Rapid Identification of *Klebsiella pneumoniae* by Matrix-Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry and Detection of Meropenem Resistance by Flow Cytometric Assay

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Background: The aim of this study was to develop a rapid detection method of carbapenem-resistant *Klebsiella pneumoniae* (CRKP) strains both MALDI-TOF MS and flow cytometry (FCM). **Methods:** A total of 174 *K. pneumoniae* strains were included in this study. Molecular characterization of carbapenemase gene was performed by PCR. Bacterial identification was performed by MALDI-TOF-MS. Meropenem susceptibility was tested at the concentrations of breakpoints described by the Clinical and Laboratory Standards Institute (CLSI) guide by FCM. **Results:** Sixty-two CRKP were positive for at least one carbapenemase gene. A total of 174 *K. pneumoniae* isolates obtained from clinically relevant material were correctly identified

by Bruker MALDI-TOF MS with log (score) >2.0. These results were 100% concordant with the Phoenix™ Automated Microbiology System (BD, MD) and conventional identification results. Based on the analysis of the receiver operating characteristic (ROC) curves, the best validity and sensitivity data were obtained with a cut-off value of 18.88% by FCM. The concordance, sensitivity, and specificity for FCM by the selected cut-off values were 99.4%, 98.9%, and 100%, respectively. **Conclusions:** We conclude that reliable results on bacterial identification and meropenem susceptibility test can be obtained within 2 hr combined by MALDI-TOF-MS and FCM. J. Clin. Lab. Anal. 30:1191–1197, 2016. © 2016 Wiley Periodicals, Inc.

Key words: flow cytometric assay; *Klebsiella pneumoniae*; MALDI-TOF MS

INTRODUCTION

Enterobacteriaceae species are among the most important human pathogens, causing community – and hospital – acquired infections (1). *Klebsiella pneumoniae* is the most common pathogenic *Klebsiella* species and belongs to the *Enterobacteriaceae* (2). In humans, *K. pneumoniae* most often colonizes the nasopharynx and the intestinal tract and is an important etiological agent of a wide variety of serious infectious diseases, including urinary tract, respiratory tract, and blood stream infections (2, 3).

Carbapenems such as ertapenem, imipenem, and meropenem are the first-line antimicrobial drugs for treatment of serious healthcare-associated infections

caused by *K. pneumoniae* (4). During the past decade, resistance to carbapenems has been increasingly reported worldwide in *K. pneumoniae*. The spread of the carbapenem-resistant *K. pneumoniae* (CRKP) in hospitals represents a serious threat to public health, since treatment options are very limited (5). Although the prevalence of carbapenem-resistant *Enterobacteriaceae* (CRE) isolates is reported relatively low, it has

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dramatically increased in recent years in Turkey. Among *Enterobacteriaceae*, high prevalence of antibiotic resistance has been reported in *K. pneumoniae* isolates (6).

Rapid identification of *K. pneumoniae* and detection of carbapenem resistance are critical for early and targeted antimicrobial therapy and infection control (7). The delay to appropriate antibiotic treatment is a major risk factor for infection associated morbidity and mortality. Conventional methods for bacterial identification and antibiotic-susceptibility test are time consuming, labor intensive, and still require 48–72 hr from sample collection to test result (8).

Matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) was first developed in the late 1980s. Since then, the system has been adapted for microorganism identification. The overall microorganism's species identification workflow is simple, time saving, and cost effective in this system (9). MALDI-TOF MS assays to detect β -lactamase activity have also been developed in the recent years (10). Flow cytometry (FCM) was first appeared in microbiology in the former 1970s. FCM is a rapid, powerful high-throughput technology for the differentiation and functional analysis of microorganisms. FCM has also been used previously for antibiotic-susceptibility testing of several clinical important microorganisms (11, 12).

The aim of this study was to develop a rapid detection method of CRKP strains using both MALDI-TOF MS and FCM.

