ORIGINAL ARTICLE

Development of a new protocol for rapid bacterial identification and susceptibility testing directly from urine samples

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Abstract

The current gold standard method for the diagnosis of urinary tract infections (UTI) is urine culture that requires 18–48 h for the identification of the causative microorganisms and an additional 24 h until the results of antimicrobial susceptibility testing (AST) are available. The aim of this study was to shorten the time of urine sample processing by a combination of flow cytometry for screening and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) for bacterial identification followed by AST directly from urine. The study was divided into two parts. During the first part, 675 urine samples were processed by a flow cytometry device and a cut-off value of bacterial count was determined to select samples for direct identification by MALDI-TOF-MS at $\geq 5 \times 10^6$ bacteria/mL. During the second part, 163 of 1029 processed samples reached the cut-off value. The sample preparation protocol for direct identification included two centrifugation and two washing steps. Direct AST was performed by the disc diffusion method if a reliable direct identification was obtained. Direct MALDI-TOF-MS identification was performed in 140 urine samples; 125 of the samples were positive by urine culture, 12 were contaminated and 3 were negative. Reliable direct identification was obtained in 108 (86.4%) of the 125 positive samples. AST was performed in 102 identified samples, and the results were fully concordant with the routine method among 83 monomicrobial infections. In conclusion, the turnaround time of the protocol described to diagnose UTI was about 1 h for microbial identification and 18–24 h for AST.

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Introduction

Urinary tract infection (UTI) is a common disease in both outpatients and inpatients [1,2]. Rapid diagnosis improves the management of patients, especially with sepsis, because inappropriate antibiotic therapy has an important impact on patient outcome and mortality rates [3]. Moreover, increasing bacterial resistance to antibiotics is a challenge for administering adequate empirical treatment.

In recent years, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) has become the reference standard for identification (ID) of microorganisms in clinical microbiology laboratories. Although direct ID from clinical specimens may be a promising application of MALDI-TOF-MS, several limitations should be considered. First, a relatively high bacterial concentration is required to obtain a valid protein spectrum. Second, a sufficient sample volume is also needed, being critical for some types of biological specimens. Additionally, some clinical samples are extremely purulent and thick due to high leucocyte concentrations,

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making it almost impossible to separate microorganisms from human cells. Previous studies have demonstrated that urine is an optimal specimen for direct MALDI-TOF-MS-based ID [4,5]. Once the pathogen is identified, an additional 18–24 h are needed to perform antimicrobial susceptibility testing (AST). Performing both ID and AST directly from urine samples could greatly shorten the time to the final microbiological report. As urine is the most common sample processed in microbiological laboratories, a screening method is required to rapidly identify positive samples suitable for direct ID and AST. Although flow cytometry is used mainly to identify negative samples that can be reported without culture [6,7], this method has also been used to rapidly identify positive urine samples to perform direct MALDI-TOF-MS ID [4,8].

The objective of our study was to elaborate a protocol for rapidly processing urine samples by a combination of flow cytometry for screening, mass spectrometry for ID followed by AST by a disc diffusion method directly from urine samples.

Material and Methods

Sample collection

Urine samples were collected during two time periods in the Department of Clinical Microbiology of a 700-bed university hospital in Barcelona, Spain. All samples were collected in 100-mL urine containers without any chemical preservatives and once received in the laboratory were processed immediately or stored at 4°C until processing. The first part of the study (Part A) lasted 5 weeks, during which 675 urine samples were collected and processed by flow cytometry to establish a cut-off value of bacterial count to select samples for direct ID by MALDI-TOF-MS. During the second part of the study (Part B), which lasted 8 weeks, 1029 urine samples were collected and processed by flow cytometry for screening. Samples achieving the cut-off value were then processed by MALDI-TOF-MS for bacterial ID followed by susceptibility testing. Ninety-six samples were needed to estimate a proportion using a 95% CI with 10% precision and assuming uncertainty (expected proportion 50%). We finally processed 140 samples by MALDI-TOF-MS for direct ID and 108 for direct susceptibility testing.

