1 **REASSESSMENT OF ROUTINE MIDSTREAM CULTURE IN DIAGNOSIS OF**

2 **URINARY TRACT INFECTION**

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4 **Authors:**

5 Sanchutha Sathiananthamoorthy[a,#], James Malone-Lee[a], Kiren Gill[a], Anna Tymon[b], Trang K.

6 Nguyen[a,*], Shradha Gurung[a,*], Linda Collins[a,*], Anthony S. Kupelian[a,*], Sheela Swamy[a],

7 Rajvinder Khasriya[a,*], David A. Spratt[b], Jennifer L. Rohn[a,#]

8 **Affiliations:**

9 a. Centre for Nephrology, Division of Medicine, University College London, Royal Free

10 Campus, Rowland Hill Street, London NW3 2PF, UK.

11 b. EDI Department of Microbial Diseases, University College London, 256 Gray's Inn

12 Road, London WC1X 8LD, UK.

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14 **Running Head:** Failure of midstream urine culture to detect infection

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16 **#Address correspondence to:**

17 Sanchutha Sathiananthamoorthy, rebmssa@ucl.ac.uk

18 Jennifer L. Rohn, j.rohn@ucl.ac.uk

19

20 ***Present addresses:**

21 Trang K Nguyen, University of Leeds, Leeds, UK

22 Shradha Gurung, University of Kent, Kent, UK

23 Linda Collins, Middlesex University, London, UK

24 Anthony S Kupelian, University College Hospital, London, UK

25 Rajvinder Khasriya, University College Hospital, London, UK

ABSTRACT

Midstream urine culture (MSU) remains the gold standard diagnostic test for confirming urinary tract infection (UTI). We previously showed that patients with chronic lower urinary tract symptoms (LUTS) below the diagnostic cut-off on MSU culture may still harbour bacterial infection, and that their antibiotic treatment was associated with symptom resolution. Here, we evaluated the results of the UK's MSU culture in symptomatic patients and controls. Next, we compared the bacterial enrichment capabilities of the MSU culture with a 50 µl uncentrifuged culture, a 30 ml centrifuged sediment culture, and 16S rRNA gene sequencing. This study was conducted on urine specimens from 33 LUTS patients attending their first clinical appointment (mean age = 49 years, standard deviation [SD] = 16.5), 30 LUTS patients on treatment (mean age = 47.8 years, SD = 16.8) whose symptoms had relapsed, and 29 asymptomatic controls (mean age = 40.7 years, SD = 15.7). We showed that the routine MSU culture, adopting the UK interpretation criteria tailored to acute UTI, failed to detect a variety of bacterial species, including recognised uropathogens. Moreover, the diagnostic MSU culture was unable to discriminate between patients and controls. In contrast, genomic analysis of urine enriched by centrifugation discriminated between the groups, generating a more accurate understanding of species richness. In conclusion, the UK's MSU protocol misses a significant proportion of bacteria, which include recognised uropathogens, and may be unsuitable for excluding UTI in patients with LUTS.

51 **KEY WORDS**

52 Midstream urine culture

53 Urinary tract infection

54 Lower urinary tract symptoms

55 Mixed growth

56 16S rRNA gene sequencing

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70 **INTRODUCTION**

71 An estimated 150-250 million individuals worldwide develop urinary tract infection (UTI)
72 every year, making it one of the most frequent types of human infections (1, 2). Midstream
73 urine culture (MSU) is the gold standard test for its diagnosis. Hospital laboratories in the UK
74 abide by the Standards for Microbiology Investigations (SMI B 41) protocol, issued and
75 updated by Public Health England (PHE), which generally recommends a threshold of $\geq 10^5$
76 colony forming units per millilitre (cfu/ml) to confirm both uncomplicated and complicated
77 UTI (3); this criterion is also adopted in many other countries, but varies considerably. The
78 10^5 cfu/ml threshold originates from a comparison conducted in the late 1950s of 74 pregnant
79 female patients with clinically determined acute pyelonephritis and 337 asymptomatic
80 women (4, 5).

81 Concerns regarding the suitability of this quantitative cut-off for patients with uncomplicated,
82 acutely dysuric lower UTI were initially raised by Stamm, for which the evidence-based
83 suggestion for lowering the colony count to 10^2 cfu/ml was concluded to be the more
84 appropriate alternative (6, 7). It is now recognised that culture, interpreted using a single
85 quantitative threshold, may not be appropriate when used to detect different clinical
86 manifestations of UTI (urethritis, cystitis, and pyelonephritis). This may be further
87 complicated by a multitude of host and microbial factors (8, 9), making it increasingly likely
88 that no single threshold is suitable for the detection of UTI in every clinical circumstance
89 (10).

90 The current quantitative MSU threshold is problematic for other reasons. First, bacterial
91 strains are known to vary in virulence (11), meaning that some strains might generate disease
92 even at lower concentrations. Second, the concentration of bacteria in urine can vary widely
93 depending on fluid intake before MSU sampling; a dilute specimen might fall below the

94 diagnostic cut-off as a result. Third, recognised uropathogens, including *Escherichia coli* and
95 *Enterococcus faecalis*, adhere to host cells and are also known to invade them to form
96 intracellular colonies (12-14). Thus, urinary supernatant may be a poor substrate for
97 recovering sequestered organisms. However, it is also known that an innate immune response
98 to UTI exacerbates the shedding of urothelial cells into urine (15-19).

99 An additional problem associated with standard MSU interpretation in the UK is the
100 dismissal of mixed growth cultures by default. Conventionally, 'mixed growth' cultures are
101 assumed to reflect contamination by organisms recognised to colonise the healthy
102 periurethral, vaginal and perianal regions (5). Although some laboratories electively consider
103 the growth of up to three organisms each at $>10^5$ cfu/ml to suggest polymicrobial infection, or
104 may proceed with a repeat assessment, many disregard mixed cultures altogether (3).
105 However, we and others have shown that polymicrobial infection is common in patients with
106 lower urinary tract symptoms (LUTS) (13, 20-22). In addition, polymicrobial urosepsis has
107 been reported in patients who demonstrated identical, mixed isolates from blood and urine
108 specimens (23, 24). Another study reported that *E. coli,* a predominant uropathogen,
109 manifested greater invasive properties when isolated from a polymicrobial culture in contrast
110 to pure growth (25). This finding is supported by recent work showing that *E. faecalis* can
111 bolster *E. coli* by exporting the nutrient L-ornithine (26).

112 Few studies have analysed the urinary microbial composition by laboratory outcome (i.e.
113 negative, mixed, positive cultures), and the majority of such work has assessed culture
114 techniques adopted in US laboratories (6, 27-30). Given the limitations of culture-based
115 microbial detection (31-33), characterisation of microbial communities in health and disease
116 is now becoming increasingly dependent on approaches, such as DNA-based identification,
117 which do not require growth in particular culture conditions (34). Using metagenomics, the
118 urinary microbiota in patients with neurogenic bladder dysfunction (35), overactive bladder

119 (36), urgency urinary incontinence (UUI) (28), stress urinary incontinence (37) and
120 uncomplicated UTI (38) have been described. Some studies have also included comparisons
121 to asymptomatic individuals (28, 33, 35, 36, 39-41).

122 Critical microbiological evaluation of the urine culture protocol adopted in the UK is long
123 overdue. Here, we chose to study MSU culture performance in patients describing LUTS,
124 including those who fall short of a positive culture and those demonstrating mixed growth,
125 since these are generally deemed not to have a *bona fide* infection. This assertion hinges on
126 standard culture-based diagnosis, and emerging data (13, 28, 42) suggest that such patients
127 harbour chronic infection. Our aim was to evaluate the MSU culture in symptomatic patients
128 and asymptomatic controls and assess its performance using both culture and molecular
129 approaches, with and without specimen enrichment.

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131 **MATERIALS AND METHODS**

132 **Subject recruitment and clinical assessment**. This study obtained ethical approval from the
133 East London & the City Research Ethics Committee, London, UK. Adult men and women
134 aged ≥18 years with LUTS were eligible for study inclusion. Pregnant subjects were not
135 included in the study. Since urine cultures are relied upon for diagnosis at initial presentation
136 and during relapse, two separate symptomatic patient groups were recruited and assessed.
137 Patients attending their first appointment at the Whittington Hospital Lower Urinary Tract
138 Symptoms (LUTS) Clinic (referred to in this study as "new patients"), who were not on
139 antibiotic treatment and had not taken antibiotics in the preceding four weeks, were evaluated
140 by the present clinician and inducted into the first patient group. Patients attending a follow-
141 up consultation with symptomatic recurrence, relapse or no response to initial antimicrobial
142 treatment (referred to as "relapsed patients") were identified following clinical assessment to
143 form the second patient group. Asymptomatic controls aged ≥18 years of either sex, with no

144 urological complications and not on antibiotic treatment were recruited from departmental
145 staff, students and the general population. All patients and controls provided written consent
146 prior to study inclusion. Figure 1 presents a workflow of all experimental procedures
147 conducted on each subject. Clinicians overseeing the care of new and relapsed patients were
148 blinded from all urinalyses results (i.e. urinary dipstick, microscopy and MSU culture test
149 results). For all downstream analysis, the culture plates were coded with a four-digit study
150 number and processed separately in large batches. The data for each technique were inputted
151 separately and comparisons did not take place until all of it had been entered and the code
152 broken.