MATERIAL AND METHODS

Bacterial strains and antibiotic-susceptibility testing

A total of 174 *K. pneumoniae* strains (87 carbapenem resistant, 87 carbapenem susceptible) isolated from various clinical specimens (blood [$n = 98$], urine [$n = 49$], wound aspirate [$n = 12$], catheter [$n = 7$], transtracheal aspirate [$n = 5$], and other samples [$n = 3$]) between May 2011 and October 2015 were included in the study. Microorganisms were firstly defined by colony morphology, microscopic morphology by Gram stain, and further phenotypic characteristics by the Phoenix System (Becton Dickinson Diagnostic Systems, Sparks, MD). All isolates were tested for resistance against carbapenems (ertapenem, imipenem, and meropenem) by the Phoenix System. Interpretation of the test results was made using the Clinical Laboratory Standards Institute (CLSI) criteria (13). The *K. pneumoniae* isolates were stored in 10% skimmed milk solution at -80°C . The stored bacterial

isolates were sub-cultured on 5% sheep blood agar and Eosin Methylene Blue agar plates (Salubris AS, Turkey) and incubated at 37°C for 48 hr. Additionally, susceptibility to ertapenem, imipenem, and meropenem was determined by *E*-test (Oxoid, Hampshire, UK) and was interpreted in accordance with the standards of CLSI (13). *K. pneumoniae* ATCC 13883 and *Escherichia coli* ATCC 25922 were used as quality control strains for susceptibility testing.

Molecular detection of resistance genes

DNA was isolated from bacterial colonies using the boiling lysis method as previously described (boiling a fresh cultured colony in 100 μl sterile distilled water for 10 min and subsequent centrifugation at $16,000 \times g$ for 5 min) (14). Two micro liters of supernatant were used as the template in a 25 μl PCR. The PCR assays were carried out to amplify the entire sequences of the *bla*_{KPC}, *bla*_{NDM-1}, *bla*_{OXA-48}, *bla*_{VIM}, and *bla*_{IMP}, genes using previously described specific oligonucleotide primers (15). Five microliters of PCR product was run in gel electrophoresis (1.5% agarose, $1 \times$ TBE, 100V) using molecular standard (K180-250 UL; Amresco, Solon, OH). The gel was stained with ethidium bromide, visualized with UV and evaluated by Gel Doc 2000 software (Bio-Rad laboratories, Hercules, CA). *K. pneumoniae* ATCC 13883 (resistance genes negative), *K. pneumoniae* ATCC 1705 (*bla*_{KPC} positive), *K. pneumoniae* ATCC BAA 2146 (*bla*_{NDM-1} positive), *K. pneumoniae* NCTC 13440 (*bla*_{VIM} positive), and *K. pneumoniae* (GATA-64) (*bla*_{OXA-48} positive) (kindly provided by Prof. Zerrin AKTAS [Istanbul University, Turkey]) were used in the study as reference strains.

Bruker's MALDI-TOF MS analysis

A single colony was smeared directly onto MALDI plate. One microliter α -cyano-4-hydroxycinnamic acid (CHCA) matrix solution (Bruker Daltonics, Billerica, MA) was added to the sample and allowed to dry. Analysis was carried out with Microflex LT MALDI-TOF MS device and Flex control 3.0 software (Bruker Daltonics, Germany) optimized for microorganism identification at the linear positive ion mode between 2.000 and 20.000 Da mass ranges. The spectrums were obtained by 60 Hz laser shots, which consist 40 pock-ets in totally 240.

Flow cytometric analysis

All bacterial strains were grown at 37°C on 5% sheep blood agar plates (Salubris AS, Istanbul,

Turkey) for 18–24 hr. Bacterial cells suspensions were prepared in Mueller Hinton broth (Salubris AS) and were incubated at 37°C with shaking until reaching the log phase (about 1 hr and 15 min), correspond with 5×10^6 CFU/ml. Subsequently, bacterial suspensions were exposed with 1 mg/l meropenem (Sigma Co., St. Louis, MO) which is MIC breakpoint of meropenem according to CLSI document (13). Cell suspension without antibiotic treatment was also served as antibiotic-free control. The suspensions were incubated for 60 min at 37°C. After incubation, the cells were washed and stained with 420 nM thiazole orange (TO) (Sigma Co., Munich, Germany) and 48 μ M propidium iodide (PI) (Sigma Co.) for 5 min at room temperature, protecting from light.

