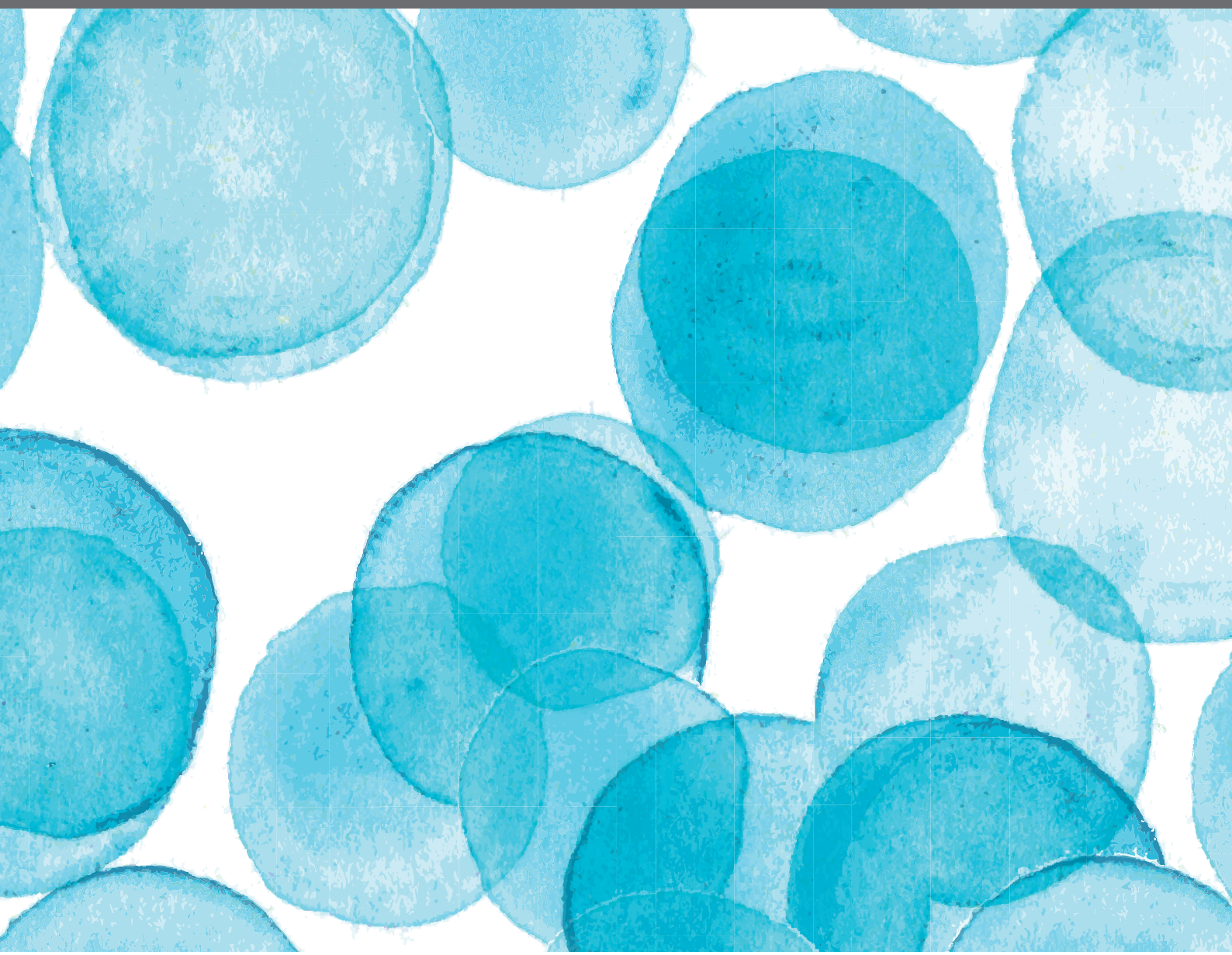
MALDI-TOF MS APPLICATION FOR SUSCEPTIBILITY TESTING OF MICROORGANISMS

EDITED BY: Karsten Becker and Sören Schubert
PUBLISHED IN: Frontiers in Microbiology



frontiers Research Topics

frontiers

ISSN 1664-8714
ISBN 978-2-88966-313-2
DOI 10.3389/978-2-88966-313-2

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to Quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: researchtopics@frontiersin.org

MALDI-TOF MS APPLICATION FOR SUSCEPTIBILITY TESTING OF MICROORGANISMS

Topic Editors:
Karsten Becker, University Medicine Greifswald, Germany
Sören Schubert, Ludwig Maximilian University of Munich, Germany

Citation: Becker, K., Schubert, S., eds. (2021). MALDI-TOF MS Application for Susceptibility Testing of Microorganisms. Lausanne: Frontiers Media SA.
doi: 10.3389/978-2-88966-313-2

Table of Contents

frontiers in Microbiology

EDITORIAL
published: 04 November 2020
doi: 10.3389/fmicb.2020.568891

Editorial: MALDI-TOF MS Application for Susceptibility Testing of Microorganisms

Karsten Becker[1]* *and Sören Schubert*[2]

[1] Friedrich Loeffler-Institute of Medical Microbiology, University Medicine Greifswald, Greifswald, Germany, [2] Faculty of Medicine, Max von Pettenkofer Institute of Hygiene and Medical Microbiology, Ludwig Maximilians University Munich, Munich, Germany

Keywords: mass spectometry, MALDI-TOF, susceptibility testing, carbapenamase, resistance detection, MRSA - Methicillin-resistant *Staphylococcus aureus*

Editorial on the Research Topic

MALDI-TOF MS Application for Susceptibility Testing of Microorganisms

In the present era of multi-/pan-resistant microorganisms, the acceleration of the microbiological diagnostics is the most pressing task and declared aim of many academic and industrial research groups. These efforts are of fundamental importance for appropriate antibiotic treatment and efficient infection control measures. Besides neglected issues of the preanalytical processes, rapid identification, and rapid susceptibility testing are essential components to be addressed (van Belkum et al., 2013, 2019; Idelevich et al., 2018a). Conventional standardized methods for susceptibility testing are usually accurate, but require long incubation times, especially for fungi (Idelevich and Becker, 2015). In the past decades the primary emphasis was on the development of nucleic acid-based molecular assays, which are only partially able to meet these demands. In particular, all DNA-based molecular assays are focused to the respective resistance genes. The presence of these, however, does not necessarily correspond to the resistance phenotype, nor is applicable in case of unknown resistance mechanisms. Consequently, universal approaches are necessary, which allow for (i) rapid and untargeted identification, (ii) fast and mechanism-independent susceptibility testing and, if needed, (iii) further characterization (e.g., typing) of a given isolate.

Although being a phenotypic approach, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS)-based analysis of the whole-organism protein mass spectra offers the closest approximation to the ribosomal sequence information. This method has been successfully established for the identification and subtyping of bacterial and fungal microorganisms (Wieser et al., 2012; Clark et al., 2013) and, meanwhile, it has become a routine method in many diagnostic laboratories contributing enormously to a reduction of the time-to-result in identification procedures (Idelevich et al., 2019). However, for susceptibility testing, rapid phenotypic approaches feasible for routine applications are still missing (Schubert and Kostrzewa, 2017). As shown in this special issue summarizing the results of the respective Frontiers Research Topic "MALDI-TOF MS Application for Susceptibility Testing of Microorganisms," this technology is able to provide solutions for rapid determination of antibiotic susceptibilities independently of the underlying resistance phenotype.

The review article by Burckhardt and Zimmermann provides an excellent entrance into the different MALDI-TOF MS approaches for specific or universal determination of antimicrobial resistances and addresses the question why we do need timely susceptibility testing. This overview is complemented by a mini-review by Florio et al. about the detection of antifungal resistance by MALDI-TOF MS. Approaches to determine bacterial and fungal resistances comprise technical

Edited by:
Bart Devreese, Ghent University, Belgium

Reviewed by:
Maurizio Sanguinetti, Catholic University of the Sacred Heart, Italy

***Correspondence:**
Karsten Becker
karsten.becker@med.uni-greifswald.de

Specialty section:
This article was submitted to Antimicrobials, Resistance and Chemotherapy, a section of the journal Frontiers in Microbiology

Received: *02 June 2020*
Accepted: *14 October 2020*
Published: *04 November 2020*

Citation:
Becker K and Schubert S (2020) Editorial: MALDI-TOF MS Application for Susceptibility Testing of Microorganisms. Front. Microbiol. 11:568891. doi: 10.3389/fmicb.2020.568891

solutions using (i) defined marker peaks to deduce susceptibility or resistance, (ii) alterations of antibiotics such as hydrolysis, decarboxylation or acetylation as read-out, (iii) peak shifts as read-out by incorporation of ^{13}C, (iv) quantification of the area under the curve as read-out and, as newest development, (v) the direct-on-target microdroplet growth assay (DOT-MGA).

The latter approach, originally described by Idelevich et al. (2018b), is represented in this issue by two articles reporting on the detection of methicillin resistance in *Staphylococcus aureus* by Nix et al. and on the rapid detection of extended-spectrum β-lactamases and AmpC β-lactamases in *Enterobacterales* by Correa-Martínez et al. Instead of targeting specific resistance mechanisms, it resembles the minimum inhibitory concentration (MIC) determination by the broth microdilution method by using MALDI-TOF MS targets directly as incubation device. Thus, the created layout of the test panel as well as the interpretation criteria applied follow the criteria of the European Committee on Antimicrobial Susceptibility Testing (EUCAST). Both articles demonstrated that these novel phenotypic methods are able to provide reliable results in a short time.

Dortet et al. present results on another innovative derivative of the MALDI-TOF MS technology, the so-called MALDIxin test, which has been recently developed by this group (Dortet et al., 2018). By directly assessing lipid A modifications in intact bacteria, this approach is able to identify polymyxin-resistant isolates in about 15 min and owns the capacity to discriminate between chromosome- and plasmid-encoded resistance as further advantage. Here, the authors showed their results of applying the MALDIxin to detect colistin-resistant *Salmonella enterica* isolates.

Further articles report on the evaluation of already existing commercially available MALDI-TOF mass spectrometry kits. Recent EUCAST guidelines for carbapenemase detection (European Committee for Antimicrobial Susceptibility Testing of the European Society of Clinical Microbiology Infectious Diseases, 2017) recommends hydrolysis assays by MALDI-TOF MS as a useful method for clinical practice. Several working groups evaluated the performances of the hydrolysis-based MBT STAR®-Carba IVD assay to detect carbapenemase-producing bacteria. The study of Cordovana et al. investigated a huge collection of diverse Italian and German *Klebsiella pneumoniae* isolates for the presence of the *Klebsiella pneumoniae* carbapenemases (KPC)-related peak, which has been confirmed in a subset by applying the MBT STAR®-Carba IVD assay. The confirmation procedure showed 100% sensitivity and specificity, both from colonies and from positive blood cultures. Using colony material, the assay as performed by Anantharajah et al. detected all included carbapenemase-producing *Enterobacteriaceae*, *Pseudomonas,* and *Acinetobacter* isolates with sensitivities and specificities of 100%. Of note, performed on positive blood cultures, the assay missed a few carbapenemase-producing *Acinetobacter* isolates, but a prolonged imipenem-incubation time of the strain pellet was able to improve the carbapenemase detection. Analyzing retrospective cultured isolates and prospective patient-derived blood cultures, Lee et al. measured the β-lactamase activities against various β-lactams including meropenem using the MBT STAR-BL module. Of interest, the assay protocol used enabled the reporting of β-lactamase-producing Gram-negative rods at ∼14 and 48 h before the interim and final reports of routine BCs processing, respectively. Oviaño et al. could show that this assay was also able to reliably detect the activity of imipenem/relebactam, a novel β-lactam-β-lactamase inhibitor combination, with a turnaround time of less than 1 h in clinical *Enterobacterales* KPC isolates.

Another facets of MALDI-TOF MS are presented by Hu et al. and Wang et al.. Hu et al. intended to evaluate an automatic *S. aureus* subtyping module, which identifies highly specific the methicillin resistance of phenol soluble modulin (PSM)-bearing MRSA through detection of a specific PSM-*mec* peak by MALDI-TOF MS. Wang et al. presented a machine learning approach to generate robust heterogeneous vancomycin-intermediate *Staphylococcus aureus* (hVISA) detection models for analyzing the complex MALDI-TOF mass spectra.

In summary, this special issue gives the reader a comprehensive overview of the state of the art of MALDI-TOF MS applications on antimicrobial susceptibility testing as well as very recent insights in cutting-edge developments. Future tasks comprise the extension of MALDI-TOF MS-targeted antimicrobial substances, the continued transfer of research results into commercial applications including their thorough evaluation and improved strategies to integrate these applications in routine diagnostics and automation solutions.

AUTHOR CONTRIBUTIONS

KB and SS managed the Research Topic MALDI-TOF MS Application for Susceptibility Testing of Microorganisms as topic editors. KB wrote the manuscript with input from SS. All authors reviewed and edited the manuscript.

FUNDING

This work was funded in part by the German Federal Ministry of Education and Research (BMBF) to KB (grant number 16GW0150).

ACKNOWLEDGMENTS

The authors gratefully acknowledge helpful discussions with Evgeny A. Idelevich.

REFERENCES

Clark, A. E., Kaleta, E. J., Arora, A., and Wolk, D. M. (2013). Matrix-assisted laser desorption ionization-time of flight mass spectrometry: a fundamental shift in the routine practice of clinical microbiology. *Clin. Microbiol. Rev.* 26, 547–603. doi: 10.1128/CMR.00072-12

Dortet, L., Bonnin, R. A., Pennisi, I., Gauthier, L., Jousset, A. B., Dabos, L., et al. (2018). Rapid detection and discrimination of chromosome- and

MCR-plasmid-mediated resistance to polymyxins by MALDI-TOF MS in *Escherichia coli*: the MALDIxin test. *J. Antimicrob. Chemother.* 73, 3359–3367. doi: 10.1093/jac/dky330

European Committee for Antimicrobial Susceptibility Testing of the European Society of Clinical Microbiology and Infectious Diseases (2017). *EUCAST Guidelines for Detection of Resistance Mechanisms and Specific Resistances of Clinical and/or Epidemiological Importance*. Version *2.0*.

Idelevich, E. A., and Becker, K. (2015). How to accelerate antimicrobial susceptibility testing. *Clin. Microbiol. Infect.* 25, 1347–1355. doi: 10.1016/j.cmi.2019.04.025

Idelevich, E. A., Reischl, U., and Becker, K. (2018a). New microbiological techniques in the diagnosis of bloodstream infections. *Dtsch. Arztebl. Int.* 115, 822–832. doi: 10.3238/arztebl.2018.0822

Idelevich, E. A., Seifert, H., Sundqvist, M., Scudeller, L., Amit, S., Balode, A., et al. (2019). Microbiological diagnostics of bloodstream infections in Europe-an ESGBIES survey. *Clin. Microbiol. Infect.* 25, 1399–1407. doi: 10.1016/j.cmi.2019.03.024

Idelevich, E. A., Sparbier, K., Kostrzewa, M., and Becker, K. (2018b). Rapid detection of antibiotic resistance by MALDI-TOF mass spectrometry using a novel direct-on-target microdroplet growth assay. *Clin. Microbiol. Infect.* 24, 738–743. doi: 10.1016/j.cmi.2017.10.016

Schubert, S., and Kostrzewa, M. (2017). MALDI-TOF MS in the microbiology laboratory: current trends. *Curr. Issues Mol. Biol.* 23, 17–20. doi: 10.21775/cimb.023.017

van Belkum, A., Bachmann, T. T., Lüdke, G., Lisby, J. G., Kahlmeter, G., Mohess, A., et al. (2019). Developmental roadmap for antimicrobial susceptibility testing systems. *Nat. Rev. Microbiol.* 17, 51–62. doi: 10.1038/s41579-018-0098-9

van Belkum, A., Durand, G., Peyret, M., Chatellier, S., Zambardi, G., Schrenzel, J., et al. (2013). Rapid clinical bacteriology and its future impact. *Ann. Lab. Med.* 33, 14–27. doi: 10.3343/alm.2013.33.1.14

Wieser, A., Schneider, L., Jung, J., and Schubert, S. (2012). MALDI-TOF MS in microbiological diagnostics-identification of microorganisms and beyond (mini review). *Appl. Microbiol. Biotechnol.* 93, 965–974. doi: 10.1007/s00253-011-3783-4

Conflict of Interest: KB is inventor of a patent application which is owned by the University of Münster and licensed to Bruker Daltonik GmbH.

The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 23 February 2018
doi: 10.3389/fmicb.2018.00334

Comprehensive Evaluation of the MBT STAR-BL Module for Simultaneous Bacterial Identification and β-Lactamase-Mediated Resistance Detection in Gram-Negative Rods from Cultured Isolates and Positive Blood Cultures

*Annie W. T. Lee[1], Johnson K. S. Lam[2], Ricky K. W. Lam[2], Wan H. Ng[2], Ella N. L. Lee[1], Vicky T. Y. Lee[3], Po P. Sze[1], Rahim Rajwani[1], Kitty S. C. Fung[4], Wing K. To[5], Rodney A. Lee[3], Dominic N. C. Tsang[2] and Gilman K. H. Siu[1]**

[1] Department of Health Technology and Informatics, The Hong Kong Polytechnic University, Hung Hom, Hong Kong, [2] Department of Pathology, Queen Elizabeth Hospital, Kowloon, Hong Kong, [3] Department of Pathology, Pamela Youde Nethersole Eastern Hospital, Hong Kong, Hong Kong, [4] Department of Pathology, United Christian Hospital, Kowloon, Hong Kong, [5] Department of Pathology, Princess Margaret Hospital, Kowloon, Hong Kong

OPEN ACCESS

Edited by:
Karsten Becker,
Universität Münster, Germany

Reviewed by:
Jaroslav Hrabak,
Charles University, Czechia
Irene Burckhardt,
University of Heidelberg, Germany

***Correspondence:**
Gilman K. H. Siu
gilman.siu@polyu.edu.hk

Specialty section:
This article was submitted to Antimicrobials, Resistance and Chemotherapy, a section of the journal Frontiers in Microbiology

Received: 31 August 2017
Accepted: 12 February 2018
Published: 23 February 2018

Citation:
Lee AWT, Lam JKS, Lam RKW, Ng WH, Lee ENL, Lee VTY, Sze PP, Rajwani R, Fung KSC, To WK, Lee RA, Tsang DNC and Siu GKH (2018) Comprehensive Evaluation of the MBT STAR-BL Module for Simultaneous Bacterial Identification and β-Lactamase-Mediated Resistance Detection in Gram-Negative Rods from Cultured Isolates and Positive Blood Cultures. Front. Microbiol. 9:334. doi: 10.3389/fmicb.2018.00334

Objective: This study evaluated the capability of a MALDI Biotyper system equipped with the newly introduced MBT STAR-BL module to simultaneously perform species identification and β-lactamase-mediated resistance detection in bacteremia -causing bacteria isolated from cultured isolates and patient-derived blood cultures (BCs).

Methods: Two hundred retrospective cultured isolates and 153 prospective BCs containing Gram-negative rods (GNR) were collected and subjected to direct bacterial identification, followed by the measurement of β-lactamase activities against ampicillin, piperacillin, cefotaxime, ceftazidime, and meropenem using the MBT STAR-BL module. The results and turnaround times were compared with those of routine microbiological processing. All strains were also characterized by beta-lactamase PCR and sequencing.

Results: Using the saponin-based extraction method, MALDI-TOF MS correctly identified bacteria in 116/134 (86.6%) monomicrobial BCs. The detection sensitivities for β-lactamase activities against ampicillin, piperacillin, third-generation cephalosporin and meropenem were 91.3, 100, 97.9, and 100% for cultured isolates, and 80.4, 100, 68.8, and 40% for monomicrobial BCs ($n = 134$) respectively. The overall specificities ranged from 91.5 to 100%. Furthermore, the MBT STAR-BL and conventional drug susceptibility test results were concordant in 14/19 (73.7%) polymicrobial cultures. Reducing the logRQ cut-off value from 0.4 to 0.2 increased the direct detection sensitivities for β-lactamase activities against ampicillin, cefotaxime and meropenem in BCs to 85.7, 87.5, and 100% respectively. The MBT STAR-BL test enabled the reporting of β-lactamase-producing GNR at 14.16 and 47.64 h before the interim and final reports of routine BCs processing, respectively, were available.

Conclusion: The MALDI Biotyper system equipped with the MBT STAR-BL module enables the simultaneous rapid identification of bacterial species and β-lactamase-mediated resistance from BCs and cultured isolates. Adjustment of the logRQ cut-off value to 0.2 significantly increased the detection sensitivities for clinically important drug-resistant pathogens.

Keywords: MBT STAR-BL, MALDI-TOF MS, drug resistance, bacterial, drug hydrolysis test, beta-lactamases, blood culture

INTRODUCTION

Sepsis is a major cause of infectious disease-associated morbidity and mortality (Fleischmann et al., 2016). Proper initial antibiotic therapy is a crucial parameter for improvement of patient outcomes (Kumar et al., 2006; Dellinger et al., 2008). Empirical treatment must be administrated at the time of sepsis diagnosis, and the regimen should be adjusted if necessary when bacterial identification and drug susceptibility results are available (Kang et al., 2005; Dellinger et al., 2008; Kumar, 2011). Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) is particularly useful for direct identification of causative agents of bacteremia, which can be inducers of sepsis, within the same day of a positive blood culture (BC) broth result (Schubert et al., 2011; Wuppenhorst et al., 2012; Chen et al., 2013; Clerc et al., 2013). However, predictions of drug susceptibility patterns based on bacterial identification alone have become inaccurate, given the increasing incidence of multidrug-resistance among Gram-negative bacteria (Paterson, 2006; Livermore, 2012).

Recent studies demonstrated the feasibility of using MALDI-TOF MS to predict β-lactam resistance through detection of hydrolytic β-lactam substrates produced by bacterial β-lactamases (Burckhardt and Zimmermann, 2011; Hrabak et al., 2011; Sparbier et al., 2012; Ghebremedhin et al., 2016). Unfortunately, in the absence of automated analysis software, previous studies have used either manual calculations (Sparbier et al., 2012; Ghebremedhin et al., 2016) or self-developed algorithms with ambiguous cut-off values (Jung et al., 2014) to analyze the peak patterns. These techniques introduce intra- and inter-observer variability to the assay and are difficult to implement in routine diagnostic workflows. Recently, Bruker Daltonik launched a software module, the MALDI Biotyper™ Selective Testing for Beta-Lactamase Activity (MBT STAR-BL), for the automatic analysis of drug hydrolysis mass spectra. This module facilitates the simultaneous bacterial identification and detection of β-lactamase-mediated resistance toward ampicillin (AMP), piperacillin (PIP), cefotaxime (CTX), ceftazidime (CAZ), meropenem (MEM), and ertapenem (ETP).

The present study aimed to evaluate the ability of the MALDI Biotyper system equipped with the MBT STAR-BL module to identify bacteremia -causing bacteria and predict β-lactam resistance from plated isolates, as well as BC broths. The time-to-results determined using MBT STAR-BL were also compared with those obtained using a conventional culture-based method.

MATERIALS AND METHODS

Sample Collection

In the first stage, 200 archived Gram-negative isolates of different species and various drug susceptibility patterns were collected and used to evaluate the ability of the MBT STAR-BL to detect β-lactamase-mediated resistance to all claimed antibiotics except ertapenem, which was not available locally. All strains were isolated from BCs previously collected at four different public hospitals throughout the territory. *Escherichia coli* strain DH5a was used as a β-lactamase-negative control strain, whereas ATCC *E. coli* strain BAA-2452, a NDM-1 carbapenemase producer, was used as a positive control in all drug hydrolysis tests.

In the second stage, 153 positive BC broths derived from patients with Gram-negative bacterial bloodstream infections were collected prospectively from January to December 2016. BACTEC™ FX (Becton Dickson, US) and BacT/Alert FA (bioMerieux, France) blood culture system are housed in hospitals. All positive BCs were subjected to direct Gram staining. If Gram-negative rods were found, a 5-mL aliquot of culture broth was transported to our laboratory for direct bacterial identification, followed by the detection of β-lactamase-mediated resistance using MBT STAR-BL within the same day.

On the following day, MALDI-TOF MS analyses were repeated using isolated colonies grown on subculture plates.

Bacterial Identification from BC Broths Using MALDI-TOF MS

For direct bacterial identification from BC broths, bacterial proteins were extracted using saponin-based protocol (Chen et al., 2013). The target plate was then analyzed using the Bruker Microflex LT system and MALDI Biotyper Compass software with the V5.0.0.0 spectra library (5989 spectra).

Preparation of Antibiotic Solutions

Solutions of AMP (3 mg/mL), PIP (0.5 mg/mL), CTX (0.5 mg/mL), CAZ (0.25 mg/mL) and MEM (1 mg/mL) were prepared in incubation buffer (10 mM ammonium bicarbonate, 10 μg/mL zinc chloride, pH 8–9).

The antibiotics used for MBT STAR-BL measurement were selected according to the bacterial identification given by MALDI-TOF MS. If Enterobacteriaceae was identified, β-lactamase activity against AMP, CTX/CAZ and MEM were studied. For non-fermentative Gram-negative rods (NFGRs), including *Acinetobacter* spp. and *Pseudomonas* spp., β-lactamase

activities against PIP, CAZ and MEM were investigated. β-lactamase activities against to all five β-lactam drugs was determined if no reliable ID was obtained.

MBT-STAR-BL Measurement

For plated isolates, sample preparation was done according to Hrabak et al. (2012). For BC broths, a bacterial pellet was isolated using saponin-based extraction method. Briefly, 1 mL of BC broth was treated with 5% saponin and subsequently washed twice with distilled water. After centrifugation, the bacterial pellet was resuspended in 50 μL of the appropriate antibiotic solution and incubated at 37°C under agitation (900 rpm) for 2 h, followed by centrifugation at 2,000 rpm for 2 min to collect the supernatant.

One microliter of supernatant was applied to the MSP96 target plate (reactions were performed in quadruplicate). The dried spots were overlaid with 1 μL of MBT STAR-BL Matrix. Automated mass spectrometric measurements were performed using the STAR-BL module (RUO version) embedded in the MALDI Biotyper Compass software. For instrument calibration, an antibiotic calibration standard (ACS; Bruker Daltonik) was measured in parallel with the samples in each run.

The STAR-BL module automatically calculated the normalized logRQ values for each sample. A value $\leq$0.2 indicated negative drug hydrolysis (i.e., reported as susceptible), whereas a value $\geq$0.4 indicated positive β-lactamase activity (i.e., reported as resistant). Normalized logQR values between 0.2 and 0.4 indicated an indeterminate hydrolysis measurement requiring retesting. If the repeated test yielded the same logQR value, the results were reported as indeterminate.

