



NOTE

Bacteriology

Use of the MALDI BioTyper system and rapid BACpro with MALDI-TOF MS for rapid identification of microorganisms causing bacterial urinary tract infection in feline urine samples

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ABSTRACT. With the increasing number of cats kept as pets, opportunities to treat cats with lower urinary tract disease (LUTD) have recently increased in the clinical veterinary field. Urine samples collected from 50 cats with bacterial cystitis brought to Maeda Veterinary Hospital between August 10, 2015 and March 31, 2017 were used in the study. Sample preparation of the urine was performed using a MALDI Sepsityper kit and rapid BACpro. To identify the isolates, MALDI-TOF MS was performed on an AutoFlex TOF/TOF mass spectrometer. MALDI-TOF MS using rapid BACpro for pretreatment was found to be a quick and reliable method for identification of bacteria from infected urine, with a shortened analysis time enabling earlier and more accurate selection of antibiotics for treatment of feline LUTD.

KEY WORDS: cat, lower urinary tract disease, matrix-assisted laser desorption ionization-time of flight mass spectrometry, urine

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The increasing number of cats kept as pets has increased opportunities to treat cats with lower urinary tract disease (LUTD) in clinical veterinary medicine. Urinary tract infection accounts for 2–10% of cases of feline LUTD [6, 7]. The causes of renal failure in young cats include renal hypoplasia, polycystic kidney, renal agenesis, and urinary tract dysplasia, whereas urinary bacterial infection-induced cystitis due to reduction of urine specific gravity accompanying glomerulonephritis and interstitial nephritis is more common in middle-aged and elderly cats [11, 12]. Bacterial infections associated with renal failure caused by urinary tract stones formed by struvite (magnesium ammonium phosphate) and calcium oxalate calculi and tumors develop regardless of age, and progression to pyonephrosis may occur. This increases the importance of identification of urinary bacteria and drug sensitivity tests. However, several days may be required to obtain results from a laboratory test center, which delays initiation of appropriate treatment with antibiotics at clinical sites.

Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) can be used to generate protein fingerprint signatures from whole bacterial cells [4]. Algorithms can then be used for rapid identification of bacteria by comparing fingerprints to a database of reference spectra [4]. Anhalt and Fenselau performed the first study using MS to identify bacteria [1]. However, MALDI-TOF MS instruments for use under routine conditions have only recently become available. For urine specimens with $\geq 10^5$ CFU/ml, the *Escherichia coli* identification rate is high (97.6–100.0%), but that for *Enterococcus faecalis* is low (60.0–66.7%), showing variation of rates among bacterial species [5, 8]. The identification rate for two-morphology colony types is also low (60%, 3/5) [5].

In this study, we performed direct identification of bacteria in urine using pretreatment kits for direct application of positive blood culture bottles to MALDI-TOF MS, with the aim of improving the low identification rates for *E. faecalis* and two-

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morphology colony types. Urine samples collected from 50 cats with bacteriuria brought to Maeda Veterinary Hospital between August 10, 2015 and March 31, 2017 (30 males and 20 females aged 0–19 years) were used in the study. Urine was collected by puncture of the urinary bladder with a 25-G needle through the skin after confirming the position of the bladder in a supine position with ultrasound. Urine was centrifuged and the precipitate was Gram-stained. Bacteriuria was diagnosed based on a positive Gram stain.

Bacterial identification with the conventional method was performed using a MicroScan WalkAway system (Siemens Healthcare Diagnostics, IL, U.S.A.). For the conventional culture, 1 μ l of well-mixed urine was inoculated and spread on blood agar plates using a sterile plastic disposable loop (Eiken Chemical Co., Tokyo, Japan). Plates were incubated in an aerobic atmosphere at 37°C for 18–24 hr. When bacterial growth was observed, the colonies on blood agar were counted, and colonies from both types of plates were identified using the MicroScan WalkAway system.

