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RESEARCH ARTICLE

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Improved bacterial identification directly from urine samples with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

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Background: Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) contributes to rapid identification of pathogens in the clinic but has not yet performed especially well for Gram-positive cocci (GPC) causing complicated urinary tract infection (UTI). The goal of this study was to investigate the possible clinical use of MALDI-TOF MS as a rapid method for bacterial identification directly from urine in complicated UTI.

Methods: MALDI-TOF MS was applied to urine samples gathered from 142 suspected complicated UTI patients in 2015-2017. We modified the standard procedure (Method 1) for sample preparation by adding an initial 10 minutes of ultrasonication followed by centrifugation at 500 *g* for 1 minutes to remove debris such as epithelial cells and leukocytes from the urine (Method 2).

Results: In 133 urine culture-positive bacteria, the rate of corresponded with urine culture in GPC by MALDI-TOF MS in urine with standard sample preparation (Method 1) was 16.7%, but the modified sample preparation (Method 2) significantly improved that rate to 52.2% (*P*=.045). Method 2 also improved the identification accuracy for Gram-negative rods (GNR) from 77.1% to 94.2% (*P*=.022). The modified Method 2 significantly improved the average MALDI score from 1.408±0.153 to 2.166±0.045 (*P*=.000) for GPC and slightly improved the score from 2.107±0.061 to 2.164±0.037 for GNR.

Conclusion: The modified sample preparation for MALDI-TOF MS can improve identification accuracy for complicated UTI causative bacteria. This simple modification offers a rapid and accurate routine diagnosis for UTI, and may possibly be a substitute for urine cultures.

KEYWORDS

bacterial identification, clinical laboratory, complicated urinary tract infection, MALDI-TOF MS, methodology, rapid diagnosis

1 | INTRODUCTION

The clinical use of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has spread as a new method of bacterial identification due to the simplicity and speed of the procedure.[1] Reportedly, MALDI-TOF MS can identify bacteria directly from culture-positive blood samples.[2] Rapid identification of causative bacteria in common infectious diseases such as upper respiratory tract infection (RTI) or urinary tract infection (UTI) are beneficial for patients.[3,4] In order to apply MALDI-TOF MS for routine clinical diagnosis, direct comparison between MALDI-TOF MS and conventional cultures is required, especially in complicated UTI cases where it is not easy to assume the causative bacteria.[5]

Several institutions have applied this method in the clinic for bacteremia but comparatively few have adopted it for UTI. This method can detect bacteria in blood, but contamination of urine by more than one kind of bacteria, cluster formation, and the smaller size of Gram-positive bacteria have made MALDI-TOF MS identification of Gram-positive bacteria in urine difficult, requiring some additional procedure for separating bacterial aggregation.[6] There are no standardized protocols for sample preparation for identifying bacteria from urine culture, which may include several kinds of bacteria, causative or not.[7] In this study, we especially focused on Gram-positive bacteria, which cause complicated UTI more often than uncomplicated UTI. We modified the standard sample preparation for MALDI-TOF MS by adding ultrasonication and centrifugation for separating bacterial aggregation and removing debris such as epithelial cells and leukocytes in urine.

2 | MATERIALS AND METHODS

2.1 | Patients

The results of bacterial identification by MALDI-TOF/MS and standard urine culture were compared for urine samples from 142 suspected complicated UTI patients managed in Kobe University Hospital in 2015 and 2017, and 133 cases were culture positive. All 133 patients included in this study were diagnosed with complicated UTI by positive urine culture; that is, all the positive urine samples analyzed were consistent with complicated UTI. Complicated UTI was defined as UTI with an underlying disease affecting the urinary tract, such as stones, cancer or benign prostate hyperplasia, or systemic disease related to the immune system such as diabetes, steroid use or chemotherapy.[8] This study was approved by the Kobe University School of Medicine institutional review board (IRB).

2.2 | Urine culture testing

Semiquantitative urine cultures were performed by procedures based on Cintron 16 recommendations using cystine lactose electrolyte deficient (CLED) agar (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan). All bacterial concentrations were determined by a single technician and were expressed as the number of colony forming units (CFU) per milliliter. Samples were considered positive if they contained $\geq 10^5$ or 10^4 to $<10^5$ CFU of urinary pathogens/mL of pure culture. The microorganisms isolated were identified by standard biochemical procedures.[9]

