RESEARCH ARTICLE

NaOH–HCl neutralized urine preparation for direct testing of uropathogens by Bruker MS

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Background: We evaluated the analytical performance of a NaOH–HCl neutralization protocol for identifying uropathogens directly from urine samples by a Bruker MS system (Bruker Daltonics, German) and sought to establish a protocol for integrating the method with conventional screening.

Methods: Among all urine samples requested for Gram staining, UF-1000i, and urine cultures by physicians, we selected samples that were positive by both Gram staining and UF-1000i testing. Urine was prepared by neutralization using 0.01M NaOH–HCl, and the samples were processed by Bruker MS. For the low detection limit, one strain each of *Escherichia coli* and *Enterococcus faecium* was inoculated in sterile deionized water and sterile urine specimens at sequential dilutions.

Results: In a total of 1270 urine samples, 125 samples (9.8%) were positive by both Gram staining and UF-1000i. Of 94 samples showing a single morphotype on agar plates, 82 samples had colony counts $\geq 10^5$ CFU/mL and most uropathogens (95.1%, 78 of 82) had UF-1000i counts $\geq 10^6$ bacteria/mL. Among them, Bruker MS correctly identified 86.6% (71/82) of all isolates, 89.2% (66/74) of Gram-negative bacteria (GNB), and 62.5% (5/8) of Gram-positive cocci (GPC) with higher average scores for GPC (mean score 2.013) and GNB (mean score 2.110).

Conclusions: Bruker MS with urine preparation by NaOH–HCl neutralization provides a simple, cost-effective, and accurate method for identifying uropathogens directly from urine. Using Bruker MS when single morphotype Gram staining and the UF-1000i count as $\geq 10^6$ bacteria/mL may improve the efficiency of bacterial identification in routine practice.

KEYWORDS

Bruker MS, direct identification, NaOH–HCl neutralization, urine screening tests

1 | INTRODUCTION

Urine processing to improve direct identification of uropathogens using matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) could improve the quantity of extracted and purified bacterial biomass,^{1–11} facilitated primarily by the high bacterial counts in infected urine.^{2,12} Overall, currently published methods for direct identification from urine have used differential

centrifugation,^{8,10} which combines MALDI-TOF MS with complex extraction protocols using pre- or post-treatment. Such protocols include the use of lysis solutions, such as special detergents (sodium dodecyl sulfate [SDS] or tween 80)^{2,3,7} or organic solvents (formic acid or acetonitrile),^{4,6,10} filter systems, such as vacuum-filter, dual-filter, or diafiltration units,^{1,5,11} or short-time bacterial cultivation on solid media.⁹ To date, the accuracy of these methods compared with conventional culture techniques has ranged from 58% to 92%. Moreover,

these complex protocols require large volumes (≥ 10 – 15 mL)^{1,11} and special equipment and are technically complex, labor intensive, and expensive for routine use in clinical practice.^{1–11}

Other drawbacks of these techniques are that insufficient bacteria are usually obtained from urine samples and that they require different thresholds for identifying microorganisms depending on the species.^{6,7} In addition, urine culture is the gold standard for microbiological confirmation of UTIs, but up to 80% of urine culture samples have been reported to be negative.¹³ Therefore, an algorithm for pre-selecting urine samples depending on the bacterial load could be of use before MALDI-TOF MS. Rapid urine screening methods such as Gram staining as well as automated urine screening devices like the UF-1000i (Sysmex Corporation, Kobe, Japan) allow the detection of the presence or absence of microorganisms and are able to identify possibly positive urine samples for bacterial cultures.^{6–9,14} However, performing Gram staining appropriately shows inter-observer variation, and the application of UF-1000i for screening has resulted in contradictory results if screening is performed with the urine flow cytometry method alone.^{14,15}

In our study, we evaluated the performance of Bruker MS (Bruker MALDI-TOF; Bruker Daltonik, Bremen, Germany) with urine prepared with NaOH–HCl neutralization for direct testing of urine samples. We also aimed to establish a protocol, combining this approach with conventional screening tests for proper workflow in clinical microbiology laboratories.