Routine microbiological processing

The routine protocol included conventional urine sample screening with a Clinitek® 500 Urine Chemistry Analyzer (Siemens Healthcare Diagnostics, Erlangen, Germany) and quantitative urine culture on cysteine-lactose electrolyte-deficient agar (Becton Dickinson GmbH, Heidelberg, Germany) at 37°C for 24–48 h in aerobiosis. The final ID was achieved with MALDI-TOF-MS (Bruker Daltonik, GmbH, Bremen, Germany). Urine samples were considered positive with >10^4/mL growth of one or two microorganisms whereas samples with more than two microorganisms were considered contaminated. Susceptibility testing was performed by a disc diffusion method and the results were interpreted according to EUCAST guidelines (http://www.eucast.org).

Flow cytometry

Flow cytometry analysis was performed by UF-500i (Sysmex, Kobe, Japan), using 4 mL of urine, as described previously [7,8].

Direct MALDI-TOF-MS-based identification

The following protocol was used to achieve direct ID by MALDI-TOF-MS. First, 10 mL of urine were centrifuged at 430 ***g*** for 5 min; then, the supernatant was centrifuged at 15 600 ***g*** for 2 min; the pellet obtained was washed twice with sterile water and used for ID by MALDI-TOF-MS. Each sample was analysed in duplicate. For this, two spots of MSP 96 target polished steel plates (Bruker Daltonik GmbH) were covered with 1 μL of the pellet obtained and air-dried. Then, one of the spots was covered with 1 μL of 70% formic acid (Sigma-Aldrich, St Louis, MO, USA) and air-dried. Thereafter, both spots were covered with 1 μL of matrix solution (cyano-4-hydroxy-cinnamic acid) in 50% acetonitrile with 2.5% trifluoroacetic acid) (Bruker Daltonik GmbH). Spectra acquisition was performed in MALDI Microflex LT (Bruker DaltoniK GmbH) using FLEXCONTROL v.3.0 software. The final ID was achieved using BIOTYPER v.3.0 software. According to the manufacturer, a score of <1.7 indicates no reliable identification (NRI), a score between 1.7 and 2.0 indicates genus identification, and a score ≥2.0 indicates species identification. Apart from a score value, MALDI-TOF-MS software provides a list of ten microorganisms with the most similar spectra. We considered the species-level ID valid if the same species with a score ≥1.7 was obtained for the first microorganism from the list of the two dropped spots or for the first two microorganisms from the list of the same spot if one of the spots was NRI or No Peaks (NP). We considered the genus-level ID valid if only the first option with a score ≥1.7 was obtained from the list of only one spot. The highest score obtained from two spots was recorded to calculate the average score value.

Direct antimicrobial susceptibility testing

Susceptibility testing was conducted with the remaining pellet after direct bacterial ID by MALDI-TOF-MS. To do this, an equivalent 0.5 McFarland suspension (0.5 McF) in sterile saline was prepared and plated on Müller–Hinton (MH) agar to perform AST by a disc diffusion method. If the pellet was insufficient to achieve 0.5 McF, an additional 10 mL of urine, if available, were centrifuged as described above. The results of

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the direct susceptibility testing were interpreted according to EUCAST guidelines and compared with those obtained using the conventional procedure.

Statistical analysis

Statistical analysis was performed using SPSS software (SPSS, Chicago, IL, USA). To compare quantitative and qualitative data the Student's *t*-test and chi-squared test were used, respectively. Differences were considered significant with a p <0.05.