2.3 | Sample preparation for MALDI-TOF Mass Spectrometry

We employed two different methods of centrifugation for MALDI-TOF MS: conventional (Method 1) for the first 53 urine samples and additional ultrasonication and low speed centrifugation for cell component removal (Method 2) for the next 80 cases. Procedures were as follows: (i) Three mL of urine was equally aliquoted into two 1.5 mL tubes. (ii) Ultrasonication by ultrasonic bath for 10 minutes to disperse bacterial cell aggregation (Method 2 only). (iii) Centrifugation at 500 *g* for 1 minutes (Method 2 only). (iv) Supernatant was placed in another 1.5 mL tube (Method 2 only). (v) Centrifugation at 15 000 *g* for 5 minutes. (vi) The supernatant was discarded and the pellet was mixed with distilled water (1 mL). (vii) Centrifugation at 15 000 *g* for 2 minutes. (viii) The supernatant was discarded and the pellet mixed with distilled water (1 mL). (ix) Centrifugation at 15 000 *g* for 2 minutes. (x) The supernatant was discarded and the pellet mixed with distilled water (300 μL) until the pellet could not be seen, followed by the addition of dehydrated ethanol (900 μL). (xi) Incubation at room temperature for 10 minutes. (xii) Centrifugation at 15 000 *g* for 2 minutes. (xiii) The supernatant was discarded and the pellet mixed with distilled water (1 mL). (xiv) The supernatant was discarded and the pellet was dried for 10 minutes. (xv) 10 μL formic acid was added and mixed well by pipetting, and then mixed by adding acetonitrile (10 μL). (xvi) Centrifugation at 15 000 *g* for 2 minutes.

2.4 | MALDI-TOF MS

After the last centrifugation, 1 μL of supernatant was applied to the MALDI target plate and air dried. After drying, the bacteria cultured in urine were overlaid with 1 μL of matrix solution (saturated solution of HCCA [a-cyano-4-hydroxy cinnamic acid] in organic solvent [50% acetonitrile and 2.5% trifluoroacetic acid]) and air dried. Measurements were acquired by the MALDI Biotyper system (Bruker Daltonics, Bremen, Germany) using the default settings. Data analysis was performed on the MALDI Biotyper software (Bruker Daltonics) at default settings. Bacterial identification scores were obtained from pattern matching of the experimental spectra with the database. According to the manufacturer's recommendations, a score ≥2.0 indicated species identification, a score between 1.7 and 2.0 indicated genus identification and a score of <1.7 indicated no identification.[10] Median scores for each isolated organism were obtained for comparison of accuracy.

2.5 | Statistical analyses

We determined significant differences using the χ^2 test or Fisher's exact test. Student's *t* test was used to compare the MALDI score between the two different methods. Differences were considered to be statistically significant at $P<.05$.

TABLE 1 Total bacteria identification by conventional urine culture and MALDI-TOF MS

Isolated organism by urine culture	Total identification by urine culture (No. of cases)	MALDI-TOF/MS identification score>1.7 (No. of cases)	MALDI-TOF/MS identification score>2.0 (No. of cases)	Mean score±SE
Escherichia coli	54	46	38	2.20±0.04
Klebsiella pneumoniae	12	11	5	2.10±0.08
Enterococcus faecalis	10	6	5	2.16±0.07
Staphylococcus aureus	10	2	2	1.54±0.29
Streptococcus agalactiae	7	3	3	1.92±0.163
Staphylococcus epidermidis	4	0	0	N/A
Enterobacter cloacae	5	5	3	2.11±0.12
Candida albicans	4	0	0	N/A
Pseudomonas aeruginosa	4	3	2	2.04±0.16
Corynebacterium striatum	3	2	2	2.17±0.03
Morganella morganii	3	3	2	2.12±0.10
Aerococcus urinae	2	1	0	1.92
Enterobacter aerogenes	2	1	1	1.83±0.44
Enterococcus faecium	2	1	0	1.93
Klebsiella oxytoca	2	2	2	2.28±0.16
Proteus mirabilis	2	1	0	1.92
Candida tropicalis	1	0	0	N/A
Citrobacter amalonaticus	1	1	1	2.49
Citrobacter freundii	1	1	1	2.17
Citrobacter koseri	1	0	0	N/A
Staphylococcus haemolyticus	1	1	0	1.95
Others	2	0	0	N/A
Total	133	90	67	

3 | RESULTS

3.1 | Urine culture

Conventional urine culture tests detected 133 bacterial cases from complicated UTI patients who were diagnosed by a single urologist (K.S.). We had four fungus isolations (3.0%), three Gram-positive rods (GPR) (2.3%), 40 Gram-positive cocci (GPC) (30.1%), and 86 Gram-negative rods (GNR) (64.7%). In detail, *Escherichia coli* was most often isolated (n=54, 40.6%) followed by *Klebsiella pneumoniae* (n=12, 9.0%), *Enterococcus faecalis* (n=10, 7.5%), and *Staphylococcus aureus* (n=10, 7.5%) (Table 1).