Flow cytometry analysis to measure the fluorescence of bacterial suspensions was performed on a BD Accuri C6 flow cytometer (Becton Dickinson, Piscataway, NJ) with 488 nM excitation light and emission collected through a 530/30 nM band pass filter for TO (FL1) and >670 nM band pass filter for PI (FL3). Usually, 10,000 cells were analyzed for each sample and light scatter and fluorescence signals were collected with log amplification.

In order to differentiate between susceptible and resistance of the strains to the meropenem, percentage of viable bacterial counts obtained from cells with meropenem were compared, through receiver operating characteristic (ROC) curve analysis, with those obtained from the antibiotic-free control cells. In this manner, the optimal threshold for discriminating between susceptibility and resistance was obtained. The susceptibility results were compared to those of the *E*-test results accepted as the gold standard.

Statistical analysis

Statistical analysis was performed with SPSS software version 20.0 (SPSS Inc. Chicago, IL) to generate the ROC curves and calculate the associated areas and specificities and sensitivities.

RESULTS

Carbapenemase genes analysis

The 87 CRKP were tested for carbapenemase genes by PCR, of which 62 were positive for at least one gene. PCR results showed that 40 *K. pneumoniae* strains harbored only *bla*_{OXA-48} gene, seven strains had only *bla*_{NDM-1} and one strain had only *bla*_{VIM} gene. Both *bla*_{OXA-48} and *bla*_{NDM-1} were present in six strains, whereas three strains had both *bla*_{NDM-1} and *bla*_{IMP} genes. Two *K. pneumoniae* were positive for

*bla*_{OXA-48}, *bla*_{NDM-1}, and *bla*_{VIM} genes, while *bla*_{OXA-48}, *bla*_{NDM-1}, and *bla*_{IMP} genes were determined in three *K. pneumoniae* strains.

MALDI-TOF MS analysis

A total of 174 *K. pneumoniae* isolates obtained from clinically relevant material were correctly identified by Bruker MALDI-TOF MS at the species level with log scores >2.0. These results were 100% concordant with the Phoenix identification results.

FCM analysis

All experiments were performed and compared to appropriate paired antibiotic-free controls. Initially to evaluate auto fluorescence, tube containing only broth stained with TO and PI and tube containing antibiotic-free cells unstained with TO and PI was differentiated by forward scatter (FSC) and side scatter (SSC) threshold from noise, medium components and cellular debris. No significant basal fluorescence was recorded stained broth and unstained antibiotic-free cells (Fig. 1).

Gates were set up based on live and dead cells. Forward scatter, side scatter and fluorescence intensities of the individual particles (labeled with TO and labeled with PI) were detected. The percentage of live bacteria were calculated based upon the gated population by forward and side scatter and the area under the obtained curve, which is automatically normalized by the FCM software (Becton Dickinson). Fluorescence from the FL1 (TO) and FL3 (PI), FSC, and SSC signals were monitored for each treatment at each point by FCM (Fig. 2).

On FSC vs. SSC panels, to distinguish bacteria cells from auto fluorescence related to “background,” we marked red color the fluorescence from TO (Fig. 2a, e, i). All particles fluoresced red were accepted as

Fig. 1. Forward scatter (FSC) vs. side scatter (SSC) signals in tube (a) containing Mueller Hinton broth stained with TO and PI to evaluate broth fluorescence, (b) containing unstained antibiotic-free cells to evaluate cells auto fluorescence.