Routine Microbiological Processing

Routine processing of BCs included the subcultivation of positive broths on Columbia Blood agar. The final identification involved a MALDI-TOF MS analysis of single isolated colonies grown on subculture plates.

Interim drug susceptibility patterns were determined by disk diffusion test directly using positive BC broths, whereas final drug susceptibilities relied on the disk diffusion testing patterns obtained from subcultured isolates (Clinical and Laboratory Standards Institute, 2013). Furthermore, the extended-spectrum β-lactamase (ESBL) and carbapenemase phenotypes were confirmed using a combined disk method (Clinical and Laboratory Standards Institute, 2013; Pournaras et al., 2013).

Strain Characterization

The presence of plasmid-mediated β-lactamases in all identified β-lactam-resistant Enterobacteriaceae was confirmed using multiplex PCR assays, followed by amplicon sequencing-based genotyping (Dallenne et al., 2010; Doyle et al., 2012). PCR sequencing was used to detect the mutations in the *ampC* promoter/attenuator region as well as in *ampD and ampR* which are associated with hyperproduction of AmpC in Enterobacteriaceae (Kaneko et al., 2005; Schmidtke and Hanson, 2006; Peter-Getzlaff et al., 2011). For NFGRs, carbapenemases were characterized using a dual-tube multiplex PCR (Kazi et al., 2015), whereas some rare β-lactamases, such as bla_{Pom} and bla_{L1}, were detected as previously described (Thaller et al., 2011; Yang et al., 2014). For cases involving discrepancy between the phenotypic DST and MBT STAR-BL assays, the minimal inhibitory concentration (MIC) was determined using an *E*-test (bioMérieux, Marcy l'Etoile, France) according to the manufacturer's guidelines.

Assessment of the Times-to-Results Using the MBT STAR-BL and Routine Culture Methods

The times-to-results were compared based on a subset of 153 monomicrobial BCs ($n = 88$) collected from a hospital adjacent to our laboratory that houses the MALDI Biotyper system with STAR-BL module. BCs that were identified as positive between 7:00 a.m. and 4:00 p.m. were collected at the hospital. MBT STAR-BL measurements were performed immediately upon sample arrival in our laboratory. Time-zero was defined as the time at which the primary Gram staining result was reported. For MALDI-TOF MS analysis, the time elapsed between time-zero and the MBT STAR-BL analysis completion time was considered the "Time-to-MBT STAR-BL." For routine microbiological processing, the total time required to obtain bacterial identification and the results of the direct (interim) disk diffusion test was defined as the "Time-to-interim report," whereas the total time required to obtain the final drug susceptibility result from subcultured isolates was defined as the "Time-to-final report" (**Figure 1**).

RESULTS

MBT STAR-BL Testing on Archived Cultured Isolates

Among 139 Enterobacteriaceae collected in this study, 103 were AMP-resistant, as determined by phenotypic DST, and 94 (91.3%) exhibited positive hydrolysis in the MBT STAR-BL module. Notably, 63/66 (95.5%) AMP-resistant *E. coli* isolates were correctly identified by MBT STAR-BL with 100% specificity (**Table 1**).

MBT STAR-BL successfully identified 47/48 (97.9%) CTX-resistant and 22/30 (73.3%) CAZ-resistant Enterobacteriaceae isolates with no false positivity (**Table 1**). Interestingly, all 26 ESBL-producing Enterobacteriaceae demonstrated positive CTX hydrolysis, whereas only 3 (11.5%) could hydrolyze CAZ.

Remarkably, all carbapenemase-producing Enterobacteriaceae ($n = 16$) isolates were correctly identified by MBT STAR-BL with 100% specificity (**Table 1**).

For NFGRs, MBT STAR-BL successfully detected PIP and MEM hydrolysis in all *A. baumannii* isolates harboring bla_{OXA-23} and bla_{OXA-51} ($n = 28$) and all *P. aeruginosa* isolates harboring bla_{VIM} ($n = 3$) (**Table 1**). However, all *A. baumannii* isolates harboring bla_{OXA-23} and bla_{OXA-51} failed to exhibit CAZ hydrolysis in MBT-STAR-BL.

The characteristics of the bacterial cultures with discrepant results are shown in **Table 4**.

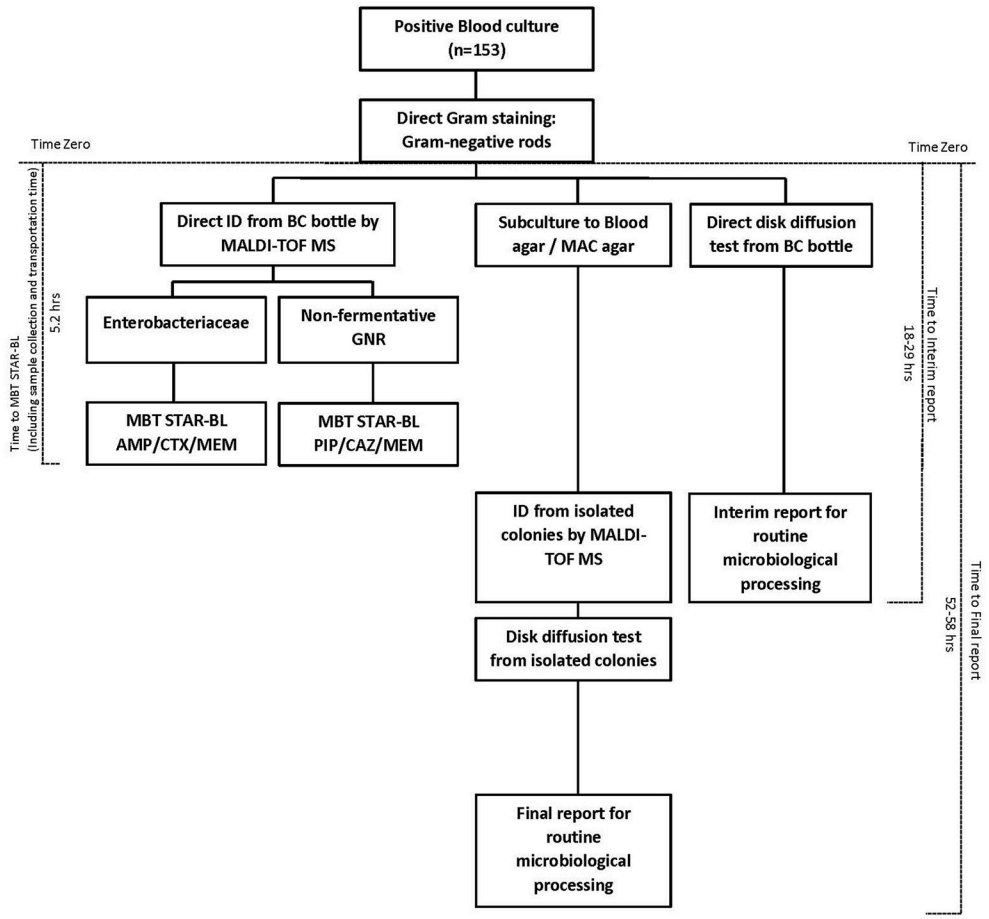


FIGURE 1 | Diagram of the workflow and turnaround time of bacterial identification and β-lactam resistance detection from positive blood cultures, using MBT STAR-BL and routine microbiological processing.

MBT STAR-BL Testing on Monomicrobial Blood Culture Broths

A total of 153 positive BC bottles, including monomicrobial ($n = 134$) and polymicrobial cultures ($n = 19$), were prospectively collected for this study.

Direct MALDI-TOF MS correctly identified bacteria in 116/134 (86.6%) monomicrobial BCs, and achieved species-level identification in 87/134 (64.9%) (Table S1).

Among 112 monomicrobial BCs harboring AMP-resistant Enterobacteriaceae, 90 (80.4%) exhibited AMP hydrolysis in MBT STAR-BL (**Table 2**). False negative results were mainly obtained from species that harbored inducible chromosomal *ampC* (**Table 4**). For *E. coli*, 58/63 (92.1%) AMP-resistant cultures were correctly identified by MBT STAR-BL, with the specificity of 92.9% (**Table 5**).

In addition, MBT STAR-BL successfully detected CTX resistant Enterobacteriaceae in 22/32 (68.8%) BCs (**Table 2**). Notably, of 28 ESBL-producing Enterobacteriaceae, 20 (71.4%) demonstrated positive CTX hydrolysis (**Table 2**). The false negative MBT STAR-BL results did not correlate with CTX-M genotypes or MIC levels (**Table 4**). Moreover, eight BCs containing CTX-susceptible Enterobacteriaceae yielded false positive hydrolysis results, resulting in a specificity of 91.5% (**Table 5**).

Unfortunately, no carbapenem-resistant Enterobacteriaceae were collected from BCs in this study.

For NFGRs, four BCs were resistant to PIP and CAZ as determined by phenotypic DST. MBT STAR-BL correctly predicted PIP resistance for all of them, but none of the BCs showed detectable CAZ hydrolysis in MBT STAR-BL. In addition, among 5 NFGRs that haboured carbapenemase genes and exhibited MEM resistance in phenotypic DST, MBT STAR-BL only detected MEM hydrolysis in 2 (40%) of the BCs (**Table 2**).

The characteristics of the BCs with discrepant results are shown in **Table 4**.

MALDI-TOF MS analyses were repeated for each sample using plated isolates subcultivated from BCs on the following day. The diagnostic sensitivities and specificities of MBT STAR-BL for the subcultured plates resembled those obtained from the archived cultured isolates (**Tables 2**, **5**).

TABLE 1 | Performance of the MBT STAR-BL module for the detection of β-lactamase-mediated resistance in cultured isolates.

Bacterial species and β-lactamase genes	No. of isolates	No. of resistant isolates confirmed by disk diffusion test				Positive hydrolysis detected by MBT STAR BL[b]			
		AMP/PIP[a]	CTX	CAZ	MEM	AMP/PIP	CTX	CAZ	MEM
Escherichia coli									
None	35	0	0	0	0	0	0	0	0
TEM-1b/1c	28	28	0	0	0	28	0	0	0
CTX-M-13 (ESBL)	7	7	7	2	0	7	7	1	0
CTX-M-14 (ESBL)	13	13	13	5	0	13	13	1	0
TEM-72 (ESBL	1	1	1	0	0	1	1	0	0
TEM-1b + OXA-15 (ESBL)	3	3	3	0	0	3	3	0	0
Hyper AmpC[c]	4	4	2	2	0	1	1	0	0
CMY-2 AmpC	1	1	1	1	0	1	1	0	0
NDM-5	6	6	6	6	6	6	6	6	6
KPC-2	3	3	3	3	3	3	3	3	3
Klebsiella pneumoniae									
SHV-1/11	16	16	0	0	0	13	0	0	0
SHV-1 + CTX-M-24 (ESBL)	1	1	1	0	0	1	1	0	0
SHV-1 + OXA (ESBL)	1	1	1	1	0	1	1	1	0
SHV-1 + DHA-1 AmpC	1	1	1	1	0	1	1	1	0
SHV-1 + NDM-1	1	1	1	1	1	1	1	1	1
Klebsiella oxytoca									
SHV-1 + IMP-8	3	3	3	3	3	3	3	3	3
Proteus mirabilis									
TEM-1b	6	6	0	0	0	6	0	0	0
***Salmonella* spp.**									
None	1	0	0	0	0	0	0	0	0
Citrobacter freundii									
Inducible AmpC + NDM-1	1	1	1	1	1	1	1	1	1
Enterobacter cloacae									
Inducible AmpC[d]	3	3	0	0	0	0	0	0	0
Inducible AmpC + CMY-2 AmpC	2	2	2	2	0	2	2	2	0
Inducible AmpC + IMP-1	2	2	2	2	2	2	2	2	2
Total *Enterobacteriaecae*	139	103	48	30	16	94	47	22	16
Pseudomonas aeruginosa									
Inducible AmpC	30	0	ND	0	0	0	ND	0	0
Inducible AmpC + VIM-4	3	3	ND	3	3	3	ND	3	3
Acinetobacter baumannii									
OXA-23, −51	28	28	ND	20	28	28	ND	0	28
Total NFGR	61	31	ND	23	31	31	ND	3	31
Total organisms	200	134	48	53	47	125	47	25	47

[a] *An AMP hydrolysis assay was applied to Enterobacteriaceae isolates, whereas a PIP hydrolysis assay was used for all NFGRs, including Pseudomonas spp. and Acinetobacter spp.*
[b] *Isolates were considered resistant only when the logRQ value was ≥0.4 in the MBT STAR-BL module.*
[c] *Hyper AmpC refers to E. coli strains with insertions of 1 or 2 bases between the 35 and 10 boxes of the ampC promoter region, which were shown to cause ampC hyperexpression (Peter-Getzlaff et al., 2011).*
[d] *Inducible AmpC refers to bacterial species that harbored an inducible chromosomal ampC gene with intact regulatory elements (i.e., no ampD and ampR mutations).*

MBT STAR-BL Testing on Polymicrobial Blood Culture Broths

Direct MALDI-TOF MS correctly identified at least 1 bacterial species in 16/19 (84.2%) polymicrobial BCs (**Table 3**).

Additionally, MBT STAR-BL successfully detected AMP hydrolysis in all 10 BCs containing AMP-resistant *E. coli* strains. Among the 7 polymicrobial BCs containing ESBL-producing Enterobacteriaceae, 5 (71.4%) exhibited CTX hydrolysis in an MBT STAR-BL test (**Table 3**).

Full concordance was yielded between the MBT STAR-BL and phenotypic DST in prediction of PIP resistance in polymicrobial BCs containing NFGRs (**Table 3**). For the BCs containing two

TABLE 2 | Performance of the MBT STAR-BL module in the detection of β-lactamase-mediated resistance in prospectively collected monomicrobial blood cultures.

Bacterial Species and β-lactamase genes	No. of cultures	No. of resistant isolates confirmed by disk diffusion test			True and (false) positive hydrolysis detected from blood culture broths by MBT STAR BL			True and (false) positive hydrolysis detected from subcultured isolates by MBT STAR BL		
		AMP/PIP[a]	CTX/CAZ[b]	MEM	AMP/PIP[a]	CTX/CAZ[b]	MEM	AMP/PIP[a]	CTX/CAZ[b]	MEM
Escherichia coli										
None	14	0	0	0	0 (1)	0 (1)	0 (1)	0	0	0
TEM-1b/1c	38	38	0	0	35	0 (2)	0	37	0	0
CTX-M-13 (ESBL)	10	10	10	0	8	6	0 (2)	10	10	0
TEM-1b + CTX-M-13 (ESBL)	4	4	4	0	4	3	0	4	4	0
TEM-1b + CTX-M-14 (ESBL)	7	7	7	0	7	5	0	7	7	0
SHV-1 + CTX-M-39 (ESBL)	1	1	1	0	1	1	0	1	1	0
CMY-2 AmpC	2	2	2	0	2	0	0	2	1	0
DHA-1 AmpC	1	1	1	0	1	1	0	1	1	0
Klebsiella pneumoniae										
SHV-1	27	27	0	0	17	0 (4)	0	22	0	0
SHV-1 + CTX-M-14 (ESBL)	4	4	4	0	4	3	0	4	4	0
DHA-1 AmpC	1	1	1	0	1	1	0	1	1	0
Klebsiella oxytoca										
SHV-1	1	1	0	0	1	0	0	1	0	0
Proteus mirabilis										
TEM-1b/1c	3	3	0	0	2	0	0	3	0	0
TEM-1b + CTX-M-14 (ESBL)	2	2	2	0	2	2	0	2	2	0
***Salmonella* spp.**										
TEM-1b	1	1	0	0	1	0	0	1	0	0
Morganella morganii										
Inducible AmpC[d]	2	2	0	0	1	0	0	1	0	0
Enterobacter cloacae										
Inducible AmpC[d]	2	2	0	0	0	0	0	0	0	0
Citrobacter freundii										
Inducible AmpC[d]	3	3	0	0	3	0 (1)	0	3	0	0
Pluralibacter gergoviae										
Inducible AmpC[d]	1	1	0	0	0	0	0	0	0	0
Raoultella ornithinolytica										
Inducible AmpC[d]	2	2	0	0	0	0	0	2[C]	0	0
Total Enterobacteriaceae	126	112	32	0	90 (1)	22 (8)	0 (3)	102	31	0
Acinetobacter baumannii										
None	2	0	0	0	0	0	0	0	0	0
OXA-23, −51	2	2	2	2	2	0	0	2	0	2
Pseudomonas aeruginosa										
Inducible AmpC[d]	1	0	0	0	0	0	0	0	0	0
Pseudomonas otitidis										
POM-1	1	0	0	1	0	0	0	0	0	1
Stenotrophomonas maltophilia										
MBL L1	2	2	2	2	2	0	2	2	0	2
Total NFGRs	8	4	4	5	4	0	2	4	0	5
Total organisms	134	116	36	5	94 (1)	22 (8)	2 (3)	106	31	4

[a] *An AMP hydrolysis assay was applied to Enterobacteriaceae, whereas a PIP hydrolysis assay was used for all NFGRs, including Pseudomonas spp. and Acinetobacter spp.*

[b] *A CTX hydrolysis assay was applied to Enterobacteriaceae, whereas a CAZ hydrolysis assay was used for all NFGRs.*

[c] *The subcultured isolates of R. ornithinolytica failed to hydrolyze AMP after a 2-h incubation. However, the hydrolysis became positive when the incubation time was extended to 4 h.*

[d] *Inducible AmpC refers to bacterial species that harbored an inducible chromosomal ampC gene with intact regulatory elements (i.e., no ampD and ampR mutations).*

TABLE 3 | Performance of the MALDI-TOF MS workflow for bacterial identification and β-lactamase-mediated resistance detection in polymicrobial blood cultures and respective subcultured isolates.

Blood culture broths	Conventional ID and DST							MALDI-TOF MS ID and MBT STAR-BL from blood culture broths				MALDI-TOF MS ID and MBT STAR-BL from subcultured isolates			
	ID	β-lactamase	AMP/PIP	CAZ	CTX	MEM	ESBL	ID	AMP/PIP	CTX/CAZ	MEM	ID	AMP/PIP	CTX/CAZ	MEM
BC6	*E. cloacae*	Inducible AmpC[a]	R	S	S	S	−	*Enterobacter sp.*	I[c]	S[c]	S	*E. cloacae*	S[c]	S[c]	S
	E. cloacae	Derepressed AmpC[b]	R	R	R	S	−					*E. cloacae*	I[c]	S[c]	S
BC11	*E. coli*	CTX-M-13	R	S	R	S	+	*E. coli*	R	R	S	*E. coli*	R	R	S
	E. coli	TEM-1c	R	S	S	S	−					*E. coli*	R	S	S
BC23	*K. pneumoniae*	SHV-1	R	S	S	S	−	No reliable ID	R	S	S	*K. pneumoniae*	R	S	S
	E. cloacae	Inducible AmpC	R	S	S	S	−					*E. cloacae*	S[c]	S	S
BC26	*E. coli*	CTX-M-13	R	R	R	S	+	*E. coli*	R	R	S	*E. coli*	R	R	S
	P. mirabilis	TEM-1b	R	S	S	S	−					*P. mirabilis*	R	S	S
BC27	*A. baumannii*	OXA-23,−45	R	R	ND	R		*A. baumannii*	R	S[c]	R	*A. baumannii*	R	S[c]	R
	A. baumannii	OXA-23,−45	R	R	ND	R						*A. baumannii*	R	S[c]	R
	P. aeruginosa	Inducible AmpC	S	S	ND	S						*P. aeruginosa*	S	S	S
BC30	*K. pneumoniae*	SHV-1	R	S	S	S	−	*E. coli*	R	S	S	*K. pneumoniae*	R	S	S
	E. coli	TEM-1b	R	S	S	S	−					*E. coli*	R	S	S
BC33	*K. pneumoniae*	TEM-1b, SHV-1	R	S	S	S	−	*Klebsiella sp.*	R	R	S	*K. pneumoniae*	R	S	*S*
	K. pneumoniae	CTX-M-39	R	R	R	S	+					*K. pneumoniae*	R	R	*S*
BC37	*P. aeruginosa*	Inducible AmpC	S	S	ND	S		*Pseudomonas sp.*	S	S	S	*P. aeruginosa*	S	S	*S*
	M. morganii	Inducible AmpC	R	S	S	S	−					*M. morganii*	S[c]	S	S
BC43	*M. morganii*	Inducible AmpC	R	S	S	S	−	*M. morganii*	I[c]	S	S	*M. morganii*	S[c]	S	S
	P. aeruginosa	Inducible AmpC	S	S	ND	S	−					*P. aeruginosa*	S	S	S
BC54	*K. pneumoniae*	SHV-1	R	S	S	S	−	No reliable ID	S	S	S	*K. pneumoniae*	R	S	I
	K. pneumoniae	SHV-1	R	S	S	S	−					*K. pneumoniae*	R	S	I
BC55	*E. coli*	CTX-M-14	R	S	R	S	+	*E. coli*	R	S[c]	S	*E. coli*	R	R	S
	E. coli	None	S	S	S	S						*E. coli*	S	S	S
BC67	*E. coli*	None	I	S	S	S	−	*E. coli*	R	S	S	*E. coli*	S	S	S
	E. coli	TEM-1b	R	S	S	S	−					*E. coli*	I[c]	S	S
BC72	*E. coli*	TEM-1b, CTX-M-13	R	S	R	S	+	*E. coli*	R	R	S	*E. coli*	R	R	S
	E. coli	CTX-M-9	R	S	R	S	+					*E. coli*	R	R	S
BC75	*K. pneumoniae*	SHV-1	R	S	S	S	−	No reliable ID	R	I	S	*K. pneumoniae*	R	S	I
	K. oxytoca	SHV-1	R	S	S	S	−					*K. oxytoca*	R	S	I
BC83	*E. coli*	TEM-1c	R	S	S	S	−	*E. coli*	R	S	S	*E. coli*	R	I	S
	E. coli	None	I	S	S	S	−					*E. coli*	S	I	S
BC86	*E. coli*	CTX-M-24	R	S	R	S	+	*E. coli*	R	I[c]	S	*E. coli*	I	R	S
	E. coli	OXA-15	R	S	R	S	+					*E. coli*	I	R	S
BC110	*E. coli*	TEM-1b	R	S	S	S	−	*E. coli*	R	S	I	*E. coli*	R	S	S
	E. coli	OXA-1	R	S	S	S	−					*E. coli*	R	S	S
PY21	*E. coli*	OXA-1, CTX-M-13 & CTX-M-37	R	R	R	S	+	*E. coli*	R	R	S	*E. coli*	R	R	S
	K. pneumoniae	SHV-1	R	S	S	S	−					*K. pneumoniae*	R	S	S
PY37	*E. coli*	None	S	S	S	S		*E. coli*	S	S	S	*E. coli*	S	S	S
	Plesiomonas Shigelloides	None	S	S	S	S	−					*Plesiomonas Shigelloides*	S	S	S

[a] *Inducible AmpC refers to bacterial species that harbored an inducible chromosomal ampC gene with intact regulatory elements (i.e., no ampD and ampR mutations).*

[b] *The E. cloacae strain was found to harbor a truncated ampD gene, which was shown to fully derepress AmpC activity (Schmidtke and Hanson, 2006).*

[c] *The underlined results indicate mismatches between conventional DST and MBT STAR-BL.*

ND, Not done; NA, Not applicable.

A. baumannii strains harboring $bla_{OXA-23\&51}$, MBT STAR-BL successfully identified MEM hydrolysis in both the BC broths and subcultured isolates (**Table 3**). Overall, the β-lactam resistance patterns predicted by MBT STAR-BL were concordant with the phenotypic DST in 14/19 (73.7%) polymicrobial BCs. Details of the mismatches are shown in **Table 4**.

TABLE 4 | The characteristics of bacterial strains with discrepant results from the phenotypic drug susceptibility test and MBT STAR-BL analysis.

Bacterial Species	β-lactamase genes	No. of strains	Source	MIC range (μg/ml), S/R	MBT STAR-BL from blood culture		MBT STAR-BL from isolates	
					logRQ value range	Results	logRQ value range	Results
MISMATCHES FOR AMP SUSCEPTIBILITY								
E. coli	Hyperexpressed AmpC[b]	3	Retrospective isolates	64 to >256, R	NA	NA	0.21 to 0.29	I
E. coli	TEM-1b/1c	3	Monomicrobial BC	>256, R	−0.23 to 0.21	S/I	0.27 to 0.47	I/R
E. coli	CTX-M-13	2	Monomicrobial BC	>256, R	0.14 to 0.24	S/I	0.82 to 1.12	R
K. pneumoniae	SHV-1	3	Retrospective isolates	>256, R	NA	NA	−0.1 to 0.23	S/I
K. pneumoniae	SHV-1	10	Monomicrobial BC	128 to >256, R	−0.41 to 0.27	S/I	−0.3 to 1.17	S/I/R
K. pneumoniae	SHV-1	2	Polymicrobial BC	>256, R	−0.34	S	1.02 to 1.15	R
P. mirabilis	TEM-1c	1	Monomicrobial BC	128, R	0.02	S	0.34	I
E. cloacae	Inducible AmpC	3	Retrospective isolates	128 to 256, R	NA	NA	−0.24 to 0.28	S/I
E. cloacae	Inducible AmpC	2	Monomicrobial BC	128, R	−0.11 to 0.15	S	−0.3 to 0.37	S
E. cloacae	Inducible AmpC	1	Polymicrobial BC	128, R	0.29	I	−0.31	S
E. cloacae	Derepressed AmpC[c]	1	Polymicrobial BC	256, R	0.29	I	0.25	I
M. morganii	Inducible AmpC	1	Monomicrobial BC	256, R	−0.16	S	0.24	I
M. morganii	Inducible AmpC	2	Polymicrobial BC	128 to 256, R	−0.43 to 0.23	S/I	−0.52 to −0.44	S
P. gergoviae	Inducible AmpC	1	Monomicrobial BC	256, R	−0.35	S	0.32	I
R. ornithinolytica	Inducible AmpC	2	Monomicrobial BC	128 to 256, R	−0.24 to 0.1	S	0.4 to 1.33	R[a]
MISMATCHES FOR CTX SUSCEPTIBILITY								
E. coli	Hyperexpressed AmpC[b]	1	Retrospective isolates	>16, R	NA	NA	0.15	S
E. coli	CTX-M-13	4	Monomicrobial BC	>16, R	−0.13 to 0.36	S/I	0.44 to 0.96	R
E. coli	TEM-1b + CTX-M-13	1	Monomicrobial BC	>16, R	0.21	I	0.51	R
E. coli	TEM-1b + CTX-M-14	2	Monomicrobial BC	>16, R	0.18 to 0.31	S/I	0.56 to 0.67	R
E. coli	CTX-M-14	1	Polymicrobial BC	>16, R	0.13	S	0.67	R
E. coli	CTX-M-24	1	Polymicrobial BC	>16, R	0.21	I	1.04	R
E. coli	CMY-2 AmpC	2	Monomicrobial BC	>16, R	0.10 to 0.20	S/I	−0.03 to 0.56	S/R
K. pneumoniae	SHV-1 + CTX-M-14	1	Monomicrobial BC	>16, R	0.12	S	0.52	R
E. cloacae	Derepressed AmpC[c]	1	Polymicrobial BC	>16, R	0.33	I	0.28	I
MISMATCHES FOR CAZ SUSCEPTIBILITY								
E. coli	CTX-M-13	1	Retrospective isolates	16 to >256, R	NA	NA	−0.32 to −0.04	S
E. coli	CTX-M-14	4	Retrospective isolates	16 to >256, R	NA	NA	−0.23 to 0.08	S
E. coli	Hyperexpressed AmpC[b]	2	Retrospective isolates	32, R	NA	NA	−0.03 to 0.15	S
E. coli	CMY-2 AmpC	1	Retrospective isolates	128, R	NA	NA	−0.13	S
A. baumannii	OXA-23, −51	28	Retrospective isolates	>256, R	NA	NA	−0.43 to 0.16	S
A. baumannii	OXA-23, −51	2	Monomicrobial BC	>256, R	−0.45 to −0.55	S	−0.53 to −0.18	S
A. baumannii	OXA-23, −51	2	Polymicrobial BC	>256, R	−0.95	S	−0.17	S
S. maltophila	MBL L1	2	Monomicrobial BC	>256, R	−1.63 to −0.25	S	−2.28 to −0.13	S
MISMATCHES FOR MEM SUSCEPTIBILITY								
A. baumannii	OXA-23, −51	2	Monomicrobial BC	>32, R	0.21–0.25	I	0.58–0.77	R
P. otitidis	POM-1	1	Monomicrobial BC	4, R	0.32	I	0.83	R

[a] *The subculture isolates of R. ornithinolytica failed to hydrolyze AMP after a 2-h incubation. However, the hydrolysis became positive when the incubation time was extended to 4 h.*

[b] *Hyper AmpC refers to E. coli strains that harbored insertions of 1 or 2 bases between the 35 and 10 boxes in the ampC promoter region, which were shown to cause ampC hyperexpression (Peter-Getzlaff et al., 2011).*

[c] *The E. cloacae strain was found to harbor a truncated ampD gene, which was previously shown to fully derepress AmpC activity (Schmidtke and Hanson, 2006).*

Assessment of the Times-to-Results Using MBT STAR-BL and Routine Microbiological Processing

The times-to-results based on MBT STAR-BL and routine microbiological processing were investigated for 88 monomicrobial BCs (**Table 6**). Among the BCs, the average time to MBT STAR-BL was 5.2 h. For Enterobacteriaceae, the MALDI-TOF MS-based workflow allowed the laboratory to identify bacterial species and β-lactamase-mediated resistance to β-lactams at averages of 13.5 and 47.5 h before the interim and final reporting of the routine processing, respectively. For NFGRs, the MBT STAR-BL assay enabled the reporting of bacterial identification and MEM hydrolysis at 23.0 and 49.9 h before the interim and final routine processing, respectively.

