution

When testing for optimal approaches to process urine, 12 urine samples (33–37.5 mL in volume) from 12 men with noticeable crystal precipitation after refrigeration were split into two aliquots of equal volume, with the first aliquot serving as the pre-treatment sample and the other aliquot diluted with buffer serving as the post-treatment sample. Filtered 0.9% saline (2–15 mL) (AirLife Modudose, CareFusion, San Diego, CA), 1X Dulbecco's Phosphate Buffered Saline (DPBS) (3–15 mL) (Gibco, Grand Island, NY), or molecular grade 1M Tris-ethylenedi-aminetetraacetate (EDTA) (0.5–1.25 mL) (Sigma Aldrich, St. Louis, MO) were each added separately to four different urine samples. The buffers were chosen due to their low cost, wide availability and routine use in clinical labs, as well as their isotonic nature with bacterial cells which would limit any cell lysis and loss of DNA. Additionally, DPBS and Tris-EDTA are buffered at a higher pH than urine, which may help in dissolution of crystals that form at lower pH such as uric acid [48,50]. We further postulated that EDTA may act as a chelating agent to reduce concentrations of Ca^{2+} in urine which would help reduce formation of calcium oxalate crystals [53]. Buffers were added in 1 mL increments (saline, DPBS) or 0.25 mL increments (Tris-EDTA) until crystal dissolution was achieved, and urine samples were brought to room temperature if necessary for complete dissolution of crystals. The urine was centrifuged post-treatment with buffers to concentrate the bacteria at $3645 \times g$ at 4˚C for 10 minutes, the supernatant discarded, and the urine pellet frozen at -80˚C. Urine pH was measured before and after buffer addition with pH strips (mColorpHast™ pH Test Strips, EMD Millipore, Darmstadt, Germany). Mean log10 concentrations of bacterial DNA in pre- and post-treatment samples for each buffer were compared using a paired t-test.

Spiking with DNA from bacterial cultures

An additional four urine specimens with crystal precipitation collected from four men were spiked with known quantities of *Escherichia coli* and *Lactobacillus jensenii* cells grown in culture to determine recovery rates of species-specific bacterial DNA using quantitative PCR (qPCR) with and without Tris-EDTA addition to urine. *E. coli* colonies were isolated on Luria Broth (LB) agar plates (Fisher Scientific, Waltham, MA) and grown aerobically in LB broth (Fisher Scientific, Waltham, MA). *L. jensenii* colonies were isolated on Brucella Blood Agar plates (Hardy Diagnostics, Santa Maria, CA) and grown aerobically in De Man, Rogosa and Sharpe (MRS) broth (Becton Dickinson and Company, Franklin Lakes, NJ). Colony Forming Units (CFU) of each culture were compared to Optical Density (OD) measurements with a spectrophotometer to estimate concentrations of bacteria in liquid cultures.

Urine specimens were divided into four aliquots of equal volume. Two aliquots were spiked to a final target concentration of 1×10^6 CFUs/mL urine each with both *E. coli* and *L. jensenii* cultures, while the remaining two aliquots were not spiked with bacterial cultures. One culture-spiked aliquot of urine and one non-spiked aliquot were processed with addition of 10% by volume Tris-EDTA. The other aliquots were not treated with Tris-EDTA. Additionally, a positive control was employed to determine the maximum DNA yield, wherein equivalent

quantities of both *E. coli* and *L. jensenii* cells were subjected to DNA extraction in the absence of urine. Comparisons of log10 concentrations of bacterial DNA between the groups were made using the paired t-test. All urine aliquots were pelleted and frozen as previously described.

Application of optimized approach to clinical samples

The optimized protocol of 10% addition by volume of 1M Tris-EDTA was applied to additional male urine specimens (n = 998 from 273 men) with urine sample volumes ranging from 30–42 mL (median = 36 mL). This set of urine samples was processed as described previously, but no pre- and post-treatment comparisons were performed. We estimated the association between NGU and log10 concentration of bacterial DNA using generalized estimating equations, specifying a Gaussian model, an identity link function, robust standard errors, and an exchangeable covariance matrix.

Molecular methods

DNA was extracted from urine pellets using the BiOstic Bacteremia DNA Isolation Kit (Mobio, Carlsbad, CA). DNA was eluted in 120 μL of half CB5 buffer and half-filtered 0.2X Tris-EDTA. Sham extraction controls were included to monitor for potential contamination during processing and DNA extraction of urine pellets. PCR inhibition was monitored using an internal amplification control (IAC) qPCR assay [54] and samples were considered inhibited if delayed by $\geq$ 2.0 cycles. Bacterial DNA concentrations were measured using a TaqMan based qPCR assay targeting a portion of the 16S rRNA gene [55] with a lower limit of detection of 5 gene copies/μL DNA. *E. coli* DNA concentrations were measured with a TaqMan based qPCR assay targeting the *E. coli* 16S rRNA gene with primers (47F 5′–GAACGGTAACAGGAA KCAGCT–3′ and 194R 5′– ATCCGATGGCAAGAGGCC –3′) and hydrolysis probe (5′– FAM–CGCATAACGTCGCAAGACCAAAGAGG–TAMRA–3′). Reactions underwent 45 cycles of amplification on the Applied Biosystems StepOnePlus Real-Time PCR System with a 95°C melt for 15 seconds and 65°C anneal/extension for 1 minute. Core reagents were supplied by Applied Biosystems (Carlsbad, CA) and master mixes contained buffer A (1X), deoxynucleotide triphosphates (1 mM), magnesium (3 mM), AmpErase uracil-N-glycosylase (0.05 U) and AmpliTaq Gold polymerase (1.0 U) per reaction. Primers were added at 0.8 μM per reaction and probe at 150 nM per reaction. *E. coli* 16S rRNA gene plasmid standards were run for each reaction with a lower limit of detection of 5 gene copies/μL DNA. *L. jensenii* DNA concentrations were measured using a TaqMan based qPCR assay targeting the 16S rRNA gene [56] with a lower limit of detection of 1.25 gene copies/μL DNA.