2 | MATERIALS AND METHODS

2.1 | Clinical samples

Ethical approval by the institutional review board of International St. Mary's Hospital, the Catholic Kwandong University College of Medicine (15IRB003, IS15EENE0006) in Korea was obtained. Among all urine samples request for conventional screening by Gram staining, automated flow cytometry (UF-1000i; Sysmex Corporation, Kobe, Japan), and conventional bacterial cultures between April and June 2015, we assessed urine samples that were positive by both Gram staining and UF-1000i in the analysis. Pedibag-type urine samples were analyzed from neonates, infants, and toddlers if they were the only samples that could be obtained.¹⁶ Gram stain was performed with leukocytes per 10 low-power fields and bacteria per 20 oil-immersion fields by two microbial experts. Samples judged to be negative, budding yeast cells, or showing more than three different microbial morphotypes by Gram stain were excluded. For automated urine flow cytometry, UF-1000i, samples with bacterial counts of $< 3 \times 10^5$ bacteria/mL reported as negative were excluded, and then samples with bacterial counts of $\geq 3 \times 10^5$ bacteria/mL were compared with bacterial counts provided according to the guidance of March Rosello et al. and the manufacturer's instructions.^{4,9}

2.2 | Conventional identification

For conventional identification, 1 μ L of urine samples was culture onto BAP and MAC plate and incubated in aerobic conditions at 37°C

for 24 hours. Subsequent microbial growth was identified using the MicroScan walkaway plus system (Beckman Coulter, Fullerton, CA, USA) or the API system (bioMérieux, Marcy l'Etoile, France). Potential uropathogens were defined as those positive for Gram-negative bacilli, *Staphylococcus aureus*, *S. saprophyticus*, β -hemolytic streptococcus, or *Enterococcus* spp., or as bi-microbial-positive urine samples with each present at $\geq 10^5$ CFU/mL. Urogenital or skin microbiota at least 10 times less than the uropathogens were ignored.¹⁶

2.3 | MALDI-TOF MS with urine preparation by NaOH–HCl neutralization

Briefly, 5 mL of urine was centrifuged for 30 seconds at 2000 g. In the previous Gram staining results, Gram-negative bacteria (GNB) were processed with 3 mL of the supernatant to remove cell debris, leukocytes, and mucus. For Gram-positive cocci (GPC), 3 mL including the pellet was assigned to avoid excessive removal of interleukocytic microorganisms. The resulting samples were centrifuged for 15 minutes at 14 000 g and then the pellet was washed with DW for twice. To the pellet, 500 μ L of 0.01 mol/L NaOH solution was added and allowed to stand for 10 minutes at room temperature, and then equal volume of 0.01 mol/L HCl solution was added for neutralization. After further centrifugation for 15 minutes at 14 000 g, and washing with DW, the pellet (10 μ L) was spotted onto the target plate and allowed to dry in an oven (56°C) for 5 minutes. For the GPC only, 1 μ L of 70% formic acid was applied to break up the cells and increase the amount of free protein. Finally, 1 μ L of matrix solution (α -cyano-4-hydroxycinnamic acid) was added. No repeat testing was performed. MALDI-TOF MS analysis was processed using Bruker MS with Flex control v. 3.0 software. A score < 1.7 indicated non-reliable ID or nonmatching identification by the manufacturer's instruction,

2.4 | The detection limit of the NaOH–HCl neutralization method

Detection limits were determined by adding the representative uropathogens, *Escherichia coli* ATCC 25922 and *E. faecium* clinical isolates into serially diluted water and sterile urine specimens with the following concentrations. After the first centrifugation step (2000 g, 30 seconds), 3 mL of the supernatant was selected and 3 mL was selected including pellet. Three independent replicates were tested for each dilution. We also compared our method with two centrifugation methods: the intact cell method protocol by Ferreira et al.^{10,17} and the 0.1% SDS–NaHCO₃ extraction method by Kim et al.³ using 5 mL of urine.

2.5 | Statistical analysis

Mean biotype confidence scores for the proprietary algorithm are reported. Data analyses were performed using SPSS v 22.0 (SPSS Inc., Chicago, IL, USA). We used chi-squared analyses and Fisher exact tests to compare proportions, as appropriate with P -values $\leq .05$ considered as statistically significant.