DNA was extracted with a MagNA Pure Compact DNA isolation kit I (Roche Molecular Biochemicals, Mannheim, Germany). Polymerase chain reaction amplification of the 16S rRNA gene was performed as described by Okazaki *et al.* [10] and Otsuka *et al.* [13], using primers 8UA (5'-AGA GTT TGA TC(A/C)TGG CTC AG-3') and 1485B (5'-TAC GGT TAC CTT GTT ACG AC-3'). Amplicons were purified and sequenced using primers 519A (5'-CAG C(A/C)G CCG CGG TAA T-3'), 519B (5'-ATT ACC GCG GC(G/T)GCT G-3'), 907A (5'-AAA CT(T/C) AAA (T/G)GA ATT GAC GG-3'), and 907B (5'-CCG TCA ATT C(A/G)TTT (A/G) A GTT T-3'). A homology search of 16S rRNA gene sequences was performed against sequences registered in GenBank/EMBL/ DDBJ using BLAST.

Urine sample preparation was performed using a MALDI Sepsityper kit (Bruker Daltonics, Bremen, Germany) [9]. In brief, urine (5 ml) was centrifuged at 2,000 \times g for 30 sec to remove leukocytes. The supernatant was centrifuged at 15,000 \times g for 10 min to collect bacteria. The pellet in distilled water (1 ml) was transferred to a 1.5 ml reaction tube (Eppendorf, Hamburg, Germany). After addition of 200 μ l of lysis buffer, the sample was mixed using a vortex shaker for 10 sec and then centrifuged for 1 min at 21,130 \times g. The supernatant was discarded and the pellet was resuspended in 1 ml of washing buffer. After a second centrifugation step (1 min at 21,130 \times g), the supernatant was discarded and the pellet was suspended in 300 μ l of distilled water. After addition of 900 μ l of ethanol, the sample was centrifuged at 21,130 \times g for 2 min. Supernatant was discarded and residual ethanol was removed after repeated centrifugation. The pellet was then suspended in 15 μ l of 70% formic acid. Acetonitrile (15 μ l) was added and the sample was mixed using a vortex shaker and briefly centrifuged prior to MALDI-TOF MS analysis.

Urine sample preparation was also performed using the rapid BACpro kit (Nittobo Medical Co., Tokyo, Japan) [2]. In brief, urine (5 ml) was centrifuged at 2,000 \times g for 30 sec to remove leukocytes. The supernatant was then centrifuged at 15,000 \times g for 10 min to collect bacteria. The pellet samples were combined with 10 μ l of reaction buffer 1, 100 μ l of reaction buffer 2, and 10 μ l of polymer suspension in sequence. The next part of the protocol required use of a desktop centrifuge (Chibitan R, Merck Millipore, Billerica, MA, U.S.A.). First, the mixture was separated by desktop centrifugation at 2,000 \times g for 30 sec, and the supernatant was discarded. Second, the resulting aggregate was resuspended in 1,000 μ l of 70% acetonitrile, and the supernatant was separated by centrifugation at 2,000 \times g for 30 sec. Third, the obtained precipitate was resuspended in 30 μ l of 70% formic acid and 100 μ l of 100% acetonitrile, and the supernatant was separated by centrifugation at 2,000 \times g for 60 sec.

The MALDI-TOF α -cyano-4-hydroxycinnamic acid matrix was prepared daily as a saturated solution in 50% acetonitrile and 2.5% trifluoroacetic acid. Subsequently, 1 μ l of the sample extract (prepared using either of the two approaches above) was spotted on a steel target plate (Bruker Daltonik) and allowed to dry. Next, 1 μ l of matrix solution was added and air dried. The target plate was then placed in the MALDI-TOF MS apparatus. To identify the isolates, MALDI-TOF MS was performed on an AutoFlex[®] TOF/TOF mass spectrometer equipped with Flexcontrol[™] software v. 3.0 (Bruker Daltonics) for automatic acquisition of mass spectra in the linear positive mode within a range of 2 to 20 kDa. Laboratory technicians performed all MALDI-TOF MS measurements in the study.