DISCUSSION

In this study, we evaluated the practicality of MALDI-TOF MS using the 5989 spectra library, followed by MBT STAR-BL as a diagnostic workflow, for the identification of bacterial species and β-lactamase-mediated resistance in cultured isolates and patient-derived BCs. In contrast to previous studies that mainly investigated origin of carbapenem resistance in Enterobacteriaceae (Hrabak et al., 2011, 2012; Papagiannitsis et al., 2015; Ghebremedhin et al., 2016; Monteferrante et al., 2016; Oviano et al., 2017), this study identified the susceptibilities of a wide spectrum of bacterial species harboring different enzymes. To the best of our knowledge, this is the most comprehensive evaluation of the MBT STAR-BL module since its official launch in 2016.

AMP is used as a surrogate marker of resistance to aminopenicillins in *E. coli* and other Enterobacteriaceae, such as *Proteus mirabilis, Salmonella* spp. *and Shigella* spp. In this study, AMP resistance was detected in *E.coli* from cultured isolates and BCs at detection sensitivities of 95.5 and 92.1%, respectively (**Table 5**), consistent with Jung et al. (2014). Although we did not observe a correlation between the logRQ values and MIC levels, we noted that all the strains with high MIC level but low logRQ value are those harboring mutations associated with hyperproduction of AmpC (**Table 4**). An analysis of the respective mass spectra revealed that the peaks corresponding to hydrolyzed AMP were weak and coexistent with the molecular peaks of intact antibiotics, indicating incomplete drug hydrolysis (**Figure 2**). We repeated this assay for all chromosomal *ampC* carriers after extending the incubation time to 4 h. Unfortunately, only two *R. ornithinolytica* isolates yielded logRQ values $\geq$ 0.4 whereas most others had values of 0.2–0.4.

Both CTX and CAZ could be used as indicator drugs for the MBT STAR-BL testing of resistance to third-generation cephalosporin. Consistent with a previous study (Mackenzie et al., 2002), the CAZ hydrolysis assay in our study yielded a high false negative rate in both cultured isolates and BCs, indicating that it might not be appropriate for predicting phenotypic drug resistance. Conversely, the CTX hydrolysis assay successfully identified 100% of ESBL- and 83.3% of AmpC-producing bacterial isolates, suggesting that CTX is a better surrogate

TABLE 5 | The overall diagnostic performances of drug hydrolysis assays for cultured isolates and blood cultures at logRQ cut-off values of 0.4 and 0.2.

	Drug hydrolysis assay											
	AMP[a]		AMP (For *E.coli* only)		PIP[b]		CTX[c]		CAZ[d]		MEM[e]	
	Sn %	Sp (%)	Sn (%)	Sp (%)	Sn (%)	Sp (%)	Sn (%)	Sp (%)	Sn (%)	Sp (%)	Sn (%)	Sp (%)
RETROSPECTIVE ISOLATES												
logRQ cut-off = 0.4	91.3 (94/103)	100 (36/36)	95.5 (63/66)	100 (35/35)	100 (31/31)	100 (30/30)	97.9 (47/48)	100 (91/91)	47.2 (25/53)	100 (147/147)	100 (47/47)	100 (153/153)
logRQ cut-off = 0.2	96.1 (99/103)	97.2 (35/36)	100 (66/66)	100 (35/35)	100 (31/31)	100 (30/30)	97.9 (47/48)	100 (91/91)	47.2 (25/53)	100 (147/147)	100 (47/47)	100 (153/153)
BLOOD CULTURE BOTTLES												
logRQ cut-off = 0.4	80.4 (90/112)	92.9 (13/14)	92.1 (58/63)	92.9 (13/14)	100 (4/4)	100 (4/4)	68.8 (22/32)	91.5 (86/94)	0 (0/4)	100 (4/4)	40 (2/5)	97.7 (126/129)
logRQ cut-off = 0.2	85.7 (96/112)	92.9 (13/14)	95.2 (60/63)	92.9 (13/14)	100 (4/4)	100 (4/4)	87.5 (28/32)	89.4 (84/94)	0 (0/4)	100 (4/4)	100 (5/5)	95.3 (123/129)
SUBCULTURED ISOLATES FROM BC												
logRQ cut-off = 0.4	91.1 (102/112)	100 (14/14)	98.4 (62/63)	100 (14/14)	100 (4/4)	100 (4/4)	96.9 (31/32)	100 (94/94)	0 (0/4)	100 (4/4)	100 (5/5)	100 (129/129)
logRQ cut-off = 0.2	96.4 (108/112)	85.7 (12/14)	100 (63/63)	85.7 (12/14)	100 (4/4)	100 (4/4)	96.9 (31/32)	93.6 (88/94)	0 (0/4)	100 (4/4)	100 (5/5)	97.7 (126/129)

[a] *In this column, the diagnostic performance of the AMP hydrolysis assay includes all Enterobacteriaceae.*

[b] *The PIP hydrolysis assay was only applied to NFGRs.*

[c] *The CTX hydrolysis assay was only applied to Enterobacteriaceae.*

[d] *In the first part of the study (retrospective isolates), a CAZ hydrolysis assay was applied to both Enterobacteriaceae and NFGR isolates, whereas in the second part of the study (blood culture), only NFGRs were included in the CAZ hydrolysis assay.*

[e] *The MEM hydrolysis assay were used for both Enterobacteriaceae and NFGR in both the cultured isolate or blood culture stages of the study.*

TABLE 6 | Assessment of the times to results, based on MBT STAR-BL and routine microbiological processing.

Organisms	No. of isolates[a]	Average time to MBT STAR-BL (h)[b]	Time to interim report by routine microbiological processing (h)[c]			Time to final report by routine microbiological processing (h)[d]		
			Average (h)	Δ Time to Result [e]	*p*-value	Average (h)	Δ Time to Result[e]	*p*-value
Enterobacteriaceae	82	5.2	18.72	13.52	<0.0001	52.68	47.48	<0.0001
ESBL producers	21	5.2	18.97	13.77	<0.0001	54.25	49.05	<0.0001
Non-ESBL producers	61	5.2	18.02	12.82	<0.0001	48.15	42.95	<0.0001
NFGR	6	5.2	28.17	22.97	<0.0001	55.08	49.88	<0.0001
Pseudomonas spp.	3	5.2	27.33	22.13	<0.0001	52	46.8	<0.0001
A. baumannii	3	5.2	29	23.8	<0.0001	58.17	52.97	<0.0001
All organisms	88	5.2	19.36	14.16	<0.0001	52.84	47.64	<0.0001

[a] *Only monomicrobial BCs with concordant results between the MBT STAR-BL and routine microbiological processing were included in the time to results assessment.*
[b] *The time to MBT STAR-BL refers to the time elapsed between the reporting of the primary Gram stain result and the completion of the MBT STAR-BL analysis (including the pending time before collection, transportation time, and times used for protein extraction, antibiotic incubation and MADLI-TOF MS measurement).*
[c] *The time to interim report refers to the time required to obtain bacterial identification and the results of a direct disk diffusion test of inoculates of BC broths in a clinical microbiology laboratory.*
[d] *The time to final report refers to the time required to obtain bacterial identification and the results of the final disk diffusion test of inoculates from colonies isolated from subcultured plates in a clinical microbiology laboratory.*
[e] *This column indicates the differences in the times to results between MBT STAR-BL and the interim and final reports of routine microbiological processing.*

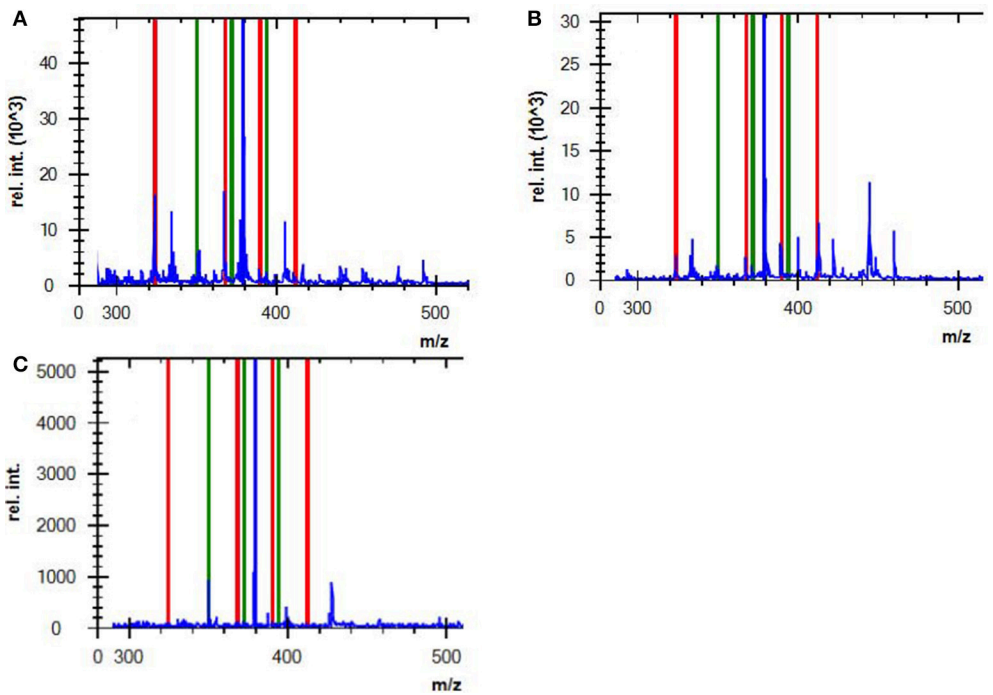


FIGURE 2 | Mass spectra of **(A)** a complete AMP hydrolysis obtained from a positive control strain (ATCC BAA-2452, NDM-1-producing *E. coli*) incubated with AMP for 2 h, **(B)** an incomplete AMP hydrolysis obtained from an *E. coli* strain that hyperexpressed *ampC*, and **(C)** non-hydrolyzed AMP from a negative control strain (DH5α). Red lines indicate the hydrolyzed antibiotic peaks, and green lines indicate intact antibiotic peaks (non-hydrolyzed).

marker for the detection of ESBL- and plasmid-mediated AmpC β-lactamase activity. However, the direct detection of ESBL from BCs yielded a CTX hydrolysis sensitivity of only 71.4%, lower than that reported by Jung et al. (2014). We attribute this disagreement to the different logRQ cut-off values used in the two studies (≥0 in Jung et al. vs. ≥0.4 in this study). In fact, 5/7 ESBL producers with false negative results in this study had logRQ values of 0.2–0.4. We note that antibiotic therapy prior to BC collection might also contribute to the occurrence of false negativity in a drug hydrolysis assay. At least two patients from whom the ESBL producers were not detected from BCs by MBT STAR-BL were receiving carbapenem treatment, which might have reduced the viable number of bacterial cells in the BC broths and led to suboptimal CTX and AMP hydrolysis.

Many previous studies involving the mass spectrometric detection of β-lactamase excluded NFGRs, as permeability- and efflux-based resistance mechanisms play major roles in drug resistance in these species (Kumar and Schweizer, 2005). However, NFGRs are important pathogens, and data regarding the ability of MBT STAR-BL to detect drug-resistant NFGR would be useful for routine diagnostic purposes. In our study, the PIP hydrolysis assay correlated perfectly with the phenotypic resistance test for all cultured isolates and BCs. Regarding the MEM hydrolysis assay, MBT STAR-BL correctly identified all carbapenem-resistant NFGRs in cultured isolates. However, in direct BCs, the results for two *A. baumannii* strains harboring $bla_{OXA-23,-51}$ and one *P. otitidis* strain harboring bla_{POM-1} were interpreted as indeterminate (logRQ: 0.2–0.4). We attributed the low logRQ values to the low recovery of bacterial cells after extraction and the reported low cell permeability of NFGR (Van Looveren et al., 2004). A previous report suggested that the inclusion of 0.005% SDS in the incubation buffer might have enhanced drug hydrolysis by perforating the membranes of NFGRs (Oviano et al., 2017).

This study also features the unique inclusion of polymicrobial BCs in a full clinical evaluation. The MBT STAR-BL module correctly predicted β-lactam resistance in 14/19 BCs, including those containing ESBL-producing Enterobacteriaceae and carbapenemase-producing NFGRs. These results indicate that the module could help to select the most appropriate antibiotic therapy for patients with polymicrobial bacteremia.

Using the current MBT STAR-BL setting, logRQ values of 0.2–0.4 indicate ambiguous drug hydrolysis and should be reported as indeterminate. However, our findings demonstrate that three AmpC-hyperproducing *E. coli* isolates, six Enterobacteriaceae harboring bla_{CTX-M} and three carbapenemase-producing NFGRs in BCs had logRQ values within this range. A reduction of the cut-off value to 0.2 could increase the detection sensitivities and identify these clinically important organisms, although the specificities would be slightly compromised. This cut-off value reduction led to increases in the sensitivities of the AMP (*E. coli* only), CTX and MEM hydrolysis assays from 92.1, 68.8, and 40% to 95.2, 87.5, and 100%, respectively, for BCs (**Table 5**). Regarding patient safety, the test aims to rapidly provide information that will lead to effective antibiotic therapy. Accordingly, the sensitivity of the test is more important than the specificity. We therefore recommended eliminating the indeterminate range and setting the logRQ cut-off value at 0.2.

We additionally modified the manufacturer's protocol by using 5% saponin instead of the MALDI Sepsityper kit for BC extraction in MBT STAR-BL test. This is the first study to apply this protocol to the direct detection of β-lactamase-mediated resistance in BCs. Our method yielded similar sensitivities and specificities for AMP, CTX and MEM hydrolysis assay as those reported in previous studies using the Sepsityper kit, if the same logRQ cut-off value was applied (Jung et al., 2014; Oviano et al., 2017). Additionally, the use of 5% saponin (<US$1) is a less expensive option for extraction, compared with the Sepsityper kit (US$17).

In this study, the MBT STAR-BL module was not installed in a clinical laboratory, but rather in an adjacent research laboratory. The time required for sample collection and transportation led to longer turnaround times for MBT STAR-BL measurements in this study (5.2 h) than the value claimed by the manufacturer (2.5 h). Nevertheless, the assay still greatly reduced the turnaround times required to identify β-lactamase-producing organisms in patient-derived BCs. Particularly, drug hydrolysis assays could confirm the presence of ESBL-producing Enterobacteriaceae and carbapenemase-producing NFGRs in BCs at an average of 14 and 48 h before the interim and final reports of routine microbiological processing, respectively, were made available. It should be noted that the time saved by MBT STAR-BL might not be as pronounced as we described for laboratories where the final antibiogram is determined by automated microbroth system or disk diffusion test prepared after short-term incubation (e.g., 6 h) on solid media.

Although MBT STAR-BL cannot provide a full antibiogram, elevated logRQ values suggest the likelihood of ESBL and carbapenemase production. On the other hand, owing to high accuracy in prediction of AMP resistance in *E. coli*, de-escalation of antibiotic therapy might be considered when AMP hydrolysis is negative as determined by MBT STAR-BL. The short assay turnaround time allows the fine-tuning of antibiotic therapy on the same day that a positive blood culture is identified.

There were two weaknesses of this study. First, we could not determine the performance of MBT STAR-BL for the direct detection of carbapenemase-producing Enterobacteriaceae (CPE) in patient-derived BCs, as no CPE were isolated from positive BCs during the study period. Considering that all the archived KPC-, NDM-, and IMP-producing Enterobacteriaceae isolates were correctly detected by MBT STAR-BL in the first part of our study, the analysis module should be capable of identifying CPE regardless of the enzyme type. Second, as it is the first evaluation study of MBT STAR-BL in our locality, the test results were not used to modify the treatment regimen in our hospitals. Therefore we could not determine the impact of the MBT STAR-BL on the patient outcome, such as change in hospital stay, sepsis related mortality, and the cost of care. A large-scale randomized controlled trial study is recommended to further investigate how the implementation of MBT STAR-BL into routine workflow can benefit the patient management.

In conclusion, the MALDI Biotyper system, when equipped with the MBT STAR-BL module, enables the rapid and simultaneous identification of bacterial species and β-lactamase-mediated resistance from BCs and cultured isolates. A reduction of the logRQ cut-off value to 0.2 significantly increased the detection sensitivities for clinically important pathogens. Finally, the low reagent costs and short turnaround time suggest that this test could be used as a tool for early therapeutic guidance in patients with infection.

ETHICS STATEMENT

The biological safety approval was obtained from Health, Safety and Environment Office of The Hong Kong Polytechnic University (Ref. number: RSA15096).

AUTHOR CONTRIBUTIONS

AL, JL, and GS conceived and designed the experiments, performed the experiments, analyzed the data and wrote the paper. RiL, WN, EL, VL, PS, and RR performed the experiments and analyzed the data. KF, WT, RoL, and DT read and approved the final version of the manuscript.

FUNDING

This work was supported by The Hong Kong Polytechnic University Large Equipment Fund (Ref# HTI-4) and HTI Seed Fund (Ref#99TX), which funded the Microflex LT biotyper instrument and reagents respectively.

ACKNOWLEDGMENTS

We would like to express our gratitude to the Bruker Daltonik specialists, Mr. Lee Chi Chung and Dr. Yeung Hoi Sze Betty, for their technical support and advice throughout the project.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.00334/full#supplementary-material

REFERENCES

Burckhardt, I., and Zimmermann, S. (2011). Using matrix-assisted laser desorption ionization-time of flight mass spectrometry to detect carbapenem resistance within 1 to 2.5 hours. *J. Clin. Microbiol.* 49, 3321–3324. doi: 10.1128/JCM.00287-11

Chen, J. H., Ho, P. L., Kwan, G. S., She, K. K., Siu, G. K., Cheng, V. C., et al. (2013). Direct bacterial identification in positive blood cultures by use of two commercial matrix-assisted laser desorption ionization-time of flight mass spectrometry systems. *J. Clin. Microbiol.* 51, 1733–1739. doi: 10.1128/JCM.03259-12

Clerc, O., Prod'hom, G., Vogne, C., Bizzini, A., Calandra, T., and Greub, G. (2013). Impact of matrix-assisted laser desorption ionization time-of-flight mass spectrometry on the clinical management of patients with Gram-negative bacteremia: a prospective observational study. *Clin. Infect. Dis.* 56, 1101–1107. doi: 10.1093/cid/cis1204

Clinical and Laboratory Standards Institute (2013). "Performance standards for antimicrobial susceptibility testing," in *23rd Informational Supplemental, CLSI M100-S23* (Wayne, PA: Clinical and Laboratory Standards Institute).

Dallenne, C., Da Costa, A., Decre, D., Favier, C., and Arlet, G. (2010). Development of a set of multiplex PCR assays for the detection of genes encoding important beta-lactamases in Enterobacteriaceae. *J. Antimicrob. Chemother.* 65, 490–495. doi: 10.1093/jac/dkp498

Dellinger, R. P., Levy, M. M., Carlet, J. M., Bion, J., Parker, M. M., Jaeschke, R., et al. (2008). Surviving Sepsis Campaign: international guidelines for management of severe sepsis and septic shock: 2008. *Crit. Care Med.* 36, 296–327. doi: 10.1097/01.CCM.0000298158.12101.41

Doyle, D., Peirano, G., Lascols, C., Lloyd, T., Church, D. L., and Pitout, J. D. (2012). Laboratory detection of Enterobacteriaceae that produce carbapenemases. *J. Clin. Microbiol.* 50, 3877–3880. doi: 10.1128/JCM.02117-12

Fleischmann, C., Scherag, A., Adhikari, N. K., Hartog, C. S., Tsaganos, T., Schlattmann, P., et al. (2016). Assessment of global incidence and mortality of hospital-treated sepsis. Current estimates and limitations. *Am. J. Respir. Crit. Care Med.* 193, 259–272. doi: 10.1164/rccm.201504-0781OC

Ghebremedhin, B., Halstenbach, A., Smiljanic, M., Kaase, M., and Ahmad-Nejad, P. (2016). MALDI-TOF MS based carbapenemase detection from culture isolates and from positive blood culture vials. *Ann. Clin. Microbiol. Antimicrob.* 15:5. doi: 10.1186/s12941-016-0120-x

Hrabak, J., Studentova, V., Walkova, R., Zemlickova, H., Jakubu, V., Chudackova, E., et al. (2012). Detection of NDM-1, VIM-1, KPC, OXA-48, and OXA-162 carbapenemases by matrix-assisted laser desorption ionization-time of flight mass spectrometry. *J. Clin. Microbiol.* 50, 2441–2443. doi: 10.1128/JCM.01002-12

Hrabak, J., Walkova, R., Studentova, V., Chudackova, E., and Bergerova, T. (2011). Carbapenemase activity detection by matrix-assisted laser desorption ionization-time of flight mass spectrometry. *J. Clin. Microbiol.* 49, 3222–3227. doi: 10.1128/JCM.00984-11

Jung, J. S., Popp, C., Sparbier, K., Lange, C., Kostrzewa, M., and Schubert, S. (2014). Evaluation of matrix-assisted laser desorption ionization-time of flight mass spectrometry for rapid detection of beta-lactam resistance in Enterobacteriaceae derived from blood cultures. *J. Clin. Microbiol.* 52, 924–930. doi: 10.1128/JCM.02691-13

Kaneko, K., Okamoto, R., Nakano, R., Kawakami, S., and Inoue, M. (2005). Gene mutations responsible for overexpression of AmpC beta-lactamase in some clinical isolates of Enterobacter cloacae. *J. Clin. Microbiol.* 43, 2955–2958. doi: 10.1128/JCM.43.6.2955-2958.2005

Kang, C. I., Kim, S. H., Park, W. B., Lee, K. D., Kim, H. B., Kim, E. C., et al. (2005). Bloodstream infections caused by antibiotic-resistant gram-negative bacilli: risk factors for mortality and impact of inappropriate initial antimicrobial therapy on outcome. *Antimicrob. Agents Chemother.* 49, 760–766. doi: 10.1128/AAC.49.2.760-766.2005

Kazi, M., Shetty, A., and Rodrigues, C. (2015). The carbapenemase menace: do dual mechanisms code for more resistance? *Infect. Control Hosp. Epidemiol.* 36, 116–117. doi: 10.1017/ice.2014.33

Kumar, A. (2011). Optimizing antimicrobial therapy in sepsis and septic shock. *Crit. Care Nurs. Clin. North Am.* 23, 79–97. doi: 10.1016/j.ccell.2010.12.005

Kumar, A., Roberts, D., Wood, K. E., Light, B., Parrillo, J. E., Sharma, S., et al. (2006). Duration of hypotension before initiation of effective antimicrobial therapy is the critical determinant of survival in human septic shock. *Crit. Care Med.* 34, 1589–1596. doi: 10.1097/01.CCM.0000217961.75225.E9

Kumar, A., and Schweizer, H. P. (2005). Bacterial resistance to antibiotics: active efflux and reduced uptake. *Adv. Drug Deliv. Rev.* 57, 1486–1513. doi: 10.1016/j.addr.2005.04.004

Livermore, D. M. (2012). Current epidemiology and growing resistance of gram-negative pathogens. *Korean J. Intern. Med.* 27, 128–142. doi: 10.3904/kjim.2012.27.2.128

Mackenzie, F. M., Miller, C. A., and Gould, I. M. (2002). Comparison of screening methods for TEM- and SHV-derived extended-spectrum beta-lactamase detection. *Clin. Microbiol. Infect.* 8, 715–724. doi: 10.1046/j.1469-0691.2002.00473.x

Monteferrante, C. G., Sultan, S., Ten Kate, M. T., Dekker, L. J., Sparbier, K., Peer, M., et al. (2016). Evaluation of different pretreatment protocols to detect accurately clinical carbapenemase-producing Enterobacteriaceae by MALDI-TOF. *J. Antimicrob. Chemother.* 71, 2856–2867. doi: 10.1093/jac/dkw208

Oviano, M., Ramirez, C. L., Barbeyto, L. P., and Bou, G. (2017). Rapid direct detection of carbapenemase-producing Enterobacteriaceae in clinical urine samples by MALDI-TOF MS analysis. *J. Antimicrob. Chemother.* 72, 1350–1354. doi: 10.1093/jac/dkw579