3 | RESULTS

3.1 | Gram stain and UF-1000i urine flow cytometry detection

Among the 1270 urine samples requested for Gram staining, UF-1000i, and urine culture by physicians during the study period, 125 (9.8%) samples were positive by both Gram staining and UF-1000i. Most of these samples were clean catch samples (88.7%, 110 of 125), with the remainder collected from pediatrics bags (8.1%, 10 of 125) or indwelling catheters (4.0%, 5 of 125). The sensitivity and specificity of Gram staining of the bacterial culture in our laboratory were 89.9% (125/139) and 94.9% (1074/1131) respectively. The diagnostic performance of UF-1000i in our laboratory was validated at 95% sensitivity and 80% specificity at a UF-1000i cut-off value of 3×10^5 bacteria/mL, according to the guidance of Broeren et al. and the manufacturer's instructions.^{14,15}

In total, 94 of the 125 samples had single colony morphologies on agar, and all but one showed a single morphotype Gram stain result. Of the 94 samples, 82 (87.2%) had colony counts $\geq 10^5$ CFU/mL, with 54 (65.8%) having UF-1000i counts $\geq 10^7$ bacteria/mL, 23 (28.0%) having counts of 10^6 – 10^7 bacteria/mL, and 5 (6.0%) having counts of 10^5 – 10^6 bacteria/mL, all with single morphotype on Gram staining. Ten samples (10 of 94, 10.6%) had counts of 10^4 – 10^5 CFU/mL, of which six were had UF-1000i counts of 10^6 – 10^7 bacteria/mL and four had counts of 10^5 – 10^6 bacteria/mL. The remaining two samples had colony counts of 10^3 – 10^4 CFU/mL, of which one had a UF-1000i count of 10^6 – 10^7 bacteria/mL and the other had a count of 10^5 – 10^6 bacteria/mL. Of the three samples yielding two different colony morphologies on agar, all had UF-1000i counts $\geq 10^6$ bacteria/mL, and two of the samples had a single morphotype on Gram staining. In 27 samples, we identified more than two colony morphologies that were considered contaminants, and 14 (51.9%) of these had UF-1000i

counts $\geq 10^6$ bacteria/mL, and 15 (55.6%) had a single morphotype on Gram staining (Table 1).

Most (99.2%, 124 of 125) samples had positive culture results. Of the 124 positive culture samples, 93 (75.0%, 93 of 124) were considered uropathogens, and all but one of these (98.9%, 92 of 93) showed a single morphotype on Gram staining, with 91.4% (85 of 93) having UF-1000i counts $\geq 10^6$ bacteria/mL. Thirty-one samples were considered to contain nonsignificant uropathogens, of which 51.6% (16/31) had UF-1000i counts $\geq 10^6$ bacteria/mL. The most frequently isolated single uropathogens were *E. coli* (75.6%, 68 of 90), *Enterococcus* spp. (7.8%, 7 of 90), and *Klebsiella pneumoniae* (6.7%, 6 of 90). The most common UF-1000i bacterial counts for *E. coli* and *Enterococcus* spp. were $\geq 10^7$ bacteria/mL (61.8%, 42 of 68) and 10^6 – 10^7 bacteria/mL (57.1%, 4 of 7), respectively. Three samples considered to have uropathogens had two different uropathogens in cultures and all samples (100%), including one with a dual morphotype on Gram staining (UF-1000i counts $\geq 10^6$ bacteria/mL). Among 31 samples considered not to have uropathogens, only 51.6% (16 of 31) had UF-1000i counts $\geq 10^6$ bacteria/mL, and there was no significant difference in the Gram staining results ($P > .05$; Table 2).