Fig. 2. Forward scatter (FSC), side scatter (SSC), FL1 (thiazole orange [TO]), and FL3 (propidium iodide [PI]) fluorescence intensities of the antibiotic-free control (a–d), meropenem-resistant *K. pneumoniae* ATCC BAA 2146 (e–h), and meropenem-susceptible *K. pneumoniae* ATCC 13883 (i–l) strains. Percentages of dead cells show in upper right (UR) quadrant in panels c, g, and k. Histograms of dead and live cell counts depicted in panels d, h, and l. The M1 area shows viable cells, and dead bacteria are in the M2 area. The proportion of dead cells recorded in panels d, h, and l were 0.9%, 1.7%, and 73.1%, respectively.

bacteria cells, because TO stains just live/dead cells; remaining fluorescents were accepted as auto fluorescence. In following, the gates were established for all *K. pneumoniae* on the basis of their TO fluorescence for antibiotic-free control (Fig. 2b), resistant (Fig. 2f) and susceptible (Fig. 2j) strains in FL1. The mean peak values on the basis of their PI fluorescence in FL3 were used to calculate of percentage of live and dead cells in antibiotic-free control (Fig. 2c), resistant (Fig. 2g) and susceptible (Fig. 2k) strains. There was no significant PI fluorescence respecting dead cells observed in antibiotic-free control cells (Fig. 2d). The

meropenem-resistant strains were not stained by PI and showed a fluorescence profile identical to the antibiotic-free control cell (Fig. 2g and h). The typical fluorescence distribution of susceptible bacterial population is depicted in Figure 2k and l.

Figure 3 shows the percentage of bacterial count curves of meropenem susceptible, resistant *K. pneumoniae* incubated with meropenem and antibiotic-free control strain. In the antibiotic-free control, percentage of bacterial count remained nearly the same after incubation for 15, 30, 45, and 60 min with meropenem. In the resistant *K. pneumoniae*, percentage of bacterial

Fig. 3. Percentage of bacterial counts of susceptible isolate (*K. pneumoniae* ATCC 13883) with and without meropenem and resistant isolate (*K. pneumoniae* ATCC BAA 2146) with meropenem at concentration of breakpoint of sensitivity (1 mg/l).

count obtained in the presence of antibiotic was nearly similar to those of the antibiotic-free control. However, meropenem-sensitive strain observed that percentage of bacterial count was significantly reduced compared with antibiotic-free control after 1 hr incubation of meropenem (considered as 100%).

In order to obtain the optimal cut-off percentage of bacterial count for discriminating between susceptible

Fig. 4. ROC curves for discriminating resistance from susceptible by flow cytometry according to established cut-off (18.88%). Area under curve: 0.990; $P < 0.001$.

TABLE 1. ROC Curve Analysis for Distinguishing Between Susceptible and Resistance Calculated by SPSS v.20.0 Software

Reduced percentage of bacterial counts in the cells compared the paired antibiotic-free control	Sensitivity	Specificity
5.0577	0.931	0.000
5.2686	0.943	0.000
5.6011	0.954	0.000
6.3434	0.966	0.000
7.0593	0.977	0.000
18.8872	0.989	0.000
31.1173	0.989	0.011
32.9062	0.989	0.023
35.0892	0.989	0.034
36.3778	0.989	0.046
37.1577	0.989	0.057

and resistance, data obtained from isolates were analyzed by ROC curve (Fig. 4). Table 1 shows a complete sensitivity/specificity report and indicates the cut-off value in bold.

This value allowed us to consider *K. pneumoniae* susceptible to meropenem by the FCM if the percentage of bacterial counts in the meropenem containing medium was reduced 18.8% or more compared to the number of *K. pneumoniae* in the paired antibiotic-free control.

The susceptibility test performed by FCM for 174 *K. pneumoniae* isolates showed 99.4% concordance with the susceptibility results obtained by *E*-test accepted as the gold standard. Only one discrepancy was found. The isolate was found susceptible by FCM method, but resistant by the *E*-test. The carbapenems have slightly different characteristics. However, we carried out the susceptibility test for just meropenem. Since we found a correlation among meropenem, ertapenem, and imipenem susceptibilities results by *E*-test, which give rise to thought flow cytometric results for the one of carbapenems give preliminary opinion for the others.