153 Symptoms of urgency urinary incontinence (UUI), voiding dysfunction, pain, and stress
154 urinary incontinence (SUI) were recorded using "yes/no" response questions in a validated
155 questionnaire (43). The context-related symptoms were summed within each of the four
156 symptom groups. Patient and control urine specimens were obtained using the clean-catch
157 MSU method and anonymised with a four-digit study number. Subjects were carefully
158 instructed in the collection technique. Each urine specimen was analysed using urinary
159 dipsticks and an automated Clinitek Status analyser (Siemens Healthcare, Germany) for
160 leukocyte esterase and nitrites. Microscopic leukocyte counts were determined using a
161 Neubauer counting chamber, loaded with 10 µl of fresh urine. Each prepared specimen was
162 examined by microscopy (x200) using an Olympus CX41 light microscope (Olympus, UK).

163 **National routine diagnostic screening.** An aliquot of each MSU specimen was submitted to
164 the Whittington Hospital Microbiology Laboratory, London, UK for routine culture. Urine
165 specimens were stored at 4°C until they were transported to the processing laboratory. These
166 samples took approximately 60 minutes to arrive and were cultured either immediately on the
167 same day, or on the next day following overnight storage at 4°C, which reflected usual
168 practice. The protocol involved inoculating ChromID CPS (now ChromID CPS Elite)

169 chromogenic culture medium (bioMérieux, France) with 1µl of uncentrifuged urine, which
170 was then placed in an ordinary incubator at 37°C. Microbial colonies were identified using
171 the manufacturer's colour criteria. A count of $\geq 10^5$ cfu/ml for one organism was interpreted
172 as a significant result. Cultures with a colony count below this threshold were reported as "no
173 significant growth". MSU cultures, with more than one organism reported as "mixed growth
174 of *n* types of organisms".

175 **Extended culture-based analysis.** Urine samples were stored at 4°C for 0.5-4.0 hours before
176 transportation to the research laboratory (University College London, UK), where they were
177 processed immediately. An identical MSU culture was performed on an aliquot of the same
178 urine specimen. Simultaneous to this culture, 50 µl of uncentrifuged urine was plated on
179 chromogenic agar. From the remaining aliquots of each specimen, 30 ml was centrifuged at
180 1400 x *g* for 10 minutes. The sediment was resuspended in 400 µl of sterilised phosphate
181 buffer saline (PBS) solution (Life Technologies, UK). Ten-fold serial dilutions were
182 performed using PBS to reveal the presence of morphologically small-sized colonies, where
183 the growth of other organisms dominated. All cultures were incubated aerobically at 37°C for
184 18-24 hours.

185 **Identification of cultured isolates**. Microbial isolates were identified using matrix-assisted
186 laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) using the
187 MicroFlex LT mass spectrometer (Bruker Daltonics, USA). In the first instance, the direct
188 colony plate method was employed for identification and each cultured isolate was inoculated
189 twice onto the target plate. The target plate was left to air-dry before applying 1µl of matrix
190 solution (Bruker Daltonics, USA) consisting of alpha-cyano-4-hydroxycinnamic acid
191 dissolved in 50% acetonitrile and 2.5% trifluoroacetic acid. The air-dried target plate was
192 inserted into the mass spectrometer and time was given for the high vacuum to be restored.
193 Using the MALDI Biotyper 3.0 software programme (Bruker Daltonics, USA), the isolate

194 identifiers were recorded onto the programme and the Bruker Taxonomy library was selected
195 for the analysis. In the event of an unsuccessful identification, an ethanol-formic acid
196 extraction protocol was adopted to assist with microbial cell wall disruption and release of
197 ribosomal proteins (44). A heavy suspension of the unidentified isolate was made in 300 µl of
198 sterile distilled water. Following the addition of absolute ethanol (900 µl) microbial
199 suspension, samples were spun down at 20,000 x *g* for 2 minutes. The resulting ethanol was
200 discarded and the centrifugation step was repeated. Residual ethanol was removed and the
201 resulting pellet was left to air-dry at room temperature for 5 minutes. The deposit was
202 resuspended in 70% formic acid (20 to 50 µl). An equal volume of acetonitrile was added to
203 this mixture, followed by centrifugation at 20,000 x *g* for 2 minutes. The supernatant was
204 then applied onto a sterilised target plate twice and left to air-dry, before resuming with the
205 direct approach protocol from the point of adding matrix solution to each dried spot.

206 **16S rRNA gene sequencing of urine.** Prior to extraction**,** urine samples for DNA sequencing
207 were stored at -80°C. Genomic DNA was extracted from each specimen using an approach
208 adapted from a previously reported phenol-chloroform-isoamyl alcohol and bead-beating
209 method (45). Specimens were spun down using a pre-cooled centrifuge at 18,000 x *g* for 5
210 minutes at 4°C. Cell pellets were resuspended in extraction buffer (500 µl) consisting of 120
211 mM potassium phosphate (K_2PO_4) buffer (pH 8.0) with 5% cetyltrimethylammonium
212 bromide (CTAB) (Sigma-Aldrich, USA) in 0.7 M NaCl. The specimen tubes were vortexed
213 and contents transferred into pre-sterilised 2 ml tubes containing zirconia/silica beads (0.1mm
214 diameter). Phenol-chloroform-isoamyl (PCI) (25:24:1) alcohol (500 µl) (Invitrogen, USA)
215 was added to each sample and kept on ice. The samples were homogenised using a RiboLyser
216 (Hybaid, Germany) for 30 seconds at 5.5 m/s and spun down at 18,000 x *g* for 15 minutes.
217 Chloroform-isoamyl (CI) alcohol (Invitrogen, USA) was added at a 1:1 ratio to the extracted
218 layer of DNA supernatant. After centrifugation at 18,000 x *g* for 2 minutes at 4°C, the DNA

219 of each sample was precipitated by adding 30% polyethylene glycol (PEG-8000) (Sigma-
220 Aldrich, USA) at a 2:1 ratio.

221 PCR was performed to amplify the V5-V7 hypervariable regions of the 16S rRNA gene using
222 the barcoded primers 785F (5'-GGATTAGATACCCBRGTAGTC-3') and 1175R (5'-
223 ACGTCRTCCCCDCCTTCCTC-3') (see Table S1) (Sigma, UK). Each 25 µl sample reaction
224 contained 0.125 µl Moltaq DNA polymerase (0.025 µM) (Molzym, VH Bio Ltd, UK), 2.5 µl
225 of Molzym buffer (x 1) (Molzym, VH Bio Ltd., UK), 0.5 mM $MgCl_2$, 0.2 mM dNTPs
226 (Bioline, UK), 1 µl of forward and reverse primer (0.4 µM) and PCR water (Molzym, VH
227 Bio Ltd., UK). The PCR reaction stages involved an initial denaturation step at 95°C for 5
228 minutes and subsequently amplified for 30 cycles at 94°C for 30 seconds (denaturation),
229 54°C for 40 seconds (annealing), 72°C for 60 seconds (elongation) followed by 72°C for 10
230 minutes and a final hold of 4°C. All sample reactions were performed in duplex. The DNA
231 concentrations were determined using the Qubit high-sensitivity DNA assay kit (Life
232 Technologies, UK) and the Qubit 2.0 fluorometer (Life Technologies, UK). The amplicons
233 were purified using Agencourt AMPure XP-PCR magnetic particles (Beckman Coulter,
234 USA) and combined in equimolar ratios using elution buffer (Qiagen, UK) to generate three
235 pooled DNA libraries (libraries 1, 2 and 3) for pyrosequencing on the MiSeq desktop
236 sequencer (Illumina Inc., USA), using the v2 reagent kit (Illumina Inc., USA). Since library 3
237 contained samples with the lowest DNA yields, the purification step was repeated for this
238 pooled library in an attempt to improve on the final DNA concentration.

239 Our own customised and commercial mock communities were incorporated into DNA
240 libraries 1 and 2 to verify the DNA sequencing protocol (see Figure S1).

241 **Statistical analysis.** All descriptive and inferential statistics were generated using SPSS
242 version 25.0 software (IBM, USA). Demographic data, age, gender, 24-hour urinary

243 frequency, nocturia, validated LUTS scores, log-transformed leukocyte counts were
244 compared across the groups using non-parametric tests. In all cases, except age, homogeneity
245 of variance was not shown so Mood's median test was used instead of the Kruskal-Wallis.
246 Hospital MSU culture data (with the outcomes: "negative", "mixed growth" and "positive
247 culture") were compared across the study groups using the Chi-squared test or Fisher's exact
248 test.

249 The 16S rRNA data was analysed using QIIME (Quantitative Insights Into Microbial
250 Ecology, qiime.org version 1.8) (46). The raw reads were demultiplexed and assigned to
251 samples using barcoded sequences. After demultiplexing, the paired-end data were joined to
252 obtain a single FASTQ file for each sample. These sequences were then clustered into
253 Operational Taxonomic Units (OTUs) using an open reference OTU picking strategy. The
254 OTU clusters were assigned to the Greengenes Reference Database
255 (http://greengenes.lbl.gov) (47) based on 97% sequence similarity (46). OTUs were then
256 filtered to remove the chimeric sequences (UCHIME) (48) and taxonomy was assigned using
257 the Ribosomal Database Project (RDP) Classifier (49). Core diversity analyses based on
258 study group and sample type were performed.