Results

Optimization of crystal dissolution

The following metrics were used to evaluate each approach: visual disappearance of crystals in urine, volume of buffer added, shifts in pH, DNA yields, and absence or reduction of PCR inhibition. Addition of saline, DPBS, and Tris-EDTA dissolved precipitated crystals in each urine sample, but required varying amounts of buffer for complete dissolution, with Tris-EDTA requiring the least amount of buffer by volume (Fig 1 and Table 1). Urine pH was not impacted by the addition of saline or DPBS but was higher after the addition of Tris-EDTA (Table 1). Only aliquots treated with Tris-EDTA demonstrated mean post-treatment bacterial DNA quantities that were significantly higher than quantities in pre-treatment aliquots (mean 4.98×10^2 copies/mL pre vs. 4.47×10^4 copies/mL post, p = 0.02). There were no significant
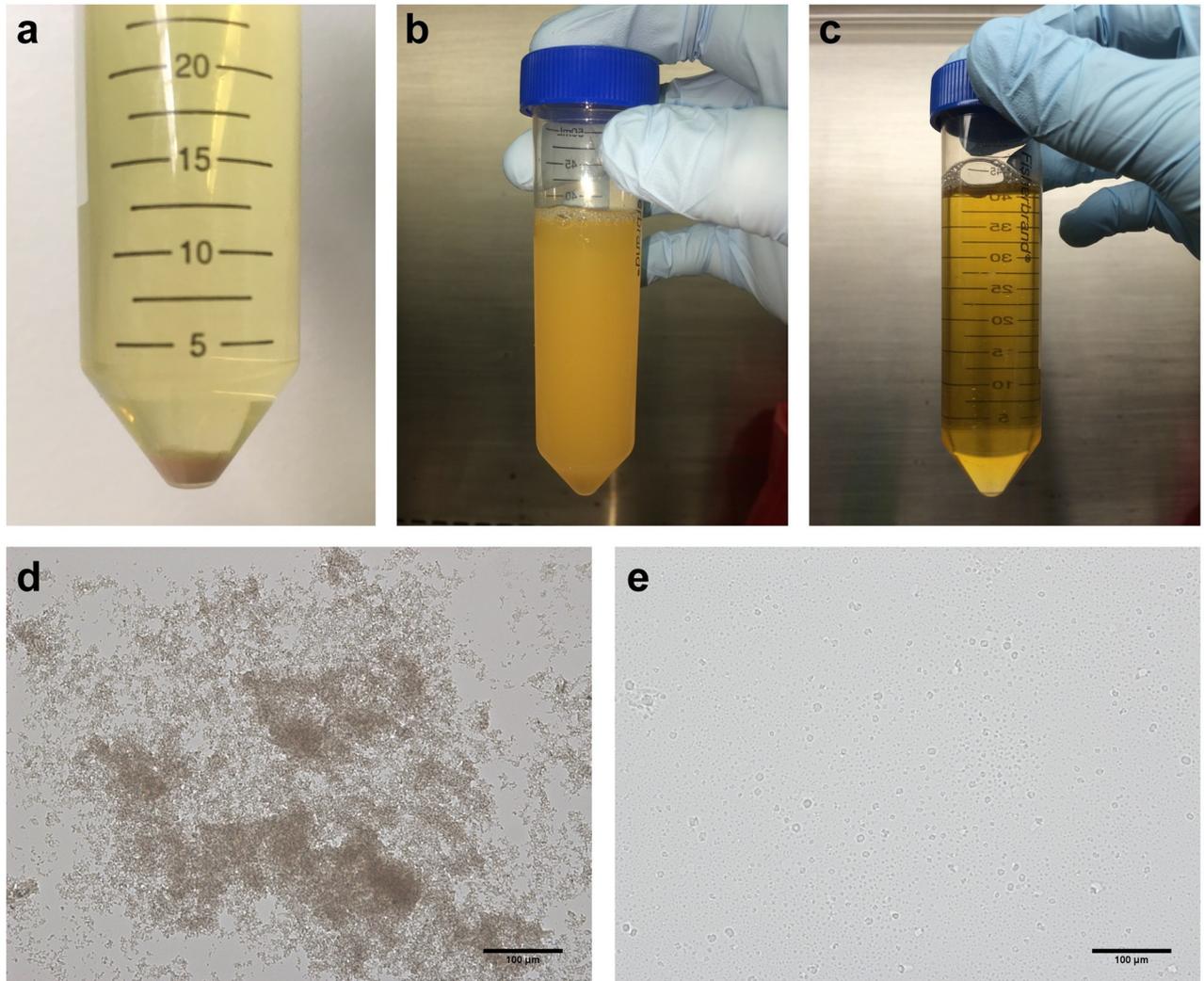
Fig 1. Images and microscopy of urine crystal precipitation and dissolution. (a) Pellet from refrigerated urine sample with crystal precipitation. After centrifugation, pellet is pink in appearance. (b) Refrigerated urine sample with crystal precipitation before and (c) three minutes after Tris-EDTA addition. (d) Microscopic view (20X) of crystals from urine pellet without and (e) with Tris-EDTA addition.

differences in aliquots treated with either saline (2.49×10^3 vs. 9.22×10^3, p = 0.26) or DPBS (8.43×10^3 vs. 2.54×10^4, p = 0.11) (Fig 2). The 16S rRNA gene copies in blank buffer samples were at or below the detection threshold of 20 copies/mL. PCR inhibition was reduced in three samples with addition of saline or Tris-EDTA, but not DPBS. PCR inhibition was not detected in any other samples.