3.2 | MALDI-TOF MS

MALDI-TOF MS identified 90 cases including both Methods 1 and 2. The frequency of identified bacteria was *E. coli* (n=46, 51.1%) followed by *K. pneumoniae* (n=11, 12.2%) and *E. faecalis* (n=10, 11.1%) and *S. aureus* (n=10, 11.1%) (Table 1). Regarding the accuracy of the MALDI-TOF MS identification, 67 cases (50.4%) were confirmed by a score ≥2.0 (at species level) and 90 cases (67.7%) by a score of ≥1.7 (at genus level) in total urine samples from both Methods 1 and 2. The distribution of corresponding ratio in total urine samples including Methods 1 and 2 was 2/3 (66.7%) in GPR; 14/35 (40.0%) in GPC; 76/87 (87.4%) in GNR and 0/4 (0%) in fungus. MALDI scoring rate by score ≥2.0 was 33.3% in GPR, 25.0% in GPC, 64.0% in GNR.

3.3 | Comparison between the 2 MALDI-TOF MS methods

Method 1 (n=53) resulted in the identification of *E. coli* (n=18, 34.0%), *K. pneumoniae* (n=4, 7.5%) (Table 2). Method 2 (n=59) results were *E. coli* (n=28, 47.5%), *E. faecalis* (n=8, 13.6%) and *K. pneumoniae* (n=7, 11.9%) (Table 3). Dispersed cell aggregation after ultrasonication in Method 2 was confirmed under a microscope (data not shown). The rates of correspondence with conventional cultures in Method 1 were GPR: 2/3 (66.7%), GPC: 2/12 (16.7%), GNR: 27/35 (77.1%) and fungi: 0/2 (0%). Rates for Method 2 were GPC: 12/23 (52.2%) (P=.045), GNR: 49/52 (94.2%) (P=.022) and fungi: 0/2 (0%) (P=1.000) (Table 4). MALDI scores for Method 1 were 1.985±0.069 in total, 2.107±0.061 in GNR, 1.408±0.153 in GPC, and 2.167±0.032 in GPR. Scores for Method 2 were 2.164±0.031 in total, 2.164±0.037 in GNR, 2.166±0.045 in GPC (Table 5). Statistical analysis showed that

TABLE 2 Bacteria identification by conventional urine culture and MALDI-TOF MS (Method 1)

Isolated organism by urine culture	Total identification by urine culture (No. of cases)	MALDI-TOF/MS identification Score>1.7 (No. of cases)	MALDI-TOF/MS identification Score>2.0 (No. of cases)	Mean score±SE
Escherichia coli	22	18	13	2.14±0.06
Klebsiella pneumoniae	5	4	3	2.25±0.14
Staphylococcus aureus	4	0	0	1.07±0.05
Corynebacterium striatum	3	2	2	2.17±0.03
Candida albicans	2	0	0	N/A
Enterococcus faecalis	2	1	0	1.848
Pseudomonas aeruginosa	2	2	1	2.19±0.14
Staphylococcus epidermidis	2	0	0	1.68
Morganella morganii	1	1	1	2.10
Citrobacter koseri	1	0	0	1.18
Staphylococcus haemolyticus	1	1	0	1.952
Candida tropicalis	1	0	0	N/A
Klebsiella oxytoca	1	1	1	2.11
Citrobacter amalonaticus	1	1	1	2.49
Enterobacter aerogenes	1	0	0	1.39
Proteus mirabilis	1	0	0	N/A
Aerococcus urinae	1	0	0	N/A
Streptococcus agalactiae	1	0	0	1.17
Enterococcus faecium	1	0	0	N/A
Total	53	31	22	

TABLE 3 Bacteria identification by conventional urine culture and MALDI-TOF MS (Method 2)

Isolated organism by urine culture	Total identification by urine culture (No. of cases)	MALDI-TOF/MS identification Score>1.7 (No. of cases)	MALDI-TOF/MS identification Score>2.0 (No. of cases)	Mean score±SE
Escherichia coli	32	28	25	2.23±0.03
Enterococcus faecalis	8	5	5	2.22±0.04
Klebsiella pneumoniae	7	7	2	2.00±0.05
Staphylococcus aureus	6	2	2	2.25±0.12
Streptococcus agalactiae	6	3	3	2.17±0.05
Enteroacter cloacae	5	5	3	2.10±0.03
Morganella morganii	2	2	1	2.13±0.17
Staphylococcus epidermidis	2	0	0	N/A
Candida albicans	2	0	0	N/A
Pseudomonas aeruginosa	2	1	1	1.90±0.03
Aerococcus urinae	1	1	0	N/A
Enterobacter aerogenes	1	1	1	2.27
Enterococus faecium	1	1	0	1.93
Citrobacter freundii	1	1	1	2.17
Klebsiella oxytoca	1	1	1	2.42
Proteus mirabilis	1	1	0	1.92
Others	2	0	0	N/A
Total	80	59	45	