The Autoflex[®] II TOF/TOF mass spectrometer was periodically calibrated using the Bruker Daltonics bacterial test standard (*E. coli* extracts containing RNase A and myoglobin). Calibration masses were as follows: RL36, 4364.3 Da; RS22, 5095.8 Da; RL34, 5380.4 Da; RLMeth, 6254.4 Da; RL32, 6315.2 Da; RL29, 7273.5 Da; RS19, 10229.1 Da; RNase A, 13682.2 Da; and myoglobin, 16952.5 Da. Automated analysis of raw spectral data was performed using MALDI BioTyper automation v.3.0 software (Bruker Daltonics) with a library of 5,989 spectra (database updated on July 31, 2015) and default settings. The whole process from MALDI-TOF MS measurement to identification was performed automatically without user intervention. In brief, the software generated a list of peaks up to 100. The threshold for peak acceptance was a signal-to-noise ratio of 10. After alignment, peaks with a mass-to-charge ratio difference of <250 ppm were considered to be identical. The peak lists generated were used for matches against the reference library by using an integrated pattern matching algorithm in the software. Pattern-matching results are expressed as scores ranging from 0 to 3, with a score <1.7 not considered to give reliable identification and a score \geq 2.0 indicating identification of a species [15].

To determine the minimal bacterial concentration allowing reliable MALDI-TOF MS identification, we inoculated 50-ml aliquots of sterile water with one strain of *E. coli* or *E. faecalis* at a bacterial count of 2×10^8 CFU/ml. Sequential dilutions were then used to achieve aliquots of each microorganism at counts of 1.0×10^7 , 5.0×10^6 , 1.0×10^6 , 5.0×10^5 , 1.0×10^5 , 5.0×10^4 and 1.0×10^4 CFU/ml. If necessary, aliquots were further diluted before spreading on a plate to obtain a countable number of colonies (50 to 500) when plated. Aliquots of 300 μ l for each dilution were plated on blood agar plates, which were incubated at 37°C in an aerobic atmosphere. Colonies were counted manually, and the mean of three aliquots at each dilution was considered to be the final count for that dilution. Samples of 10 ml for each dilution were used for preparation for MALDI-TOF MS, as described above.

MALDI-TOF MS requires high bacterial counts to provide reliable scores. Inoculation with at least 1.0×10^5 CFU/ml was

Table 1. MALDI-TOF MS scores for different *Escherichia coli* and *Enterococcus faecalis* bacteria counts

Kit	Microorganism	MALDI-TOF MS score for bacteria count (CFU/ml)						
		1.0×10^7	5.0×10^6	1.0×10^6	5.0×10^5	1.0×10^5	5.0×10^4	1.0×10^4
MALDI Sepsityper	<i>E. coli</i>	2.321	2.301	2.284	2.184	2.105	1.988	1.904
	<i>E. faecalis</i>	2.122	2.115	1.986	1.862	1.792	1.662	1.624
Rapid BACpro	<i>E. coli</i>	2.398	2.321	2.299	2.186	2.134	1.991	1.972
	<i>E. faecalis</i>	2.182	2.182	2.162	2.084	2.051	1.742	1.722

Table 2. Urine culture results of 39 specimens analyzed by MALDI-TOF MS

Urine culture (No. of isolates)	MALDI sepsityper (No. of isolates)	Rapid BACpro (No. of isolates)
Growth of colonies (N=37)		
1-colony morphology (N=29)	Positive with same identification (25) ^{a)}	Positive with same identification (25) ^{a)}
	Not reliable identification (4) ^{b)}	Not reliable identification (4) ^{b)}
2-colony morphology (N=8)	Not reliable identification (8)	<i>Enterococcus faecalis</i> and <i>Citrobacter freundii</i> (2)
		<i>Enterococcus faecalis</i> and <i>Enterobacter cloacae</i> (2)
		<i>Enterococcus faecalis</i> and <i>Klebsiella pneumoniae</i> (3)
		Not reliable identification (1) ^{c)}
No growth (N=13)		

a) A total of 25 correct identifications at the species level. b) Four cases were cultures with $<1.0 \times 10^5$ CFU/ml. c) Cases of *E. faecalis* and *E. cloacae* were cultures with $<1.0 \times 10^5$ CFU/ml.