Results

The characteristics of the samples from study groups A and B are shown in Table 1. To establish a cut-off value of bacterial count in Part A all the samples processed (*n* = 675) were divided into ten groups according to the bacterial count provided by flow cytometry. Table 2 shows the correlation between the urine culture results (positive, negative or contaminated) and the bacterial count obtained by UF-500i. The cut-off value was determined to be $\geq 5 \times 10^6$ bacteria/mL since 94% (110/117) of the samples achieving or exceeding the cut-off value were positive by urine culture with only 6% (7/117) of contaminated samples. We also compared the characteristics of the samples achieving the cut-off value with those that did not from the part B of the study (Table 3).

During Part B, 1029 consecutive urine samples were tested by UF-500i and 163 (15.8%) achieved the bacterial count cut-off (Table 2), with 140 out of 163 (85.9%) samples being processed by MALDI-TOF-MS for direct ID and 23 samples not having sufficient volume to be processed. On analysing the urine culture results, 125 of the 140 samples processed were positive, 12 samples were contaminated and three were negative.

MALDI-TOF-MS direct ID results

A valid ID was obtained in 108 (86.4%) of the 125 samples positive by culture, while 17 (13.6%) samples were NP (*n* = 6) or NRI (*n* = 11). Interestingly, species-level ID was obtained in 104 of these 108 samples. Table 4 summarizes the results of direct MALDI-TOF-MS ID and conventional urine culture in the 125 culture-positive samples. None of the samples was misidentified with direct MALDI-TOF-MS compared with the routine method. Nineteen of the 108 samples identified were positive for two pathogens by standard culture. MALDI-TOF-MS correctly identified one of the two pathogens, most likely the predominant pathogen. The average score of direct MALDI-TOF-MS ID was 2.122 (min 1.753; max 2.420). The dominating pathogen was *Escherichia coli* (68/108, 63%), followed by *Klebsiella pneumoniae* (22/108, 20.4%). Of the 125 positive samples, 124 yielded growth of $\geq 10^5$ CFU/mL and only one sample showed growth between 10^4 and 10^5 CFU/mL on culture plates.

Among the 12 samples categorized as contaminated, five were NRI, one was NP and in six samples a reliable ID was obtained, including two *E. coli* (at the species level), two *K. pneumoniae* (at the genus level), one *K. oxytoca* (at the genus level) and one *Aerococcus urinae* (at the genus level).

On analysing three samples that were negative by conventional culture, two were NP and one was NRI. The turnaround time of direct MALDI-TOF-MS ID was about 1 h.

Direct AST results

Direct AST was performed in 102 out of 108 samples in which bacteria were identified, including three Gram-positive and 99 Gram-negative bacteria (GNB). In six samples the pellet obtained for MALDI-TOF-MS was not sufficient to achieve a 0.5 McF suspension, and no additional urine sample was available for further processing. The results of direct and routine susceptibility testing were fully concordant in 83 monomicrobial infections, including 3 Gram-positive bacteria and 80 GNB. Among 80 monomicrobial GNB samples, 14 (17.5%) showed extended-spectrum β-lactamase-producing GNB (nine *E. coli* and five *K. pneumoniae*) and one AmpC β-lactamase-hyper-producing *Enterobacter cloacae*.

Regarding the 19 samples in which two pathogens were isolated, *Enterococcus* spp. and GNB co-infection were identified in five cases, and the results of direct ID and AST corresponded to GNB. Nevertheless, enterococcal growth was detected on MH plates. The remaining 14 samples were positive for two Gram-negative bacteria and showed mixed growth on MH plates. Routine testing detected one extended-spectrum β-lactamase-producing *E. coli*, one cefamicinase-producing *E. coli* and one multi-resistant *Pseudomonas aeruginosa*. Interestingly, these resistance patterns were suspected in direct AST despite the presence of the second pathogen.

Direct AST was also performed in five of the six directly identified samples, which were later reported to be contaminated by the routine procedure, being of mixed growth on MH plates.