259

260 RESULTS

261 **Clinical characteristics of the patient cohorts providing samples.** As previous work
262 suggested that the gold standard MSU culture was not an optimal diagnostic tool to detect
263 infection in LUTS patients, we set out to directly compare it with two alternative culture
264 techniques. We collected clean-catch MSU specimens from 33 untreated new patients (mean
265 age: 49 years, standard deviation [SD] = 16.5) and 30 patients experiencing a symptomatic
266 relapse (mean age: 47.8 years, SD = 16.8). Since urine cultures are relied upon for diagnosis

267 at initial presentation and during relapse, these two patient groups were recruited and
268 assessed separately. For comparison, we also recruited 29 asymptomatic controls (mean age:
269 40.7 years, SD = 15.7). Table 1 details the clinical characteristics of each study cohort.
270 Statistically similar age distributions were observed for all three study groups ($\chi^2 = 4.4$, df =
271 2, $P = 0.113$). The majority of patients were female, which reflected the well-known
272 demographics of UTI.

273 Firstly, we inspected the differences between patient and control cohorts. None of the
274 controls reported any LUTS, thus differing significantly from both patient cohorts studied. A
275 review of the symptomatic history of new patients ($n = 30$, duration of symptoms was not
276 recorded for three patients) revealed 1 patient (3.0%) to experience symptoms for <1 year, 16
277 patients (48.5%) for 1-4 years, 9 patients (27.3%) for 5-10 years and 4 patients (12.1%) for
278 ≥10 years. The median number of daytime and night-time urinary episodes differed across the
279 three groups, with pairwise post-hoc comparisons identifying a higher median frequency for
280 new patients and relapsed patients compared with controls (Table 1). We proceeded to
281 examine the symptomatic differences between new and relapsed patients. Comparisons of
282 symptom presentation between patient groups revealed that the number of new patients
283 reporting UUI and SUI symptoms was significantly higher than that of relapsed patients. The
284 proportion of new and relapsed patients that reported voiding symptoms and pain symptoms
285 were statistically similar (Table 1).

286 In addition to observing the presence and absence of symptoms, we also calculated a
287 symptom score to determine the magnitude of LUTS within each patient group. The number
288 of symptoms was summed for each of the four categories to provide a score and compared
289 across the groups using Mood's median test. The median symptom scores for UUI, SUI,
290 voiding and pain symptoms categories differed across the three study groups. Post-hoc
291 analysis revealed higher median UUI, SUI and voiding scores for new patients than relapsed

292 patients. However, both patient cohorts had similar pain scores and total symptom scores
293 (Table 1). Statistical comparison of the respective frequencies for each assessed LUTS
294 between new and relapsed patient groups are provided within the supplementary material
295 (Table S2).

296 **Urinary microscopy and dipstick analysis.** We performed urinary leukocyte counts to
297 determine whether patient urine manifested evidence of infection independent of bacterial
298 assessment. As shown in Table 2, there was a significant difference in $\log_{10}$ leukocyte counts
299 among the three study groups ($\chi^2 = 6.2$, df = 2, $P < 0.05$). Post-hoc analysis using
300 Bonferroni's correction identified significant differences between controls and new patients,
301 and between controls and relapsed patients. No significant difference in $\log_{10}$ leukocyte
302 counts was identified between new patients and relapsed patients.

303 Analysis of the urinary dipstick results showed 13 (39.4%) new patients, 11 (36.7%) relapsed
304 patients and 6 (20.7%) controls tested positive for leukocyte esterase with only 1 (3.0%) new
305 patient testing positive for urinary nitrite. The results were unsurprising, as previous work
306 suggested that the dipstick is not sensitive enough to diagnose infection (leukocyte esterase =
307 46-66%, nitrite = 6-18%), particularly in patients with non-acute symptoms of UTI (50-52).

308 Although LUTS can be driven by non-infectious causes, the association between patient
309 symptoms and leukocyte recruitment demonstrates urinary tract inflammation. This is critical
310 if an infective aetiology for LUTS is being considered.

311 **Hospital MSU culture results.** Next, we determined the relationship between patient
312 symptoms and their standard MSU results. Table 2 summarises the data, in which the
313 majority of new patients, relapsed patients and controls were reported to have a negative
314 MSU culture. Six (9.5%) symptomatic patients showed a positive culture result as did two
315 (6.9%) controls. A comparison of the hospital MSU culture outcomes revealed that the MSU

316 culture was unable to discriminate between the three study groups (χ^2 = 1.7, df = 4, P =
317 0.787). The hospital reports showed that all four new patients with positive cultures grew *E.*
318 *coli*, whereas *Enterococcus* (n = 1) and a coliform belonging to the
319 *Klebsiella/Enterobacter/Serratia* group (n = 1) were reported for the positive MSU cultures
320 of relapse patients. *Proteus* (n = 1) and *Streptococcus agalactiae* (n = 1) were cultivated from
321 controls.

322 Further pairwise statistical comparisons of the number of positive and negative MSU cultures
323 using the Fisher's exact test were performed. For this particular analysis, "mixed growth" and
324 "no significant growth" cultures were under the category of "negative" as both results are
325 traditionally dismissed as insignificant. This analysis indicated that even with the exclusion
326 of the relapsed group, the MSU culture was still unable to discriminate between
327 asymptomatic controls and new patients (χ^2 = 0.539, df = 1, P = 0.674). The same analysis for
328 relapsed patients and controls also showed no significant difference (χ^2 = 0.0, df = 1, P =
329 1.0). Likewise, comparison of new patients and relapsed patients showed no difference (χ^2 =
330 0.539, df = 1, P = 0.674). These results further highlight the inability of the MSU culture to
331 discriminate between the three study groups.

332 Since the hospital laboratory did not report the microbial composites of mixed growth and
333 negative MSU cultures, we inspected the 1 µl loop MSU cultures that were replicated in our
334 own research laboratories subjecting colonies grown on chromogenic agar to MALDI-TOF
335 MS analysis for identification. The pooled percentage frequencies of organisms identified
336 from the polymicrobial MSU cultures of new patients (n = 7), relapsed patients (n = 4) and
337 controls (n = 5) are shown in Figure S2. No colonies on the research laboratory MSU culture
338 were observed for 17 (58.6%) controls, 12 (36.4%) new patients, and 13 (43.3%) relapsed
339 patients. Whilst 19 isolates were grown from 12 asymptomatic control MSU cultures, 39
340 isolates were identified from 21 new patient cultures and 25 isolates were grown from 17

341 relapsed patient cultures. With this replicated diagnostic culture, *Enterococcus* and
342 *Escherichia* were predominantly cultured from new patients and relapsed patients, whereas
343 *Streptococcus* and *Staphylococcus* were most frequently isolated from controls. Within this
344 sample size, Fisher's exact test confirmed a significantly higher frequency of *Escherichia* (χ^2
345 = 5.4, df = 1, $P < 0.05$) and *Enterococcus* ($\chi^2 = 8.9$, df = 1, $P < 0.05$) isolated from new
346 patient cultures than from controls. The relapsing patient group was also observed to produce
347 a significantly higher number of *Enterococcus* isolates than the control group ($\chi^2 = 6.4$, df =
348 1, $P < 0.05$). In summary, the quantitative MSU had no discriminatory diagnostic power, but
349 the microbial communities isolated in the process showed some differences between patients
350 and controls.

351 **Comparison of culture techniques.** We compared the discriminatory power of the hospital
352 MSU culture with other methods, specifically plating a larger volume of uncentrifuged urine,
353 and plating samples enriched by centrifugation. As shown in Figure 2, microbial growth was
354 categorised as "no growth", "one isolate" and "≥ two isolates" for comparison. Of note, as the
355 amount of bacterial input increased (from 1 µl supernatant to 50 µl supernatant to centrifuged
356 sediment); regardless of patient group, more polymicrobial growths were revealed. Using this
357 alternative categorisation, the MSU culture replicated within our research laboratory was still
358 unable to discriminate between the three study groups ($\chi^2 = 4.6$, df = 4, $P = 0.326$).
359 Additionally, culturing more supernatant (50 µl) or the sediment did not improve
360 differentiation across the three cohorts ($\chi^2 = 11.7$, df = 4, $P = 0.02$ [no significant differences
361 identified with post-hoc analysis] and $\chi^2 = 8.4$, df = 4, $P = 0.078$. respectively), likely because
362 as we and others have reported, healthy bladders also harbour polymicrobial growths. These
363 results suggest that quantitative microbiology is not an adequate diagnostic tool for patients
364 experiencing LUTS.

365 **Patient and control urinary bacterial communities.** Figures 3 illustrates pairwise study
366 group comparisons of the genera and respective percentage frequencies identified on the
367 MSU culture (reproduced in-house), 50 µl urine unspun culture and 30 ml sediment cultures
368 (See figure S3 for species-level characterisation across study groups). As seen with the MSU
369 culture results, differences were noted among the various groups on sediment culture. Whilst
370 15 different genera were grown from symptomatic patient sediment cultures (n = 13 from
371 new patients, n = 11 from relapsed patients) eight genera were isolated from control sediment
372 cultures. Seven genera were shared by both patient and control groups, which were
373 *Corynebacterium*, *Enterococcus*, *Escherichia*, *Klebsiella*, *Proteus*, *Staphylococcus* and
374 *Streptococcus*. Organisms that were cultivated from patient samples that were not isolated
375 from control specimens were *Candida*, *Citrobacter*, *Enterobacter*, *Lactobacillus*, *Leclercia*,
376 *Morganella* and *Pseudomonas*. In both symptomatic patient groups, *Enterococcus*,
377 *Staphylococcus* and *Escherichia* were the most abundant organisms. In asymptomatic
378 controls, *Staphylococcus*, *Enterococcus* and, *Streptococcus* were most frequently isolated.
379 However, *Staphylococcus* (χ^2 = 3.7, df = 1, P = 0.064) and *Streptococcus* (χ^2 = 2.4, df = 1, P
380 = 0.2) were present at statistically similar frequencies in patients. Fisher's exact test revealed
381 a significantly higher frequency of *Enterococcus* isolates cultivated from the new patients
382 than from asymptomatic controls (χ^2 = 6.2, df = 1, P < 0.05). No significant difference was
383 observed with any other genus between new patients and controls. Comparison of frequencies
384 between control and relapsed patient groups using Fisher's exact test revealed a significantly
385 higher frequency of *Staphylococcus* (χ^2 = 6.9, df = 1, P < 0.05), and *Streptococcus* (χ^2 = 7.0,
386 df = 1, P < 0.05) in controls than relapsed patients. No significant differences in the number
387 of genera were identified between new and relapsed patient groups.