3.2 | MALDI-TOF analysis using the 0.01 mol/L NaOH–HCl neutralization method

Of a total of 125 urine samples tested with urine culture and Bruker MS, 94 samples had a single colony morphology on agar. Eighty-two samples had colony counts $\geq 10^5$ CFU/mL and most uropathogens (95.1%, 78 of 82) had UF-1000i counts $\geq 10^6$ bacteria/mL. Among them, Bruker MS correctly identified 71 samples (86.6%) with a higher mean identification score (>2 , thereby exceeding the criteria for reliability) (Figure 1). The percentage of correctly identified GNB results was greater than that of GPC. However, among the bacteria correctly identified by Bruker MS, higher average scores were seen for Gram-positive bacteria (mean score 2.013) and GNB (mean score 2.110)

TABLE 1 Culture results of 125 urine samples positive by both Gram staining and UF1000i flow cytometry

Conventional identification (total no. of isolates)	Gram stain results, no. of isolates (%)							
	Single morphotype				Two morphotypes			
	No.	UF1000i, bacteria/mL			No.	UF1000i, bacteria/mL		
		10 ⁵ -10 ⁶	10 ⁶ -10 ⁷	>10 ⁷		10 ⁵ -10 ⁶	10 ⁶ -10 ⁷	>10 ⁷
Growth (124)								
1 colony types (94)	93	10	29	54	1	-	1	-
≥10 ⁵ CFU/mL (82)	82	5	23	54	-	-	-	-
10 ⁴ -10 ⁵ CFU/mL (10)	9	4	5	-	1	-	1	-
10 ³ -10 ⁴ CFU/mL (2)	2	1	1	-	-	-	-	-
2 colony types (3)	2	-	1	1	1	-	-	1
3 colony types (27)	15	9	6	-	12	4	7	1
No growth (1)	1	-	1	-	-	-	-	-
Total (125)	111	19	37	55	14	4	8	2

CFU, colony forming unit.

TABLE 2 Bacteria present among the 124 urine samples positive by Gram staining, UF1000i flow cytometry, and urine cultures

Conventional identification (total no. of isolates)	Gram stain results, no. of isolates (%)							
	Single morphotype				Two morphotypes			
	No.	UF1000i, bacteria/mL			No.	UF1000i, bacteria/mL		
		10 ⁵ -10 ⁶	10 ⁶ -10 ⁷	>10 ⁷		10 ⁵ -10 ⁶	10 ⁶ -10 ⁷	>10 ⁷
Uropathogens (93)	92	8	29	55	1	-	-	1
<i>S. aureus</i> (1)	1	-	-	1	-	-	-	-
<i>E. faecalis</i> (2)	2	-	2	-	-	-	-	-
<i>E. faecium</i> (4)	4	1	2	1	-	-	-	-
<i>E. durans</i> (1)	1	1	-	-	-	-	-	-
<i>S. agalactiae</i> (1)	1	-	1	-	-	-	-	-
<i>E. coli</i> (68)	68	6	20	42	-	-	-	-
<i>K. pneumoniae</i> (6)	6	-	-	6	-	-	-	-
<i>E. aerogenes</i> (3)	3	-	2	1	-	-	-	-
<i>E. cloacae</i> (1)	1	-	-	1	-	-	-	-
<i>P. mirabilis</i> (1)	1	-	1	-	-	-	-	-
<i>P. aeruginosa</i> (2)	2	-	-	2	-	-	-	-
Bi-microbial [†] (3)	2	-	1	1	1	-	-	1
<i>E. coli</i> + <i>P. mirabilis</i>	-	-	-	1	-	-	-	-
<i>E. coli</i> + <i>Streptococcus</i> spp.	-	-	1	-	-	-	-	-
<i>K. pneumoniae</i> + <i>E. faecalis</i>	-	-	-	-	-	-	-	1
Non-uropathogens (31)	18	11	7	-	13	4	8	1
<i>Streptococcus</i> spp. (3)	2	1	1	-	1	-	1	-
<i>Lactobacillus</i> spp. (1)	1	1	-	-	-	-	-	-
Contaminant (27)	15	9	6	-	12	4	7	1
Total (124)	110	19	36	55	14	4	7	2

[†]Samples having two different uropathogens in cultures.