DISCUSSION

K. pneumoniae is an important etiological agent for serious hospital—or community—acquired bacterial infections all around the world. Carbapenem antibiotics are the first line antibiotic class for the treatment of serious hospital-acquired infection caused by *K. pneumoniae* (4, 16). Since its first detection in 1996 in North Carolina, CRKP has become an increasingly serious problem worldwide. Clinicians have very limited antibiotic options to use for treatment of infections, since CRKP is resistant to almost all of the antibiotics (17). Infections caused by CRKP increase

in mortality, total hospital cost, and length of stay. Therefore, rapid identification of *K. pneumoniae* and detection of carbapenem resistance are critical steps for the infection control team to implement strict infection control practice (7, 17). However, detection of CRKP strains in the clinical laboratory remained a difficult task (18).

MALDI-TOF MS method has been used as a new method for rapid and inexpensive identification of bacteria in routine clinical microbiology laboratories throughout the world. The system has been applied in the identification of microorganisms for the first time in 1975. MALDI-TOF MS has the potential to replace traditional phenotypic methods for identification of bacteria in most clinical bacteriology laboratory because it is rapid and inexpensive. Two commercially available MALDI-TOF MS systems (Bruker MS [Bruker Daltonics] and VITEK MS [bioMérieux, Marcy l'Etoile, France]) have been developed to use in clinical microbiology laboratories (19). MALDI-TOF MS assays have also been used for the rapid detection of carbapenemase activity, at a low cost. However, it is unlikely to be usable currently. The method should be investigated further and validated (10, 20, 21). There are only a few recent studies of the use of MALDI-TOF MS for the rapid identification and typing of *K. pneumoniae* strains. They reported MALDI-TOF MS was a rapid method to identify clinical *K. pneumoniae* strains (22–24). In the present study, all *K. pneumoniae* strains were identified with log score values >2.0 using the Bruker Biotyper software in MALDI-TOF MS. Observed concordance between MALDI-TOF MS and BD Phoenix™ was 100%. Faster results were obtained in a few minutes from MALDI-TOF MS rather than at least 4–6 hr from BD Phoenix™.

Flow cytometry is a new simple and rapid approach in accurate assessment of the susceptibility of microorganisms to many antibiotics (12, 25–28). Here, we presented a study to determine the susceptibility of *K. pneumoniae* strains to one of the carbapenem antibiotics, meropenem. The viable bacteria percentage obtained from the cells incubated with meropenem were compared, using ROC curves, to the viable bacterial percentage from paired antibiotic-free control. It was evaluated that the viable bacterial percentage obtained from *K. pneumoniae* after 1 hr of incubation made it possible to define whether a *K. pneumoniae* isolate was resistant or susceptible to meropenem (11). This incubation time is similar to other susceptibility studies performed in *Enterobacteriaceae* (11, 26, 29). Based on the analysis of the ROC curves, the best validity and sensitivity data were obtained with a cut-off value of 18.88%. The concordance, sensitivity, and

specificity data for FCM vs. *E*-test method using the selected cut-off values were 99.4%, 98.9%, and 100%, respectively. Interestingly, we found only one very major error (30), with regard to the *E*-test results accepted gold standard. FCM was repeated twice and the same result was found for this strain. This *K. pneumoniae* isolate would be reported as sensitive by FCM, although it is resistant to meropenem. We did not obtain any minor error. Similar findings have been reported by a few researchers. Nuding and Zabel performed a susceptibility test in plate-grown colonies of 67 bacterial strains including *K. pneumoniae* by FCM. Ninety-five percent of the FCM susceptibility results were concordant with results derived by the agar diffusion method and 94% with the results obtained with the WalkAway-96 (Dade-Behring, Marburg, Germany). The authors concluded that FCM offered a suitable technique for the rapid detection and susceptibility testing of bacteria with great potential (12). March et al. performed susceptibility test in *Enterobacteriaceae* showed 97% concordance between the results obtained by FCM and the results obtained by the commercial methods. They considered *Enterobacteriaceae* resistant to antibiotic tested if in order to obtain a bacterial count reduction of at least 14% compared to paired antibiotic-free control after 1 hr of incubation. They concluded that they obtained reliable results on bacterial antibiotic-susceptibility test and confirmed that FCM proposed method allows obtaining a rapid antibiotic-susceptibility test (11).