Spiking with bacterial cultures

E. coli and *L. jensenii* were not detected by qPCR in any non-spiked urine aliquots. *E. coli* detection in spiked samples was higher in urine processed with Tris-EDTA compared to samples without (mean 1.9×10^6 copies/mL with Tris-EDTA vs. 5.0×10^4 copies/mL, p = 0.08) (Fig 3). Similarly, *L. jensenii* detection was significantly higher in urine processed with Tris-EDTA (mean 2.8×10^6 copies/mL with Tris-EDTA vs. 6.1×10^2 copies/mL, p = 0.04) (Fig 3). When compared to positive controls of spiked culture, which we considered as 100% efficient, mean

Table 1. Volume of buffer required to dissolve urine crystals and urine pH pre- and post-treatment.

| Sample ID	Buffer	Volume Buffer Required (mL)	Percent Buffer Required (v/v)*	Pre-Treatment Urine pH	Post-Treatment Urine pH
Sample 1	Saline	2.0	12.1	5.5	5.5
Sample 2		15.0	88.2	5.5	5.5
Sample 3		5.0	27.0	6.5	6.5
Sample 4		8.0	44.4	5.5	5.5
Sample 5	DBPS	4.0	24.2	5.5	5.5
Sample 6		5.0	29.4	5.3	5.3
Sample 7		15.0	85.7	5.5	5.5
Sample 8		3.0	16.7	5.5	5.5
Sample 9	Tris-EDTA	0.5	2.7	5.5	6.5
Sample 10		0.75	4.2	5.5	6.1
Sample 11		1.0	5.6	5.3	6.5
Sample 12		1.25	6.7	5.3	6.5

*v/v indicates volume/volume.

percent yield was 57.5% (range 38.3–68.7%) for *E. coli* in samples treated with Tris-EDTA compared to 1.8% (range 0–3.5%) in samples not treated with Tris-EDTA. Mean percent yield of *L. jensenii* was 33.5% (range 0.11–89.0%) for samples treated with Tris-EDTA compared to 0.02% (range 0–0.06%) for samples not treated with Tris-EDTA.

Application of optimized approach to clinical samples

Based on the metrics that were laid out, processing of urine samples with Tris-EDTA was selected as the optimal approach because addition of only 6.7% of the urine volume was
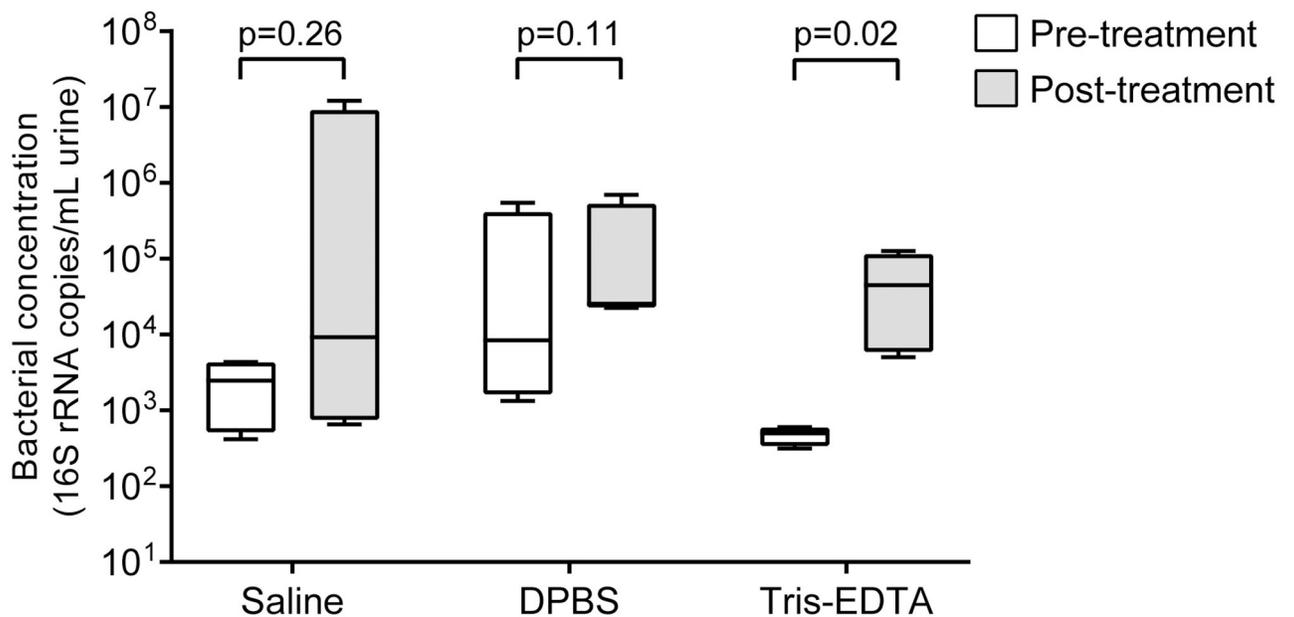
Fig 2. Bacterial DNA concentrations in urine samples before and after treatment with saline, DPBS, or Tris-EDTA. Data shown as box and whisker plots with whiskers representing maximum and minimum values (16S rRNA gene copies/mL of urine). Lines within each box represent median values. Mean bacterial concentrations in post-treatment samples treated with Tris-EDTA were found to be significantly higher.
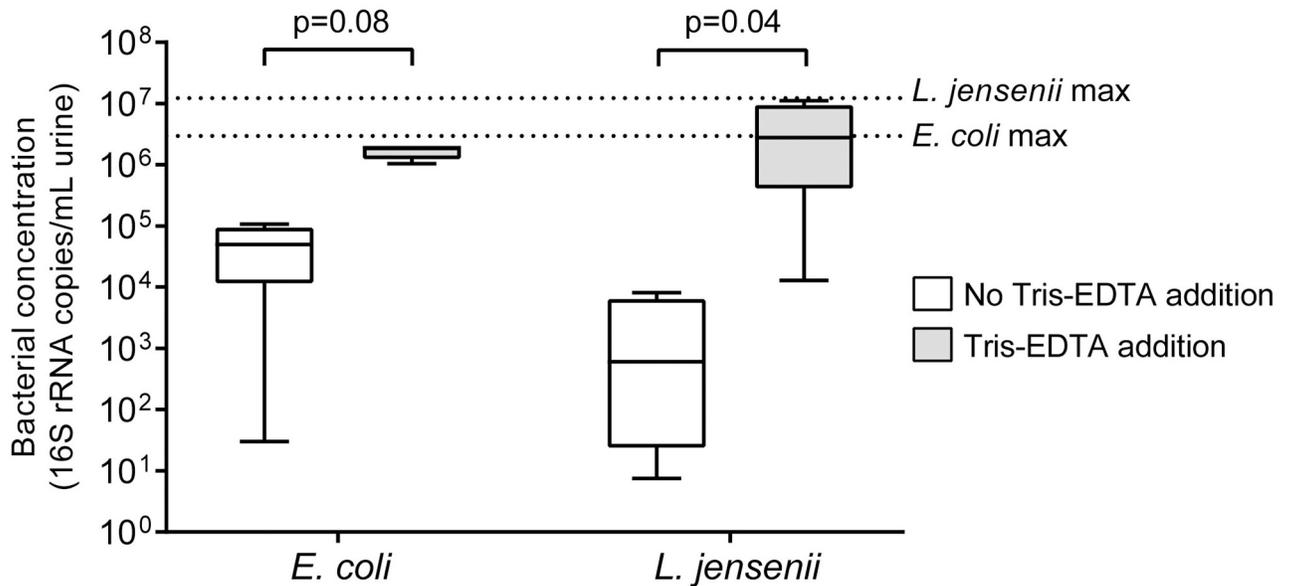
Fig 3. Bacterial DNA recovery from spiked urine with and without Tris-EDTA addition. Data shown as box and whisker plots with whiskers representing maximum and minimum values (16S copies/mL urine). Lines within each box represent median values. Dotted lines represent 100% yield based on spiked positive controls. Mean *Escherichia coli* and *Lactobacillus jensenii* DNA recovery in samples treated with Tris-EDTA were found to be higher than in samples not treated with Tris-EDTA.