388 We went on to analyse urinary bacteria in unprocessed urine (1 ml uncentrifuged) versus 30
389 ml of urine enriched by centrifugation in new patient versus control study groups using next-

390 generation sequencing based on rDNA. The percentage of identified sequencing reads or
391 reads that passed filter (PF) (Q30 >70%) for loaded DNA libraries 1, 2 and 3 were: (i) 77.9%
392 (10,122,606 PF reads), (ii) 80.0% (14,910,030 PF reads) and (iii) 68.3% (10,129,563 PF
393 reads) respectively. Bacterial DNA was detected in the urine samples of 32 (97.0%) of 33
394 recruited new patients (30 uncentrifuged urine samples and 30 centrifuged samples), 26
395 (89.7%) of 29 control samples (22 uncentrifuged samples and 23 centrifuged samples).
396 Samples that yielded low DNA concentrations were salvaged with an extended protocol.
397 Beyond this, bacterial DNA was undetectable in one new patient and three controls.

398 Figure 4 presents the relative abundance of the 20 most abundant taxa identified from the
399 uncentrifuged and centrifuged urine samples of new patients and controls (see supplementary
400 Table S3 for hierarchical classifications). The 20 most abundant taxa made up 81.0% of new
401 patient sequences and 79.0% of control sequences. The most abundant taxa of the
402 uncentrifuged urinary bacterial community of new patients from highest to lowest were
403 *Enterobacteriaceae* (32.3%), followed by *Lactobacillus* (15.5%) and *Streptococcus* (8.7%)
404 and *Enterococcus* (8.0%), whereas the most abundant taxa of the new patient urinary
405 microbial community represented by centrifuged samples were *Enterobacteriaceae* (26.9%),
406 *Enterococcus* (12.8%), *Psychrobacter* (9.3%) and *Streptococcus* (8.3%). The most abundant
407 taxa identified from uncentrifuged control samples were *Streptococcus* (21.5%),
408 *Enterobacteriaceae* (20.1%), *Lactobacillus* (11.6%) and *Gardnerella* (7.5%). In contrast, the
409 most abundant taxa identified from centrifuged control urine samples were *Streptococcus*
410 (15.8%), *Staphylococcus* (14.8%), *Enterobacteriaceae* (11.5%) and *Lactobacillus* (9.2%).
411 From these data, a decrease in overall abundance of *Lactobacillus* (15.5% uncentrifuged,
412 7.8% centrifuged) and an increase in abundance of *Enterococcus* (8.0% uncentrifuged, 12.8%
413 centrifuged) were observed with the new patient centrifuged urinary community when
414 compared with the new patient uncentrifuged community. On the other hand, the control

415 centrifuged community showed an increase in overall abundance of *Staphylococcus* (1.9%
416 uncentrifuged, 14.8% centrifuged) compared with the control uncentrifuged urinary
417 community. Centrifugation of samples therefore has a strong influence on species recovery.
418 Moreover, this approach also highlights differences in microbial composition between
419 patients and controls (similar to what was seen in our sediment culture data [Figure 3]).

420 Table 3 presents the richness and diversity measures of new patient and control urinary tract
421 bacterial communities. Richness was assessed by comparing the mean number of OTUs and
422 mean Chao1 estimator values using the Welch's two-sample *t* test. Diversity was assessed by
423 comparing the mean Shannon index and mean inverse Simpson's index using the Welch's
424 two-sample *t* test. Pairwise comparisons indicated no significant difference in the mean
425 number of observed OTUs and Chao1 estimator between patient and control uncentrifuged
426 samples, centrifuged samples and both combined. Additionally, no significant difference was
427 observed in the mean Shannon's index and inverse Simpson's index between patients and
428 control communities.

429 Overall, our analyses show a clear difference between patients with LUTS and controls,
430 namely that the *Enterobacteriaceae* was the most abundant taxa associated with disease, and
431 *Streptococcus*, with health. However, it is clear the method of sample processing enriches for
432 different taxa. Specifically, centrifugation enriched *Enterococcus* in patient samples, and
433 *Staphylococcus* in controls.

434 **Comparison of sediment culture and sequencing approaches.** As shown in Figure 5,
435 colour-coded matrices consisting of all cultured bacterial taxa that were detected by
436 centrifuged sediment culture and 16S rRNA gene sequencing were generated for new patient
437 centrifuged samples and control centrifuged urine samples. Among all centrifuged samples
438 37 (59.7%) of 62 contained bacteria that were detected by both culture and sequencing

439 methods. Three (9.1%) patient and four (13.8%) control centrifuged samples were sequence-
440 negative, but culture-positive. Two (6.1%) patient and three (10.3%) centrifuged control
441 samples were culture-negative, but sequence-positive *Enterococcus* was the most frequently
442 identified bacterium from both sediment culture and sequencing methods. These genera were
443 identified from 22 (66.7%) new patient samples. *Staphylococcus* was the most frequently
444 identified bacterium by both methods from control samples. This genus was identified from
445 11 (37.9%) centrifuged samples. *Escherichia* demonstrated the most cases (n = 14) in which
446 it was detected by sediment culture, but remained undetected by sequencing. One new patient
447 sample (NL5) grew *Leclercia* on sediment culture, but this particular taxa was not detected
448 by sequencing. This analysis shows that the sequencing method is vastly superior to culture
449 techniques for bacterial detection, and that culture methods do not recover a large number of
450 bacterial species, including recognised uropathogens.

451

452 **DISCUSSION**

453 A recent commentary in *J Clin Micro* emphasised how the diagnostic landscape for UTI is
454 becoming increasingly challenging in the face of alternatives to standard culture (53). The
455 primary purpose of this study was to evaluate the routine MSU culture performed in UK
456 diagnostic laboratories. This evaluation is crucial, since culture is considered the gold
457 standard diagnostic test for confirming or excluding UTI in symptomatic patients, although
458 evidence suggests that it may be deficient (6, 7). In the UK, symptom-based empirical
459 antibiotic treatment for acute UTI is recommended (54). The evidence suggests that the
460 diagnostic accuracy of acute symptoms in previously asymptomatic subjects outperforms
461 routine culture-based diagnosis (55). By contrast, relapsing patients with LUTS are not as
462 straightforward and may demonstrate a distinct microbiota under the influence of prescribed

463 antimicrobials. The cohort of relapsing patients was consequently excluded from genomic
464 analysis to aid in clarifying the differences in urinary microbial communities between first-
465 visit patients with LUTS and health. Despite testing negative on routine MSU, such patients
466 have been shown to harbour uropathogens when their urine is examined with more sensitive
467 methods (13, 28, 29, 36, 42). Furthermore, symptomatic resolution has been observed
468 following antibiotic treatment in this type of patient (56). Therefore, we questioned whether
469 current routine MSU cultures with a $>10^5$ cfu/ml threshold were sensitive enough to detect
470 UTI in patients presenting with LUTS.

471 Following a symptomatic assessment of LUTS patients, we scrutinised the microbial
472 composition of each patient and control MSU culture and compared these to the results
473 reported by the hospital diagnostic laboratory, as well as to organisms identified using other
474 alternative culture and molecular methods. Crucially, we found that the routine MSU culture
475 results reported by the diagnostic laboratory failed to discriminate between patients and
476 control groups. Fewer than 10% of patients with a clinically suspected UTI had a positive
477 MSU culture, with only four of 33 new patient cultures producing monomicrobial growth at
478 10^5 cfu/ml. In contrast, bacterial DNA sequencing revealed bacteria including recognised
479 uropathogens in 30 of 32 new patients, including symptomatic patients who were reported to
480 have a negative MSU culture. In this study, 16S rRNA gene sequencing was not applied as a
481 diagnostic test, but rather, it was used to determine whether recognised uropathogens were
482 missed with routine culture techniques, and to characterise symptomatic and healthy urinary
483 bacterial communities.

484 Our second key finding was that MSU culture grossly underestimated the amount of
485 polymicrobial growth in patient urine. For example, 36.4% of new patient urines grew mixed
486 growths; this number rose to 63.6% when more uncentrifuged urine was plated, and to 84.8%
487 in the case of centrifuged sediment cultures. Although it is now recognised that even healthy

488 urine contains bacteria (41), which certainly complicates the diagnostic landscape, our
489 enhanced culture and genomic analyses showed a clear difference between the taxa isolated
490 between patients and controls. Specifically, *Enterobacteriaceae* was the dominant family in
491 new patients, whereas *Streptococcus* was foremost in asymptomatic controls. These findings,
492 taken together with other reports, suggest that automatically discounting polymicrobial
493 cultures may dismiss symptomatic patients in need of treatment.