Papagiannitsis, C. C., Studentova, V., Izdebski, R., Oikonomou, O., Pfeifer, Y., Petinaki, E., et al. (2015). Matrix-assisted laser desorption ionization-time of flight mass spectrometry meropenem hydrolysis assay with NH4HCO3, a reliable tool for direct detection of carbapenemase activity. *J. Clin. Microbiol.* 53, 1731–1735. doi: 10.1128/JCM.03094-14

Paterson, D. L. (2006). Resistance in gram-negative bacteria: enterobacteriaceae. *Am. J. Infect. Control* 34, S20–S28. discussion: S62–S70. doi: 10.1016/j.ajic.2006.05.238

Peter-Getzlaff, S., Polsfuss, S., Poledica, M., Hombach, M., Giger, J., Bottger, E. C., et al. (2011). Detection of AmpC beta-lactamase in *Escherichia coli*: comparison of three phenotypic confirmation assays and genetic analysis. *J. Clin. Microbiol.* 49, 2924–2932. doi: 10.1128/JCM.00091-11

Pournaras, S., Zarkotou, O., Poulou, A., Kristo, I., Vrioni, G., Themeli-Digalaki, K., et al. (2013). A combined disk test for direct differentiation of carbapenemase-producing enterobacteriaceae in surveillance rectal swabs. *J. Clin. Microbiol.* 51, 2986–2990. doi: 10.1128/JCM.00901-13

Schmidtke, A. J., and Hanson, N. D. (2006). Model system to evaluate the effect of ampD mutations on AmpC-mediated beta-lactam resistance. *Antimicrob. Agents Chemother.* 50, 2030–2037. doi: 10.1128/AAC.01458-05

Schubert, S., Weinert, K., Wagner, C., Gunzl, B., Wieser, A., Maier, T., et al. (2011). Novel, improved sample preparation for rapid, direct identification from positive blood cultures using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. *J. Mol. Diagn.* 13, 701–706. doi: 10.1016/j.jmoldx.2011.07.004

Sparbier, K., Schubert, S., Weller, U., Boogen, C., and Kostrzewa, M. (2012). Matrix-assisted laser desorption ionization-time of flight mass spectrometry-based functional assay for rapid detection of resistance against beta-lactam antibiotics. *J. Clin. Microbiol.* 50, 927–937. doi: 10.1128/JCM.05737-11

Thaller, M. C., Borgianni, L., Di Lallo, G., Chong, Y., Lee, K., Dajcs, J., et al. (2011). Metallo-beta-lactamase production by *Pseudomonas otitidis*: a species-related trait. *Antimicrob. Agents Chemother.* 55, 118–123. doi: 10.1128/AAC.01062-10

Van Looveren, M., Goossens, H., and Group, A. S. (2004). Antimicrobial resistance of *Acinetobacter* spp. in Europe. *Clin. Microbiol. Infect.* 10, 684–704. doi: 10.1111/j.1469-0691.2004.00942.x

Wuppenhorst, N., Consoir, C., Lorch, D., and Schneider, C. (2012). Direct identification of bacteria from charcoal-containing blood culture bottles using matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry. *Eur. J. Clin. Microbiol. Infect. Dis.* 31, 2843–2850. doi: 10.1007/s10096-012-1638-2

Yang, Z., Liu, W., Cui, Q., Niu, W., Li, H., Zhao, X., et al. (2014). Prevalence and detection of Stenotrophomonas maltophilia carrying metallo-beta-lactamase blaL1 in Beijing, China. *Front. Microbiol.* 5:692. doi: 10.3389/fmicb.2014.00692

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

REVIEW
published: 06 August 2018
doi: 10.3389/fmicb.2018.01744

Susceptibility Testing of Bacteria Using Maldi-Tof Mass Spectrometry

Irene Burckhardt and Stefan Zimmermann*

Department of Infectious Diseases, Medical Microbiology and Hygiene, University of Heidelberg, Heidelberg, Germany

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) was introduced into the microbiological routine more than 10 years ago. Since then it has almost replaced biochemical identification. It is unrivaled in terms of accuracy and cost. From a laboratory's perspective it would be an ideal method to replace classic susceptibility testing, that is Kirby-Baur agardiffusion or determination of minimal inhibitory concentrations (MICs). First reports on possible assays for susceptibility testing are more than 10 years old. However, the developments during the last 5 years were substantial. This review focuses with some exceptions on the progress, which was achieved during the last decade.

Keywords: MALDI-TOF MS, susceptibility testing, bacteria, time to report, treatment

OPEN ACCESS

Edited by:
Karsten Becker,
Universität Münster, Germany

Reviewed by:
Jette Sophia Jung,
Max von Pettenkofer-Institut, Ludwig-Maximilians-Universität München, Germany
Jozsef Soki,
University of Szeged, Hungary

****Correspondence:***
Irene Burckhardt
irene.burckhardt@med.uni-heidelberg.de

Specialty section:
This article was submitted to Antimicrobials, Resistance and Chemotherapy, a section of the journal Frontiers in Microbiology

Received: *24 May 2018*
Accepted: *12 July 2018*
Published: *06 August 2018*

Citation:
Burckhardt I and Zimmermann S (2018) Susceptibility Testing of Bacteria Using Maldi-Tof Mass Spectrometry. Front. Microbiol. 9:1744. doi: 10.3389/fmicb.2018.01744

BACKGROUND AND AIM OF THE REVIEW: WHY DO WE NEED SUSCEPTIBILITY TESTING FOR BACTERIA AND WHY IS TIME-TO-RESULT SUCH AN ISSUE?

According to the World Health Report 2015 infectious diseases cause more than 30% of all deaths worldwide (WHO, 2015) and rapid and correct antibiotic therapy is the most important single factor for the survival of patients with bacterial infections (Kumar et al., 2009). However, initial therapy is based on the experience of the physician, his knowledge about the most common bacteria causing the disease and its statistically most common susceptibility profile. That means initial therapy is rapid but guided by experience from past cases not evidence from the actual case. The choice of empirical therapy can be terribly wrong. Ideally identification and susceptibility testing could be done shortly after the respective patient samples were taken. In reality bacteria have to be cultured on agar plates in laboratories specialized in medical microbiology before they can be identified and tested against antibiotics. In general this takes 1 day for growth and an additional day for identification and susceptibility testing. During the last 10 years we could witness the triumph of MALDI-TOF MS over biochemistry for bacterial identification from bacterial cultures (solid or liquid) (Clark et al., 2013; Patel, 2015; Singhal et al., 2015; Angeletti, 2016; Arca-Suárez et al., 2017; Tré-Hardy et al., 2017) or directly from patient material (Sandalakis et al., 2017). One of its main advantages is that identification using MALDI-TOF MS takes minutes. Identification using biochemistry necessitates growth of bacteria and often takes at least 1 day. However, the gold standard for susceptibility testing still is the determination of the minimal inhibitory concentrations (MICs) toward a selection of antibiotics. Again, growth of bacteria is essential and often takes at least 1 day. Accelerating susceptibility testing is the next big goal in medical microbiology and MALDI-TOF MS is the promising technology for achieving it. This review will focus on important technical developments and the progress toward rapid susceptibility testing using MALDI-TOF MS. Literature published until March 2018 was included into the review (see **Table 1** for a list of references in alphabetical order and organism/resistance studied, spectrometer used, matrix used and range (m/z) studied).

TABLE 1 | List of references in alphabetical order and organism/resistance studied, spectrometer used, matrix used and range (m/z) studied.

References	Organism/resistance studied	Spectrometer	Matrix	Range studied (m/z)
Bernardo et al., 2002	*S. aureus*/methicillin resistance	Reflex III MS[§]	sinapinic acid	1,000–10,000
Burckhardt and Zimmermann, 2011	gram-negative bacteria/carbapenem resistance	microflex LT[§]	HCCA*	440–530
Calderaro et al., 2017	Enterobacteriaceae/carbapenem resistance	microflex LT	DHB	100–1,200
Du et al., 2002	*S. aureus*/methicillin resistance	linear MALDI-TOF MS[$]	5-chloro-2 mercapto-benzothialzole	600–3,500
Edwards-Jones et al., 2000	*S. aureus*/methicillin resistance	Kompact MALDI 2 linear TOF MS[#]	5-chloro-2 mercapto-benzothialzole	500–10,000
Griffin et al., 2012	*E. faecium*/vancomycin resistance (*vanB*)	microflex LT	HCCA	2,000–20,000
Hrabák et al., 2011	gram-negative bacteria/carbapenem resistance	microflex LT	2,5-dihydroxybenzoid acid (DHB)	360–600
Idelevich et al., 2018	*K.pneumoniae, P. aeruginosa,* meropenem resistance	microflex LT	HCCA	2,000–20,000
Johansson et al., 2014a	*B. fragilis*/carbapenem resistance	microflex LT	HCCA	400–600
Johansson et al., 2014b	*B. fragilis*/carbapenem resistance	microflex LT	HCCA	400–600
Josten et al., 2014	*S. aureus*/methicillin resistance	Biflex II MS[§] Vitek MS[&]	HCCA	n.a.
Jung et al., 2014a	P. aeruginosa/carbapenem resistance, aminoglycoside resistance, quinolone resistance	microflex LT	HCCA	2,000–10,000
Jung et al., 2016	gram-negative bacteria/beta-lactam resistance, aminoglycoside resistance, quinolone resistance	microflex LT	HCCA	2,000–20,000
Justesen et al., 2018	*B. fragilis*, clindamycin, meropenem, metronidazole resistance	microflex LT	HCCA	2,000–20,000
Lange et al., 2014	*Klebsiella* spp./carbapenem resistance	microflex LT	HCCA	2,000–20,000
Lau et al., 2014	*K. pneumoniae*/carbapenem resistance	microflex LT	HCCA	2,000–20,000
Nagy et al., 2011	*B. fragilis*/carbapenem resistance	microflex LT	HCCA	2,000–20,000
Nakano et al., 2014	*E. faecium*/vancomycin resistance (*vanA*)	microflex LT	HCCA	2,000–20,000
Oviaño et al., 2016	Enterobacteriaceae, *P. aeruginosa*, *A. baumannii*, carbapenem resistance		HCCA	300–600
Papagiannitsis et al., 2015	gram-negative bacteria/carbapenem resistance	microflex LT	2,5-dihydroxybenzoid acid (DHB)	360–600
Pardo et al., 2016	gram-negative bacteria/quinolone resistance	Vitek MS	HCCA	270–420
Pranada et al., 2016	*S. aureus*/methicillin resistance	microflex LT	HCCA	2,000–20,000
Ramos et al., 2016	Enterobacteriaceae, *P. aeruginosa*, *A. baumannii*, carbapenem resistance	Vitek MS	HCCA	400–600
Rotova et al., 2017	Enterobacteriaceae, *P. aeruginosa,* carbapenem resistance	microflex LT	HCCA, DHB	300–600
Sparbier et al., 2012	gram-negative bacteria/beta-lactam resistance (penicillins, cephalosporins, carbapenems)	microflex LT	HCCA	2,000–20,000
Sparbier et al., 2013	*S. aureus*/methicillin resistance	microflex LT	HCCA	2,000–20,000
Szabados et al., 2012	*S. aureus*/methicillin resistance	microflex LT	HCCA	2,000–20,000

[§]*manufactured by Bruker Daltonic GmbH, Bremen, Germany.*
**HCCA:α-cyano-4-hydroxycinnamic acid.*
[$]*manufactured by Micromass, Waters Corporation, USA.*
[&]*manufactured by bioMérieux, Nürtingen, Germany.*
[#]*manufactured by Kratos Analytical, Shimadzu Corporation, Japan.*
n.a.: not available.

ASSAYS USING DEFINED MARKER PEAKS TO DEDUCE SUSCEPTIBILITY OR RESISTANCE (SINGLE PEAKS, CLUSTER OF PEAKS, WHOLE SPECTRA)

Methicillin-Resistance-MRSA

At the turn of the millennium the most famous and most feared bacterium was methicillin-resistant *Staphylococcus aureus* (MRSA). Therefore, it is not surprising that the first attempts to use MALDI-TOF MS for susceptibility testing were made with *S. aureus*. The aim was to determine whether a specific *S. aureus* isolate was susceptible or resistant toward methicillin, i.e., MSSA or MRSA. As early as 2000 Edwards-Jones and colleagues (Edwards-Jones et al., 2000) studied 20 different staphylococcal strains: 7 MSSA (all reference strains from official strain collections), 7 MRSA (clinical isolates only) and 6 non-*S. aureus* staphylococcal strains (1 clinical isolate and five reference strains). They analyzed peaks between 500 and 10,000 m/z. With this approach they identified 2 MSSA specific peaks (2,548 and 2,647 m/z) and 5 MRSA specific peaks (581, 1,140, 1,165, 1,229, and 2,127 m/z). However, the authors did not verify their findings with a prospective evaluation of clinical samples.

Two years later Du et al. (2002) analyzed 76 clinical *S. aureus* strains. They studied a mass range of 600–3,500 m/z. With this method only 74% of the above mentioned 76 *S. aureus* strains could be identified as *S. aureus*. However, clustering of the spectra revealed two main clusters with a good correlation to susceptibility or resistance, i.e., MSSA or MRSA. But concordance was not 100%. 7 strains, which were *mecA* negative (PCR) clustered into the MRSA group. Unfortunately the authors did not trace back the differences between MSSA and MRSA spectra to the individual peaks but published two "typical" spectra of MSSA and MRSA. A close look at these spectra shows that one of the different peaks is the peak at 2,413 m/z, which we now know is related to PSM-mec peptide (Josten et al., 2014).

Again in 2002 Bernardo and colleagues (Bernardo et al., 2002) studied two well-characterized reference strains, ATCC 29213 (MSSA) and ATCC 43300 (MRSA), and compared their spectra to clinical isolates. They studied a mass range of 1,000–10,000 m/z and in contrast to all other studies mentioned in this review they used the reflector mode not the linear mode of the MALDI-TOF MS. They could not find an MRSA-specific fingerprint.

Ten years later in 2012 the group of S. Gatermann (Szabados et al., 2012) studied a pair of isogenic *S. aureus* strains, which harbored or lacked a certain SCCmec (staphylococcal chromosome *mec*). They did not find evidence for a characteristic spectrum or peak associated with methicillin-resistance. Unfortunately the authors neither mentioned the SCC cassette type present in the resistant strain nor published a peak list for further reference.

In Josten et al. (2014) screened a very diverse collection of 220 MRSA strains for the presence of a special peak at 2415 m/z. Using the RNA antisense technology they first established that the peak at 2,415 m/z in a *S. aureus* spectrum was correlated with the expression of PSM-mec. PSM-mec is a small peptide, which is encoded on three different SCCmec cassette types (i.e., type II, III, and VIII). Because PSMmec is encoded on the SCCmec cassette its expression is associated with a methicillin resistant phenotype in *S. aureus*. Afterwards they prepared spectra of 220 strains and visually checked for the presence of a peak at 2,415 m/z. Their conclusion was that as soon as the peak was discernible in a spectrum the strain most probably was an MRSA. However, during analysis they had to realize that sample preparation was extremely important for the detection of the peak. As soon as the background of the measurement was high it was difficult to identify the peak. In Pranada et al. (2016) used prototype software for automated detection of a peak between 2,411 and 2,415 m/z in *S. aureus* spectra generated during routine identification. They reevaluated 1304 spectra of *S. aureus* isolates from their microbiology routine, which had been acquired from 2011 to 2014. For each of these isolates the information on methicillin resistance was available (MICs determined using VITEK2, bioMerieux). Of 211 MRSA strains 49 (23.2%) had a peak at 2,411–2,415 m/z. Importantly none of the 1093 MSSA strains had a peak at 2,411–2,415 m/z, which makes this peak extremely specific for MRSA (see **Figure 1**).

During ECCMID 2017 confirmatory data were presented with a positivity rate for PSM-mec of 15.7% (Timke et al., 2017).

Vancomycin-Resistance: VRE–vanA and vanB

However, MRSA was not the only focus of work for rapid susceptibility testing. From a clinical perspective vancomycin-resistant enterococci (*vanA* or *vanB* positive) are in the focus of infectious disease specialists, too. In 2012 Griffin and co-workers (Griffin et al., 2012) published a paper on the discrimination between *vanB* and *non-vanB* carrying enterococci in Australia using MALDI-TOF. The group had collected 67 consecutive vancomycin resistant *Enterococcus faecium* isolates (*vanB* postive, confirmed by PCR) from the beginning of January 2009 to the end of June 2010. Among the controls were 8 strains, which were phenotypically resistant to vancomycin but were *vanB* and *vanA* negative by PCR. The mechanism of non-susceptibility to vancomycin of the latter strains was not investigated. Five differentiating peaks were identified using the program ClinPro Tools version 2.0 (2,211, 4,717, 5,095, 5,946, and 8,328 m/z). For a prospective validation of these findings reference spectra of *vanB* positive and negative strains were added to the database. Phenotypically vancomycin resistant enterococci not carrying the *vanB* gene were designated as VRE negative in the database. Unfortunately the total number of added spectra was not mentioned in the original publication. From January 2012 to February 2012 strains from 129 patient samples were used for validation. Altogether 281 different colonies were tested. Compared to phenotypic methods 97.5% were correctly identified and 2.5% were incorrectly identified, 1.1% were false positive and 1.4% false negative.

Two years later the group of Nakano (Nakano et al., 2014) published a paper on the discrimination between *vanA*-positive and *vanA*-negative *Enterococcus faecium*. They studied 61 *vanA*-positive strains from a surveillance program (rectal swabs and stool, 2005–2013) and 71 *vanA*-negative strains from blood

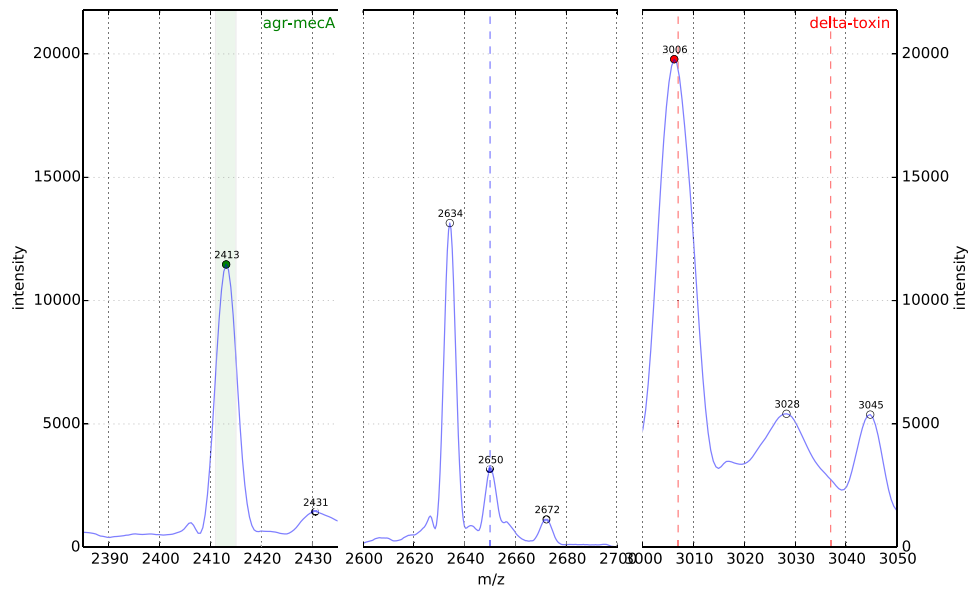


FIGURE 1 | MALDI-TOF MS of *S. aureus* (ATCC33591, MRSA); the peak analysis shows the PSM-mec related peak in the detection window m/z 2411-15 (origin: DGHM 2016, poster 139, A. Pranada and co-workers "Optimization and Evaluation of MRSA Detection by Peak Analysis of MALDI-TOF Mass Spectra").

cultures (2009–2013). All but two strains were isolated from patients from the Kyoto region in Japan during the mentioned time periods. Using classification models (genetic algorithm, supervised neural network, QuickClassifier) revealed five peaks differentiating between the two entities: 3,184, 5,702, 7,415, 7,445, and 12,662 m/z. Unfortunately the authors neither published typical spectra nor further elaborated which peak was characteristic for which genotype. Additionally they did not try to clear up the identity of the peak, i.e., which peptide or protein caused the peak. This is especially unfortunate because all strains stem from one region. The discriminating peaks might be an artifact due to clonality.

Carbapenem-Resistance: MRGN and *B. fragilis*

In Lau et al. (2014) studied 38 isolates of *Klebsiella pneumoniae*, which carried the pKpQIL plasmid. This plasmid contains the bla_{KPC} gene, which encodes a carbapenemase called KPC (*K. pneumoniae* carbapenemase). KPC causes high carbapenem resistance. They started by comparing spectra from bla_{KPC} positive and negative strains as determined by PCR. They worked with 19 bla_{KPC} positive isolates (ATCC BAA-1705 and 18 clinical isolates from an outbreak in 2011) and 19 bla_{KPC} negative isolates (ATCC BAA-1706 and 18 clinical isolates). Visual comparison of the spectra revealed a peak at 11.109 m/z, which was only present in the bla_{KPC} positive isolates. Using transformation and additional proteomic methods they could confirm the identity of the protein causing the peak. Most importantly for laboratory routine they could show that this peak can be detected directly from blood-cultures, which had been artificially inoculated with two different bla_{KPC} positive isolates. In 2016 the group of Paolo Gaibani in Bolognia tested this assay with 34 KPC-producing *K. pneumoniae* strains of which 30 (88.2%) were positive for the 11.109 m/z peak. Further genetic analysis revealed that the 4 strains negative for the 11.109 m/z peak could be explained by different isoforms of Tn*4401*. Only TN*4401a* is commonly associated with the 11.109 m/z peak (Gaibani et al., 2016). In 2018 the same group published a study on 140 well-characterized *K. pneumoniae* strains collected between 2011 and 2017 and found an overall accuracy of 98%, a positive predictive value of 98% and a negative predictive value of 97% (Gaibani et al., 2018).

In Nagy et al. (2011) from Hungary described the separation of *Bacteroides fragilis* strains into two divisions using MALDI-TOF MS spectra: division I (all strains were *cfiA*-negative) and division II (all strains were *cfiA*-positive). Clinically these divisions are of interest because the presence of the *cfiA*-gene is associated with carbapenem resistance in *B. fragilis*. The study included 38 different clinical *B. fragilis* strains with known *cfiA* gene status (determined by PCR) and two reference strains: NCTC 9343 (*cfiA* negative) and TAL 3636 (*cfiA* positive). The clinical strains originated from Europe and the US. Especially in the mass range between 4,000 and 5,500 m/z they could identify characteristic differences in the respective spectra using ClinPro Tools v. 2.2. Altogether they identified 20 peaks, which differentiated between divisions I and II (division I (*cfiA*-negative): 4,711, 4,817, 5,017, 5,204, 5,268, 7,292, 9,421, 9,631, 10,404, and 10,530 m/z; division II (*cfiA*-positive): 4,688, 4,826, 5,002, 5,189, 5,282, 7,321, 9,375, 9,649, 10,374, and 10,558 m/z). Unfortunately the identities of the peaks were not studied. Therefore, we do not know whether at least one of the peaks is resistance mechanism

correlated or whether the peak differences are due to clonality. A further analysis of spectra from 277 clinical samples previously acquired showed that some of these peaks could be found in spectra from clinical routine. They correlated 100% with PCR positivity. Finally the authors generated a specialized database consisting of 2 MSPs only (*cfiA* +, *cfiA*-) and challenged it with spectra from 9 *cfiA*-positive and 19 *cfiA*-negative strains. Matching was correct in 100%. These findings were confirmed in 2011 and 2014. A group from Belgium studied 248 isolates of *B. fragilis* collected between 2003 and 2011 with very good differentiation between *cfiA*-positive and negative strains (Wybo et al., 2011) and in 2014 Nagy and co-workers confirmed their original findings with 60 *B. fragilis* isolates from polymicrobial or severe infections (Fenyvesi et al., 2014). From a laboratory perspective the differentiation between divisions II and I is interesting, because until today only the division II strains harbor the *cfiA* gene. This gene does not correlate 100% with carbapenem resistance. However, it can serve as an indicator for the necessity to perform further susceptibility testing. Only *cfiA* positive isolates showed imipenem MICs >4 mg/L (EUCAST cut-off for imipenem resistance). On the other hand from the data presented it is justified to call division I strains susceptible to carbapenems without further susceptibility testing. But if this workflow is implemented one should be aware of the obvious risk that other carbapenem resistance mechanisms are overlooked.

All of the described methods have the same big advantage for the clinical routine. All of these peaks can be identified in the spectra, which are generated during the normal identification workflow. No additional assay is necessary; no additional incubation time is needed. The peaks can be detected visually or by software tools, which already exist but are not routinely available yet. However, all of the papers (except two) share the same incompleteness. They do not correlate the peaks with the peptides or proteins they represent. Therefore, it cannot be excluded that these peaks are discriminatory only in the strain collections used and are an artifact from clonality rather than truly discriminatory worldwide.

Assays Using Alterations of Antibiotics as Read-Out (Hydrolysis, Decarboxylation, Acetylation)

The most diverse class of antibiotics is the class of the beta-lactams. This class contains penicillins, cephalosporins, and carbapenems. Their spectrum of antibacterial activity is versatile; however, they share a common feature. They can be inactivated by hydrolysis. In the clinical setting this hydrolysis is caused by enzymes produced by bacteria. Thousands of these bacterial enzymes (i.e., beta-lactamases) have been described and their number is still rising. The mechanism of hydrolysis is always exactly the same. The enzymes can break the beta-lactam ring of the beta-lactams open and a single H_2O is linked to the molecule. Depending on the buffer the resulting hydrolysate is unstable and a spontaneous decarboxylation can take place. In terms of susceptibility testing this degradation process can be monitored with MALDI-TOF. The addition of water increases the original antibiotic mass by 18 Da and the decarboxylation decreases the molecular mass by 44 Da. Taken together this results in an absolute loss of 26 Da (44–18 Da) compared to the original mass of the antibiotic. To be able to visualize this degradation a bacterial suspension with the antibiotic in question has to be prepared and incubated for various amounts of time.