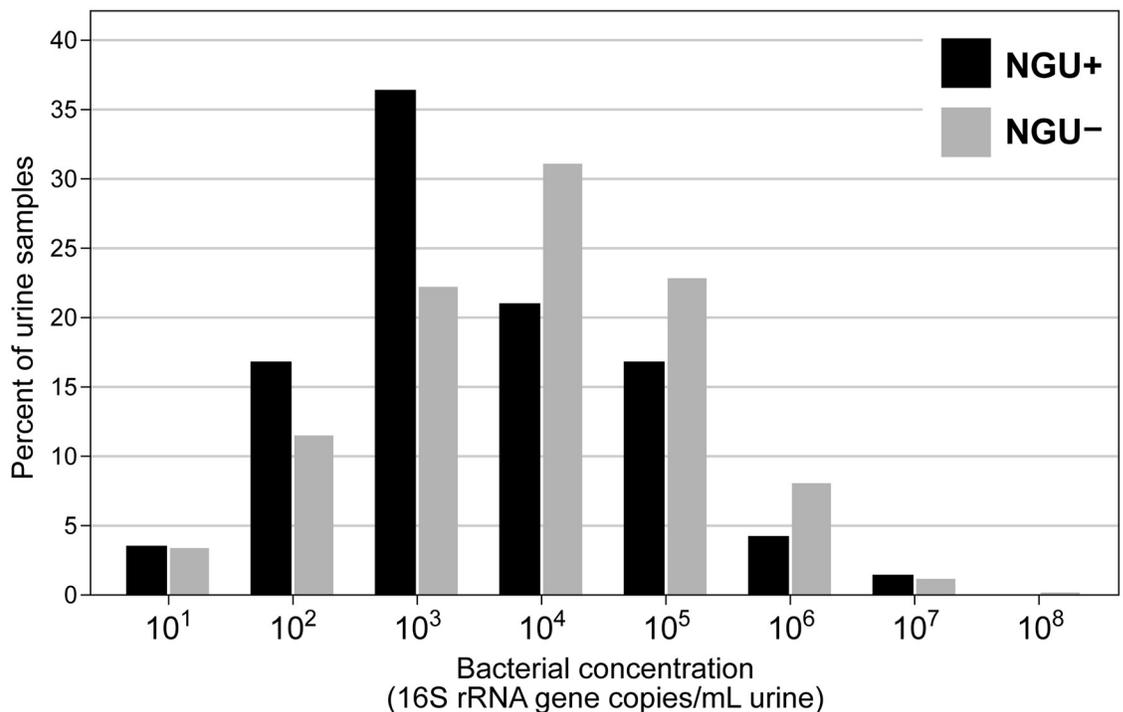https://doi.org/10.1371/journal.pone.0222962.g003

Fig 4. Distribution of bacterial DNA extracted from urine samples treated with 10% v/v Tris-EDTA. Data shown for 955 urine specimens including 143 specimens that were from NGU+ men and 812 specimens that were from NGU- men. An additional 43 samples had bacterial concentrations below the lower limit of detection (16 = NGU+ men and 27 = NGU- men). Bacterial concentrations were significantly lower in men with NGU.

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required to dissolve crystals in the most concentrated sample tested (Table 1). To account for urine samples with potentially more crystal precipitation and to improve throughput, we increased the amount of buffer added to 10% by volume and applied this protocol to an additional 998 urine specimens. Of 998 specimens, 159 (15.9%) were collected at time points when men had NGU. Bacterial concentrations in all samples ranged from 1.66×10^1–1.05×10^8 gene copies/mL urine, with a mean of 7.13×10^5 gene copies/mL urine and a median of 2.38×10^4 gene copies/mL urine in 955 samples with concentrations above the lower limit of detection (Fig 4). Buffer controls were included with every batch of DNA extraction from 998 urine samples to determine if there was any contaminant DNA from water or extraction reagents. These negative controls contained 30 mL filtered water and 3 mL Tris-EDTA which were extracted in the same manner as the test urine specimens. Mean 16S rRNA gene copies in these extraction controls was 5.87 copies/mL, which is just above detection threshold. PCR inhibition was detected in 9.1% of samples and was alleviated when DNA was diluted (up to 1:20 dilution) in all but two samples. Bacterial concentrations in urine from NGU positive men ranged from 2.24×10^1–2.22×10^7 gene copies/mL urine with a mean of 4.81×10^5 gene copies/mL urine and a median of 7.30×10^3 gene copies/mL urine, while bacterial concentrations in urine from NGU negative men ranged from 1.66×10^1–1.05×10^8 gene copies/mL urine with a mean of 7.53×10^5 gene copies/mL urine and a median of 2.77×10^4 gene copies/mL urine (Fig 4). Bacterial DNA concentrations of urine from men with NGU was 0.61 times lower than concentrations in urine from men without NGU (95% confidence interval = 0.38–0.97, p = 0.04).