Table 3. Identification by MALDI-TOF MS of 16S rRNA and conventional identification of 45 microorganisms causing cystitis with bacterial counts $\geq 1.0 \times 10^5$ CFU/ml

Conventional method (No. of isolates)	MALDI-TOF MS method		16S rRNA method (No. of isolates)
	MALDI sepsityper kit (No. of isolates)	Rapid BACpro (No. of isolates)	
<i>Citrobacter freundii</i> (2)	No reliable identification (2)	<i>Citrobacter freundii</i> (2)	<i>Citrobacter freundii</i> (2)
<i>Enterobacter cloacae</i> (4)	<i>Enterobacter cloacae</i> (3)	<i>Enterobacter cloacae</i> (3)	<i>Enterobacter cloacae</i> (4)
	No reliable identification (1)	No reliable identification (1)	
<i>Escherichia coli</i> (22)	<i>Escherichia coli</i> (22)	<i>Escherichia coli</i> (22)	<i>Escherichia coli</i> (22)
<i>Klebsiella pneumoniae</i> (3)	<i>Klebsiella pneumoniae</i> (3)	<i>Klebsiella pneumoniae</i> (3)	<i>Klebsiella pneumoniae</i> (3)
Total gram-negative bacteria (31)	90.3% (28/31)	96.7% (30/31)	100.0% (31/31)
<i>Enterococcus faecalis</i> (9)	No reliable identification (9)	<i>Enterococcus faecalis</i> (8)	<i>Enterococcus faecalis</i> (9)
		No reliable identification (1)	
<i>Staphylococcus aureus</i> (5)	<i>Staphylococcus aureus</i> (3)	<i>Staphylococcus aureus</i> (3)	<i>Staphylococcus aureus</i> (5)
	No reliable identification (2)	No reliable identification (2)	
Total gram-positive cocci (14)	21.4% (3/14)	78.5% (11/14)	100.0% (14/14)
Total (45)	68.8% (31/45)	91.1% (41/45)	100.0% (45/45)

required to obtain reliable scores for *E. coli* after preparation with the MALDI Sepsityper Kit and rapid BACpro, and the required level was higher for *E. faecalis* using the MALDI Sepsityper Kit (5.0×10^6 CFU/ml) and rapid BACpro (1.0×10^5 CFU/ml) (Table 1). Of the 50 urine specimens, growth of colonies was observed in 37 specimens: 29 with single-colony morphology, and 8 with two-colony morphology. Thirteen specimens did not grow in culture, and MALDI-TOF MS did not identify a significant protein profile in any of these cases (Table 2).

In specimens with single-colony morphology, MALDI-TOF MS correctly identified 25 isolates (86.2%) using the MALDI Sepsityper Kit and 25 isolates (86.2%) with rapid BACpro. In the 4 cases in which identification was not possible, the bacterial count was $\leq 1.0 \times 10^5$ CFU/ml, which was below the detection limit. In specimens with two-colony morphology, MALDI-TOF MS gave correct results in only one specimen (*Citrobacter freundii*, *Enterobacter cloacae*, *Klebsiella pneumoniae*) using the MALDI Sepsityper Kit, whereas with rapid BACpro 2 species were detected in 7 urine specimens. No bacterium were identified with rapid BACpro in one case, in which *E. faecalis* and *E. cloacae* were present and the *E. cloacae* count was 1.0×10^5 CFU/ml, which was below the detection limit.

Thirty-two strains were identified using the conventional method, including 31 enteric Gram-negative bacteria (GNB) and 14 gram-positive cocci (GPC) (Table 3). MALDI-TOF MS correctly identified 28 enteric GNB (90.3%) and 3 GPC (21.4%)

with the MALDI Sepsityper Kit, and 29 enteric GNB (96.7%) and 11 GPC (78.5%) with rapid BACpro. Overall, among the 45 specimens with colony counts $\geq 1.0 \times 10^5$ CFU/ml, microorganism identification coincided with the MALDI Sepsityper Kit in 31 cases (68.8%) and rapid BACpro in 40 cases (91.1%) (Table 3). Of the GNB, *C. freundii*, *E. cloacae*, *E. coli*, and *K. pneumoniae* were correctly identified in 0 (0.0%) and 2 (100.0%), 3 (75.0%) and 3 (75.0%), 22 (100.0%) and 22 (100.0%), and 3 (100.0%) and 3 (100.0%) samples using the MALDI Sepsityper Kit and rapid BACpro, respectively. Of the GPC, *E. faecalis* and *S. aureus* were correctly identified in 0 (0.0%) and 8 (88.8%), and 3 (60.0%) and 3 (60.0%) using the MALDI Sepsityper Kit and rapid BACpro, respectively. The *E. faecalis* strain, which could not be identified, was present at 1.0×10^5 CFU/ml, which was below the detection limit. The 45 strains identified by the conventional method were subjected to 16S rRNA analysis, and identification was consistent for all strains.