(Table 3). In 10 samples with colony counts of 10⁴-10⁵ CFU/mL, Bruker MS correctly identified 40% (4/10) (two *E. coli*, one *K. pneumoniae*, and one *E. faecium*), but in neither of two samples with colony counts of 10³-10⁴ CFU/mL were the bacteria correctly identified. Among three samples with two colony morphologies, Bruker MS correctly identified one of the mixed isolates in two samples with colony counts of ≥10⁵ CFU/mL on agar (one *E. coli* and one *K. pneumoniae*). Among 28 samples with three colony morphologies or no growth on agar, Bruker MS was unable to correctly identify any of the bacteria.

3.3 | Low detection limit in Bruker MS

Our 0.01 mol/L NaOH-HCl neutralization method for protein extraction showed the detection limit with a confidence score of the genus identification level of 10⁷ CFU/mL for *E. coli* in the sterile saline and urine specimens; it also yielded a limit of 10⁷ CFU/mL for *E. faecium* using both diluents in pellets. Using the 0.1% SDS-NaHCO₃ extraction method described by Kim et al.³, the detection limit was 10⁷ CFU/mL for *E. coli* only in the sterile saline and for *E. faecium* using only pellets in the sterile saline. Using the intact cell method, neither *E. coli* nor *E. faecium* was detected at colony counts of 10⁷ CFU/mL.

4 | DISCUSSION

We proposed the MALDI-TOF MS method in which urine was prepared for bacterial protein extraction by NaOH-HCl neutralization. To more accurately predict bacteriuria, we prospectively studied MALDI-TOF analysis performed on the same day as urine culture. Several previous studies on the direct identification of uropathogens by MALDI-TOF MS have been retrospectively performed using primarily culture-positive specimens.^{8,11,17} These study designs could be subjected to bias as the use of already defined clinical specimens could result in false-positive results, such as identifying clinically insignificant organisms, contamination with mixed flora, or a relatively high prevalence of positive cultures.

In addition, all samples for which Gram staining, UF-1000i, and urine culture were performed were requested by a physician. Gram staining is particularly helpful for inpatient care because it can diagnose the cause of sepsis before blood cultures are positive. However, its reported accuracy when used to diagnose UTI is inconsistent, with different microorganism counts being reported in different samples.^{7,18,19} It should also only be performed on request, because there is little

FIGURE 1 Flow diagram of direct testing of uropathogens by Bruker MS

value to be gained from direct smears of urine samples without clinical justification.^{16,20} Automated urine screening devices like UF-1000i is both rapid and simple to use, but it only report bacterial counts, and do not differentiate clinically significant uropathogens.^{6,15} Contradictory results have been reported, indicating either that it was not useful as a screening test because of false-negative results¹⁵ or that it reduced unnecessary cultures by 43%.¹⁴ In terms of the diagnostic accuracy for UTI, Gram staining has been reported to have 82.2%-97.9% sensitivity and 66.0%-95.0% specificity, while UF-1000i with a cut-off value of 230 bacteria/mL with a colony count $\geq 10^4$ CFU/mL has 95% sensitivity and 80% specificity.^{7,15} In our study, the diagnostic performance of Gram staining and UF-1000i is comparable with previously that in published results.^{7,15} Thus, to reduce costs and time and to seek the proper clinical algorithm for routine practice among this population, we designed a study that preselected samples based on positivity by Gram staining and UF-1000i screening.

By preselecting samples based on positivity by Gram staining and UF-1000i screening, the positive culture rate was very high (99.2%). Previous studies based on UF-1000i screening or Gram staining alone have reported 91.6% and 93.6% culture positivity, respectively.^{3,10} When assessing the likelihood of uropathogens being causative for clinically relevant bacteriuria, UF-1000i counts $\geq 10^6$ bacteria/mL and single morphotype Gram staining might represent an appropriate cut-off point for preselecting urine samples before direct MALDI-TOF MS (Table 1). Considering significant uropathogens, 91.4% (85 of 93) had

UF-1000i counts $\geq 10^6$ bacteria/mL; however, counts at this level were only observed in 51.6% (16 of 31) of the nonsignificant uropathogens. We observed that *E. coli* and *Enterococcus* spp. had UF-1000i bacterial counts of $\geq 10^7$ bacteria/mL (88.2%, 60 of 68) and 10^6 - 10^7 bacteria/mL (57.1%, 4 of 7) respectively. This may be due to the higher bacterial titers associated with GNB compared with GPC in some circumstances.