In 2011 two European groups published this phenomenon simultaneously for the detection of carbapenem degrading enzymes (i.e., carbapenemases). The group of Burckhardt and Zimmermann (2011) studied 47 clinical isolates carrying different carbapenemases (KPC, NDM, IMP, VIM) and ertapenem resistance (MIC ≥4 mg/L). They included 30 clinical strains carrying other resistance mechanisms (ESBL, K1), which did not cause ertapenem resistance. They studied a mass range of 440–530 m/z. A spectrum of ertapenem as it is used for patient therapy showed 4 peaks: 476 m/z (ertapenem without sodium), 498 m/z (monosodium salt), 521 m/z (disodium salt) and 450 m/z (hydrolyzed and decarboxylated ertapenem) (see **Figure 2**). Depending on the enzyme the mixtures of bacteria and ertapenem had to be cultivated between 1 and 2.5 h. The read-out they used was total disappearance of the ertapenem peaks at 476, 498, and 521 m/z. Discrimination between carbapenemase carrying and non-carrying strains was 100%. In this proof-of-principle study the authors used a reaction volume of 1 ml, a 10 µl loop full of bacteria, a concentration of 0.5 g/L of ertapenem in 0.9% NaCl and α-cyano-4-hydroxycinnamic acid as matrix.

At the same time the group of Hrabák et al. (2011) studied the degradation of meropenem. They used 124 different strains of which 30 carried carbapenemases (KPC, NDM, IMP, and VIM). All the other strains were controls carrying other resistance mechanisms resulting in elevated carbapenem MICs (55 strains) or were completely susceptible to meropenem and imipenem (39 strains). They looked at masses from 360 to 600 m/z. The spectrum of meropenem showed three characteristic peaks: 383 m/z (meropenem without sodium), 405 and 427 m/z (sodium salts). After hydrolysis the following peaks were discernible: 401, 423, 445, and 468 m/z (sodium salts). They used 1 ml of a McFarland (McF) 8 bacterial solution, a 0.1 mM meropenem solution in 20 mM Tris-HCl (pH 6.8) and incubated in a final reaction volume of 50 µl for 3 h. Of the three different matrices used (α-cyano-4-hydroxycinnamic acid, 2,5-dihydroxybenzoid acid, DHB and 2,5-dihydroxyacetophenone, DHAP) DHB worked best under the described circumstances. Read out was the disappearance of at least one of the following peaks: 383 or 405 m/z. Their analysis of the strains using the above mentioned workflow produced one false negative and two false-positive results. In 2015 the same group published a modification of their initial method (Papagiannitsis et al., 2015). Addition of 50 mM NH_4HCO_3 (pH 7.0) to the original reaction mix improved detection of OXA-48 producing strains from 3 of 19 strains to 19 of 19 strains. In 2017 the same group compared meropenem and imipenem for the detection of carbapenemases. The imipenem assay achieved a higher sensitivity (97%) and specificity (100%) for the testing of *P. aeruginosa* (250 strains tested), whereas the meropenem assay achieved a higher sensitivity (99%) and specificity (100%) for Enterobacteriaceae (124 strains tested) (Rotova et al., 2017). In

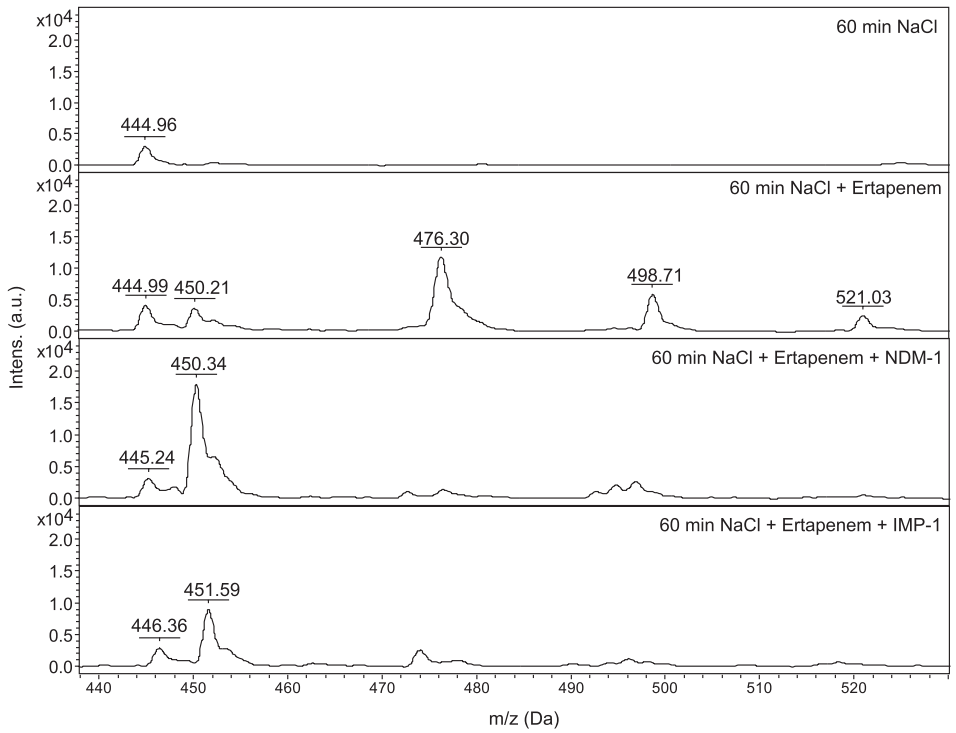


FIGURE 2 | Ertapenem degradation: NDM-1 carrying *K. pneumoniae*, IMP-1 carrying *P. aeruginosa*, 60 min of incubation at 36°C, NaCl: 0.45%, ertapenem concentration: 0.5 g/l, x-axis: mass per charge in Dalton (m/z, Da), y-axis: intensity: arbitrary units. Data are representative of more than three independent experiments. JCM, 2011, 49, 3321–3324, doi: 10.1128/JCM.00287-11, original Figure 1, reproduced with permission from American Society for Microbiology.

2017 another group published results for a slightly modified meropenem assay testing 1185 enterobacterial strains from Italy, which carried mainly KPC or VIM enzymes (Calderaro et al., 2017). It showed that the integrity of meropenem is an important factor in the analysis of the read-out and the mere presence and absence of meropenem specific peaks is not suitable as the only read-out.

In Sparbier et al. (2012) published another version of the beta-lactamase hydrolysis assay. It was extended to ampicillin, piperacillin, ceftazidime, cefotaxime and imipenem and results for ertapenem and meropenem were confirmed. In this study the authors used a reaction volume of 10 µl, a 1 µl loop full of bacteria and an incubation time of up to 3 h. Concentrations were different for all substances: ampicillin (10 mg/ml), piperacillin (1 mg/ml), ceftazidime (0.25 mg/ml), cefotaxime (0.5 mg/ml), imipenem (0.5 mg/ml), ertapenem (0.5 mg/ml), meropenem (0.5 mg/ml). They studied a mass range between 290 and 600 m/z. Only 10 different strains were used in this study including *E. coli* DH5α and 9 different clinical isolates carrying different beta-lactamases (AmpC, ESBL and KPC). But they were the first to use beta-lactamase inhibitors for inhibition of beta-lactamases in this kind of assay. Interpretation of data was done visually and the assay was considered positive for the presence of a beta-lactamase if the intensities of the peaks of the hydrolyzed forms represented 80% or more of the intensities of the non-hydrolyzed plus the hydrolyzed forms of the respective antibiotic. In 2014 Jung and co-authors confirmed that the assay works for 3rd-generation cephalosporins/Enterobacteriaceae and aminopenicillins/*E. coli* directly from blood culture (Jung et al., 2014b). In 2016 it was shown that the hydrolysis assay (imipenem only) works directly from positive blood cultures for Enterobacteriaceae, *P. aeruginosa* and *A. baumannii* (Oviaño et al., 2016) In 2018 another group successfully used the hydrolysis assay on blood cultures with ampicillin, piperacillin, cefotaxime, ceftazidime and meropenem (Lee et al., 2018). Interestingly the hydrolysis assay seems to be influenced by the agar type used for cultivation of strains. Strains grown on MacConkey agar tended to give false negative results in a study in 2016 (Ramos et al., 2016). This effect was mainly observed for *A. baumannii*.

In 2014 the ESCMID Study Group on Anaerobic Infections confirmed that the ertapenem hydrolysis assay worked with *cfiA* positive *B. fragilis* strains, too (Johansson et al., 2014a). Johansson and co-workers studied a group of 28 different *B. fragilis* strains of which 16 carried the *cfiA* gene and had different levels of elevated ertapenem MICs (≥2 mg/L). These elevated ertapenem MICs correlated in 10/16 cases with elevated imipenem MICs (≥2 mg/L). They used the pellet of 1.5 ml McF 4 bacterial solutions and 20 µl of a 10 mM ammonium hydrogen citrate buffer for incubation with 0.5 mg/mL of ertapenem. All of the 16 *cfiA* positive strains hydrolyzed ertapenem after 2.5 h of incubation. This hydrolysis could be blocked by 2,6-Pyridinecarboxylic acid (DPA), a metallo-beta-lactamase inhibitor. All 12 *cfiA* negative strains did not show any hydrolysis of ertapenem. The same year the same group could show that the assay also worked with pellets

from positive blood culture bottles despite the presence of blood components (Johansson et al., 2014b).

Beta-lactams are not the only antibiotics, which can be inactivated by enzymes. The plasmid-encoded acetyltransferase AAC(6')-Ib-cr inactivates quinolones via acetylation. In 2016 Pardo and co-workers (Pardo et al., 2016) described a MALDI-TOF MS based method to detect acetylation of norfloxacin. Acetylation increases the mass of the acetylated substance by 42 Da. They studied a collection of 113 ESBL-producing Enterobacteriaceae of French origin. 102 of these 113 strains were phenotypically resistant to norfloxacin, however, only 64 strains carried the *aac(6′)-IB-cr* gene. They used a 1 μl loop full of bacteria and incubated them in 10 μl of a 0.03 or 0.5 mg/mL norfloxacin solution for 4 h at 35°C. They studied the mass region between 270 and 420 m/z. Norfloxacin produced peaks at 320 m/z (norfloxacin without sodium) and 342 m/z (mono sodium salt). Acetylation should increase the weight of the respective substance by 42 Da. And after incubation of norfloxacin with an AAC(6′)-Ib-cr producing strain peaks at 362 m/z (acetylated norfloxacin) and 384 m/z (acetylated mono sodium norfloxacin) could be seen in the respective spectra. As read-out they calculated the areas under the curve (AUCs) for the respective peaks. Optimal cut-offs for positivity were determined using receiver operating characteristics (ROC) curve analysis. The lower concentration of norfloxacin (0.03 mg/mL) seemed to be better suited for the analysis. They authors claimed that their assay had a sensitivity of 98% and a specificity of 100% for the detection of AAC(6')-Ib-cr.

Assays Using Peak Shift as Read-Out (Incorporation of ^{13}C)

In February 2013 Demirev and co-workers described a very universal method to determine susceptibility in bacteria (Demirev et al., 2013). They used two different culture media to grow bacteria. The two media differed by their carbon component. One medium contained ^{12}C, in the other medium 98% of all carbon atoms were ^{13}C, i.e., heavier. The idea was to monitor whether the bacterium was still capable of growing in the presence of the antibiotic or not. This could be deduced from the spectrum generated from bacteria grown in a solution containing ^{13}C and antibiotic. If the bacterium was resistant it could grow in the presence of the antibiotic and would incorporate ^{13}C and the spectrum would shift to higher m/z. In theory this principle could be applied to all antibiotics and should work irrespective of resistance mechanisms.

In August 2013 Sparbier and colleagues published data created with *S. aureus* and media either containing ^{12}C or ^{13}C-labeled lysine (Sparbier et al., 2013). The authors called this assay MBT-RESIST (MALDI Biotyper resistance test with stable isotope-labeled amino acids). They started with 10 MSSA and 10 MRSA strains and further evaluated their findings with 28 *S. aureus* strains from patient samples. As antibiotics they used oxacillin (60 mg/L) or cefoxitin (40 mg/L). Bacteria were incubated at 37°C for 3 h in a volume of 100 μl and a final concentration of 3.5×10^6 cells/mL. Each test consisted of three tubes. Tube 1 contained ^{12}C medium and no antibiotic. Tube 2 contained ^{13}C medium and no antibiotic. Tube 3 contained ^{13}C medium *and* antibiotic. Tube 1 and 2 served as controls. The spectrum created from tube 3 decided whether a strain was rated susceptible or resistant against the respective antibiotic (see **Figure 3**).

The spectrum of a resistant strain in tube 3 would resemble more the spectrum from tube 2 than from tube 1. The spectrum of a susceptible strain in tube 3 would resemble more the spectrum from tube 1 than from tube 2. However, depending on the strain studied the acquired spectra were ambiguous. Therefore, the authors decided to use automated spectra analysis for the interpretation of the test. With this workflow one susceptible strain was falsely considered to be resistant using the oxacillin set-up. Three strains were wrongly classified using the cefoxitin set-up. Two main factors were responsible for false classification; a) the quality of the spectra; a noisy background led to false classifications; b) growth kinetics of strains; strains growing slowly were difficult to correctly classify after only 3 h of incubation.

In November 2013 the same group published that this approach works not only for *S. aureus* and oxacillin/cefoxitin but also for *Pseudomonas aeruginosa* and meropenem, tobramycin and ciprofloxacin (Jung et al., 2014a). In contrast to their initial publication they used a reaction volume of 300 μl and looked at a mass range of 2,000–1,0000 m/z. The authors used 10 strains of *P. aeruginosa* to establish their workflow and validation sets of 30 strains for each antibiotic (15 susceptible and 15 resistant strains each). To ease the interpretation of the spectra meropenem had to be added 30 min before the ^{13}C lysine to the reaction tube. According to the authors even strains with MIC values close to the breakpoint were classified correctly. Unfortunately neither sensitivity/specificity nor positive/negative predictive values were explicitly mentioned in the publication.

Assays Using Quantification of the Area Under the Curve as Read-Out

In Lange et al. (2014) published a completely new MALDI-TOF MS method for susceptibility testing of bacteria. The authors used the abbreviation MBT-ASTRA (MALDI Biotyper antibiotic susceptibility test rapid assay) for this test. They determined the susceptibility of 108 *Klebsiella* spp. isolates (see **Figure 4**) against meropenem using relative growth. Each test consisted of two tubes. Tube 1 contained meropenem and tube 2 did not contain meropenem. They used meropenem at a concentration of 8 mg/L, a reaction volume of 200 μl (BHI, 0.5 McFarland solution) and an incubation time of 1 h. The resulting spectra were normalized to the maximum peak and the resulting spectrum subdivided into 100 equally spaced thresholds (relative intensity range). The number of peaks above each threshold was counted and plotted against the threshold. The area under this curve, not under the initial spectrum (AUC) was determined for each measurement. Finally the relative growth was calculated as follows: AUC (+meropenem)/AUC (–meropenem). A relative growth of >0.4 was indicative of resistance to meropenem. This protocol was tested against 94 *K. pneumoniae* and 14 *K. oxytoca* strains and gave five false positive and one false negative result. All other 102 results were correct. The strain, which gave the false negative

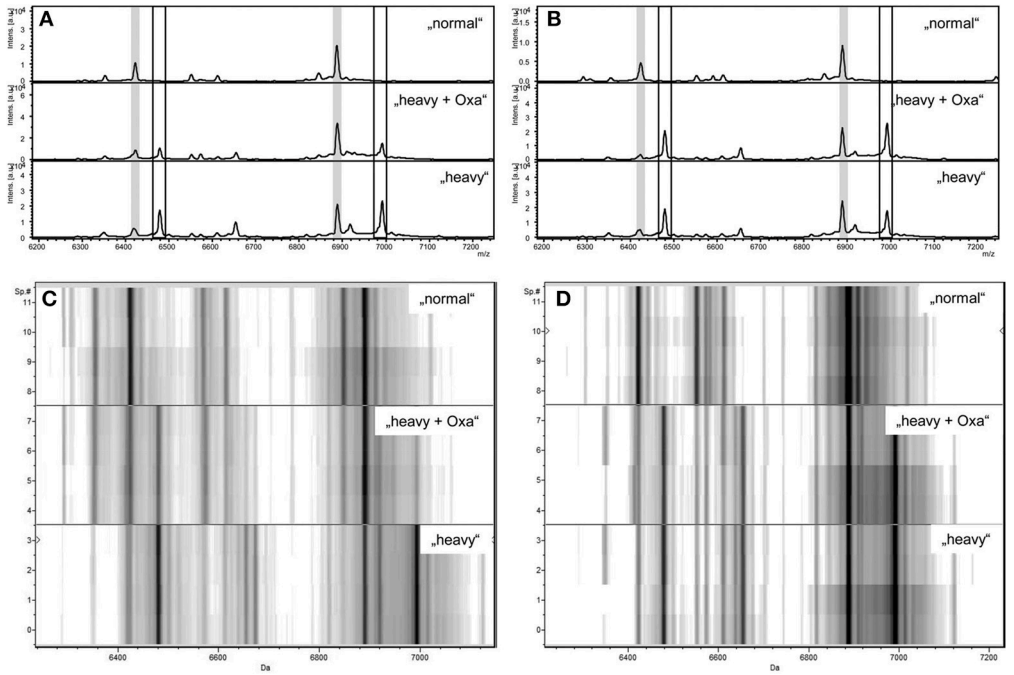


FIGURE 3 | Zoom of MALDI-TOF MS spectra displayed in flexAnalysis **(A,B)** and ClinProTools **(C,D)** in the mass range between 6.200 and 7.200 Da of a susceptible *S. aureus* strain **(A,C)** and a resistant *S. aureus* strain **(B,D)** after incubation with normal lysine ("normal"), with "heavy" lysine ("heavy"), or with "heavy" lysine and oxacillin ("heavy + Oxa"). Peaks correspondig to "normal" proteins are highlighted in light gray **(A,B)**. Peaks corresponding to "heavy" proteins are indicated by the boxes **(A,B)**. y axes give the numbers of multiple measurements **(C,D)**. Intes. [a.u.], intensity [arbitrary units]; Sp.#, spectrum number. JCM, 2013, 51, 3741–3748, doi: 10.1128/JCM.01536-13, original Figure 1, reproduced with permission from American Society for Microbiology.

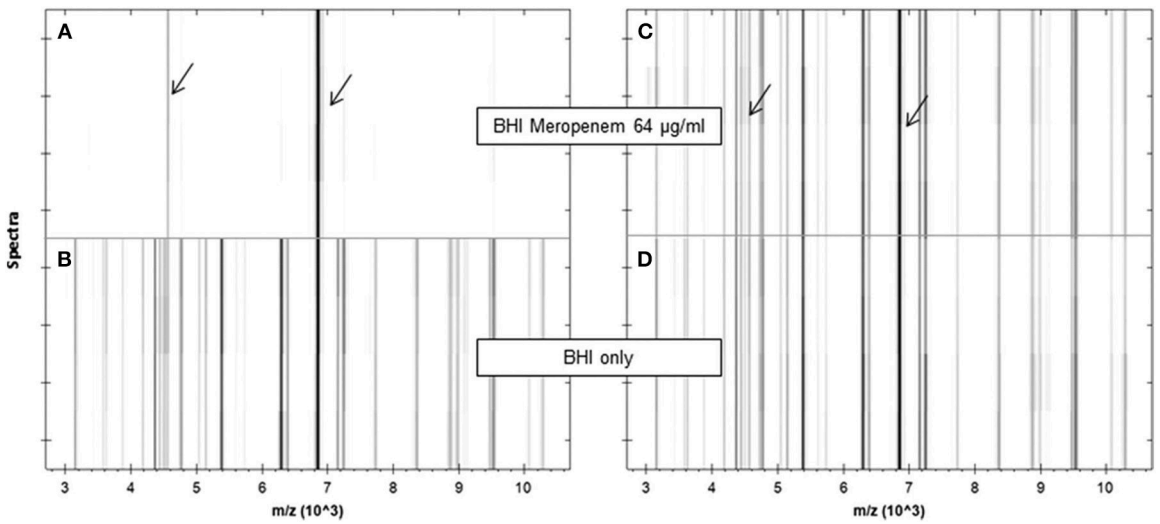


FIGURE 4 | Pseudogel views of the mass range between 3 and 10 kDa of a susceptible **(A,B)** and a resistant **(C,D)** *K. pneumoniae* strain after incubation in the absence (lower panels) or presence (upper panels) of meropenem (64 µg/ml) for 1 h. For each incubation, four spectra acquired from two different spots are shown. Internal standard peaks are marked by arrows. JCM, 2014, 52, 4155–4162, doi: 10.1128/JCM.01872-14, original Figure 1, reproduced with permission from American Society for Microbiology.

result, was a strain expressing heterogeneous resistance. An explanation for the false positive results could not be given. A repetition of the assay for these five strains gave the correct results. Additionally this assay worked with artificially inoculated blood culture bottles. 17 of 18 *Klebsiella* sp. were correctly classified.

Two years later Jung and co-workers published a slightly changed version of the MBT-ASTRA for gentamicin,

ciprofloxacin, cefotaxime and piperacillin-tazobactam (Jung et al., 2016). In contrast to the first description of the assay they used 200 µl of liquid Mueller-Hinton broth (OD_{600} of 0.007; 5×10^6 cfu/mL) instead of BHI broth and incubated for up to 3 h. First they spiked 30 blood culture bottles (BD BACTEC Plus Aerobic/F and Anaerobic) with different Enterobacteriaceae (*E. coli*, *Enterobacter* spp., *Klebsiella* spp. and *P. mirabilis*) and used gentamicin (4 mg/L) and ciprofloxacin (1 mg/L) as antibiotics. In a second step they tested 99 real-time patient blood-cultures (mainly *E. coli* and *Klebsiella* spp.) for non-susceptibility to ciprofloxacin (1 mg/L), cefotaxime (2 mg/L) and piperacillin-tazobactam (16/4 mg/L). Both parts of the study showed that all strains, which were fully susceptible or fully resistant to any of the tested antibiotics were accurately classified. Problems with correct classification were seen in case of poor growth in the tube without antibiotic, too short incubation times and with strains with MICs near the antibiotic concentration used in the assay.

In 2018 a first study of *B. fragilis* and MBT-ASTRA was published (Justesen et al., 2018). In this proof of principle study the authors demonstrated the suitability of MBT-ASTRA for the susceptibility prediction for clindamycin, meropenem and metronidazole.

New Developments

In 2017 the direct-on-target microdroplet growth assay was described (Idelevich et al., 2018). In this proof-of-principle study Idelevich and co-workers studied meropenem susceptible and resistant strains of *K. pneumoniae* and *P. aeruginosa* (12 susceptible and 12 resistant strains of each species). Bacterial suspensions with or without 2 mg/L of meropenem were applied to a target and incubated for up to 18 h on the target. The subsequent MALDI-TOF MS analysis showed a successful identification for meropenem-resistant isolates only. Meropenem-susceptible strains showed spectra with the result "no identification" (see **Figure 5**).

What Can We Hope for in the Future?

From a patient's perspective susceptibility testing should be accurate and rapidly available. Under ideal circumstances the time to report for rapid susceptibility testing would be a few minutes and availability of test results would be 24/7. Accuracy would mean that the susceptible/intermediate/resistant (SIR) result had a 100% positive predictive value for successful therapy, i.e., if an antibiotic is classified susceptible the patient should be successfully treated with it in 100 of 100 cases.

In reality time to report for susceptibility testing is >18 h for agardiffusion and >6–24 h for MIC determination after successful cultivation of the bacterium. In general test results are available during daytime and generating large data for positive predictive values of SIR determination is hardly feasible with patients.

The fastest possible susceptibility testing with MALDI-TOF MS is the simultaneous detection of one or more characteristic "resistance" or "susceptibility" peaks in the spectra generated for identification of the respective strain. Automated detection of these peaks is already feasible but what is largely missing is the identification of the protein behind this/these peak(s). This set-up would help very much during clinical routine in cases where for one particular bacterium the susceptibility to one single antibiotic is needed. Ideally the peak is caused by a protein causing resistance or at least correlated to the dominant resistance mechanism (e.g., MRSA and PSM-mec).

However, what infectious disease specialists wish for are rapid and reliable antibiograms displaying results for >10 antibiotics comparable to what is available today with MIC determination. The degradation method could be part of this set-up if the resistance mechanism to the antibiotic tested is enzymatic degradation. However, MBT-RESIST and/or MBT-ASTRA seem to be more suitable to achieve that goal. They are able to detect non-susceptibility due to different resistance mechanisms (e.g., efflux, target modification and degradation). With the lack of new antibiotic substances for therapy the ID specialist must optimize therapy by using the MIC values. With the current MIC assays it is possible to report the MIC values and use them for therapy optimization. Nothing comparable to an MIC was published for bacteria and susceptibility testing with MALDI-TOF MS. All assays use a single defined concentration of an antibiotic. However, for *Candida albicans*

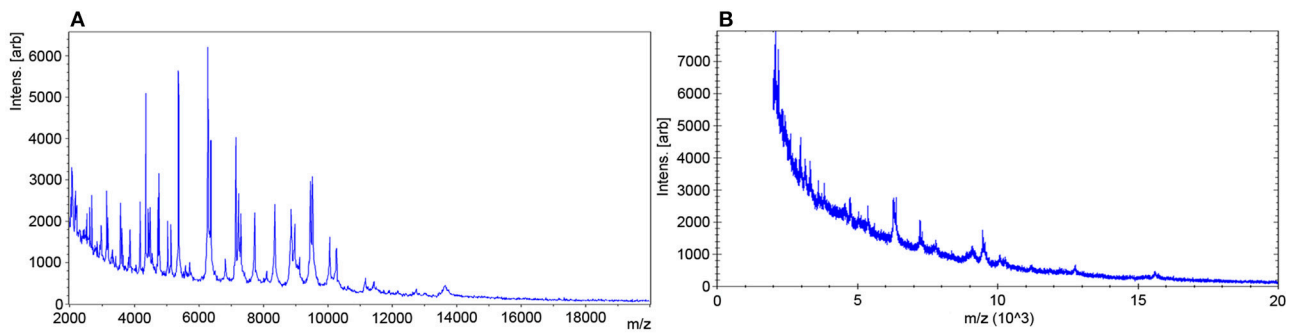


FIGURE 5 | Evaluation of the MALDI-TOF MS data **(A)** Meropenem-resistant isolate, MALDI Biotyper finding: *K. pneumoniae*. **(B)** Meropenem-susceptible isolate, MALDI Biotyper finding: No identification. With permission from Idelevich, E. A., Sparbier, K., Kostrzewa, M., and Becker, K. Rapid detection of antibiotic resistance by MALDI-TOF mass spectrometry using a novel direct-on-target microdroplet growth assay. Clin. Microbiol. Infect. 24, 738–743, Elsevier, © 2018.