In conclusion, Bruker MALDI-TOF MS offers easy, rapid, and inexpensive system for identification of clinical relevant *K. pneumoniae* strains. FCM provides rapid, simple, and accurate results compared with conventional antibiotic-susceptibility methods. Both methods together might easily be implemented to the clinical microbiology laboratory during daily routine. The implementation of MALDI-TOF MS in combination with FCM can lead to decreased turnaround times for identification of clinically important *K. pneumoniae* strains and the detection of antibiotic susceptibility, which result in decreased mortality and morbidity associated with infections, as well spreading of infectious agent. According to our results, we also suggest that 18.88% on the analysis of the ROC curves may be accepted cut-off value, and the dead ratio over this threshold should be evaluated as susceptible.

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REFERENCES

- Nordmann P, Naas T, Poirel L. Global spread of Carbapenemase-producing *Enterobacteriaceae*. *Emerg Infect Dis* 2011;17:1791–1798.
- Pitout JD, Nordmann P, Poirel L. Carbapenemase-Producing *Klebsiella pneumoniae*, a Key Pathogen Set for Global Nosocomial Dominance. *Antimicrob Agents Chemother* 2015;59:5873–5884.
- Candan ED, Aksöz N. *Klebsiella pneumoniae*: Characteristics of carbapenem resistance and virulence factors. *Acta Biochim Pol* 2015;62:867–874.
- Hu F, Chen S, Xu X, et al. Emergence of carbapenem-resistant clinical *Enterobacteriaceae* isolates from a teaching hospital in Shanghai, China. *J Med Microbiol* 2012;61:132–136.
- Dortet L, Cuzon G, Plésiat P, Naas T. Prospective evaluation of an algorithm for the phenotypic screening of carbapenemase-producing *Enterobacteriaceae*. *J Antimicrob Chemother* 2015;71:135–140.
- Kilic A, Baysallar M. The First *Klebsiella pneumoniae* Isolate Co-Producing OXA-48 and NDM-1 in Turkey. *Ann Lab Med* 2015;35:382–383.
- Adams-Sapper S, Nolen S, Donzelli GF, et al. Rapid induction of high-level carbapenem resistance in heteroresistant KPC-producing *Klebsiella pneumoniae*. *Antimicrob Agents Chemother* 2015;59:3282–3289.
- Nakano R, Nakano A, Ishii Y, et al. Rapid detection of the *Klebsiella pneumoniae* carbapenemase (KPC) gene by loop-mediated isothermal amplification (LAMP). *J Infect Chemother* 2015;21:202–206.
- Hu YY, Cai J-C, Zhou H-W, Zhang R, Chen G-X. Rapid detection of porins by matrix-assisted laser desorption/ionization-time of flight mass spectrometry. *Front Microbiol* 2015;6:784.
- Mirande C, Canard I, Buffet Croix Blanche S, et al. Rapid detection of carbapenemase activity: Benefits and weaknesses of MALDI-TOF MS. *Eur J Clin Microbiol Infect Dis* 2015;34:2225–2234.
- March GA, García-Loygorri MC, Simarro M, Gutiérrez MP, Orduña A, Bratos MA. A new approach to determine the susceptibility of bacteria to antibiotics directly from positive blood culture bottles in two hours. *J Microbiol Methods* 2015;109:49–55.
- Nuding S, Zabel LT. Detection, identification, and susceptibility testing of bacteria by flow cytometry. *J Bacteriol Parasitol* 2013; S5:005.
- Clinical and Laboratory Standards Institute (CLSI). Performance standards for antimicrobial susceptibility testing; 22nd informational supplement. CLSI M100-S22. Clinical and Laboratory Standards Institute, Wayne, PA, 2012.