In this study, NaOH and HCl were used as pretreatment lysing solutions for bacterial protein extraction instead of special detergents or organic solvents. NaOH and HCl are the widely available reagents in clinical laboratories. Different methods have been used by researchers to dissociate target microorganisms from contaminating molecular entities, either by exposure to chemicals or by altering the environmental conditions. In this study, the efficacy of alkali treatment of the bacterial pellets was evaluated as a potential step for extracting host proteins that are tightly bound to the microbes before Bruker MS.

Kwon et al.²¹ were proposed that samples with colony counts $\geq 10^5$ CFU/mL in agar plate should be reported because they showed significant correlation with 73.9 times with UTIs. With the cutoff for UTI being defined as a monobacterial culture with counts $\geq 10^5$ CFU/mL on agar,²¹ Bruker MS correctly identified 86.6% (71 of 82) of samples, with identification scores above the predefined threshold of 1.7 for reliability (mean scores as 2.105). Consistent with previous studies,^{1,3,6,8,10} the identification rate was much higher for GNB than for GPC (85.1% and 62.5% respectively). Indeed, GPC and GNB isolates both showed higher mean identification scores of 2.127 and 2.013

TABLE 3 Comparison of conventional culture and Bruker MS among 82 mono-bacterial urine samples with counts of $>10^5$ CFU/mL on agar

Conventional identification	Total samples			UF1000i, bacteria/mL, 10^5 - 10^6			UF1000i, bacteria/mL, 10^6 - 10^7			UF1000i, bacteria/mL, $\geq 10^7$		
	Correct ID n/N [†] (%)	Mean score		Correct ID n/N [†] (%)	Mean score		Correct ID n/N [†] (%)	Mean score		Correct ID n/N [†] (%)	Mean score	
GPC	5/8 (62.5)	2.013		0/1 (0)	-		3/4 (75.0)	2.009		2/3 (66.7)	2.019	
<i>S. aureus</i>	1/1 (100)	2.084		-	-		-	-		1/1 (100)	2.084	
<i>E. faecalis</i>	2/2 (100)	2.072		-	-		2/2 (100)	2.072		-	-	
<i>E. faecium</i>	2/4 (50)	1.912		-	-		1/2 (50.0)	1.885		1/2 (50)	1.954	
<i>E. durans</i>	0/1 (0)	-		0/1 (0)	-		-	-		-	-	
GNB	66/74 (89.2)	2.110		3/4 (75.0)	1.952		16/19 (84.2)	2.114		47/51 (92.2)	2.180	
<i>E. coli</i>	55/61 (90.2)	2.124		3/4 (75.0)	1.952		13/16 (81.3)	2.116		39/41 (95.1)	2.182	
<i>K. pneumoniae</i>	5/6 (83.3)	2.005		-	-		-	-		5/6 (83.3)	2.116	
<i>E. aerogenes</i>	3/3 (100)	2.267		-	-		2/2 (100)	2.346		1/1 (100)	2.093	
<i>E. cloacae</i>	1/1 (100)	2.072		-	-		-	-		1/1 (100)	2.072	
<i>P. mirabilis</i>	1/1 (100)	1.706		-	-		1/1 (100)	1.706		-	-	
<i>P. aeruginosa</i>	1/2 (50)	1.988		-	-		-	-		1/2 (50)	1.988	
Total	71/82 (86.6)	2.105		3/5 (60.0)	1.952		19/23 (81.8)	2.002		50/54 (90.9)	2.162	

[†]No. of samples correctly identified in MALDI-TOF MS/no. of samples showing monobacterial uropathogens in conventional cultures.

respectively. In the method described by March Rosello et al., preincubation in brain-heart infusion broth was required to get an adequate bacteria counts for tests when UF-1000i counts $<10^7$ bacterial/mL were obtained. By contrast, UF-1000i bacterial counts $\geq 10^7$ bacterial/mL were sufficient to allow direct uropathogens identification with a sensitivity of 87.5%.⁹ Using the NaOH-HCl preparation method in this study, we showed that 89.1% (69/77) of samples were correctly identified by Bruker MS when UF-1000i bacterial counts were $\geq 10^6$ bacteria/mL (with scores >2 for GPC and GNB as well). Compared with pellet from GNB, the GPC pellet tended to be less well formed partly due to differences in the cell walls and being more resistant to cell lysis between the two organisms.^{1,10} Neutralization of urine with 0.01 mol/L NaOH and 0.01 mol/L HCl could effectively cause cell lysis and promote the release of microorganism from leukocytes, thereby increasing sensitivity. Perhaps more importantly, though, NaOH and HCl solutions are common reagents used in clinical microbiology laboratories, making them cheap, easy to obtain, and easy to use.