Discussion

Urine is a convenient sample that is useful for defining the genital tract microbiota of men using molecular approaches. Obtaining high concentrations of quality DNA is essential to maximize bacterial detection and identification. Urine is frequently refrigerated or frozen to prevent bacterial overgrowth and to prevent degradation of nucleic acids. However, subjecting urine to low temperatures can lead to precipitation of crystals, which may reduce DNA yields and cause inhibition in downstream molecular applications. Heating urine to dissolve crystals is an approach that is often recommended. For example, the APTIMA Combo 2® Assay protocol for *C. trachomatis* and *N. gonorrhoeae* testing recommends heating urine to 37°C for up to 5 minutes to dissolve any visible urine precipitates if present (Protocol 502446 Rev. 002, Marlborough, MA). However, heating the urine can potentially lead to bacterial lysis, DNA degradation, or overgrowth of bacteria. It has also been reported that sufficient mixing at room temperature can dissolve crystals formed in urine [53,57], but we found that this approach cannot fully dissolve all crystals in every sample. In this study, we developed an efficient method that can be applied to most urine samples to dissolve crystals prior to urine centrifugation and DNA extraction, resulting in higher bacterial DNA yields and reduced PCR inhibition.

Crystal formation in urine is linked to urine pH, temperature, and concentrations of particular ions. Crystals such as uric acid and amorphous urate crystals are pH dependent and tend to form in acidic urine [47,48], and these crystals typically form pink pellets on centrifugation [47,49,53] which we noted in our study as well (Fig 1). We hypothesized that increasing the pH would result in dissolution of these crystals. We used small volumes of Tris-EDTA to raise urine pH, resulting in crystal dissolution (Fig 1). In contrast, larger volumes of saline and DPBS were required for dissolution of crystals and no increase of pH was noted (Table 1), suggesting that any salutary effect was likely due to dilution alone. Calcium oxalate crystals can form with sufficient Ca^{2+} concentrations in urine and this process is not pH dependent [50]. Saetun et al. found that calcium oxalate and larger amorphous crystals were common in frozen urine samples, and that addition of EDTA to urine pellets reduced pellet size to ~25% of the

starting amount [53]. EDTA is a known chelating agent that can remove cations such as Ca^{2+} and Mg^{2+} from solution and may aid in dissolving calcium oxalate crystals by reducing calcium concentrations in urine. Calcium and magnesium ions can also form urate crystals [47], and the chelation of these cations could be important in reversing crystal formation. Moreover, calcium is a known PCR inhibitor that can compete with magnesium to bind to DNA polymerases [58]. Hence, we used Tris-EDTA with the dual purpose of increasing pH and chelating Ca^{2+} and Mg^{2+} ions. We found that the buffer was also effective in reducing PCR inhibition suggesting that chelation of Ca^{2+} and Mg^{2+} ions prior to DNA extraction may be an important step in urine sample processing.

In this study, we processed 998 urine samples with addition of 10% v/v Tris-EDTA and found that bacterial concentrations in these samples ranged from 1.66×10^1–1.05×10^8 16S rRNA gene copies/mL urine with a mean of 7.13×10^5 gene copies/mL urine and a median of 2.38×10^4 gene copies/mL urine (Fig 4). All urine in this study was obtained from adult men given that this was a study of NGU in men. Some men were diagnosed with NGU, and samples obtained from these men had a lower concentration of bacterial DNA (Fig 4). Our goal for this study was to optimize DNA extraction of urine samples from men, and hence we did not test any samples from women in this study. This protocol will need to be validated if applied to urine samples collected from women. In addition to measuring total bacterial concentrations, we also monitored PCR inhibition in the samples. Of the 998 samples that we tested, 9.1% exhibited PCR inhibition, although inhibition was overcome with up to a 1:20 dilution of DNA. One potential inhibitor in urine is urea, which can be removed from urine samples by dialysis or ultrafiltration [45], but the approach is not high-throughput and hence not tested in this study.

Extraction efficiency of our optimized approach was determined by spiking urine samples with cultures from a Gram-negative bacterium, *E. coli*, and a Gram-positive bacterium, *L. jensenii*. DNA recovery from both bacteria was found to be higher in urine aliquots treated with Tris-EDTA than in those without (Fig 3). These data suggest that both Gram-negative and Gram-positive bacteria can be recovered with greater efficiency using our optimized approach. One important limitation is that we did not perform broad-range amplicon PCR and deep sequencing on matched samples to determine if overall bacterial community representation is improved with the addition of Tris-EDTA buffer. Hence, we cannot conclude that this approach will improve yields for all bacteria present in urine. Another limitation of this test was that we compared efficiencies in only four urine samples because urine was collected primarily for questions related to the microbiota and NGU, restricting samples available for optimization of DNA extraction. Despite the limited number of samples tested for extraction efficiency, we showed increased concentrations of bacterial isolate DNA in each of the four samples treated with Tris-EDTA compared to samples that were not treated.

Low DNA yield and PCR inhibition are common problems when extracting bacterial DNA from urine and refrigerating or freezing urine can lead to crystal precipitation that exacerbates these problems. Adding Tris-EDTA to urine samples to increase pH and reduce concentrations of inhibitors prior to DNA extraction is a cost effective, high-throughput solution for improving recovery of bacterial DNA for diagnostic testing and molecular applications aimed at increasing our understanding of bacterial communities in the genital tract.