TABLE 1. Characteristics of the patients and urine samples from Part A (*n* = 675) and Part B (*n* = 1029) of the study

Characteristics	Part A, *n* (%)	Part B, *n* (%)	p[a]
Female patients	413 (61.2)	634 (61.6)	0.859
Age (years), mean, SD	56.6, 19.3	54.9, 19.8	0.079
Outpatients	516 (76.4)	797 (77.5)	0.628
Urine catheter	100 (14.8)	135 (13.1)	0.321
Positive samples	198 (29.3)	289 (28.1)	0.577
Contaminated samples	164 (24.3)	241 (23.4)	0.678
Negative samples	313 (46.4)	499 (48.5)	0.391

[a]Differences were considered significant with a p <0.05.

TABLE 2. Correlation between urine culture results and flow cytometer bacterial count among urine samples processed during Part A and Part B of the study

	Bacterial count (bacteria/mL) by UF-500i										
Urine culture	$<5 \times 10^3$ *n* (%)	$5 \times 10^3 – 10^4$ *n* (%)	$10^4 – 5 \times 10^4$ *n* (%)	$5 \times 10^4 – 10^5$ *n* (%)	$10^5 – 5 \times 10^5$ *n* (%)	$5 \times 10^5 – 10^6$ *n* (%)	$10^6 – 5 \times 10^6$ *n* (%)	$5 \times 10^6 – 10^7$ *n* (%)	$10^7 – 10^8$ *n* (%)	$\geq 10^8$ *n* (%)	Total *n* (%)
Urine samples processed during Part A of the study											
Positive	0 (0)	5 (6.9)	18 (11.6)	8 (14.5)	18 (18)	11 (26.2)	28 (45.9)	**16 (80)**	**90 (96.8)**	**4 (100)**	198 (29.3)
Contaminated	7 (9.6)	7 (9.7)	29 (18.7)	17 (30.9)	46 (46)	22 (52.4)	29 (47.5)	4 (20)	3 (3.2)	0	164 (24.3)
Negative	66 (90.4)	60 (83.3)	108 (69.7)	30 (54.5)	36 (36)	9 (21.4)	4 (6.6)	0	0	0	313 (46.4)
Total	73 (100)	72 (100)	155 (100)	55 (100)	100 (100)	42 (100)	61 (100)	20 (100)	93 (100)	4 (100)	675 (100)
Urine samples processed during Part B of the study											
Positive	2 (1.6)	5 (4.6)	20 (9.7)	12 (15.6)	34 (21.1)	23 (32.9)	51 (43.6)	**29 (72.5)**	**112 (91.8)**	**1 (100)**	289 (28.1)
Contaminated	5 (4)	12 (11)	34 (16.4)	28 (36.4)	66 (41)	32 (45.7)	46 (39.3)	8 (20)	10 (8.2)	0	241 (23.4)
Negative	118 (94.4)	92 (84.4)	153 (73.9)	37 (48.1)	61 (37.9)	15 (21.4)	20 (17.1)	3 (7.5)	0	0	499 (48.5)
Total	125 (100)	109 (100)	207 (100)	77 (100)	161 (100)	70 (100)	117 (100)	40 (100)	122 (100)	1 (100)	1029 (100) (100)

Positive samples with bacterial count greater than or equal to the established cut-off in the two study groups are shown in bold.