Marinach and co-workers published the concept of the minimal profile change concentration (MPCC) (Marinach et al., 2009). Instead of using a single concentration of fluconazole they performed a serial dilution from 128 to 0.125 µg/mL. The MPCC was defined as the lowest drug concentration at which a mass spectrum profile change was detected. This concept was further evaluated with 16 strains of *C. albicans*. MICs had been determined following the CLSI guidelines. MPCC and MIC results were highly correlated (94–100%). An essential prerequisite for the success of these assays is their automation, miniaturization and standardization to make large-scale studies possible. Aspects that still have to be addressed during these studies are inoculums, time of incubation, concentration(s) of antibiotics, optimal matrices, background reduction and automated interpretation of results. Hopefully cost for all of these necessary developments will be manageable and the final test will prove to be cost effective. Cost will be an important factor determining or even deciding whether MALDI-TOF MS based susceptibility testing will be the upcoming technique for susceptibility testing. The main competitor for MALDI-TOF MS for susceptibility testing currently is the detection of known resistance determinants using PCR, that is genome based assays not proteome based assays. Even whole genome sequencing for "susceptibility testing" is under discussion. Very recently Greninger published an excellent review covering the pros and cons, the benefits and pitfalls of diagnostic metagenomics (Greninger, 2018) and therefore this subject is not further discussed here.

During the ongoing evaluation of MALDI-TOF MS for susceptibility testing it will be necessary to reach a broad consensus on two important aspects of susceptibility testing with MALDI-TOF MS. First we need to account for resistance mechanisms, which take time to take action. For example some enzymes are very slow. If incubation times are too short the susceptible/resistant classification will be wrong. For these mechanisms we need reaction conditions with which they can be detected within a few hours. Second, we need to agree on whether correlation of MALDI-TOF MS susceptibility results to MIC values is enough or whether we need *in vivo* therapy studies. Today we observe cases of therapeutic failure despite susceptible MIC that is *in vitro* susceptibility. One possible reason for therapeutic failure is that the initial *in vitro* susceptibility testing result does not mirror the *in vivo* activity of the antibiotic. Especially for strains with a divergent MALDI-TOF MS and MIC classification it would be interesting to determine the *in vivo* action, *in vivo* veritas. The breakthrough for antibiotic susceptibility testing with MALDI-TOF MS would be to demonstrate that results from MALDI-TOF MS susceptibility testing correlate better with successful therapy than results from traditional susceptibility testing (agardiffusion, E-Test, MIC-determination using automated systems).

If we find good solutions for these aspects the MALDI-TOF MS technology undoubtedly is the most promising type of assay for rapid and reliable susceptibility testing within the next 10 years.

AUTHOR CONTRIBUTIONS

IB drafting of manuscript, writing of manuscript, revision of manuscript. SZ revision of manuscript.

REFERENCES

Angeletti, S. (2016). Matrix assisted laser desorption time of flight mass spectrometry (MALDI-TOF MS) in clinical microbiology. *J. Microbiol. Methods* 138, 20–29. doi: 10.1016/j.mimet.2016.09.003

Arca-Suárez, J., Galán-Sánchez, F., Marin-Casanova, P., and Rodríguez-Iglesias, M. A. (2017). Direct identification of microorganisms from thioglycolate broth by MALDI-TOF MS. *PLoS ONE* 12:e0185229. doi: 10.1371/journal.pone.0185229

Bernardo, K., Pakulat, N., Macht, M., Krut, O., Seifert, H., Fleer, S., et al. (2002). Identification and discrimination of *Staphylococcus aureus* strains using matrix-assisted laser desorption/ionization-time of flight mass spectrometry. *Proteomics* 2, 747–753. doi: 10.1002/1615-9861(200206)2:6<740::AID-PROT740>3.0.CO;2-M

Burckhardt, I., and Zimmermann, S. (2011). Using matrix-assisted laser desorption ionization-time of flight mass spectrometry to detect carbapenem resistance within 1 to 2.5 hours. *J. Clin. Microbiol.* 49, 3321–3324. doi: 10.1128/JCM.00287-11

Calderaro, A., Buttrini, M., Piergianni, M., Montecchini, S., Martinelli, M., Covan, S., et al. (2017). Evaluation of a modified meropenem hydrolysis assay on a large cohort of KPC and VIM carbapenemase-producing Enterobacteriaceae. *PLoS ONE* 12:e0174908. doi: 10.1371/journal.pone.0174908

Clark, A. E., Kaleta, E. J., Arora, A., and Wolk, D. M. (2013). Matrix-assisted laser desorption ionization-time of flight mass spectrometry: a fundamental shift in the routine practice of clinical microbiology. *Clin. Microbiol. Rev.* 26, 547–603. doi: 10.1128/CMR.00072-12

Demirev, P. A., Hagan, N. S., Antoine, M. D., Lin, J. S., and Feldman, A. B. (2013). Establishing drug resistance in microorganisms by mass spectrometry. *J. Am. Soc. Mass Spectrom.* 24, 1194–1201. doi: 10.1007/s13361-013-0609-x

Du, Z., Yang, R., Guo, Z., Song, Y., and Wang, J. (2002). Identification of *Staphylococcus aureus* and determination of its methicillin resistance by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Anal. Chem.* 74, 5487–5491. doi: 10.1021/ac020109k

Edwards-Jones, V., Claydon, M. A., Evason, D. J., Walker, J., Fox, A. J., and Gordon, D. B. (2000). Rapid discrimination between methicillin-sensitive and methicillin-resistant *Staphylococcus aureus* by intact cell mass spectrometry. *J. Med. Microbiol.* 49, 295–300. doi: 10.1099/0022-1317-49-3-295

Fenyvesi, V. S., Urban, E., Bartha, N., Abrok, M., Kostrzewa, M., Nagy, E., et al. (2014). Use of MALDI-TOF/MS for routine detection of cfiA gene-positive Bacteroides fragilis strains. *Int. J. Antimicrob. Agents* 44, 474–475. doi: 10.1016/j.ijantimicag.2014.07.010

Gaibani, P., Ambretti, S., Tamburini, M. V., Vecchio Nepita, E., and Re, M. C. (2018). Clinical application of Bruker Biotyper MALDI-TOF/MS system for real-time identification of KPC production in *Klebsiella pneumoniae* clinical isolates. *J. Glob. Antimicrob. Resist.* 12, 169–170. doi: 10.1016/j.jgar.2018.01.016

Gaibani, P., Galea, A., Fagioni, M., Ambretti, S., Sambri, V., and Landini, M. P. (2016). Evaluation of matrix-assisted laser desorption ionization-time of flight mass spectrometry for identification of kpc-producing *Klebsiella pneumoniae*. *J. Clin. Microbiol.* 54, 2609–2613. doi: 10.1128/JCM.01242-16

Greninger, A. L. (2018). The challenge of diagnostic metagenomics. *Expert Rev. Mol. Diagn.* 18, 1–11. doi: 10.1080/14737159.2018.1487292

Griffin, P. M., Price, G. R., Schooneveldt, J. M., Schlebusch, S., Tilse, M. H., Urbanski, T., et al. (2012). Use of matrix-assisted laser desorption ionization-time of flight mass spectrometry to identify vancomycin-resistant enterococci and investigate the epidemiology of an outbreak. *J. Clin. Microbiol.* 50, 2918–2931. doi: 10.1128/JCM.01000-12

Hrabák, J., Walkova, R., Studentova, V., Chudackova, E., and Bergerova, T. (2011). Carbapenemase activity detection by matrix-assisted laser desorption

ionization-time of flight mass spectrometry. *J. Clin. Microbiol.* 49, 3222–3227. doi: 10.1128/JCM.00984-11

Idelevich, E. A., Sparbier, K., Kostrzewa, M., and Becker, K. (2018). Rapid detection of antibiotic resistance by MALDI-TOF mass spectrometry using a novel direct-on-target microdroplet growth assay. *Clin. Microbiol. Infect.* 24, 738–743. doi: 10.1016/j.cmi.2017.10.016

Johansson, A., Nagy, E., and Soki, J. (2014a). Detection of carbapenemase activities of Bacteroides fragilis strains with matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS). *Anaerobe* 26, 49–52. doi: 10.1016/j.anaerobe.2014.01.006

Johansson, Å., Nagy, E., and Soki, J. (2014b). Instant screening and verification of carbapenemase activity in Bacteroides fragilis in positive blood culture, using matrix-assisted laser desorption ionization–time of flight mass spectrometry. *J. Med. Microbiol.* 63, 1105–1110. doi: 10.1099/jmm.0.075465-0

Josten, M., Dischinger, J., Szekat, C., Reif, M., Al-Sabti, N., Sahl, H. G., et al. (2014). Identification of agr-positive methicillin-resistant *Staphylococcus aureus* harbouring the class A mec complex by MALDI-TOF mass spectrometry. *Int. J. Med. Microbiol.* 304, 1018–1023. doi: 10.1016/j.ijmm.2014.07.005

Jung, J. S., Eberl, T., Sparbier, K., Lange, C., Kostrzewa, M., Schubert, S., et al. (2014a). Rapid detection of antibiotic resistance based on mass spectrometry and stable isotopes. *Eur. J. Clin. Microbiol. Infect. Dis.* 33, 949–955. doi: 10.1007/s10096-013-2031-5

Jung, J. S., Hamacher, C., Gross, B., Sparbier, K., Lange, C., Kostrzewa, M., et al. (2016). Evaluation of a semi-quantitative MALDI-TOF MS method for rapid antimicrobial susceptibility testing of positive blood cultures. *J. Clin. Microbiol.* doi: 10.1128/JCM.01131-16

Jung, J. S., Popp, C., Sparbier, K., Lange, C., Kostrzewa, M., and Schubert, S. (2014b). Evaluation of matrix-assisted laser desorption ionization-time of flight mass spectrometry for rapid detection of beta-lactam resistance in Enterobacteriaceae derived from blood cultures. *J. Clin. Microbiol.* 52, 924–930. doi: 10.1128/JCM.02691-13

Justesen, U. S., Acar, Z., Sydenham, T. V., and Johansson, A. (2018). Antimicrobial susceptibility testing of Bacteroides fragilis using the MALDI Biotyper antibiotic susceptibility test rapid assay (MBT-ASTRA). *Anaerobe* doi: 10.1016/j.anaerobe.2018.02.007

Kumar, A., Ellis, P., Arabi, Y., Roberts, D., Light, B., Parrillo, J. E., et al. (2009). Initiation of inappropriate antimicrobial therapy results in a fivefold reduction of survival in human septic shock. *Chest* 136, 1237–1248. doi: 10.1378/chest.09-0087

Lange, C., Schubert, S., Jung, J., Kostrzewa, M., and Sparbier, K. (2014). Quantitative matrix-assisted laser desorption ionization-time of flight mass spectrometry for rapid resistance detection. *J. Clin. Microbiol.* 52, 4155–4162. doi: 10.1128/JCM.01872-14

Lau, A. F., Wang, H., Weingarten, R. A., Drake, S. K., Suffredini, A. F., Garfield, M. K., et al. (2014). 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.* 52, 2804–2812. doi: 10.1128/JCM.00694-14

Lee, A. W. T., Lam, J. K. S., Lam, R. K. W., Ng, W. H., Lee, E. N. L., Lee, V. T. Y., et al. (2018). Comprehensive evaluation of the mbt star-bl module for simultaneous bacterial identification and beta-lactamase-mediated resistance detection in gram-negative rods from cultured isolates and positive blood cultures. *Front. Microbiol.* 9:334. doi: 10.3389/fmicb.2018.00334

Marinach, C., Alanio, A., Palous, M., Kwasek, S., Fekkar, A., Brossas, J. Y., et al. (2009). MALDI-TOF MS-based drug susceptibility testing of pathogens: the example of Candida albicans and fluconazole. *Proteomics* 9, 4627–4631. doi: 10.1002/pmic.200900152

Nagy, E., Becker, S., Soki, J., Urban, E., and Kostrzewa, M. (2011). Differentiation of division I (cfiA-negative) and division II (cfiA-positive) Bacteroides fragilis strains by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *J. Med. Microbiol.* 60, 1584–1590. doi: 10.1099/jmm.0.031336-0

Nakano, S., Matsumura, Y., Kato, K., Yunoki, T., Hotta, G., Noguchi, T., et al. (2014). Differentiation of vanA-positive Enterococcus faecium from vanA-negative E. faecium by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry. *Int. J. Antimicrob. Agents* 44, 256–259. doi: 10.1016/j.ijantimicag.2014.05.006

Oviaño, M., Sparbier, K., Barba, M. J., Kostrzewa, M., and Bou, G. (2016). Universal protocol for the rapid automated detection of carbapenem-resistant Gram-negative bacilli directly from blood cultures by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF/MS). *Int. J. Antimicrob. Agents* 48, 655–660. doi: 10.1016/j.ijantimicag.2016.08.024

Papagiannitsis, C. C., Študentová, V., Izdebski, R., Oikonomou, O., Pfeifer, Y., Petinaki, E., et al. (2015). Matrix-assisted laser desorption ionization-time of flight mass spectrometry meropenem hydrolysis assay with NH_4HCO_3, a reliable tool for direct detection of carbapenemase activity. *J. Clin. Microbiol.* 53, 1731–1735. doi: 10.1128/JCM.03094-14

Pardo, C. A., Tan, R. N., Hennequin, C., Beyrouthy, R., Bonnet, R., and Robin, F. (2016). Rapid detection of AAC(6')-Ib-cr production using a MALDI-TOF MS strategy. *Eur. J. Clin. Microbiol. Infect. Dis.* 35, 2047–2051. doi: 10.1007/s10096-016-2762-1

Patel, R. (2015). MALDI-TOF MS for the diagnosis of infectious diseases. *Clin. Chem.* 61, 100–111. doi: 10.1373/clinchem.2014.221770

Pranada, A. B., Bienia, M., and Kostrzewa, M. (2016). "Optimization and Evaluation of MRSA Detection by Peak Analysis of MALDI-TOF Mass Spectra," in *DGHM 2016* (Ulm).

Ramos, A. C., Carvalhaes, C. G., Cordeiro-Moura, J. R., Rockstroh, A. C., Machado, A. M., and Gales, A. C. (2016). Influence of culture media on detection of carbapenem hydrolysis by matrix-assisted laser desorption ionization-time of flight mass spectrometry. *J. Clin. Microbiol.* 54, 1896–1898. doi: 10.1128/JCM.00749-16

Rotova, V., Papagiannitsis, C. C., Skalova, A., Chudejova, K., and Hrabak, J. (2017). Comparison of imipenem and meropenem antibiotics for the MALDI-TOF MS detection of carbapenemase activity. *J. Microbiol. Methods* 137, 30–33. doi: 10.1016/j.mimet.2017.04.003

Sandalakis, V., Goniotakis, I., Vranakis, I., Chochlakis, D., and Psaroulaki, A. (2017). Use of MALDI-TOF mass spectrometry in the battle against bacterial infectious diseases: recent achievements and future perspectives. *Expert Rev. Proteomics* 14, 253–267. doi: 10.1080/14789450.2017.1282825

Singhal, N., Kumar, M., Kanaujia, P. K., and Virdi, J. S. (2015). MALDI-TOF mass spectrometry: an emerging technology for microbial identification and diagnosis. *Front. Microbiol.* 6:791. doi: 10.3389/fmicb.2015.00791

Sparbier, K., Lange, C., Jung, J., Wieser, A., Schubert, S., and Kostrzewa, M. (2013). MALDI biotyper-based rapid resistance detection by stable-isotope labeling. *J. Clin. Microbiol.* 51, 3741–3748. doi: 10.1128/JCM.01536-13

Sparbier, K., Schubert, S., Weller, U., Boogen, C., and Kostrzewa, M. (2012). Matrix-assisted laser desorption ionization-time of flight mass spectrometry-based functional assay for rapid detection of resistance against beta-lactam antibiotics. *J. Clin. Microbiol.* 50, 927–937. doi: 10.1128/JCM.05737-11

Szabados, F., Kaase, M., Anders, A., and Gatermann, S. G. (2012). Identical MALDI TOF MS-derived peak profiles in a pair of isogenic SCCmec-harboring and SCCmec-lacking strains of *Staphylococcus aureus*. *J. Infect.* 65, 400–405. doi: 10.1016/j.jinf.2012.06.010

Timke, M., Trojahn, M., Kostrzewa, M., and Wasner, M. (2017). "Instant Detection of PSM Positive MRSA in MALDI Biotyper Workflow," in *ECCMID 2017* (Vienna).

Tré-Hardy, M., Lambert, B., Despas, N., Bressant, F., Laurenzano, C., Rodriguez-Villalobos, H., et al. (2017). MALDI-TOF MS identification and antimicrobial susceptibility testing directly from positive enrichment broth. *J. Microbiol. Methods* 141, 32–34. doi: 10.1016/j.mimet.2017.07.012

WHO (2015). *The World Health Statistics 2015*. Geneva: WHO. (Accessed Oct 24, 2016).

Wybo, I., De Bel, A., Soetens, O., Echahidi, F., Vandoorslaer, K., Van Cauwenbergh, M., and Pierard, D. (2011). Differentiation of cfiA-negative and cfiA-positive Bacteroides fragilis isolates by matrix-assisted laser desorption ionization-time of flight mass spectrometry. *J. Clin. Microbiol.* 49, 1961–1964. doi: 10.1128/JCM.02321-10

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 11 October 2018
doi: 10.3389/fmicb.2018.02393

Rapid Detection of Heterogeneous Vancomycin-Intermediate *Staphylococcus aureus* Based on Matrix-Assisted Laser Desorption Ionization Time-of-Flight: Using a Machine Learning Approach and Unbiased Validation

Hsin-Yao Wang[1,2], *Chun-Hsien Chen*[3], *Tzong-Yi Lee*[1,4,5,6,7], *Jorng-Tzong Horng*[1,8,9], *Tsui-Ping Liu*[1], *Yi-Ju Tseng*[1,3*] and *Jang-Jih Lu*[1,10,11*]

[1] Department of Laboratory Medicine, Chang Gung Memorial Hospital at Linkou, Taoyuan, Taiwan, [2] Ph.D. Program in Biomedical Engineering, Chang Gung University, Taoyuan, Taiwan, [3] Department of Information Management, Chang Gung University, Taoyuan, Taiwan, [4] Department of Computer Science and Engineering, Yuan Ze University, Taoyuan, Taiwan, [5] Innovation Center for Big Data and Digital Convergence, Yuan Ze University, Taoyuan, Taiwan, [6] Warshel Institute for Computational Biology, Chinese University of Hong Kong, Shenzhen, China, [7] School of Science and Engineering, Chinese University of Hong Kong, Shenzhen, China, [8] Department of Bioinformatics and Medical Engineering, Asia University, Taichung, Taiwan, [9] Department of Computer Science and Information Engineering, National Central University, Taoyuan, Taiwan, [10] School of Medicine, Chang Gung University, Taoyuan, Taiwan, [11] Department of Medical Biotechnology and Laboratory Science, Chang Gung University, Taoyuan, Taiwan

OPEN ACCESS

Edited by:
Karsten Becker, Universität Münster, Germany

Reviewed by:
Irene Burckhardt, Universität Heidelberg, Germany
João Nobrega De Almeida Júnior, Universidade de São Paulo, Brazil

***Correspondence:**
Yi-Ju Tseng
yjtseng@mail.cgu.edu.tw
Jang-Jih Lu
janglu45@gmail.com

Specialty section:
This article was submitted to Antimicrobials, Resistance and Chemotherapy, a section of the journal Frontiers in Microbiology

Received: *29 June 2018*
Accepted: *18 September 2018*
Published: *11 October 2018*

Citation:
Wang H-Y, Chen C-H, Lee T-Y, Horng J-T, Liu T-P, Tseng Y-J and Lu J-J (2018) Rapid Detection of Heterogeneous Vancomycin-Intermediate Staphylococcus aureus Based on Matrix-Assisted Laser Desorption Ionization Time-of-Flight: Using a Machine Learning Approach and Unbiased Validation. Front. Microbiol. 9:2393. doi: 10.3389/fmicb.2018.02393

Heterogeneous vancomycin-intermediate *Staphylococcus aureus* (hVISA) is an emerging superbug with implicit drug resistance to vancomycin. Detecting hVISA can guide the correct administration of antibiotics. However, hVISA cannot be detected in most clinical microbiology laboratories because the required diagnostic tools are either expensive, time consuming, or labor intensive. By contrast, matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) is a cost-effective and rapid tool that has potential for providing antibiotics resistance information. To analyze complex MALDI-TOF mass spectra, machine learning (ML) algorithms can be used to generate robust hVISA detection models. In this study, MALDI-TOF mass spectra were obtained from 35 hVISA/vancomycin-intermediate *S. aureus* (VISA) and 90 vancomycin-susceptible *S. aureus* isolates. The vancomycin susceptibility of the isolates was determined using an Etest and modified population analysis profile–area under the curve. ML algorithms, namely a decision tree, k-nearest neighbors, random forest, and a support vector machine (SVM), were trained and validated using nested cross-validation to provide unbiased validation results. The area under the curve of the models ranged from 0.67 to 0.79, and the SVM-derived model outperformed those of the other algorithms. The peaks at m/z 1132, 2895, 3176, and 6591 were noted as informative peaks for detecting hVISA/VISA. We demonstrated that hVISA/VISA could be detected by

analyzing MALDI-TOF mass spectra using ML. Moreover, the results are particularly robust due to a strict validation method. The ML models in this study can provide rapid and accurate reports regarding hVISA/VISA and thus guide the correct administration of antibiotics in treatment of *S. aureus* infection.

Keywords: heterogeneous vancomycin-intermediate *Staphylococcus aureus*, matrix-assisted laser desorption ionization (MALDI) mass spectrometry, vancomycin intermediate *S. aureus* (VISA), machine learning, rapid detection

INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) infection remains an intractable clinical problem (Liu et al., 2011). Although vancomycin was formerly the drug of choice against MRSA, the unprecedented increase in the number and spread of organisms with reduced susceptibility to this drug, including two major phenotypes—vancomycin-intermediate *S. aureus* (VISA) and heterogeneous VISA (hVISA)—has brought this conventional treatment into question (Zhang et al., 2015). The prevalence of hVISA and VISA was reported in a systematic review to have increased worldwide from 4.68 and 2.05% (2006) to 7.01 and 7.93% (2014), respectively (Zhang et al., 2015). In Taiwan, the prevalence of hVISA also increased from 0.7% (2003) to 10.0% (2013) and that of VISA from 0.2% (2003) to 2.7% (2013) (Huang et al., 2016). Despite adequate doses of vancomycin, patients with severe hVISA or VISA infection persistently suffer from bacteremia (Howden et al., 2010). In particular, hVISA infection is associated with increased risk of treatment failure (van Hal and Paterson, 2011; Hu et al., 2015). Longer bacteremia and culture-positive periods lead to longer hospital stays and durations of vancomycin therapy, establishing a vicious circle in the growth of staphylococcal resistance to vancomycin (Sakoulas et al., 2006; Fong et al., 2009). Therefore, early and accurate detection of potentially non-susceptible staphylococcal strains is essential for hampering misuse of vancomycin and directing appropriate antibiotic therapy.

The Clinical and Laboratory Standards Institute defines VISA as an isolate with a minimal inhibitory concentration (MIC) of vancomycin between 4 and 8 μg per mL. The MIC of hVISA is within the susceptible range (≤2 μg per mL), but a subpopulation of the isolate's cells belong to a vancomycin-intermediate range (Rybak and Akins, 2001). Clinical physicians rely largely on antibiotics susceptibility tests (ASTs) to guide correct administration of antibiotics against *S. aureus* infection. However, MIC determination for *S. aureus* takes around 10 h, agar diffusion necessitates an incubation time of 18–20 h. The long turnaround time of ASTs inevitably delays accurate clinical decision-making regarding suitable antibiotics. Moreover, hVISA infection cannot be detected by routine AST methods because of its low-level vancomycin resistance and a small resistant fraction of the inoculum. hVISA can be detected by satellite colonies in the vancomycin inhibition zone and the ETest zone; it can't be reliably detected with automated MIC determination methods. The screening tests for hVISA are Etest glycopeptide resistance detection, the Etest macromethod, and brain heart infusion screening agar plates. These three screening tests vary in sensitivity and specificity, and single use of any one test results in poor accuracy (Satola et al., 2011). Population analysis profile–area under the curve is the gold standard of determining hVISA, but the process is cumbersome, time consuming, not commonly used in most clinical microbiology laboratories, and thus impractical for laboratory diagnosis (Chang et al., 2015).

Various proteins contribute to the resistance of *S. aureus* against vancomycin (Lin et al., 2018). The proteomic pattern of isolates can be analyzed in a rapid, comprehensive, and cost-effective manner using matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) in clinical microbiology laboratories (Hrabák et al., 2013; Idelevich et al., 2017). MALDI-TOF MS produces large sets of complex data. Manual interpretation of MALDI-TOF MS data is unreliable; therefore, an informatics approach is necessary for effective and accurate interpretation. Machine learning (ML) can help automatic diagnosis and make the process less time consuming (Swan et al., 2013). The application of ML to detecting vancomycin-susceptible *S. aureus* (VSSA) in hVISA/VISA has not been widely discussed or validated (Rishishwar et al., 2014; Mather et al., 2016), although several studies have demonstrated successful application of ML in clinical practice (Wang et al., 2016; Lin et al., 2018). In the present study, we used a data processing method that facilitated the application of an ML algorithm in analysis of MALDI-TOF MS data (Wang et al., 2018). Its performance in distinguishing VSSA from hVISA/VISA was validated using nested cross-validation for a minimally biased estimation of performance (Varma and Simon, 2006; Filzmoser et al., 2009; Krstajic et al., 2014). By using the proposed ML models, we can rapidly detect hVISA/VISA and guide the use of glycopeptide for patients with MRSA infection.

MATERIALS AND METHODS

Study Design

The overall study flow is presented in **Figure 1**. MRSA isolates were cultivated from a bacterial bank (Wang et al., 2018). In the study, the 125 MRSA isolates had been collected from 2009 to 2014 at the Linkou branch of Chang Gung Memorial Hospital (CGMH), Taiwan. The specimen type was blood specimen. The MALDI-TOF MS spectra of these isolates were then obtained and relevant features selected for distinguishing VSSA from hVISA/VISA. The performance of the proposed models for rapid detection of hVISA/VISA was evaluated using a nested cross-validation approach.