- Chen L, Mediavilla JR, Endimiani A, et al. Multiplex real-time PCR assay for detection and classification of *Klebsiella pneumoniae* carbapenemase gene (blaKPC) variants. *J Clin Microbiol* 2011;49:579–585.
- Poirel L, Walsh TR, Cuvillier V, Nordmann P. Multiplex PCR for detection of acquired carbapenemase genes. *Diagn Microbiol Infect Dis* 2011;59:119–123.
- Rajabnia R, Asgharpour F, Shahandashti EF, Moulana Z. Nosocomial emerging of (VIM1) carbapenemase-producing isolates of *Klebsiella pneumoniae* in North of Iran. *Iran J Microbiol* 2015;7:88–93.
- Hamzan NI, Yean CY, Rahman RA, Hasan H, Rahman ZA. Detection of blaIMP4 and blaNDM1 harboring *Klebsiella pneumoniae* isolates in a university hospital in Malaysia. *Emerg Health Threats J* 2015;10:26011.
- Wang L, Gu H, Lu X. A rapid low-cost real-time PCR for the detection of *Klebsiella pneumoniae* carbapenemase genes. *Ann Clin Microbiol Antimicrob* 2012;11:9.
- Biswas S, Rolain J-M. Use of MALDI-TOF mass spectrometry for identification of bacteria that are difficult to culture. *J Microbiol Methods* 2013;92:14–24.
- Hrabák J, Walková R, Studentová V, Chudácková E, Bergerová T. Carbapenemase activity detection by matrix-assisted laser desorption ionization-time of flight mass spectrometry. *J Clin Microbiol* 2011;49:3222–3227.
- Johansson A, Ekelöf J, Giske CG, Sundqvist M. The detection and verification of carbapenemases using ertapenem and Matrix Assisted Laser Desorption Ionization-Time of Flight. *BMC Microbiol* 2014;14:89.
- Angeletti S, Dicuonzo G, Lo Presti A, et al. MALDI-TOF mass spectrometry and blaKPC gene phylogenetic analysis of an outbreak of carbapenem-resistant *K. pneumoniae* strains. *New Microbiol* 2015;38:541–550.
- Berrazeg M, Diene SM, Drissi M, et al. Biotyping of multidrug-resistant *Klebsiella pneumoniae* clinical isolates from France and Algeria using MALDI-TOF MS. *PLoS ONE* 2013;8:e61428.
- Lau AF, Wang H, Weingarten RA, et al. A rapid matrix-assisted laser desorption ionization-time of flight mass spectrometry-based method for single-plasmid tracking in an outbreak of carbapenem-resistant *Enterobacteriaceae*. *J Clin Microbiol* 2014;52:2804–2812.
- Fredricks BA, De Coster DJ, Kim Y, Sparks N, Callister SM, Schell RF. Rapid pyrazinamide susceptibility testing of *Mycobacterium tuberculosis* by flow cytometry. *J Microbiol Methods* 2006;67:266–272.
- Huang T-H, Ning X, Wang X, Murthy N, Tzeng Y-L, Dickson RM. Rapid cytometric antibiotic susceptibility testing utilizing adaptive multidimensional statistical metrics. *Anal Chem* 2015;87:1941–1949.
- O’Gorman MR, Hopfer RL. Amphotericin B susceptibility testing of *Candida* species by flow cytometry. *Cytometry* 1991;12:743–747.
- Ordóñez JV, Wehman NM. Rapid flow cytometric antibiotic susceptibility assay for *Staphylococcus aureus*. *Cytometry* 1993;14:811–818.
- Faria-Ramos I, Espinar MJ, Rocha R, et al. A novel flow cytometric assay for rapid detection of extended-spectrum beta-lactamases. *Clin Microbiol Infect* 2013;19:E8–E15.
- Klein Breteler KB, Rentenaar RJ, Verkaart G, Sturm PD. Performance and clinical significance of direct antimicrobial susceptibility testing on urine from hospitalized patients. *Scand J Infect Dis* 2011;43:771–776.