We included 30 mixed culture samples. Of the three samples with two colony types, two showing single populations with colony counts $\geq 10^5$ CFU/mL were correctly identified as *E. coli* (score 1.684) and *K. pneumoniae* (score 1.693). Of the 27 samples with three colony morphologies, which were considered contaminants, 48.2% (13 of 27) had UF-1000i counts of 10^5 - 10^6 bacteria/mL and none had the bacteria reliably identified by Bruker MS.

To compare urine preparation by NaOH-HCl neutralization with other methods of bacterial protein extraction, we evaluated the detection limits of our method when *E. coli* and *E. faecium* were added to sterile de-ionized water and sterile urine specimens. Our method yielded a detection limit of 10^7 CFU/mL for both *E. coli* and *E. faecium*. For the latter organism, only specimens with 3 mL urine samples, including the pellet, could reliably detect microorganisms at 10^7 CFU/mL. It is possible that the 7%-8% of samples for which identification failure occurs in culture-independent direct MALDI-TOF MS is associated with the removal of interleukocytic microorganisms in the pellets after initial processing.² Therefore, after the first centrifugation step, we selected 3 mL samples of urine that included pellets instead of supernatant with the GPC isolates. It was hoped that this would improve the collection of *S. aureus* bacteria. The intact cell protocol has been reported to require at least 8×10^4 CFU/mL for *E. coli* and 1.5×10^5 CFU/mL for *E. faecalis* for inoculation when using sterile de-ionized water and sterile urine specimens with sequential dilution.^{10,17} When the intact cell protocol was performed, using 5 mL of urine^{10,17} or sterile DW containing $<10^7$ CFU/mL of bacteria, the resultant pellet was miniscule or nonvisible and we failed to recover any material for transfer onto the MALDI target plate. This finding is consistent with a previous report by DeMarco et al.¹, indicating that the detection limit for the intact cell method was at colony counts of 10^5 CFU/mL with a mean confidence score of <1.7 (1.5 ± 1.3).

There are important benefits to our method. Of note, the turnaround time of the procedure was less than 1 hour, all incubation steps were performed in room temperature, and samples with single morphotype by Gram stain or UF-1000i counts of 10^{6-7} bacterial/mL did not require preincubation in brain-heart infusion broth.⁹ Therefore,

our method was simpler than the methods reported in previous studies, which required either incubation in a water bath³ or short-time subcultures.⁹ Moreover, the need for urine volumes of only 5 mL could be clinically relevant given that conventional urinalysis requires at least 10 mL of urine. Our method could allow direct urine preparation and diagnosis without the need for further urine sampling after Gram staining, UF-1000i analysis, conventional cultures, and Bruker MS analysis. However, in the period with prospective analysis of urine samples, positive samples with Gram staining and UF 1000i analysis were less than 10% of all specimens. Therefore, we encountered a few positive urine results by Gram stain and UF-1000i. In addition, Bruker MS only correctly identified in a limited percentage (60%) for GPC-positive specimens, implying that a better pretreatment step is needed to identify GPC directly from urine specimens.

5 | CONCLUSION

Bruker MS with protein extraction by 0.01 mol/L NaOH–HCl neutralization allows for rapid, simple, cost-effective, and accurate bacterial identification directly from infected urine samples, especially when major uropathogens such as *E. coli* and *E. faecalis* are involved. Using Bruker MS after the identification of a single morphotype on Gram staining and when there is a UF-1000i count $\geq 10^6$ bacteria/mL may improve the efficiency of bacterial identification in routine practice.

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