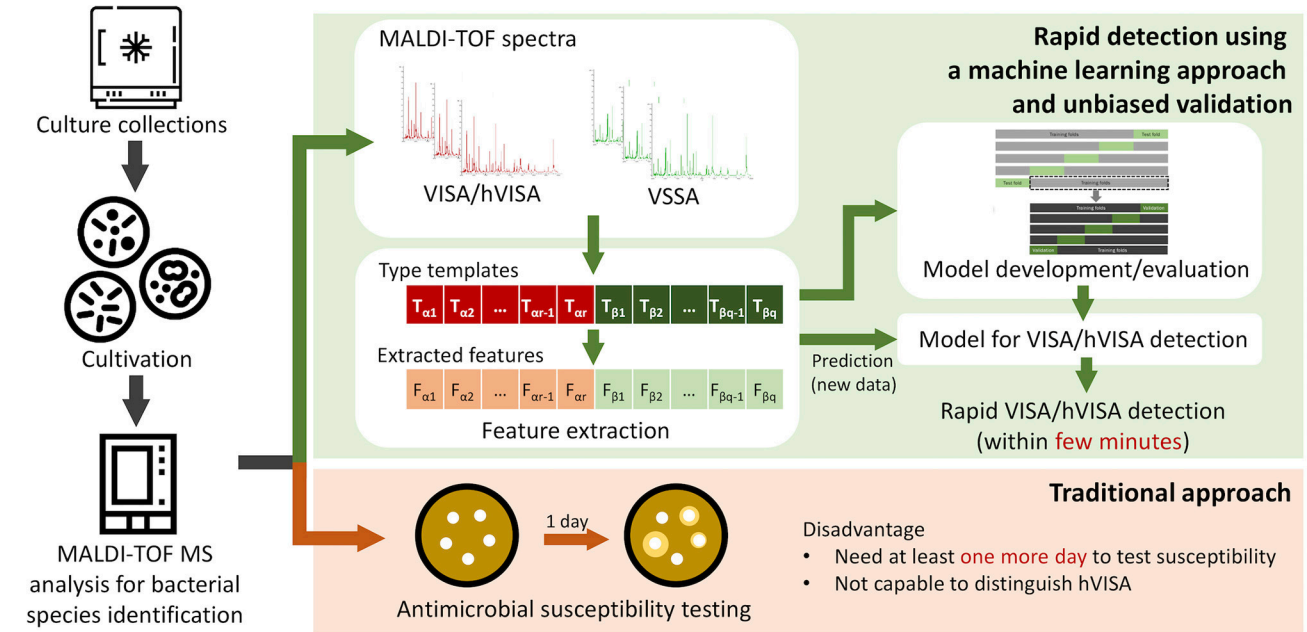


FIGURE 1 | The study flow of rapid detection of hVISA based on MALDI-TOF.

Bacterial Isolates

The bacterial strains were stored at −70°C until use (Wang et al., 2018). The strains were cultured on a blood agar plate (Becton Dickinson, MD, USA) in a 5% CO_2 incubator for 16–18 h. A colony morphology inspection, catalase test, and coagulase test were performed, and the results were in line with the characteristics of *S. aureus*. Single colonies from the blood agar plate were selected and spread onto a steel target plate (Bruker Daltonik GmbH, Bremen, Germany), followed by application of 1 mL of 70% formic acid. After being dried in ambient air, an additional 1 mL matrix solution (50% acetonitrile containing 1% α-cyano-4-hydroxycinnamic acid and 2.5% trifluoroacetic acid) was applied before analytical measurement was conducted using a Microflex LT mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany). The conditions of the Microflex LT mass spectrometer were as follows: linear positive mode; accelerating voltage: +20 kV; laser frequency: 60 Hz; and laser shots per colony: up to 240. The Bruker Daltonics Bacterial Test Standard was used as an external calibration for each batch. The species of *S. aureus* was reconfirmed according to the identification results provided by Biotyper 3.1 (Bruker Daltonik GmbH, Bremen, Germany). ASTs of oxacillin were performed according to Clinical and Laboratory Standards Institute M100 S27 guideline (CLSI., 2017). A cefoxitin disc was used for testing oxacillin susceptibility. A method of multiplex polymerase chain reactions for staphylococcal cassette chromosome mec (*SCCmec*) was used for determining *SCCmec* type and detecting *mecA* to confirm MRSA (Kondo et al., 2007). The MIC of vancomycin was determined using an Etest (bioMérieux, Marcy-l'Étoile, France) according to the manufacturer's instruction. In brief, bacterial isolates were inoculated with concentration of 0.5 McFarland on Mueller Hinton agar plates (Creative Media Plate, New Taipei City, Taiwan), followed by placing vancomycin Etest strips. The MRSA isolates were screened by Etest and those with MICs ≥2–4 μg/mL were selected for modified population analysis profile–area under the curve (PAP-AUC) analyzes to be classified as either VSSA, hVISA, or VISA(Wootton, 2001). For multilocus sequence typing (MLST), seven housekeeping genes were sequenced, including carbamate kinase (*arcC*), shikimate dehydrogenase (*aroE*), glycerol kinase (*glpF*), guanylate kinase (*gmk*), phosphate acetyltransferase (*pta*), triosephosphateisomerase (*tpi*), and acetyl coenzyme A acetyltransferase (*yqiL*). The MLS typing result was determined by comparing the sequence results to the *S. aureus* MLST database (http://saureus.mlst.net/) (Enright et al., 2000).

Analysis of MALDI-TOF MS Spectra

The quality of the MS spectra was defined by the log score provided by Biotyper 3.1 (Bruker Daltonik GmbH, Bremen, Germany). MS spectra with a log score larger than 2.00 were considered acceptable quality. A spectral range from 0 to 20,000 Da was collected. Before further analysis, the MALDI-TOF MS spectra were preprocessed using Flexanalysis 3.4 (Bruker Daltonik GmbH, Bremen, Germany), as reported in a study (Wang et al., 2018). Features were extracted from the MALDI-TOF MS spectra after preprocessing. The aim of feature extraction was to standardize and facilitate the application of ML algorithms for analyzing complicated MS spectra. Feature extraction was performed on the basis of a study (Wang et al., 2018). First, type templates were constructed based on the occurrence frequency of specific peaks in the MALDI-TOF MS spectra. In the present study, the type templates of VSSA

and hVISA/VISA were obtained using this approach. Features were then extracted from the MALDI-TOF MS spectra by aligning an individual spectrum onto the type templates. After the alignments, matched vectors for each type template could be obtained and an integrated vector of individual bacterial strain was generated. Supervised ML algorithms could be trained and validated according to the integrated vectors and their corresponding labels.

Relevant Feature Selection

To include only the relevant features for use in the model development, we performed a feature selection step before constructing the predictive models. In each training task, a mean decrease in accuracy, obtained from the random forest algorithm (Liaw and Wiener, 2002), was employed to select the most crucial features from the training dataset. The mean decrease in accuracy was generated by measuring the effect of each feature on the accuracy of the model, permuting the values of each feature, and measuring the decrease in accuracy.

Development of Predictive Models

We used random forest, a support vector machine (SVM) with a radial basis function kernel, k-nearest neighbors, and a decision tree to develop the models. Random forest is an ensemble classifier proposed by Breiman (2001) that uses random feature selection and comprises numerous classification trees. The frequency of a feature's appearance in the classification trees represents the importance of the feature. The library "randomForest" in R software (version 3.4.4, R Foundation for Statistical Computing, http://www.r-project.org/) was used for implementing the random forest classifier (Liaw and Wiener, 2002). An SVM is a data-mining method that constructs a classification model for a binary-class problem. It uses nonlinear mapping to transform the data into a higher dimension. Through appropriate nonlinear mapping to a sufficiently high dimension, data from two classes are separated by a hyperplane (Cortes and Vapnik, 1995). The library "e1071" was used for implementing the SVM classifier (Meyer et al., 2017). A simple algorithm, k-nearest neighbors stores all available cases and predicts the numerical target based on a similarity measure; it was implemented using a "class" library (Venables and Ripley, 2002). A decision tree is a recursive partitioning approach. The classification and regression trees algorithm splits each input node into two child nodes, and the same process is applied to each child node. Splitting is halted when the algorithm detects that no further gain can be made (Breiman et al., 1984). We applied the classification and regression trees algorithm to our dataset by using the "rpart" library (Therneau and Atkinson, 2018).

Predictive Model Evaluation

To develop predictive models for distinguishing VSSA from hVISA/VISA strains, we applied a nested 5-fold cross-validation approach to train and evaluate the models (**Figure 2**). In the outer 5-fold cross-validation loop, we divided the data into training (4-folds) and test (1-fold) datasets to evaluate the performance of the models with an untouched test set. In each training step in the outer fold, repeated inner 5-fold cross-validation was applied to tune and select the optimal models. The nested 5-fold cross-validation process was repeated six times to ensure our evaluation results were robust.

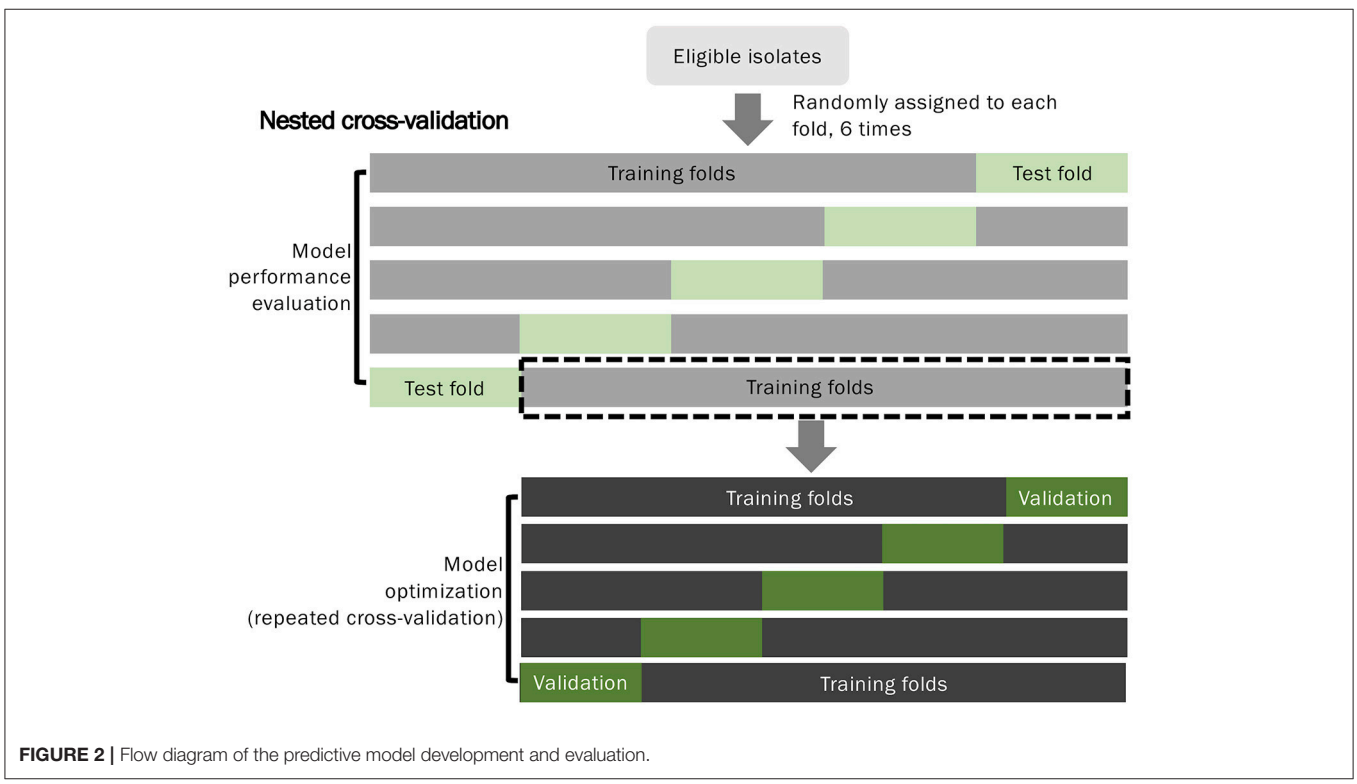


FIGURE 2 | Flow diagram of the predictive model development and evaluation.

In each fold of outer cross-validation, we selected the features and constructed the models using data from the training set and then evaluated the performance of the models using the data in the untouched test set. The area under the receiver operating characteristic curve (AUC) was used to evaluate the performance of the models. Furthermore, we used Youden's J statistic—a single statistic that captures the performance of a dichotomous diagnostic test—to generate sensitivity and specificity for further analysis of prediction performance.

Statistical Analysis

The Mann–Whitney *U*-test was used to analyze MALDI-TOF MS spectra peak characteristics. We performed analysis of variance (ANOVA) and Tukey honestly significant difference *post-hoc* analyses on the AUC values of the predictive models. All analyses were performed using the R software. All statistical tests were two-sided, and statistical significance was defined as $p < 0.05$.

Data Availability

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

RESULTS

MALDI-TOF MS Spectra of MRSA Isolates

A total of 125 MRSA isolates, namely 35 hVISA/VISA and 90 VSSA strains, were used to develop the proposed predictive models. For each isolate, 127 peaks were extracted from a mass spectrum. The peak characteristics and their intensities for these MRSA isolates are presented in **Supplementary Table 1**. Among these peaks, the intensities of 13 peaks were differed significantly between the hVISA/VISA and VSSA strains (**Table 1**).

TABLE 1 | MALDI-TOF MS spectra peak characteristics that their intensities differed significantly between the hVISA/VISA and VSSA strains.

Peak, m/z	Intensity		*P*-value[d]
	hVISA[a]/VISA[b] strains (median [IQR])	VSSA[c] strains (median [IQR])	
118	12.97 [11.58, 14.32]	11.39 [0.00, 13.20]	0.005
119	12.97 [11.58, 14.32]	11.41 [0.00, 13.23]	0.005
680	0.00 [0.00, 0.00]	0.00 [0.00, 0.00]	0.006
852	0.00 [0.00, 12.26]	12.37 [0.00, 13.46]	0.005
948	0.00 [0.00, 11.87]	0.00 [0.00, 0.00]	0.006
1132	0.00 [0.00, 11.42]	0.00 [0.00, 0.00]	< 0.001
1266	0.00 [0.00, 11.62]	0.00 [0.00, 0.00]	0.009
2429	12.37 [0.00, 13.31]	0.00 [0.00, 12.44]	0.004
2895	0.00 [0.00, 11.82]	0.00 [0.00, 0.00]	< 0.001
3176	10.65 [0.00, 11.18]	0.00 [0.00, 10.64]	0.001
6351	10.86 [10.58, 11.15]	10.62 [2.51, 10.94]	0.009
6591	10.54 [10.10, 11.02]	0.00 [0.00, 10.70]	< 0.001
9625	12.66 [12.32, 12.92]	12.30 [11.77, 12.81]	0.01

[a] *Heterogeneous Vancomycin-intermediate S. aureus.*
[b] *Vancomycin-intermediate S. aureus.*
[c] *Vancomycin-susceptible S. aureus.*
[d] *Mann–Whitney U test.*

Relevant Features for Distinguishing VSSA From hVISA/VISA

We defined relevant peak features as peaks with importance greater than 1.9 based on the random forest algorithm results. The importance was defined as z-score of mean decrease in accuracy obtained from the random forest algorithm (Liaw and Wiener, 2002). Among the 109 relevant features selected from 30 feature selection results based on repeated nested 5-fold cross-validation (**Supplementary Table 2**), four peak features were selected in more than 90% of the models. **Figure 3** shows the distribution based on kernel density estimation of the importance of four peak features. The peak at m/z 6591 was selected as a relevant feature in all the training tasks ($n = 30$) and identified as the most crucial feature for distinguishing VSSA from hVISA/VISA.

Performance of the Predictive Models

Regarding model performance for distinguishing VSSA strains from hVISA/VISA strains among the MRSA isolates, the optimal predictive model for the test set was the model constructed using the SVM classifier with a radial basis function kernel and with AUC = 0.790. The model constructed using the random forest algorithm had similar performance, with AUC = 0.763 ($p = 0.30$). The AUCs for the models constructed using k-nearest neighbors and a decision tree were 0.722 and 0.668, respectively (**Figure 4**), which were lower than those of the optimal predictive models ($p < 0.01$). Based on the maximum value of Youden's J statistic, the average sensitivity and specificity of the SVM classifier were 0.770 and 0.814, respectively (**Figure 5**). The validation results of all the classifiers using nested 5-fold cross-validation, repeated 6 times, were presented in **Supplementary Table 3**.

DISCUSSION

In the present study, we demonstrated that the ML-based approach can successfully distinguish VSSA from hVISA/VISA on the basis of MALDI-TOF MS data. The preliminary AST obtained from the ML-based approach can yield an accurate and rapid administration of correct antibiotics against MRSA infection.

To distinguish hVISA/VISA from VSSA, a local ML model can be established using the proposed strategy, and local clinical microbiologists can easily acquire ML models that adequately fit their own population. The prevalence of hVISA differs among countries and areas (Zhang et al., 2015). Up to 50% of isolates reported as susceptible to vancomycin can harbor hVISA clones (Horne et al., 2009). Moreover, the composition of isolates potentially varies among different areas. Consequently, a localized ML model trained by locally relevant data would offer superior performance to a general model. The strengths of the ML models proposed in this study are their rapidness and low cost. A vancomycin susceptibility test report could be obtained using MALDI-TOF MS alone without other testing methods. Clinical microbiologists could provide preliminary

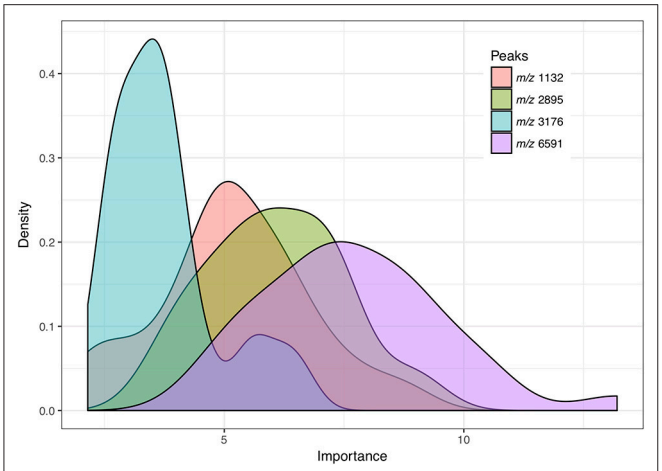


FIGURE 3 | Distribution of importance based on kernel density estimation of peak features selected by more than 90% of the predictive models. Importance: z-score of mean decrease in accuracy obtained from the random forest algorithm.

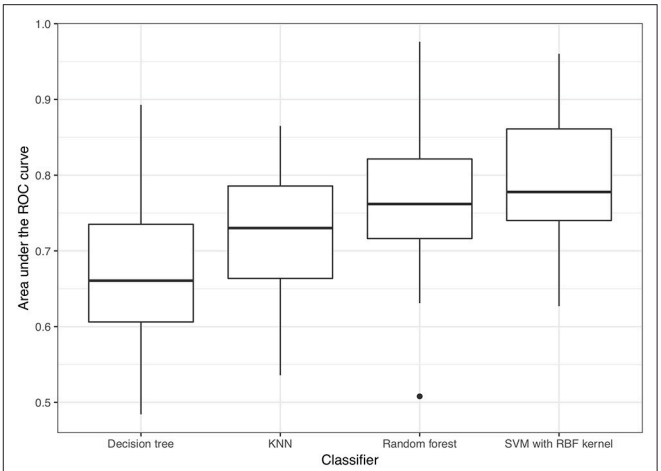


FIGURE 4 | Performance of predictive models for distinguishing VSSA from hVISA/VISA isolates. KNN: k-nearest neighbor; SVM, support vector machine; RBF kernel, radial basis function kernel.

but accurate vancomycin susceptibility days prior to PAP-AUC, which is regarded as time-consuming and expensive. Although various other hVISA screening tools have been developed, these methods are typically culture-dependent and require a long incubation time (Riederer et al., 2011; van Hal et al., 2011). Moreover, the ML models do not require additional hVISA screening tests such as glycopeptide resistance detection, the Etest macromethod, or brain heart infusion screening agar plate screening to report hVISA. Therefore, the cost of diagnosis could be considerably decreased. The MALDI-TOF MS used in this study was performed with direct deposit of bacteria onto a steel plate rather than extracting it and placing in a tube. Direct deposition is used in routine practice because it is rapid and not labor-intensive. We used typical sample processing methods so that the proposed ML models could cope with MALDI-TOF MS data in real-world applications.

Incorporating ML algorithms into prediction of antibiotics susceptibility is a promising application of ML. However, its associated issues have not been widely addressed. One study reported detection of hVISA/VISA using ML to analyze MALDI-TOF MS data. The authors used an SVM and correctly identified 100% of VISA and 97% of VSSA isolates with an overall classification accuracy of 98% (Mather et al., 2016). The performance was promising, and the authors also demonstrated that the performance did not result from the specific composition of the bacterial isolates (Mather et al., 2016). However, bias may still have existed, because only 21 VISA, 21 hVISA, and 38 VSSA isolates were used in the study. Moreover, the feature selection (essential peak selection) and model optimization steps appeared to be conducted within all the datasets, not within an independent training dataset, which may have resulted in overfitting and thus perfect performance. Another study also detailed a promising model with 99% sensitivity and 88% specificity for classifying VSSA, VISA, and hVISA (Asakura et al., 2018). The study provided a graphical user interface with fully public release code, which could truly benefit health care and research teams. However, due to the study's selection of multiple colonies from one hVISA strain and the use of leave-one-out validation, the model was also likely to be overfitted. Given the high fidelity of MALDI-TOF MS (Croxatto et al., 2012), we did not replicate each isolate by performing multiple MALDI-TOF analyses as did by other study (Asakura et al., 2018). Oversampling by direct replicating the isolates may result in overfitting bias (Kubat and Matwin, 1997; Kegelmeyer et al., 2002; Guo et al., 2008). By contrast, we used nested cross-validation to avoid overfitting. The feature selection step and model tuning were conducted within an independent training dataset in each iteration (**Figure 2**). Consequently, the selected feature compositions were different (**Supplementary Table 2**). The importance of the features could be determined by their frequency of occurrence in the nested cross-validation. As shown in **Supplementary Table 2** and **Figure 3**, the ions at m/z 1132, 2895, 3176, and 6591 were selected as the essential peaks and were selected in more than 90% of the predictive models. The ions at m/z 6887 and 9625, were selected with moderate frequency (**Supplementary Table 2**), whereas the ion at m/z 3006 was selected as an essential peak in only a few iterations (**Supplementary Table 2**). The results indicated the necessity of selecting features using an independent training dataset. A peak may be mistaken as an essential peak when iteration is not used. We confirmed the importance of characteristic peaks by using nested cross-validation. In this work, we analyzed the region from 0 to 20000 m/z because we did not presume that a characteristic peak cannot be found under 2000 m/z. We just included all the data and discover meaningful information by a data mining technique (i.e., feature selection process in this study). In most of studies, region 2000 to 20000 m/z was used for analysis, and some irregular peaks from the agar medium may show up within the region below 2000 m/z. To avoid an irregular peak being selected as a characteristic peak, random forest algorithm was applied to estimate the importance of each peak in discriminating VSSA

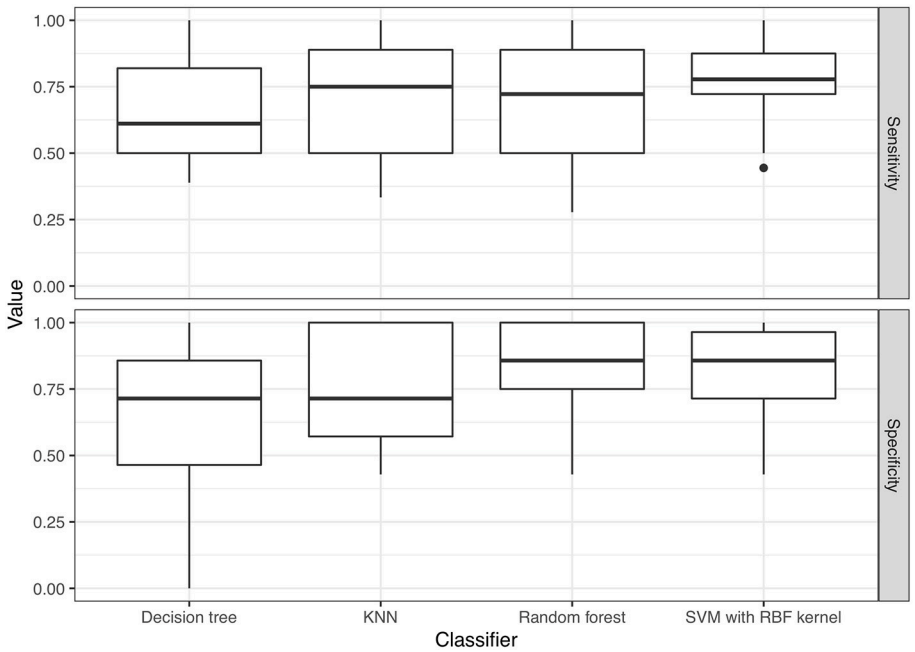


FIGURE 5 | Sensitivity and specificity of the predictive models, calculated based on the maximum value of Youden's J statistic. KNN, k-nearest neighbor; SVM, support vector machine; RBF kernel, radial basis function kernel.

from hVISA/VISA, under the scheme of nested cross validation (**Figure 2**). Characteristic peaks would be selected through the unbiased method.

The ions at m/z 1132, 2895, 3176, and 6591 were the crucial features in distinguishing VSSA from hVISA/VISA in the present study (**Supplementary Table 2** and **Figure 3**). Lu et al. reported that the ions at m/z 1835 and 1863 were characteristic peaks for hVISA and VISA (Lu et al., 2012). However, Mather et al. revealed that ions at m/z 4540 and 8258 were characteristic for VISA and VSSA, respectively (Mather et al., 2016). This discordance may be due to several reasons. First, the bacterial isolates were acquired from different locations and at different times. Second, the extraction methods were also different; tube extraction was used in these two studies (Lu et al., 2012; Mather et al., 2016), whereas we used direct deposition, which is the method used in routine practice. Third, the aforementioned difference in the methods of selecting essential peaks could also account for the discordance. In the previous studies, the characteristic peaks were selected on the basis of either descriptive statistics (Lu et al., 2012) or multiple regression (Mather et al., 2016). By contrast, we selected characteristic peaks by using random forest feature importance and confirmed the importance of the peaks in multiple iterations. In the present study, the ion at m/z 6591 was detected in 85.7 and 41.1% of the hVISA/VISA and VSSA groups, respectively. Previous studies have demonstrated m/z 6591 as a characteristic peak of clonal complex 8 (CC8) MRSA isolates (Wolters et al., 2011; Boggs et al., 2012; Josten et al., 2013; Camoez et al., 2016). In the first study conducted by Wolters et al. a model was demonstrated with the ability to discriminate five major CCs (CC5, CC8, CC22, CC30, and CC45) by using 13 peaks, including m/z 6591, which appeared to be specific to CC8 isolates (Wolters et al., 2011). In another study, m/z 6591 was adopted as one of the three peaks of a classifier constructed from 47 USA300/CC8 and 77 non-USA300 MRSA isolates. The classifier had an 87.9% overall accuracy on a validation dataset (Boggs et al., 2012). In 2013, Josten et al. analyzed the peak pattern of 401 MRSA and MSSA strains, revealing that the peak protein at m/z 6592 provided a sensitivity of 0.889 and specificity of 0.996 for CC8 (Josten et al., 2013). In 2016, a supervised neural network model constructed by Camoez et al. on the basis of data covering a 20 years period suggested m/z 6591.84 as a unique biomarker of CC8 isolates (Camoez et al., 2016). In our data, m/z 6591 was also noted in 56 of 62 CC8 and ST239 strains (90.3%). Our results are consistent with those of previous studies conducted in Europe and the United States, which suggests that despite geographical and racial diversity, peak protein m/z 6591 can provide valuable classification information regarding MRSA in Asian populations. Although ions at m/z 1132, 2895, and 3176 were also selected as informative features in the present study, the significance and relation of these features in the resistance of VISA and hVISA have not yet been reported.