Supporting information

S1 Data. 16S rRNA gene copies/mL of urine for Figs 2 and 3 and 16S rRNA gene copies with NGU status for Fig 4.
(XLSX)

Acknowledgments

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References

1. Nelson DE, Van Der Pol B, Dong Q, Revanna KV, Fan B, Easwaran S, et al. Characteristic male urine microbiomes associate with asymptomatic sexually transmitted infection. PLoS One. 2010; 5:1–7. https://doi.org/10.1371/journal.pone.0014116 PMID: 21124791

2. Siddiqui H, Nederbragt AJ, Lagesen K, Jeansson SL, Jakobsen KS. Assessing diversity of the female urine microbiota by high throughput sequencing of 16S rDNA amplicons. BMC Microbiol. 2011; 11:1–12.

3. Wolfe AJ, Toh E, Shibata N, Rong R, Kenton K, Fitzgerald M, et al. Evidence of uncultivated bacteria in the adult female bladder. J Clin Microbiol. 2012; 50:1376–1383. https://doi.org/10.1128/JCM.05852-11 PMID: 22278835

4. Lewis DA, Brown R, Williams J, White P, Jacobson SK, Marchesi J, et al. The human urinary microbiome; bacterial DNA in voided urine of asymptomatic adults. Front Cell Infect Microbiol. 2013; 3:41. https://doi.org/10.3389/fcimb.2013.00041 PMID: 23967406

5. Hilt EE, McKinley K, Pearce MM, Rosenfeld AB, Zilliox MJ, Mueller ER, et al. Urine is not sterile: use of enhanced urine culture techniques to detect resident bacterial flora in the adult female bladder. J Clin Microbiol. 2014; 52:871–876. https://doi.org/10.1128/JCM.02876-13 PMID: 24371246

6. Price TK, Dune T, Hilt EE, Thomas-White KJ, Kliethermes S, Brincat C, et al. The clinical urine culture: enhanced techniques improve detection of clinically relevant microorganisms. J Clin Microbiol. 2016; 54:1216–1222. https://doi.org/10.1128/JCM.00044-16 PMID: 26962083

7. Thomas-White K, Brady M, Wolfe AJ, Mueller ER. The bladder is not sterile: History and current discoveries on the urinary microbiome. Curr Bladder Dysfunct Rep. 2016; 11:18–24. https://doi.org/10.1007/s11884-016-0345-8 PMID: 27182288

8. Thomas-White K, Forster SC, Kumar N, Van Kuiken M, Putonti C, Stares MD, et al. Culturing of female bladder bacteria reveals an interconnected urogenital microbiota. Nat Commun. 2018; 9:1557. https://doi.org/10.1038/s41467-018-03968-5 PMID: 29674608

9.   Papp JR, Schachter J, Gaydos CA, Van Der Pol B. Recommendations for the laboratory-based detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae*—2014. MMWR. Recomm. Rep. 2014; 63:1–19. Available from: http://www.cdc.gov/mmwr/preview/mmwrhtml/rr6302a1.htm

10. Meyer T. Diagnostic procedures to detect *Chlamydia trachomatis* infections. Microorganisms. 2016; 4:25. https://doi.org/10.3390/microorganisms4030025 PMID: 27681919

11. Napierala M, Munson E, Wenten D, Phipps P, Gremminger R, Schuknecht MK, et al. Detection of *Mycoplasma genitalium* from male primary urine specimens: an epidemiologic dichotomy with *Trichomonas vaginalis*. Diagn Microbiol Infect Dis. 2015; 82:194–198. https://doi.org/10.1016/j.diagmicrobio.2015.03.016 PMID: 25934156

12. Van Der Pol B. Clinical and laboratory testing for *Trichomonas vaginalis* infection. J Clin Microbiol. 2016; 54:7–12. https://doi.org/10.1128/JCM.02025-15 PMID: 26491181

13. Mckechnie ML, Hillman R, Couldwell D, Kong F, Freedman E, Wang H, et al. Simultaneous identification of 14 genital microorganisms in urine by use of a multiplex PCR-based reverse line blot assay. J Clin Microbiol. 2009; 47:1871–1877. https://doi.org/10.1128/JCM.00120-09 PMID: 19357202

14. Schmidt K, Mwaigwisya S, Crossman LC, Doumith M, Munroe D, Pires C, et al. Identification of bacterial pathogens and antimicrobial resistance directly from clinical urines by nanopore-based metagenomic sequencing. J Antimicrob Chemother. 2017; 72:104–114. https://doi.org/10.1093/jac/dkw397 PMID: 27667325

15. Nienhouse V, Gao X, Dong Q, Nelson DE, Toh E, McKinley K, et al. Interplay between bladder microbiota and urinary antimicrobial peptides: mechanisms for human urinary tract infection risk and symptom severity. PLoS One. 2014; 9:e114185. https://doi.org/10.1371/journal.pone.0114185 PMID: 25486068

16. Horwitz D, McCue T, Mapes AC, Ajami NJ, Petrosino JF, Ramig RF, et al. Decreased microbiota diversity associated with urinary tract infection in a trial of bacterial interference. J Infect. 2015; 71:358–367. https://doi.org/10.1016/j.jinf.2015.05.014 PMID: 26048203

17. Liu F, Ling Z, Xiao Y, Yang Q, Zheng L, Jiang P, et al. Characterization of the urinary microbiota of elderly women and the effects of type 2 diabetes and urinary tract infections on the microbiota. Oncotarget. 2017; 8:100678–100690. https://doi.org/10.18632/oncotarget.21126 PMID: 29246012

18. Moustafa A, Li W, Singh H, Moncera KJ, Torralba MG, Yu Y, et al. Microbial metagenome of urinary tract infection. Sci Rep. 2018; 8:4333. https://doi.org/10.1038/s41598-018-22660-8 PMID: 29531289