TABLE 4 Correspondence rate MALDI-TOF MS identification and conventional cultures

	Method 1 No. of cases (%)	Method 2 No. of cases (%)	*P*-value
Gram-positive rods	2/3 (66.7%)	N/A	
Gram-positive cocci	2/12 (16.7%)	12/23 (52.2%)	*P*=.045
Gram-negative rods	27/35 (77.1%)	49/52 (94.2%)	*P*=.022
Fungi	0/2 (0%)	0/2 (0%)	*P*>.99

TABLE 5 Comparison of MALDI scores between the two methods

	Method 1	Method 2	*P*-value
Total	1.985±0.069	2.164±0.031	*P*=.009
Gram-negative rods	2.107±0.061	2.164±0.037	*P*=.401
Gram-positive cocci	1.408±0.153	2.166±0.045	*P*=.000
Gram-positive rods	2.167±0.032	N/A	*P*>.99

Method 2 significantly improved the total score (*P*=.009) and GPC score (*P*=.000) compared with Method 1.

3.4 | Correlation between cell number and MALDI score

To investigate the sensitivity of MALDI-TOF MS for UTI causative bacteria, we compared the results of colony count in culture and MALDI score in both sample preparation methods (Methods 1 and 2). As the results, MALDI-TOF MS which was prepared with Method 2 significantly detected UTI causative bacteria in species and genus level when bacterial count was more than 1×10^5 CFU/mL in the urine culture for GPC (Table 6). For GNR, Method 2 significantly improved the identification rate compared with Method 1 in genus level when bacterial count was more than 1×10^5 CFU/mL in the urine culture for GPC. When the bacterial count was less than 1×10^4 CFU/mL, Method 2 substantially improved the identification rate for both GPC and GNR, but the differences were not significant.

4 | DISCUSSION

Several rapid detection methods of bacterial identification have been reported and established. For example, *16S-rRNA* gene sequencing, real-time PCR using melting curve analysis, multiplex PCR, fluorescence in situ hybridization (FISH), and denaturing high-performance liquid chromatography (DHPLC) have been used to detect pathogens in urine cultures.[11,12] These DNA or molecular-based methods are useful for rapid detection and have been demonstrated to have complementary value, but they are less practical for routine clinical work than conventional culture due to their relatively high cost (>25 USD/sample) and time-consuming procedures. Also, the specific sequences or probes for detection and highly skilled personnel are required for accurate identification using these technically challenging methods.[11]

MALDI-TOF MS has been introduced in clinical laboratories as a novel bacterial identification method. This mass-spectrometry-based technology can directly and accurately identify bacteria within 15 minutes at moderate cost (<1 USD/sample), using only a small amount of a colony and a drop of matrix solution.[13] Also, MALDI-TOF MS is a highly automated procedure requiring ordinary practice skills. These advantages can allow clinical laboratories to rapidly provide accurate data to clinicians for UTI diagnosis in advance of the culture results.

We found high correspondence rates between conventional urine culture results from complicated UTI patients and routine standard MALDI-TOF MS in GNR, but not yeasts and some kinds of GPC. Some authors have reported that yeasts such as *Candida* spp. and some GPC could not be easily identified by MALDI-TOF MS at the species level, and required some modification in sample preparation methods such as protein extraction because of their thicker cell wall structure.[9] As

TABLE 6 Correlation between colony count and MALDI-TOF MS identification

GPC		Method 1	Method 2	
Bacterial count	**MALDI score**	**No. of samples (%)**	**No. of samples (%)**	***P*-value**
$<10^5$ CFU/mL	Score >2	0/1 (0.0%)	1/7 (14.3%)	*P*=.225
	Score >1.7	0/1 (0.0%)	1/7 (14.3%)	*P*=.225
$\geq10^5$ CFU/mL	Score >2	0/11 (0.0%)	9/20 (45.0%)	*P*=.008
	Score >1.7	2/11 (18.2%)	11/20 (55.0%)	*P*=.036
GNR		**Method1**	**Method2**	
Bacterial count	**MALDI score**	**No. of samples (%)**	**No. of samples (%)**	***P*-value**
$<10^5$ CFU/mL	Score >2	1/6 (17.7%)	6/9 (66.7%)	*P*=.084
	Score >1.7	3/6 (50.0%)	7/9 (77.9%)	*P*=.287
$\geq10^5$ CFU/mL	Score >2	19/29 (65.5%)	29/43 (67.4%)	*P*=.531
	Score >1.7	21/29 (72.4%)	40/43 (93.0%)	*P*=.044

CFU, colony forming unit.

mentioned above, we modified our original method (Method 1) by adding ultrasonication and centrifugation (Method 2) to disperse the aggregated cells, in particular GPC such as *S. aureus* which generally form aggregations, and to remove urine debris such as epithelial cells and leukocytes.