Urine from animals with LUTD contains many white and red blood cells and bacteria. In pretreatment, urine samples were centrifuged at $2,000 \times g$ for 30 sec to remove white blood cells, and then at $14,100\text{--}15,500 \times g$ for 5–10 min to collect bacteria [5, 8]. A sufficient bacterial count is essential for identification by MALDI-TOF MS, and our findings support a previous report showing that the bacterial count needed for good discrimination by MALDI-TOF MS is higher for GPC ($2.5\text{--}5 \times 10^5$ CFU/ml) than for GNB (about 6×10^4 CFU/ml) [17]. Rapid BACpro improved the collection, quantity and purity of the collected bacteria. The *E. faecalis* identification rate was 0% using the MALDI Sepsityper Kit, but 88.8% using rapid BACpro, and the GPC identification rate was 24.1% using the MALDI Sepsityper Kit, but 78.5% using rapid BACpro. The bacteria harvesting efficiency of rapid BACpro was high and this improved the identification rate. The concordance rate of the two-morphology colony-type urine samples was 31.8–60.0%, and 2 species could be identified.

We attempted to improve the identification rate by using pretreatment kits for direct bacterial identification from positive blood culture bottles: MALDI Sepsityper Kit and rapid BACpro. The MALDI Sepsityper Kit collects bacteria by lysing blood cells in a lysis buffer [14], whereas rapid BACpro isolates bacteria from urine through selective binding of bacteria to a cationic polymer suspension [2]. The concordance rates of identification using MALDI-TOF MS with pretreatment with the MALDI Sepsityper Kit and rapid BACpro relative to the conventional method were 68.8 and 91.1%, respectively (Table 3). These rates are similar to those reported previously [5]. The concordance rates for identification of *E. faecalis* and *E. cloacae* by MALDI-TOF MS using rapid BACpro were 88.8% and 75.0%, respectively, showing improvement of these rates. The *E. faecalis* detection sensitivity of MALDI-TOF MS using rapid BACpro was 1.0×10^5 CFU/ml (Table 1), and the *E. faecalis* count was 1.0×10^5 CFU/ml, which was below the detection limit. The limit of detection of *E. cloacae* was also 1.0×10^5 CFU/ml, which is a low bacteria count. Thus, MALDI-TOF MS identification of bacteria using rapid BACpro pretreatment is possible at a bacterial level $\geq 1.0 \times 10^5$ CFU/ml.

In two-morphology colony-type urine samples, the concordance rates for MALDI-TOF MS using the MALDI Sepsityper Kit and rapid BACpro relative to the conventional method were 0.0 and 87.5%, respectively (Table 2). The one non-identifiable case rapid BACpro contained *E. faecalis* and *E. cloacae*, and the *E. cloacae* count was low (1.0×10^5 CFU/ml). MALDI-TOF MS spectra of the two-morphology colony types were complex and the samples contained components other than bacteria, which reduced the concordance rate. Since the MALDI Sepsityper Kit lyses blood cells with lysis buffer and collects bacteria, complete removal of blood cell components is difficult. In contrast, samples treated with rapid BACpro contain very few non-bacterial components because this method isolates bacteria through selective binding of bacteria to a cationic polymer suspension without lysing blood cells [2].

Urine culture (i.e., quantitative culture of urine specimens on solid medium followed by biochemical characterization of isolates), which is the gold standard for diagnosis, requires 24–72 hr until the results are available [3]. In contrast, MALDI-TOF MS of urine specimens prepared using rapid BACpro and the MALDI Sepsityper Kit can identify bacterial species using MALDI BioTyper automation software within about 30 min, and rDNA analysis requires about 1 hr. The transition from conventional microbiological methods to MALDI-TOF MS also resulted in significant laboratory cost savings, estimated to be 51.7% of total costs during a 12-month study [16].

In conclusion, MALDI-TOF MS using rapid BACpro for pretreatment is a quick and reliable method for identification of bacteria in infected urine. The shortened analysis time enables earlier and more accurate selection of antibiotics for treatment of feline bacteriuria.

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