19. Thomas-White KJ, Gao X, Lin H, Fok CS, Ghanayem K, Mueller ER, et al. Urinary microbes and postoperative urinary tract infection risk in urogynecologic surgical patients. Int Urogynecol J. 2018; 29:1797–1805. https://doi.org/10.1007/s00192-018-3767-3 PMID: 30267143

20. Fok CS, Gao X, Lin H, Thomas-White KJ, Mueller ER, Wolfe AJ, et al. Urinary symptoms are associated with certain urinary microbes in urogynecologic surgical patients. Int Urogynecol J. 2018; 29:1765–1771. https://doi.org/10.1007/s00192-018-3732-1 PMID: 30116843

21. Chen Z, Phan M-D, Bates LJ, Peters KM, Mukerjee C, Moore KH, et al. The urinary microbiome in patients with refractory urge incontinence and recurrent urinary tract infection. Int Urogynecol J. 2018; 29:1775–1782. https://doi.org/10.1007/s00192-018-3679-2 PMID: 29946828

22. Pearce MM, Zilliox MJ, Thomas-White KJ, Richter HE, Nager CW, Visco AG, et al. The female urinary microbiota in urgency urinary incontinence. Am J Obstet Gynecol. 2015; 213:347.e1–347.e11. https://doi.org/10.1016/j.ajog.2015.07.009 PMID: 26210757

23. Karstens L, Asquith M, Davin S, Stauffer P, Fair D, Gregory WT, et al. Does the urinary microbiome play a role in urgency urinary incontinence and its severity? Front Cell Infect Microbiol. 2016; 6:78. https://doi.org/10.3389/fcimb.2016.00078 PMID: 27512653

24. Thomas-White KJ, Hilt EE, Fok C, Pearce MM, Mueller ER, Kliethermes S, et al. Incontinence medication response relates to the female urinary microbiota. Int Urogynecol J. 2016; 27:723–733. https://doi.org/10.1007/s00192-015-2847-x PMID: 26423260

25. Thomas-White KJ, Kliethermes S, Rickey L, Lukacz ES, Richter HE, Moalli P, et al. Evaluation of the urinary microbiota of women with uncomplicated stress urinary incontinence. Am J Obstet Gynecol. 2017; 216:55.e1–55.e16. https://doi.org/10.1016/j.ajog.2016.07.049 PMID: 27498309

26. Komesu YM, Richter HE, Carper B, Dinwiddie DL, Lukacz ES, Siddiqui NY, et al. The urinary microbiome in women with mixed urinary incontinence compared to similarly aged controls. Int Urogynecol J. 2018; 29:1785–1795. https://doi.org/10.1007/s00192-018-3683-6 PMID: 29909556

27. Wu P, Chen Y, Zhao J, Zhang G, Chen J, Wang J, et al. Urinary microbiome and psychological factors in women with overactive bladder. Front Cell Infect Microbiol. 2017; 7:488. https://doi.org/10.3389/fcimb.2017.00488 PMID: 29230385

28. Siddiqui H, Lagesen K, Nederbragt AJ, Jeansson SL, Jakobsen KS. Alterations of microbiota in urine from women with interstitial cystitis. BMC Microbiol. 2012; 12:205. https://doi.org/10.1186/1471-2180-12-205 PMID: 22974186

29. Abernethy MG, Rosenfeld A, White JR, Mueller MG, Lewicky-Gaupp C, Kenton K. Urinary microbiome and cytokine levels in women with interstitial cystitis. Obstet Gynecol. 2017; 129:500–506. https://doi.org/10.1097/AOG.0000000000001892 PMID: 28178051

30. Gottschick C, Deng Z-L, Vital M, Masur C, Abels C, Pieper DH, et al. The urinary microbiota of men and women and its changes in women during bacterial vaginosis and antibiotic treatment. Microbiome. 2017; 5:99. https://doi.org/10.1186/s40168-017-0305-3 PMID: 28807017

31. Frølund M, Wikström A, Lidbrink P, Abu Al-Soud W, Larsen N, Harder CB, et al. The bacterial microbiota in first-void urine from men with and without idiopathic urethritis. PLoS One. 2018; 13:e0201380. https://doi.org/10.1371/journal.pone.0201380 PMID: 30052651

32. Wang H, Altemus J, Niazi F, Green H, Calhoun BC, Sturgis C, et al. Breast tissue, oral and urinary microbiomes in breast cancer. Oncotarget. 2017; 8:88122–88138. https://doi.org/10.18632/oncotarget.21490 PMID: 29152146

33. Shrestha E, White JR, Yu S-H, Kulac I, Ertunc O, De Marzo AM, et al. Profiling the urinary microbiome in men with positive versus negative biopsies for prostate cancer. J Urol. 2018; 199:161–171. https://doi.org/10.1016/j.juro.2017.08.001 PMID: 28797714

34. Wu P, Zhang G, Zhao J, Chen J, Chen Y, Huang W, et al. Profiling the urinary microbiota in male patients with bladder cancer in China. Front Cell Infect Microbiol. 2018; 8:167. https://doi.org/10.3389/fcimb.2018.00167 PMID: 29904624

35. Bučević Popović V, Šitum M, Chow C-ET, Chan LS, Roje B, Terzić J. The urinary microbiome associated with bladder cancer. Sci Rep. 2018; 8:12157. https://doi.org/10.1038/s41598-018-29054-w PMID: 30108246

36. Alanee S, El-Zawahry A, Dynda D, Dabaja A, McVary K, Karr M, et al. A prospective study to examine the association of the urinary and fecal microbiota with prostate cancer diagnosis after transrectal biopsy of the prostate using 16sRNA gene analysis. Prostate. 2019; 79:81–87. https://doi.org/10.1002/pros.23713 PMID: 30117171

37. Liu F, Ling Z, Xiao Y, Yang Q, Wang B, Zheng L, et al. Alterations of urinary microbiota in type 2 diabetes mellitus with hypertension and/or hyperlipidemia. Front Physiol. 2017; 8:126. https://doi.org/10.3389/fphys.2017.00126 PMID: 28316574