494 There is of course a formal possibility that the mixed growths reported here could simply be
495 the result of contamination by skin bacteria during sample collection. In the UK, this
496 possibility is addressed by the UK Standards for Microbiology Investigations, which
497 recommends the counting of squamous epithelial cells in the urine as a marker for
498 contamination. However, their utility for this purpose is complicated by the fact that
499 exfoliation of urothelial cells as an innate immune response to UTI is a recognised
500 phenomenon, which is widely used as a surrogate marker of infection in both mice and
501 humans (14, 21, 57-65). Indeed, using antibodies against uroplakin III, a highly specific
502 marker for cells originating from the urinary tract, we previously demonstrated that the
503 majority of epithelial cells shed in the urine of symptomatic patients originate from the
504 bladder (14). Finally, were the presence of mixed growths merely the result of contamination,
505 we would not expect their presence to cluster statistically with symptoms as they have done
506 in this study.

507 Of note, some UTI-associated organisms were shared at the culture level by symptomatic and
508 asymptomatic patients: *Corynebacterium, Enterococcus*, *Escherichia*, *Klebsiella*, *Proteus*,
509 *Staphylococcus* and *Streptococcus*. The presence of “uropathogenic" species in controls could
510 reflect a lack of the appropriate virulence factor expression (11), or immune differences in the
511 host (for e.g. (66)). However, similar community profiles in the bladder have been seen by
512 others (13, 28, 29, 42, 67), which makes it likely that the ability to be pathogenic is highly

513 context-dependent, and could therefore be influenced by the presence of other species. Such
514 pathobiont relationships are common in the microbial world (68) and in the case of UTI, have
515 been reported in mixed infections with *E. coli* and *Enterococcus* (25, 26). Furthermore,
516 voided urine passes through the urethra as well as the bladder; the microbial ecology of the
517 urethra is poorly understood in the case of UTI (69-71), and further studies are needed to
518 understand what role such urethral communities might play.

519 Further afield, microbial community inhabitants in adjacent niches such as the vagina and
520 perianal region may also influence what species induce UTI pathophysiology. For example,
521 recent work by Gilbert *et al.* showed that transient exposure to the vaginal organism
522 *Gardnerella vaginalis* could induce dysbiosis, reactivating dormant *E. coli* in previously
523 infected mouse bladders (64). Other interactions could be protective; for example, the vaginal
524 commensal *Lactobacillis crispatus*, which is known to make the vagina less hospitable to
525 certain Gram-negative perianal uropathogens, may decrease the instance of recurrent UTI
526 (72, 73). Indeed, our results showing that *Lactobacillus* is a dominant organism among the
527 control samples, consistent with reports from other groups comparing the urine of patients
528 with LUTS with asymptomatic controls, supports this notion (28). Further research, including
529 detailed sequence comparisons, is required to understand the mobility and interspecies effects
530 of the various microbial inhabitants in the region perianal and urethra region. This could
531 further our understanding of the significance of polymicrobial communities in UTI.

532 Of interest, the process of centrifugation had a major effect on which taxa dominated,
533 enriching *Enterococcus* in patient samples, and *Staphylococcus* in controls. This is perhaps
534 unsurprising, given that uropathogens are known to strongly adhere to and, in some cases,
535 invade urothelial cells (14). These cells are shed into the urine via an innate immune response
536 that is yet to be fully characterised (63, 74). Therefore, a significant proportion of bacteria in
537 such specimens – and perhaps those most relevant for pathophysiology – could be cell-

538 associated, not planktonic. The MSU culture samples a small amount of urine supernatant
539 and does not access the cell-rich sediment that settles by gravity. Several reports from the
540 Brubaker group also recognised the limitations of standard culture and developed the
541 expanded quantitative urine culture (EQUC) protocol. This involves inoculating
542 uncentrifuged urine onto a broader selection of culture media for optimal species isolation
543 (28, 36, 42). The approach demonstrated that the routinely performed standard quantitative
544 culture missed 67% of recognised uropathogens, with the authors suggesting that the method
545 be used to supplement the standard culture (42). They did not however analyse centrifuged
546 sediment with this protocol.

547 In addition to factors beyond control, such as the volume of fluid intake before urine
548 sampling, even the use of a broader selection of culture media has its limitations (31). The
549 cultured urinary community depends on the types of specimens analysed, techniques
550 employed, nutrient medium used, laboratory conditions (e.g. pH, temperature, O_2
551 concentration), as well as the viability, facultative and fastidious natures of urinary tract
552 organism. Such factors can distort the species richness of the urinary tract community.
553 Additionally, bacteria may exist in the viable but not cultivable (VBNC) state, also referred to
554 as conditionally viable environmental (CVEC) cells, active but not cultivable (ABNC) or
555 dormant cells (75). Such species are more reliably represented by nucleic acid-based
556 approaches.

557 In their studies, Brubaker *et al*. examined bladder urine specimens (specifically collected by a
558 transurethral catheter [CSU] or suprapubic aspiration [SPA]) (28, 33, 36, 42). In our study,
559 we used a clean-catch MSU specimen collection technique for several reasons. The aim of
560 this study was to evaluate the routine MSU culture technique, which is the primary method in
561 the clinic for collecting a urine sample for culture. In symptomatic patients, catheterisation is
562 potentially an invasive, uncomfortable and painful method of collection that has also been

563 reported to increase the risk of developing an infection (76). Furthermore, catheterisation may
564 bypass organisms colonising the urethra, which may represent an early stage UTI or
565 contribute to the infection. Hooton *et al.* discussed this as a possible reason for why a lower
566 threshold is usually applied to CSU cultures and a higher count is considered for MSU
567 cultures, since it is unclear if the latter collection method has sampled bacteria from the
568 bladder and/or the urethral region (27).

569 Female subjects formed the majority of patients and controls, in line with the demographics
570 of UTI. Given that a previous study found some differences between the microbial ecology in
571 male and female bladders (77), it would be interesting to further explore the effect of sex on
572 symptomatic urinary microbiota.

573 The lack of statistical difference between patient and control uncentrifuged samples was
574 consistent with other studies (28, 78) and could be attributed to the small study cohort.
575 However statistical comparison of all patient samples (uncentrifuged and centrifuged) to all
576 control samples (uncentrifuged and centrifuged) using the non-parametric Kruskal-Wallis test
577 did confirm a significantly higher median number of OTUs in patients than controls. This
578 suggests that incorporating centrifuged urine samples may clarify the distinction between
579 patient and control bacterial community richness.

580 While region-specific 16S rRNA gene sequencing using the MiSeq platform provided a less
581 biased representation of the urinary bacterial community, it is important to acknowledge the
582 limitations of this method. Due to the short-read sequencing approach used, approximately
583 30% (approximately 500 nucleotides) of the 16S rRNA gene was amplified, which makes
584 identification at the genus level possible, but reliable taxonomic assignment of reads at the
585 species level elusive. Our comparison of taxa identified by culture and 16S rRNA gene
586 sequencing revealed that the sequencing method was more capable of identifying the

587 cultivable bacterial taxa from patient and control samples compared with the centrifuged
588 sediment culture technique. However, the sediment culture method permitted bacterial
589 identification at the species level, which could not be achieved reliably by 16S rRNA gene
590 sequencing directly from urine. Of note, *Escherichia* demonstrated one the highest rates for
591 detection by culture but was often undetected by DNA sequencing among centrifuged and
592 uncentrifuged samples. This genus belongs to the *Enterobacteriaceae* family, which are
593 reported to be challenging to identify reliably at the genus level due to the high degree of
594 sequence similarities (79). Given that *E. coli* is a prominent uropathogen, improvements are
595 needed. A molecular species-level characterisation could be achieved using the more recently
596 developed third generation sequencing technologies, including the MinION platform series
597 (80). The potential to reconstruct more than 90% of the 16S rRNA gene makes species-level
598 characterisation possible.

599 This study underlines the potential weaknesses of the MSU culture for diagnosing UTI in
600 patients affected by LUTS, which should now be assessed further in a larger, multi-centre
601 study. These patients appear to harbour chronic infection, missed by quantitative cultures and
602 most effectively demonstrated by non-culture techniques. These methods shed light on the
603 complexity of the bacterial communities within these patients, whose symptoms and the
604 urinary white blood cell count may be the best indicators of infection until the disease
605 landscape is better understood. In the meantime, those responsible for UTI detection,
606 diagnosis and patient care, including clinicians and microbiologists, may wish to use caution
607 when interpreting a negative or mixed growth MSU result in symptomatic patients, as well as
608 reassess reporting and treatment guidelines to arrive at a solution most appropriate for
609 patients, in a way that also preserves antibiotic stewardship efforts (53). Ultimately, a point-
610 of-care test based on a sound understanding of the relevant microbiomes using emerging

611 portable genomic technology would revolutionise the diagnostic landscape for this common
612 affliction.

613

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632

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FIGURE LEGENDS

Figure 1. Experimental workflow of the study. Abbreviations: MALDI-TOF MS = Matrix assisted laser desorption/ionisation time-of-flight mass spectrometry.

Figure 2. Composition types (no growth, pure and mixed cultures) achieved with three different techniques (MSU culture (1 µl), unspun culture (50 µl) and sediment culture (30 ml) across study groups.

Figure 3. Pairwise comparisons of the percentage frequencies of organisms identified from the urine specimens of new patients (n = 33), relapsed patients (n = 30) and controls (n = 29) using three different culture techniques: routine midstream urine (MSU) culture, unspun culture of a 50 µl sample volume and a spun sediment culture of a 30 ml sample volume. **3A**: new patients versus controls, **3B**: relapsed patients versus asymptomatic controls and **3C**: new patients versus controls. Data are presented as percentages of the total number of isolates identified.