Comparative studies of MALDI-TOF MS have mostly been performed in uncomplicated UTI cases where it is comparatively easier to estimate the causative bacteria hypothesize data.[14–16] MALDI-TOF MS has not shown good performance in identification of GPC which often cause complicated UTI. As an improved method for much better bacterial detection, ultrasonication and centrifugation (Method 2) resulted in higher correspondence rates with urine culture than the original method (Method 1) in our study. Our data were derived from a more difficult clinical setting using consecutive complicated UTI samples taken from cystitis outpatients with comparatively smaller numbers of bacteria than hospitalized cases with pyelonephritis or prostatitis. We therefore found significantly different MALDI scores between Method 1 (conventional) and Method 2 (modified) both in total bacteria and Gram-positive bacteria. Further experiments for additional improvement will be undertaken.

Some kinds of bacteria detected by urine culture, especially GPC such as *E. faecalis* or *S. aureus*, were not fully diagnosed by standard MALDI-TOF MS (Method 1). Identification of these two particularly common kinds of bacteria was significantly improved in our revised method (Method 2). Previously, Cherkaoui et al. reported that the majority of bacteria not identified by MALDI-TOF MS were Gram-positive bacteria when the direct colony method was used,[17] suggesting that preparatory protein extraction using formic acid could improve the MALDI-TOF MS identification for GPC compared to direct colony methods.[10,18] Also, bacterial identification by MALDI-TOF-MS is mainly based on 16S-ribosomal protein. Therefore, it tends to be difficult to distinguish among bacteria which have similar *16S-rRNA* sequences, such as certain GPCs.[17] This problematically poor performance was also observed in our first 53 cases (Method 1), but could be overcome by centrifugation in Method 2. Direct MALDI-TOF MS diagnosis from Gram-positive samples presents unique challenges to identification due to the permeability barrier posed by their thick and highly anionic cell walls. Our revised method (Method 2) addressed this problem successfully, and we will undertake experiments for further improvements in identification of the bacteria. Further refinements in examination accuracy and data base construction for other GPC targets also need to be performed.

Regarding the correlation between colony count on bacterial culture and MALDI-TOF MS performance, Ferreira et al. suggested that direct identification of bacteria MALDI -TOF MS from urine samples were available for especially GNR with species level when bacterial culture was more than 1×10^5 CFU/mL, but not good for GPC such as *E. faecalis* even in the higher bacterial concentration.[7] Our results showed that Method 2 improved the identification rate and score for GPC such as *S. aureus* and *E. faecalis* in species level (score>2.0) as well as GNR (Tables 2 and 3). Thus, our additional ultrasonication and centrifugation in sample preparation for MALDI-TOF MS could improve the performance for direct identification from urine samples.

We would like to emphasize the study limitations. First, our new method still has a nonidentification rate with GPC. We are now planning further revisions in method for a higher identification rate in GPC. Second, the number of cases in this single center study is not large enough to draw definitive conclusions. Third, the study does not include data on the antimicrobial susceptibilities of the bacteria and cannot discriminate antibiotic resistant strains. For the establishment of a more accurate diagnostic tool, further studies need to be performed to improve protein extraction methods, systematically report MALDI-TOF MS results, and construct improved databases specially designed for the clinically significant pathogens. However, this study from 133 consecutive complicated UTI patient samples is closer to daily clinical situations and the results can be helpful to physicians caring for complicated UTI patients.

5 | CONCLUSIONS

Our modified method (Method 2) with additional ultrasonication and centrifugation in MALDI-TOF MS could directly identify causative bacteria (both GNR and GPC) in complicated UTI with higher correspondence rates than the conventional method (Method 1). Further modifications could offer higher rates of correspondence with cultures, resulting in a high-performance method of rapid and accurate bacterial identification that may possibly be substituted for culture eventually.

ETHICAL APPROVAL

The study was approved by the Kobe University School of Medicine institutional review board (IRB). All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

INFORMED CONSENT

Informed consent was obtained from all individual participants included in the study.

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