Discussion

Urinary tract infection is the second most common infection and is a common cause of sepsis [9]. The aim of this study was to elaborate a protocol for rapid urine sample processing to shorten the time of microbial ID and susceptibility testing results. We used a high urine volume (10 mL) for MALDI-TOF-MS ID as the pellet obtained was also needed for direct AST. This could explain the higher score values of direct ID compared with other studies [4,5,8]. However, the use of a high input volume could be a limitation for laboratories that receive urine in 10-mL urine containers, especially to perform both direct identification and susceptibility testing, and considering that an additional 4 mL are needed for flow cytometry screening. Another approach to increase bacterial concentrations involves the addition of a short incubation step before MALDI-TOF-MS ID [10]. Nevertheless, the incubation time of at least 4 h needed to obtain significantly better results obviously prolongs the time of urine processing, being more laborious and cumbersome. In concordance with our results, the authors concluded that the Sysmex UF-1000i bacterial count of $>10^7$ bacterial/mL allows direct ID without previous incubation, using a centrifugation-based procedure, while a lower bacterial count was frequently insufficient to obtain a reliable ID. To improve the detection limits of MALDI-TOF-MS, the diafiltration method was also proposed [11]. Although this method allows identification of 10^5 CFU/mL, its turnaround time is about 2–3 h, whereas the centrifugation protocol requires <1 h. Moreover, additional materials (centrifugal filters) for diafiltration are required. Sánchez-Juanes *et al.* improved direct ID from urine with pretreatment with SDS to enhance cell lysis and the release of microorganisms [12]. A protein extraction procedure with the bacterial pellet obtained has been reported to slightly improve direct MALDI-TOF-MS-based ID [4]. Nevertheless, since bacteria were also needed for direct AST, a protein extraction protocol could not have been used in the present study.

In our study, no valid direct ID was obtained in 17 (13.6%) samples with counts $\geq 10^5$ CFU/mL by culture and $\geq 5 \times 10^6$ bacteria/mL by flow cytometry. The lower identification scores obtained in five cases could be explained by the presence of two pathogens, probably in equal proportion. The remaining failures may be due to the presence of urine proteins not removed during washing steps [13] or to the presence of contaminant bacteria in 10 mL of urine processed, that could not be isolated by routine culture, in which only 1 μL of urine is used [10]. Samples achieving the established cut-off value were more likely from older patients and patients with a urine catheter.

An interesting finding of our study was the direct ID of *Clostridium perfringens*, the obligate anaerobe that does not grow on cysteine-lactose electrolyte-deficient medium with standard incubation conditions. In this case, after direct MALDI-TOF-MS ID, the standard protocol was changed and this sample was cultured on Schaedler medium in anaerobiosis. After 18 h a pure culture was obtained with $>10^5$ CFU/mL of *C. perfringens*, whereas the standard aerobic culture was negative after 48 h of incubation. As anaerobic bacteria are rarely involved in UTI, most laboratories do not include an anaerobic culture in the standard urine culture protocol. Nevertheless, UTI caused by anaerobic bacteria has been previously reported [14,15], being particularly relevant in patients with anatomical abnormalities.

TABLE 3. Characteristics of the patients and urine samples from Part B ($n = 1029$) of the study according to the cut-off value of bacterial count

Characteristics	$<5 \times 10^6$ bacteria/mL, *n* (%)	$\geq 5 \times 10^6$ bacteria/mL, *n* (%)	p [a]
Female patients	529 (61.1)	105 (64.4)	0.422
Age (years, mean, SD)	53.8, 19.4	60.3, 20.6	**<0.001**
Outpatients	665 (76.8)	132 (81)	0.240
Urine catheter	103 (11.9)	32 (19.6)	**0.007**

[a] Differences were considered significant with a $p < 0.05$. Statistically significant differences are indicated in bold.

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TABLE 4. Results of direct MALDI-TOF-MS identification and conventional urine culture of 125 positive urine samples