Figure 4. Percentage sequence abundances of the 20 most abundant taxa detected in both new patient and control cohorts when categorised by sample type.

Figure 5. Comparison of genus-level taxa detected by sediment culture and 16S rRNA gene sequencing for new patient (NL) spun samples (n = 33) and asymptomatic control (AC) spun samples (n = 29). Interpretation: bacteria identified by culture only (pink), sequencing only (blue), both (purple) and neither (cream).

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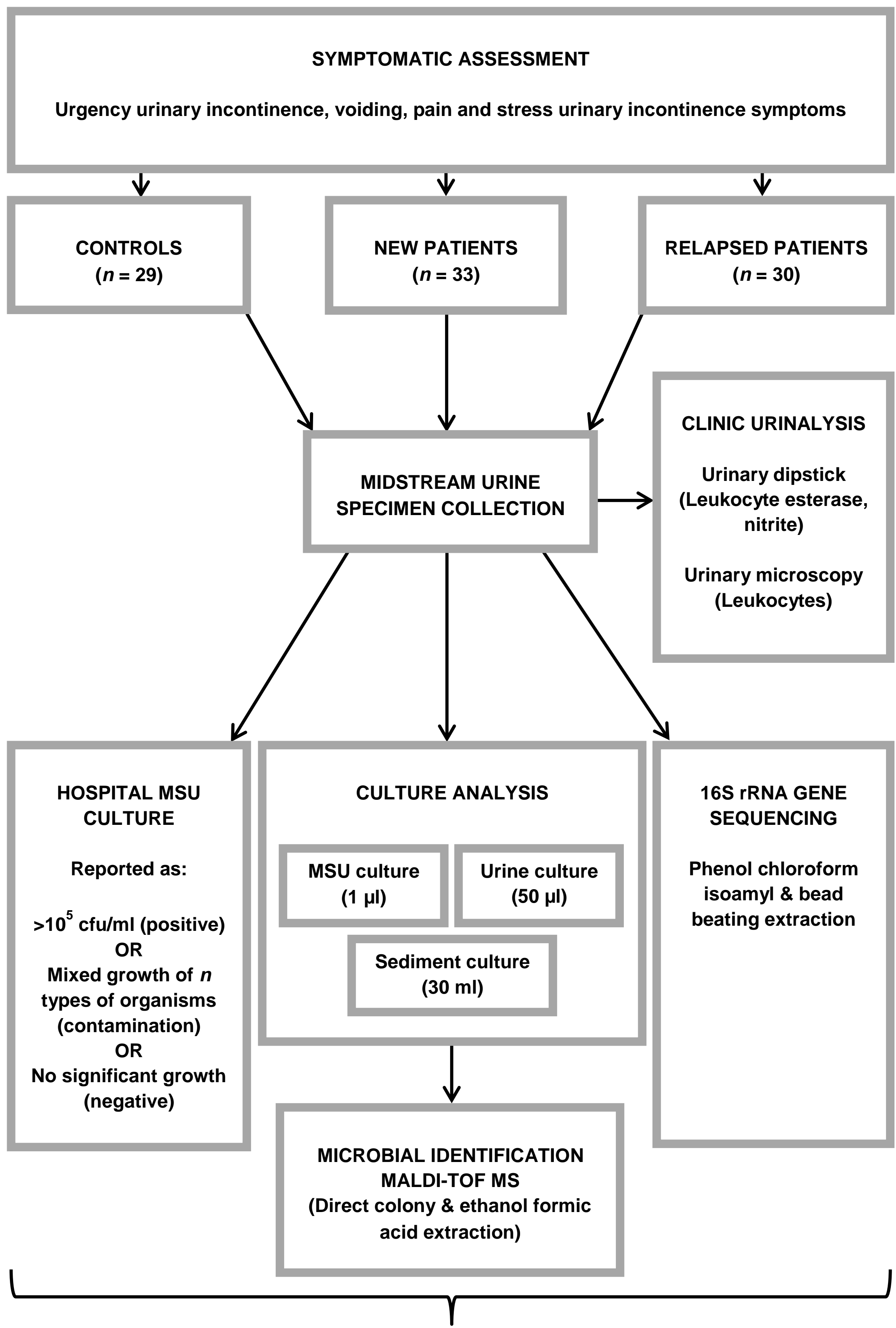

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942 **Figure 1.**

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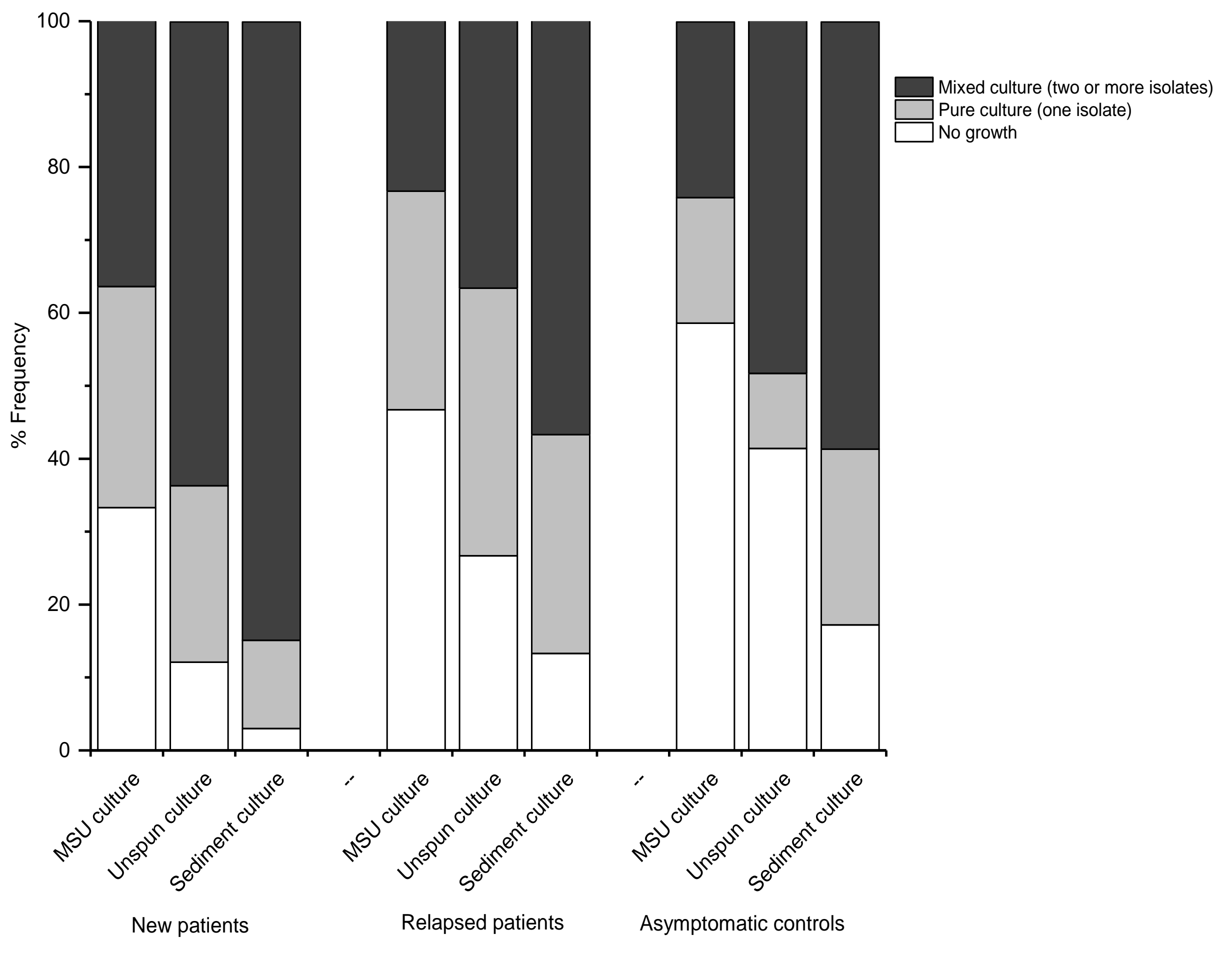

960 **Figure 2.**

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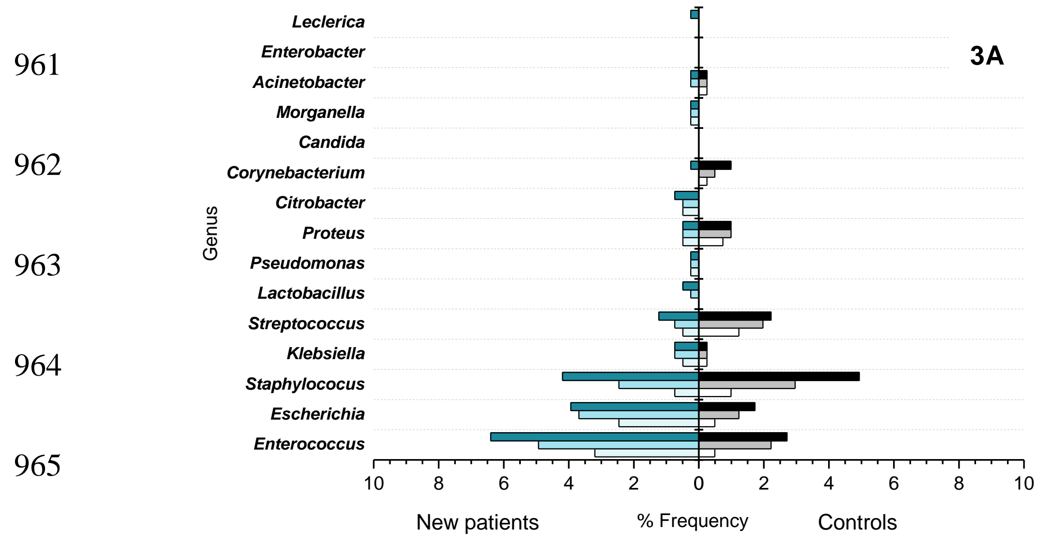