38. Ling Z, Liu F, Shao L, Cheng Y, Li L. Dysbiosis of the urinary microbiota associated with urine levels of proinflammatory chemokine interleukin-8 in female type 2 diabetic patients. Front Immunol. 2017; 8:1032. https://doi.org/10.3389/fimmu.2017.01032 PMID: 28943876

39. Kramer H, Kuffel G, Thomas-White K, Wolfe AJ, Vellanki K, Leehey DJ, et al. Diversity of the midstream urine microbiome in adults with chronic kidney disease. Int Urol Nephrol. 2018; 50:1123–1130. https://doi.org/10.1007/s11255-018-1860-7 PMID: 29651696

40. Rani A, Ranjan R, McGee HS, Andropolis KE, Panchal DV, Hajjiri Z, et al. Urinary microbiome of kidney transplant patients reveals dysbiosis with potential for antibiotic resistance. Transl Res. 2017; 181:59–70. https://doi.org/10.1016/j.trsl.2016.08.008 PMID: 27669488

41. Wu JF, Muthusamy A, Al-Ghalith GA, Knights D, Guo B, Wu B, et al. Urinary microbiome associated with chronic allograft dysfunction in kidney transplant recipients. Clin Transplant. 2018; 32:e13436. https://doi.org/10.1111/ctr.13436 PMID: 30372560

42. Apoola A, Herrero-Diaz M, FitzHugh E, Rajakumar R, Fakis A, Oakden J. A randomised controlled trial to assess pain with urethral swabs. Sex Transm Infect. 2011; 87:110–113. https://doi.org/10.1136/sti.2010.042861 PMID: 21131307

43. Dong Q, Nelson DE, Toh E, Diao L, Gao X, Fortenberry JD, et al. The microbial communities in male first catch urine are highly similar to those in paired urethral swab specimens. PLoS One. 2011; 6:1–5. https://doi.org/10.1371/journal.pone.0019709 PMID: 21603636

44. Karstens L, Asquith M, Caruso V, Rosenbaum JT, Fair DA, Braun J, et al. Community profiling of the urinary microbiota: considerations for low-biomass samples. Nat Rev Urol. 2018; 15:735–749. https://doi.org/10.1038/s41585-018-0104-z PMID: 30315209

45. Khan G, Kangro HO, Coates PJ, Heath RB. Inhibitory effects of urine on the polymerase chain reaction for cytomegalovirus DNA. J Clin Pathol. 1991; 44:360–365. https://doi.org/10.1136/jcp.44.5.360 PMID: 1646235

46. Mahony J, Chong S, Jang D, Luinstra K, Faught M, Dalby D, et al. Urine specimens from pregnant and nonpregnant women inhibitory to amplification of *Chlamydia trachomatis* nucleic acid by PCR, ligase chain reaction, and transcription-mediated amplification: identification of urinary substances associated with inhibition. J Clin Microbiol. 1998; 36:3122–3126. PMID: 9774550

47. Brunzel NA. Fundamentals of Urine and Body Fluid Analysis. 4th ed. St. Loius: Elsevier; 2018.

48. Fogazzi GB. Crystalluria: a neglected aspect of urinary sediment analysis. Nephrol Dial Transplant. 1996; 11:379–387. https://doi.org/10.1093/oxfordjournals.ndt.a027276 PMID: 8671802

49. Ackerman AL, Anger JT, Khalique MU, Ackerman JE, Tang J, Kim J, et al. Optimization of DNA extraction from human urinary samples for mycobiome community profiling. PLoS One. 2019; 14: e0210306. https://doi.org/10.1371/journal.pone.0210306 PMID: 31022216

50. Daudon M, Frochot V. Crystalluria. Clin Chem Lab Med. 2015; 53:s1479–s1487. https://doi.org/10.1515/cclm-2015-0860 PMID: 26509782

51. Simerville J, Maxted W, Pahira J. Urinalysis: a comprehensive review. Am Fam Physician. 2005; 71:1153–1162. PMID: 15791892

52. Chambers LC, Morgan JL, Lowens MS, Robinson TS, Romano SS, Leipertz GL, et al. Cross-sectional study of urethral exposures at last sexual episode associated with non-gonococcal urethritis among STD clinic patients. Sex Transm Infect. 2018;sextrans-2018-053634. https://doi.org/10.1136/sextrans-2018-053634 PMID: 30181326

53. Saetun P, Semangoen T, Thongboonkerd V. Characterizations of urinary sediments precipitated after freezing and their effects on urinary protein and chemical analyses. Am J Physiol Renal Physiol. 2009; 296:F1346–F1354. https://doi.org/10.1152/ajprenal.90736.2008 PMID: 19339629

54. Khot PD, Ko DL, Hackman RC, Fredricks DN. Development and optimization of quantitative PCR for the diagnosis of invasive aspergillosis with bronchoalveolar lavage fluid. BMC Infect Dis. 2008; 8:1–13.

55. Srinivasan S, Hoffman NG, Morgan MT, Matsen FA, Fiedler TL, Hall RW, et al. Bacterial communities in women with bacterial vaginosis: high resolution phylogenetic analyses reveal relationships of microbiota to clinical criteria. PLoS One. 2012; 7:1–15. https://doi.org/10.1371/journal.pone.0037818 PMID: 22719852

56. Srinivasan S, Liu C, Mitchell CM, Fiedler TL, Thomas KK, Agnew KJ, et al. Temporal variability of human vaginal bacteria and relationship with bacterial vaginosis. PLoS One. 2010; 5:e10197. https://doi.org/10.1371/journal.pone.0010197 PMID: 20419168

57. Haber MH, Ward PCJ. Urine. In: McClatchey KD, editor. Clinical Laboratory Medicine. 2nd ed. Philadelphia: Lippincott Williams and Wilkins; 2002. pp. 533–534.

58. Schrader C, Schielke A, Ellerbroek L, Johne R. PCR inhibitors—occurrence, properties and removal. J Appl Microbiol. 2012; 113:1014–1026. https://doi.org/10.1111/j.1365-2672.2012.05384.x PMID: 22747964