Direct MALDI-TOF-MS identification	Number of samples	Urine culture results (number of samples)	Medium score of direct MALDI-TOF-MS identification	Direct susceptibility testing performed
Escherichia coli	68	*E. coli* (59)	2.100	63
		E. coli and other microorganism (9)[a]	2.102	
Klebsiella pneumoniae	22	*K. pneumoniae* (16)	2.175	22
		K. pneumoniae and other microorganism (6)[b]	2.082	
Proteus mirabilis	5	*Proteus mirabilis* (3)	2.276	5
		Proteus mirabilis and *E. coli* (2)	2.115	
Klebsiella oxytoca	4	*K. oxytoca* (4)	2.163	4
Pseudomonas aeruginosa	3	*P. aeruginosa* (1)	1.996	3
		P. aeruginosa and *K. pneumoniae* (1)	2.325	
		P. aeruginosa and *Enterococcus faecalis* (1)	2.168	
Morganella morganii	1	*M morganii* (1)	2.276	1
Enterobacter cloacae	1	*Enterobacter cloacae* (1)	1.881	1
Streptococcus agalactiae	1	*Streptococcus agalactiae* (1)	2.203	1
Staphylococcus aureus	1	*S. aureus* (1)	1.946	1
Enterococcus faecalis	1	*Enterococcus faecalis* (1)	2.257	1
Clostridium perfringens	1	*C. perfringens* (1)	2.289	—
No peaks	6	*E. coli* (2), *K. pneumoniae* (1), *Proteus mirabilis* (1), *P. aeruginosa* and *M. morganii* (1), *P. aeruginosa* and *Enterococcus faecalis* (1)	—	—
No reliable identification	11	*E. coli* (4), *K. pneumoniae* (1), *M. morganii* (1), *Enterobacter cloacae* (1), *K. pneumoniae* and *Enterococcus faecalis* (1), *E. coli* and *Enterococcus faecalis* (1), *E. coli* and *Citrobacter freundii* (1), *Enterococcus faecalis* (1)	—	—

Abbreviations: MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight mass spectrometry.
[a]Nine mixed cultures included *E. coli* and *K. pneumoniae* (2), *Enterococcus faecalis* (1), *Serratia marcescens* (1), *P. aeruginosa* (1), *Enterococcus hirae* (1), *Proteus mirabilis* (1), *Enterobacter aerogenes* (1) and a different strain of *E. coli* (1).
[b]Six mixed cultures included *K. pneumoniae* and *E. coli* (2), *Enterococcus faecalis* (2), *Proteus mirabilis* (1) and *Citrobacter koseri* (1).

Interestingly, this urine was from a patient with Crohn's disease who had presented several episodes of UTI in the previous 6 months. Therefore, the presence of complications, such as an enterovesicular fistula, should be ruled out [16].

Importantly, the results of direct AST were fully concordant among monomicrobial infections. The small number of directly identified GPB bacteria may be explained by the high cut-off value of the bacterial count used, which is more frequently achieved by GNB than by Gram-positive microorganisms [17].

The main limitation of our protocol was contaminated samples in which a valid direct ID ($n = 6$) could be reported as a true positive. This occurred in 4.3% (6/140) of the total number of urine samples processed, representing 5.3% (6/114) of samples with a valid direct ID. Although the number of directly identified contaminated samples was small, the clinical impact of reporting these samples as positive must be taken into account. On the other hand, since the results of direct AST were available simultaneously with traditional urine culture, contaminated samples were detected and the corresponding AST could be ruled out. Another limitation of this study is the high sample volume (minimum 14 mL) required to perform the protocol, which could be a problem if smaller urine collection containers are used. Regarding a future application of the protocol proposed, two main points should be considered. First, urine is the most common sample processed in microbiology laboratories, whereas sample preparation for MALDI-TOF-MS requires time and dedicated personnel. Second, the patients who would most benefit from the application of the protocol should be determined. Direct ID and susceptibility testing should probably be reserved for patients with pyelonephritis or prostatitis and those with complicated UTI.

In conclusion, the protocol described provides rapid discrimination of positive urine samples suitable for direct ID and AST. This procedure shortens the time of urine sample processing by 24–48 h in cases of monomicrobial infection. Bacterial ID was available the same day as sample reception in the microbiology laboratory, thereby facilitating the implementation of appropriate empirical treatment, whereas AST results were available in 18–24 h, allowing treatment modification, if necessary. Nevertheless, the possible risk of reporting some contaminated samples as positive requires further study.

Transparency Declaration

The authors declare that they have no conflicts of interest.

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