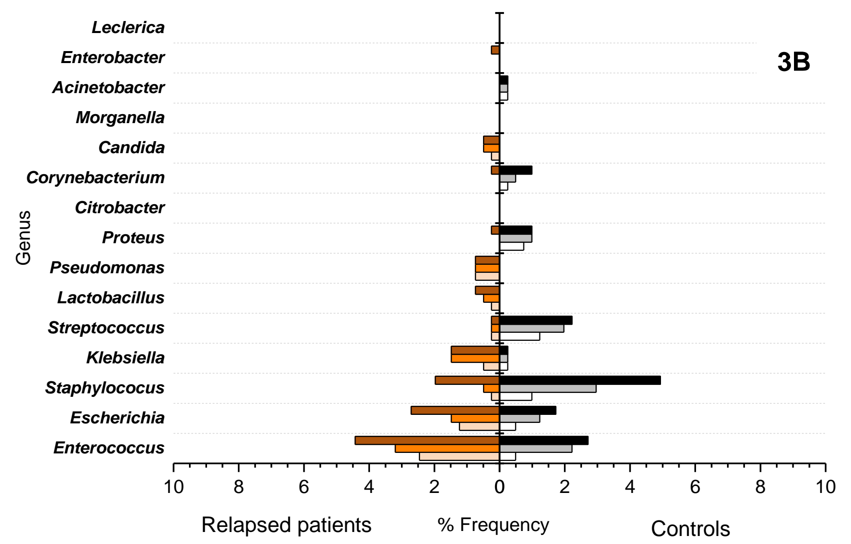

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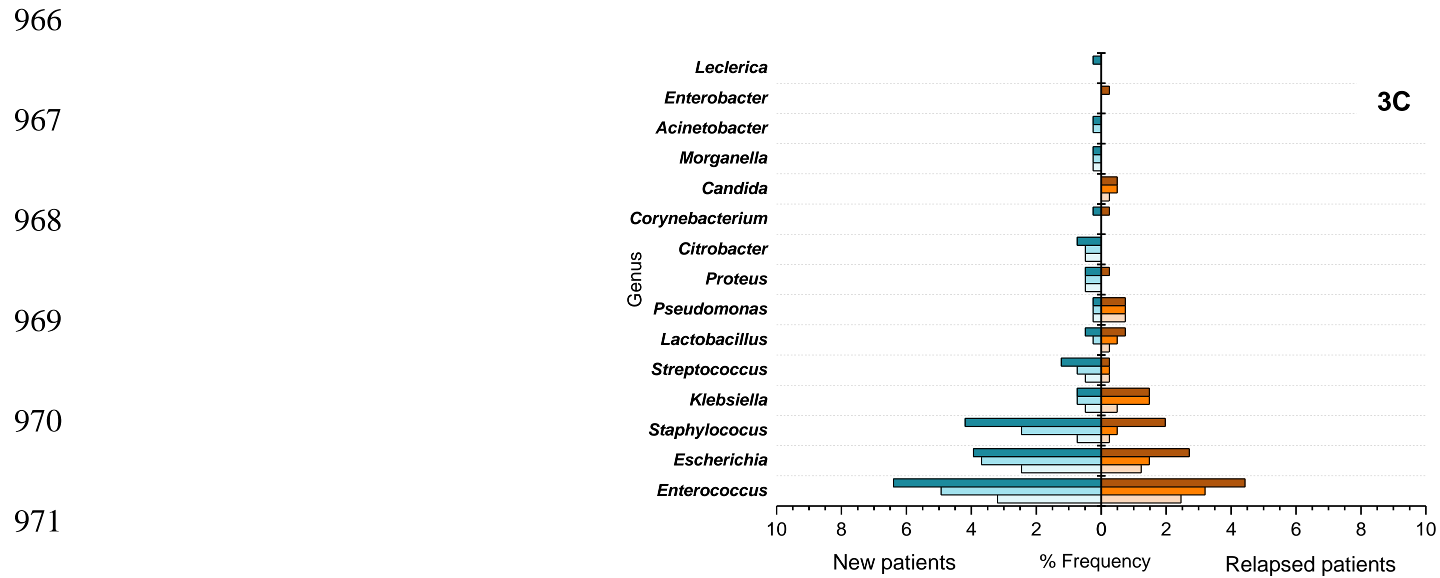

972 **Figure 3.**

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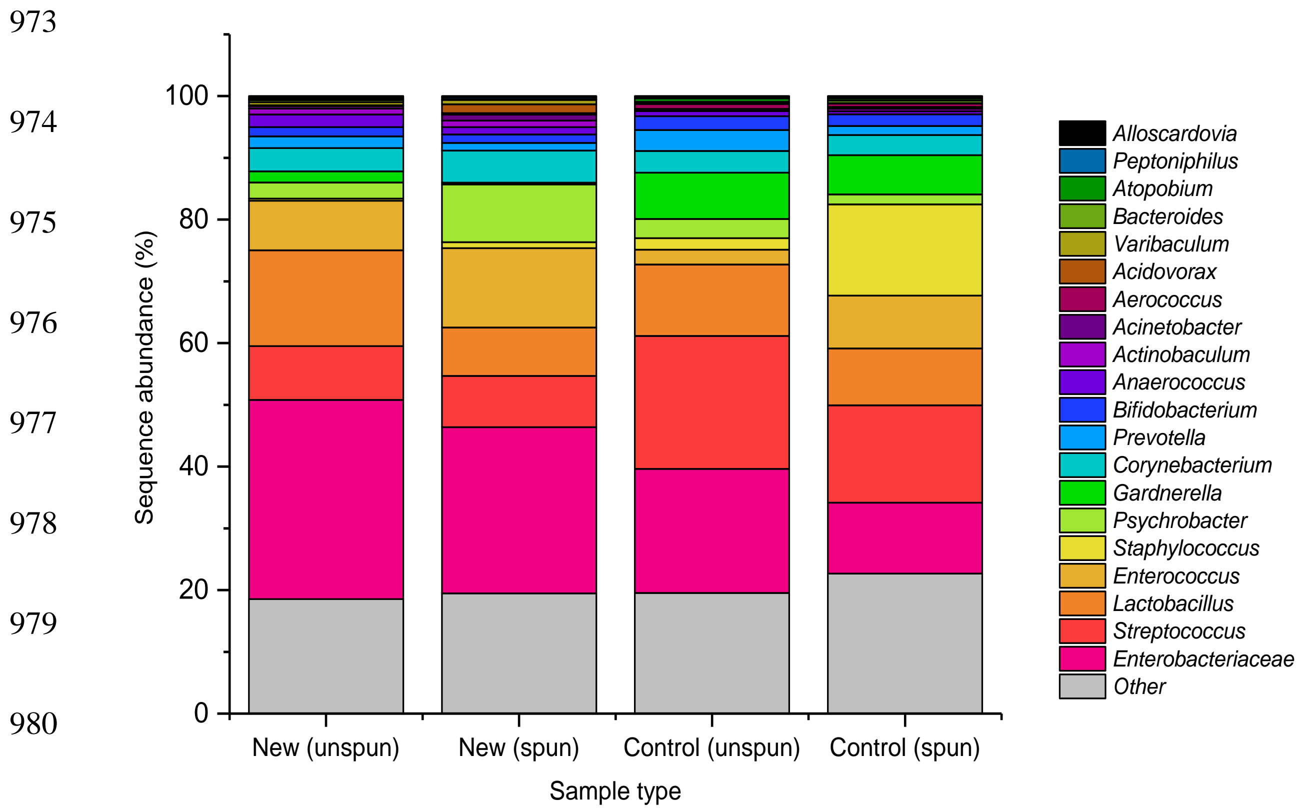

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991 **Figure 4.**

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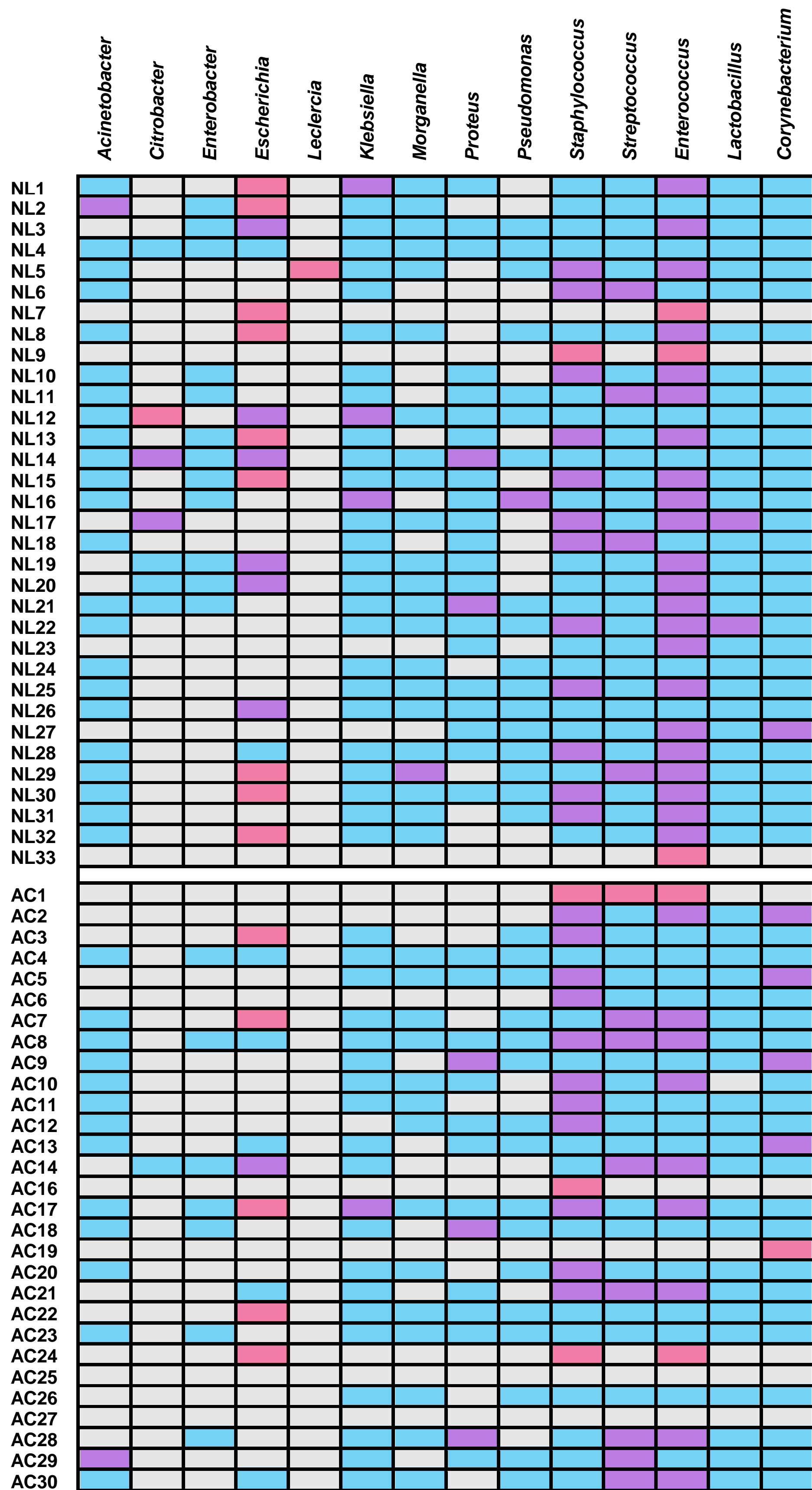

1010 **Figure 5.**

1011 **TABLES**

1012 **Table 1.** Clinical characteristics of each study group. Abbreviations: CI = confidence
1013 interval, SD = standard deviation, SUI = stress urinary incontinence, UUI = urgency urinary
1014 incontinence. Superscripts: a = Kruskal-Wallis test, b = Mood's median test, c = Chi-squared
1015 test. $ = statistical comparison between new patient and relapsed patient groups only.

1016 **Table 2.** Descriptive measures of in-house (clinic) microscopic leukocyte counts and reported
1017 routine hospital midstream urine culture results for each study group. Abbreviations: wbc =
1018 white blood cell.

1019 **Table 3.** Richness and diversity measures determined for new patients and controls

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1031 **Table 1.**

CHARACTERISTIC	NEW PATIENTS (*n* = 33)			RELAPSED PATIENTS (*n* = 30)			CONTROLS (*n* = 29)			*P*-VALUE
Demographics										
Female (%)	32 (97.0)			27 (90.0)			26 (89.7)			
Male (%)	1 (3.0)			3 (10.0)			3 (10.3)			
Mean age in years (SD)	48.7 (16.5)			47.8 (16.5)			40.7 (15.7)			0.113[a]
Age range	18-77			24-78			20-76			
Urinary Patterns (24 hours)	**Mean**	**SD**	**Median (95% CI)**	**Mean**	**SD**	**Median (95% CI)**	**Mean**	**SD**	**Median (95% CI)**	
Frequency	8.8	5.1	6.5 (5.5-10.5)	8.5	3.7	8.0 (6.5-9.5)	5.9	1.5	5.5 (5.5-6.5)	**< 0.05[b]**
Nocturia	1.7	1.7	1.5 (0.5-2.0)	1.6	1.9	1.0 (0.0-2.5)	0.3	0.4	0.0 (0.0-0.5)	**< 0.05[b]**
Symptoms[$] [Yes/No] (% within group)										
UUI (%)	25 (75.8)			12 (40.0)			0 (0.0)			**< 0.05[c]**
Pain (%)	24 (72.7)			26 (86.7)			0 (0.0)			0.172 [c]
Voiding (%)	29 (87.9)			21 (70.0)			0 (0.0)			0.080 [c]
SUI (%)	17 (51.5)			2 (6.7)			0 (0.0)			**< 0.001 [c]**
Number of symptoms	**Mean**	**SD**	**Median (95% CI)**	**Mean**	**SD**	**Median (95% CI)**	**Mean**	**SD**	**Median (95% CI)**	
UUI symptoms	3.4	2.9	3.0 (1.0-4.0)	1.4	2.2	0.0 (0.0-2.0)	0.0	0.0	0.0 (0.0-0.0)	**< 0.001[b]**
Pain symptoms	3.1	2.7	3.0 (1.0-4.0)	3.6	2.9	3.0 (2.0-4.0)	0.0	0.0	0.0 (0.0-0.0)	**< 0.001[b]**
Voiding symptoms	4.1	2.8	4.0 (2.0-6.0)	3.6	2.8	4.0 (2.0-5.0)	0.0	0.0	0.0 (0.0-0.0)	**< 0.001[b]**
SUI symptoms	0.7	0.8	1.0 (0.0-1.0)	0.3	1.0	0.0 (0.0-0.0)	0.0	0.0	0.0 (0.0-0.0)	**< 0.001[b]**
Total symptom score	11.3	5.9	12.0 (9.0-14.0)	8.9	5.2	9.0 (6.0-11.0)	0.0	0.0	0.0 (0.0-0.0)	**< 0.001[b]**

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1035 **Table 2.**

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DIAGNOSTIC TEST	NEW PATIENTS (n = 33)	RELAPSED PATIENTS (n = 30)	CONTROLS (n = 29)
CLINIC MICROSCOPIC LEUKOCYTES (log_{10}wbc/µl)			
Mean log_{10} cell count, SD (95% CI)	1.7, 0.9 (0.3-1.0)	0.7, 1.0 (0.4-1.1)	0.2, 0.4 (0.1-0.3)
Median log_{10} cell count (95% CI)	0.3 (0.0-0.3)	0.5 (0.0-0.8)	0.0 (0.0-0.8)
Frequencies (% of group)			
zero wbc/µl	15 (45.5)	12 (40.0)	20 (69.0)
1-9 wbc/µl	11 (33.3)	11 (36.7)	8 (27.6)
≥10 wbc/µl	7 (21.2)	7 (23.3)	1 (3.4)
HOSPITAL MIDSTREAM URINE CULTURE			
No significant growth[$]	21 (63.6)	23 (76.7)	22 (75.9)
Mixed growth[*] of two organisms	3 (9.1)	2 (6.7)	4 (13.8)
Mixed growth of three organisms	4 (12.1)	2 (6.7)	1 (3.4)
$>10^5$ cfu/ml of one organism	4 (12.1)	2 (6.7)	2 (6.9)

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1046 Footnotes:

1047 $ = $<10^5$ organisms/ml, **or** growth of ≥2 organisms at <100 colonies

1048 * = >100 colonies

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1051 **Table 3.**

METRIC	NEW PATIENTS			CONTROLS		
	Unspun (*n* = 30)	Spun (*n* = 30)	Both (*n* = 60)	Unspun (*n* = 22)	Spun (*n* = 23)	Both (*n* = 45)
OTU Numbers						
Mean (SD)	237.5 (122.4)	243.5 (145.1)	247.0 (137.7)	271.8 (271.8)	264.7 (235.2)	276.2 (214.0)
Median	251.0	224.0	247.5	247.0	217.5	230
Min-Max	4-510	7-606	4-619	10-639	6-1016	6-1016
Chao1 estimator						
Mean (SD)	310.2 (147.7)	317.0 (159.7)	333.3 (164.4)	341.6 (205.0)	336.9 (253.0)	362.5 (255.9)
Median	340.9	283.4	334.2	315.1	295.6	321.1
Min-Max	7-570.5	7.2-706.0	7-740.6	20.5-780.2	7.5-1085.7	7.5-1193.4
Shannon's index						
Mean (SD)	1.6 (0.8)	1.7 (0.9)	1.7 (0.9)	1.8 (0.8)	1.7 (0.8)	1.7 (0.8)
Median	1.5	1.8	1.7	1.9	1.7	1.8
Min-Max	0.3-3.3	0.5-3.3	0.3-3.3	0.4-3.2	0.3-3.4	0.3-3.4
Inverse Simpson's Index						
Mean (SD)	3.4 (2.8)	4.0 (3.2)	3.7 (3.0)	3.9 (2.6)	3.9 (3.5)	3.9 (3.0)
Median	2.6	3.0	2.7	3.3	2.5	3.1
Min-Max	1.1-14.6	1.2-13.2	1.1-14.6	1.1-11.6	1.1-17.0	1.1-17.0