This study had several limitations. First, bacterial composition affected the performance of the ML models. The performance of ML can be compromised by a complex bacterial composition. In this study, the bacterial composition of the isolates was analyzed using multilocus sequence and *SCCmec* typing. The bacterial composition results revealed a non-restricted bacterial distribution, for which classification problems are not generally simple (**Supplementary Figure 1**). The ML models and results may not be generalized directly to other countries or areas.

The MLS type of most MRSA isolates in this study are ST239 (62/125), followed by ST5 and ST59 (**Supplementary Figure 1**). This is the distinct composition of MRSA isolates in Taiwan (Sheng et al., 2009), and the characteristic peaks and the models created based on the cohort may be only used for the population in this region. In this study, we demonstrated a ML-based methodology for detecting hVISA/VISA. Through using the workflow proposed in this study, other clinical microbiology laboratories could obtain their own ML models specific for detecting hVISA/VISA in their region. We did not aim to and may not possibly generalize the ML models but we proposed a methodology which may help others generating a specific model fitting their populations more properly than do a generalized model. Second, the ML performance reported in this study is not as high as that reported in other studies that evaluated model performance using leave-one-out cross-validation (Rishishwar et al., 2014; Mather et al., 2016). This lower performance may have resulted from the stricter validation method applied in the present study. We used direct deposition instead of in-tube extraction. The direct deposition method offers a rapid turnaround time and requires less labor; however, the reproducibility and quality of MALDI-TOF MS data may be compromised (Goldstein et al., 2013; Mather et al., 2016). Compromised MALDI-TOF data may reduce ML model performance because non-susceptible *S. aureus* is relatively rare (10^{-5}-10^{-6}) in hVISA (van Hal and Paterson, 2011); more sensitive MALDI-TOF data could facilitate detection of subtle changes during MS. Third, although the performance of the ML models was validated using a minimally biased method, the models should undergo external validation in other Taiwanese institutes. Fourth, the primary aim of this study is to demonstrate and validate an unbiased methodology to detect hVISA/VISA by analyzing MALDI-TOF MS spectra through a ML-based approach. We focus more on the aspect of clinical application in this work. The validated ML model is ready to be used in our clinical practice and hopefully the proposed method can help generate clinically useful ML model in other local clinical microbiology laboratories. Besides, identifying protein/peptide behind the peaks is essential for understanding the causative proteins/mechanisms for vancomycin resistance, which is worthy further investigation in the future. In general, the present study successfully demonstrated the use of an ML approach for detecting hVISA/VISA. The negative predictive value of detecting vancomycin-non-susceptible *S. aureus* was 0.9695 when the prevalence of hVISA was 10%. Additionally, the absolute reduction of risk of administering inadequate glycopeptide dose in treating vancomycin-non-susceptible *S. aureus* was 0.0695 under the prevalence setting.

In conclusion, the proposed ML models, validated by a robust model evaluation method, successfully distinguished emerging superbugs (hVISA/VISA) from VSSA, which cannot be detected in most clinical microbiology laboratories. By utilizing cost-effective MALDI-TOF and ML technologies, providers have the opportunity to offer rapid and accurate treatment for MRSA.

AUTHOR CONTRIBUTIONS

H-YW and Y-JT had full access to all the data in the study and take responsibility for the integrity of the data, and the accuracy of the data analysis. H-YW and Y-JT analyzed/interpreted the data, performed experiments, designed the study, and wrote the paper. C-HC, T-YL, J-TH, T-PL, and J-JL reviewed/edited the manuscript for important intellectual content and provided administrative, technical, or material support. Y-JT and J-JL obtained funding and supervised the study.

FUNDING

This work was supported by Chang Gung Memorial Hospital (CMRPG3F1722, CMRPD3G0111, CORPG3H0451, and CORPG3H0431) and the Ministry of Science and Technology, Taiwan (MOST 104-2320-B-182A-005-MY3 and MOST 107-2636-E-182-001).

ACKNOWLEDGMENTS

This manuscript was edited by Wallace Academic Editing.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.02393/full#supplementary-material

REFERENCES

Asakura, K., Azechi, T., Sasano, H., Matsui, H., Hanaki, H., Miyazaki, M., et al. (2018). Rapid and easy detection of low-level resistance to vancomycin in methicillin-resistant *Staphylococcus aureus* by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. *PLoS ONE* 13:e0194212. doi: 10.1371/journal.pone.0194212

Boggs, S. R., Cazares, L. H., and Drake, R. (2012). Characterization of a *Staphylococcus aureus* USA300 protein signature using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *J. Med. Microbiol.* 61(Pt 5), 640–644. doi: 10.1099/jmm.0.037978-0

Breiman, L. (2001). Random forests. *Mach. Learn.* 45, 5–32. doi: 10.1023/A:1010933404324

Breiman, L., Friedman, J., Stone, C. J., and Olshen, R. A. (1984). *Classification and Regression Trees*. New York, NY: Routledge; Taylor and Francis.

Camoez, M., Sierra, J. M., Dominguez, M. A., Ferrer-Navarro, M., Vila, J., and Roca, I. (2016). Automated categorization of methicillin-resistant *Staphylococcus aureus* clinical isolates into different clonal complexes by MALDI-TOF mass spectrometry. *Clin. Microbiol. Infect.* 22, 161 e1–e7. doi: 10.1016/j.cmi.2015.10.009

Chang, S. C., Liu, T. P., Chen, C. J., Lin, L. C., and Lu, J. J. (2015). Detection of heterogeneous vancomycin-intermediate *Staphylococcus aureus* isolates using a combination of delta-hemolysis assay and Etest. *Diagn. Microbiol. Infect. Dis.* 81, 246–250. doi: 10.1016/j.diagmicrobio.2014.12.006

CLSI. (2017). *Performance Standards for Antimicrobial Susceptibility Testing. 27th Edn*. CLSI supplement M100. Wayne, PA: Clinical and Laboratory Standards Institute.

Cortes, C., and Vapnik, V. (1995). Support-vector networks. *Mach. Learn.* 20, 273–297. doi: 10.1007/BF00994018

Croxatto, A., Prod'hom, G., and Greub, G. (2012). Applications of MALDI-TOF mass spectrometry in clinical diagnostic microbiology. *FEMS Microbiol. Rev.* 36, 380–407. doi: 10.1111/j.1574-6976.2011.00298.x

Enright, M. C., Day, N. P., Davies, C. E., Peacock, S. J., and Spratt, B. G. (2000). Multilocus sequence typing for characterization of methicillin-resistant and methicillin-susceptible clones of *Staphylococcus aureus*. *J. Clin. Microbiol.* 38, 1008–1015.

Filzmoser, P., Liebmann, B., and Varmuza, K. (2009). Repeated double cross validation. *J Chemometr.* 23, 160–171. doi: 10.1002/cem.1225

Fong, R. K., Low, J., Koh, T. H., and Kurup, A. (2009). Clinical features and treatment outcomes of vancomycin-intermediate *Staphylococcus aureus* (VISA) and heteroresistant vancomycin-intermediate *Staphylococcus aureus* (hVISA) in a tertiary care institution in Singapore. *Eur. J. Clin. Microbiol. Infect. Dis.* 28, 983–987. doi: 10.1007/s10096-009-0741-5

Goldstein, J. E., Zhang, L., Borror, C. M., Rago, J. V., and Sandrin, T. R. (2013). Culture conditions and sample preparation methods affect spectrum quality and reproducibility during profiling of *Staphylococcus aureus* with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Lett. Appl. Microbiol.* 57, 144–150. doi: 10.1111/lam.12092

Guo, X., Yin, Y., Dong, C., Yang, G., and Zhou, G. (2008). "On the class imbalance problem," in *Proceedings of the Fourth International Conference on Natural Computation* (Jinan: IEEE), 192–201. doi: 10.1109/ICNC.2008.871

Horne, K. C., Howden, B. P., Grabsch, E. A., Graham, M., Ward, P. B., Xie, S., et al. (2009). Prospective comparison of the clinical impacts of heterogeneous vancomycin-intermediate methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-susceptible MRSA. *Antimicrob. Agents Chemother.* 53, 3447–3452. doi: 10.1128/AAC.01365-08

Howden, B. P., Davies, J. K., Johnson, P. D., Stinear, T. P., and Grayson, M. L. (2010). Reduced vancomycin susceptibility in *Staphylococcus aureus*, including vancomycin-intermediate and heterogeneous vancomycin-intermediate strains: resistance mechanisms, laboratory detection, and clinical implications. *Clin. Microbiol. Rev.* 23, 99–139. doi: 10.1128/CMR.00042-09

Hrabák, J., Chudácková, E., and Walková, R. (2013). Matrix-assisted laser desorption ionization-time of flight (maldi-tof) mass spectrometry for detection of antibiotic resistance mechanisms: from research to routine diagnosis. *Clin. Microbiol. Rev.* 26, 103–114. doi: 10.1128/CMR.00058-12

Hu, H. C., Kao, K. C., Chiu, L. C., Chang, C. H., Hung, C. Y., Li, L. F., et al. (2015). Clinical outcomes and molecular typing of heterogenous vancomycin-intermediate *Staphylococcus aureus* bacteremia in patients in intensive care units. *BMC Infect. Dis.* 15:444. doi: 10.1186/s12879-015-1215-2

Huang, S. H., Chen, Y. C., Chuang, Y. C., Chiu, S. K., Fung, C. P., Lu, P. L., et al. (2016). Prevalence of vancomycin-intermediate *Staphylococcus aureus* (VISA) and heterogeneous VISA among methicillin-resistant S. aureus with high vancomycin minimal inhibitory concentrations in Taiwan: a multicenter surveillance study, 2012-2013. *J. Microbiol. Immunol. Infect.* 49, 701–707. doi: 10.1016/j.jmii.2015.07.003

Idelevich, E. A., Sparbier, K., Kostrzewa, M., and Becker, K. (2017). Rapid detection of antibiotic resistance by MALDI-TOF mass spectrometry using a novel direct-on-target microdroplet growth assay. *Clin. Microbiol. Infect.* 24, 738–743. doi: 10.1016/j.cmi.2017.10.016

Josten, M., Reif, M., Szekat, C., Al-Sabti, N., Roemer, T., Sparbier, K., et al. (2013). Analysis of the matrix-assisted laser desorption ionization-time of flight mass spectrum of *Staphylococcus aureus* identifies mutations that allow differentiation of the main clonal lineages. *J. Clin. Microbiol.* 51, 1809–1817. doi: 10.1128/JCM.00518-13

Kegelmeyer, W. P., Hall, L. O., Bowyer, K. W., and Chawla, N. V. (2002). SMOTE: Synthetic Minority Over-sampling Technique. *J. Artific. Intell. Res.* 16:321–357. doi: 10.1613/jair.953

Kondo, Y., Ito, T., Ma, X. X., Watanabe, S., Kreiswirth, B. N., Etienne, J., et al. (2007). Combination of multiplex PCRs for staphylococcal cassette chromosome mec type assignment: rapid identification system for mec, ccr, and major differences in junkyard regions. *Antimicrob. Agents Chemother.* 51, 264–274. doi: 10.1128/AAC.00165-06

Krstajic, D., Buturovic, L. J., Leahy, D. E., and Thomas, S. (2014). Cross-validation pitfalls when selecting and assessing regression and classification models. *J. Cheminform.* 6:10. doi: 10.1186/1758-2946-6-10

Kubat, M., and Matwin, S. (1997). *Addressing the Curse of Imbalanced Training Sets: One-Sided Selection*. Nashville, TN: Icml.

Liaw, A., and Wiener, M. (2002). Classification and regression by randomForest. *R News* 2, 18–22. Available online at: https://www.r-project.org/doc/Rnews/Rnews_2002-3.pdf

Lin, L. C., Chang, S. C., Ge, M. C., Liu, T. P., and Lu, J. J. (2018). Novel single-nucleotide variations associated with vancomycin resistance in vancomycin-intermediate *Staphylococcus aureus*. *Infect. Drug Resist.* 11, 113–123. doi: 10.2147/IDR.S148335

Lin, W. Y., Chen, C. H., Tseng, Y. J., Tsai, Y. T., Chang, C. Y., Wang, H. Y., et al. (2018). Predicting post-stroke activities of daily living through a machine learning-based approach on initiating rehabilitation. *Int. J. Med. Inform.* 111, 159–164. doi: 10.1016/j.ijmedinf.2018.01.002

Liu, C., Bayer, A., Cosgrove, S. E., Daum, R. S., Fridkin, S. K., Gorwitz, R. J., et al. (2011). Clinical practice guidelines by the infectious Diseases Society Of America for the treatment of methicillin-resistant *Staphylococcus aureus* infections in adults and children. *Clin. Infect. Dis.* 52, e18-55. doi: 10.1093/cid/ciq146

Lu, J. J., Tsai, F. J., Ho, C. M., Liu, Y. C., and Chen, C. J. (2012). Peptide biomarker discovery for identification of methicillin-resistant and vancomycin-intermediate *Staphylococcus aureus* strains by MALDI-TOF. *Anal. Chem.* 84, 5685–5692. doi: 10.1021/ac300855z

Mather, C. A., Werth, B. J., Sivagnanam, S., SenGupta, D. J., and Butler-Wu, S. M. (2016). Rapid detection of vancomycin-intermediate *Staphylococcus aureus* by matrix-assisted laser desorption ionization-time of flight mass spectrometry. *J. Clin. Microbiol.* 54, 883–890. doi: 10.1128/JCM.02428-15

Meyer, D., Dimitriadou, E., Hornik, K., Weingessel, A., and Leisch, F. (2017). *e1071: Misc Functions of the Department of Statistics, Probability Theory Group (Formerly: E1071), TU Wien.* R package version 16-8. Available online at: https://cran.r-project.org/package=e1071

Riederer, K., Shemes, S., Chase, P., Musta, A., Mar, A., and Khatib, R. (2011). Detection of intermediately vancomycin-susceptible and heterogeneous *Staphylococcus aureus* isolates: comparison of Etest and Agar screening methods. *J. Clin. Microbiol.* 49, 2147–2150. doi: 10.1128/JCM.01435-10

Rishishwar, L., Petit, R. A. III, Kraft, C. S., and Jordan, I. K. (2014). Genome sequence-based discriminator for vancomycin-intermediate *Staphylococcus aureus*. *J. Bacteriol.* 196, 940–948. doi: 10.1128/JB.01410-13

Rybak, M. J., and Akins, R. L. (2001). Emergence of methicillin-resistant *Staphylococcus aureus* with intermediate glycopeptide resistance: clinical significance and treatment options. *Drugs* 61, 1–7. doi: 10.2165/00003495-200161010-00001

Sakoulas, G., Gold, H. S., Cohen, R. A., Venkataraman, L., Moellering, R. C., and Eliopoulos, G. M. (2006). Effects of prolonged vancomycin administration on methicillin-resistant *Staphylococcus aureus* (MRSA) in a patient with recurrent bacteraemia. *J. Antimicrob. Chemother.* 57, 699–704. doi: 10.1093/jac/dkl030

Satola, S. W., Farley, M. M., Anderson, K. F., and Patel, J. B. (2011). Comparison of detection methods for heteroresistant vancomycin-intermediate *Staphylococcus aureus*, with the population analysis profile method as the reference method. *J. Clin. Microbiol.* 49, 177–183. doi: 10.1128/JCM.01128-10

Sheng, W. H., Wang, J. T., Lauderdale, T. L., Weng, C. M., Chen, D., and Chang, S. C. (2009). Epidemiology and susceptibilities of methicillin-resistant *Staphylococcus aureus* in Taiwan: emphasis on chlorhexidine susceptibility. *Diagn. Microbiol. Infect. Dis.* 63, 309–313. doi: 10.1016/j.diagmicrobio.2008.11.014

Swan, A. L., Mobasheri, A., Allaway, D., Liddell, S., and Bacardit, J. (2013). Application of machine learning to proteomics data: classification and biomarker identification in postgenomics biology. *OMICS* 17, 595–610. doi: 10.1089/omi.2013.0017

Therneau, T., and Atkinson, B. (2018). *rpart: Recursive Partitioning and Regression Trees*. R package version 4.1–13. Available online at:https://CRAN.R-project.org/package=rpart

van Hal, S. J., and Paterson, D. L. (2011). Systematic review and meta-analysis of the significance of heterogeneous vancomycin-intermediate

Staphylococcus aureus isolates. *Antimicrob. Agents Chemother.* 55, 405–410. doi: 10.1128/AAC.01133-10

van Hal, S. J., Wehrhahn, M. C., Barbagiannakos, T., Mercer, J., Chen, D., Paterson, D. L., et al. (2011). Performance of various testing methodologies for detection of heteroresistant vancomycin-intermediate *Staphylococcus aureus* in bloodstream isolates. *J. Clin. Microbiol.* 49, 1489–1494. doi: 10.1128/JCM.02302-10

Varma, S., and Simon, R. (2006). Bias in error estimation when using cross-validation for model selection. *BMC Bioinformatics* 7:91. doi: 10.1186/1471-2105-7-91

Venables, W. N., and Ripley, B. D. (2002). *Modern Applied Statistics with S. 4th Edn*. New York, NY: Springer.

Wang, H. Y., Hsieh, C. H., Wen, C. N., Wen, Y. H., Chen, C. H., and Lu, J. J. (2016). Cancers screening in an asymptomatic population by using multiple tumour markers. *PLoS ONE* 11:e0158285. doi: 10.1371/journal.pone.0158285

Wang, H. Y., Lee, T. Y., Tseng, Y. J., Liu, T. P., Huang, K. Y., Chang, Y. T., et al. (2018). A new scheme for strain typing of methicillin-resistant *Staphylococcus aureus* on the basis of matrix-assisted laser desorption ionization time-of-flight mass spectrometry by using machine learning approach. *PLoS ONE* 13:e0194289. doi: 10.1371/journal.pone.0194289

Wolters, M., Rohde, H., Maier, T., Belmar-Campos, C., Franke, G., Scherpe, S., et al. (2011). MALDI-TOF MS fingerprinting allows for discrimination of major methicillin-resistant *Staphylococcus aureus* lineages. *Int. J. Med. Microbiol.* 301, 64–68. doi: 10.1016/j.ijmm.2010.06.002

Wootton, M. (2001). A modified population analysis profile (PAP) method to detect hetero-resistance to vancomycin in *Staphylococcus aureus* in a UK hospital. *J. Antimicrob. Chemother.* 47, 399–403. doi: 10.1093/jac/47.4.399

Zhang, S., Sun, X., Chang, W., Dai, Y., and Ma, X. (2015). Systematic review and meta-analysis of the epidemiology of vancomycin-intermediate and heterogeneous vancomycin-intermediate *Staphylococcus aureus* isolates. *PLoS ONE* 10:e0136082. doi: 10.1371/journal.pone.0136082

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

MINI REVIEW
published: 30 October 2018
doi: 10.3389/fmicb.2018.02577

MALDI-TOF MS Applications to the Detection of Antifungal Resistance: State of the Art and Future Perspectives

*Walter Florio[1], Arianna Tavanti[2], Emilia Ghelardi[1] and Antonella Lupetti[1]**

[1] *Dipartimento di Ricerca Traslazionale e delle Nuove Tecnologie in Medicina e Chirurgia, Università di Pisa, Pisa, Italy,*
[2] *Dipartimento di Biologia, Università di Pisa, Pisa, Italy*

MALDI-TOF MS technology has made possible revolutionary advances in the diagnosis of infectious diseases. Besides allowing rapid and reliable identification of bacteria and fungi, this technology has been recently applied to the detection of antimicrobial resistance. Several approaches have been proposed and evaluated for application of MALDI-TOF MS to antimicrobial susceptibility testing of bacteria, and some of these have been or might be applied also to yeasts. In this context, the comparison of proteomic profiles of bacteria/yeasts incubated with or without antimicrobial drugs is a very promising method. Another recently proposed MALDI-TOF MS-based approach for antifungal susceptibility testing is the application of the semi-quantitative MALDI Biotyper antibiotic susceptibility test rapid assay, which was originally designed for antimicrobial susceptibility testing of bacteria, to yeast isolates. Increasingly effective and accurate MS tools and instruments as well as the possibility to optimize analytical parameter settings for targeted applications have generated an expanding area in the field of clinical microbiology diagnostics, paving the way for the development and/or optimization of rapid methods for antifungal susceptibility testing in the near future. In the present study, the state of the art of MALDI-TOF MS applications to antifungal susceptibility testing is reviewed, and cutting-edge developments are discussed, with a particular focus on methods allowing rapid detection of drug resistance in pathogenic fungi causing systemic mycoses.

Keywords: MALDI-TOF mass spectrometry, antimicrobial resistance, antifungal susceptibility testing, blood culture, rapid AFST

OPEN ACCESS

Edited by:
Karsten Becker,
Universität Münster, Germany

Reviewed by:
Jozsef Soki,
University of Szeged, Hungary
Giovanni Gherardi,
Università Campus Bio-Medico, Italy

***Correspondence:**
Antonella Lupetti
antonella.lupetti@med.unipi.it

Specialty section:
This article was submitted to Antimicrobials, Resistance and Chemotherapy, a section of the journal Frontiers in Microbiology

Received: *21 June 2018*
Accepted: *09 October 2018*
Published: *30 October 2018*

Citation:
Florio W, Tavanti A, Ghelardi E and Lupetti A (2018) MALDI-TOF MS Applications to the Detection of Antifungal Resistance: State of the Art and Future Perspectives. Front. Microbiol. 9:2577. doi: 10.3389/fmicb.2018.02577

INTRODUCTION

During the last two decades, MALDI-TOF MS technology has rapidly evolved toward new applications in the field of clinical diagnostic microbiology, generating a novel, robust and accurate tool for rapid identification of bacteria and yeasts (Kliem and Sauer, 2012; Richter et al., 2012; Spanu et al., 2012; Szabados et al., 2012; Idelevich et al., 2014; Barnini et al., 2015;

Florio et al., 2018b). In parallel, major efforts have been made to expand and apply the enormous potentialities of this technology to another important issue: testing resistance and, in some cases, susceptibility of microorganisms to antimicrobial drugs. The emergence and diffusion of bacterial and fungal pathogens resistant to multiple drugs and the high incidence of hospital-acquired bloodstream infections (BSIs) have made the purpose of developing new methods for rapid and reliable antimicrobial susceptibility testing an urgent issue. In this context, considerable efforts have been made in order to apply MALDI-TOF MS technology to the detection of antimicrobial resistance, especially for multi-drug resistant (MDR) microorganisms causing systemic infections (Nordmann et al., 2011; Cantòn et al., 2012; Patel and Bonomo, 2013; Lamoth et al., 2018), which represent a major concern for public health (Walsh, 2010; Walsh and Gamaletsou, 2013; Lee et al., 2016; Barchiesi et al., 2017). Several MALDI-TOF MS-based approaches have been attempted for the detection of antibiotic resistance in bacteria. These include: (i) the assessment of β-lactamase activity by MALDI-TOF MS detection of hydrolysis products of β-lactam/carbapenem antibiotics (Hooff et al., 2012; Hrabák et al., 2012; Jung et al., 2014; Oviano et al., 2016); (ii) identification in mass spectra of genes (Lau et al., 2014; Rodríguez-Baño et al., 2018) or peptides specifically involved in antibiotic resistance mechanisms (Gaibani et al., 2016; Chang et al., 2018); (iii) identification of biomarkers somehow correlated with, though not responsible for drug resistance, such as the phenol-soluble protein toxin (PSM-mec), produced by a subset of methicillin-resistant *Staphylococcus aureus* strains (Chatterjee et al., 2011), and detectable by MALDI-TOF as a 2415 ± 2.00 m/z peak (Rhoads et al., 2016); and (iv) assays based on discrimination of mass spectra of resistant from susceptible microbial isolates after exposure to breakpoint concentrations of antimicrobial agents directly on the target plate for MALDI-TOF MS analysis (Idelevich et al., 2017). This latter approach seems very promising since, theoretically, it might be applied to any microbial species and antimicrobial agent independently from the underlying resistance mechanisms, and it may be suitable for laboratory automation and simultaneous testing of a panel of different antimicrobial drugs.

As multi-drug resistance is an increasing widespread problem also in fungal infections, there is a pressing need for rapid methods allowing to obtain timely and reliable information on antifungal susceptibility/resistance of fungal infectious agents, especially those causing systemic mycoses. In spite of advances in diagnosis and treatment, the incidence of invasive fungal infections has dramatically increased during the last two decades (Barchiesi et al., 2016; Benedict et al., 2017; Cornely et al., 2017; Guo et al., 2017; Pana et al., 2017), with only a few classes of antifungal drugs being available (Pfaller, 2012; Pierce and Lopez-Ribot, 2013; Patil and Majumdar, 2017). The growing elderly population, frequently suffering from co-morbidities, high colonization rate by *Candida albicans*, immunosuppressive treatment after organ transplantation, and prolonged antibiotic therapies have substantially contributed to such an increase in fungal infections (Guimarães et al., 2012; Wang et al., 2014; Barchiesi et al., 2017). Although *C. albicans* remains the most frequently isolated species, other fungal pathogens have been isolated with remarkably increasing frequency; these include *Candida glabrata* (Cleveland et al., 2012; Pfaller et al., 2014), the emerging pathogen *Candida auris* (Bao et al., 2018; Kohlenberg et al., 2018; Kordalewska et al., 2018), *Trichosporon* spp. (Davies and Thornton, 2014), and filamentous fungi, such as *Scedosporium* spp., mucoralean fungi, and *Fusarium* spp. (Walsh and Gamaletsou, 2013; Davuodi et al., 2015).

Systemic infections sustained by *Candida* spp. as well as by other fungal pathogens are associated with mortality rates that can be higher than 60%, depending on the patient category (Kett et al., 2011; Kollef et al., 2012; Barchiesi et al., 2016, 2017; Guo et al., 2017). Since timely administration of effective antifungal therapy is of vital importance for the outcome of patients affected by these infections (Garey et al., 2006) and susceptibility profiles to antifungal agents vary greatly among fungi, rapid species identification and antifungal susceptibility testing (AFST) is fundamental to reduce mortality and improve patients' outcome. MALDI-TOF MS, extensively used for identification of bacteria, has been increasingly proposed also for rapid identification of fungal pathogens directly in positive BCs (Ferreira et al., 2011; Yan et al., 2011; Spanu et al., 2012; Idelevich et al., 2014; Vecchione et al., 2018). Yeast identification by itself provides relevant clinical information since different fungal species may differ in virulence and drug resistance. For example, the antimicrobial susceptibility profile of *Candida parapsilosis* and *C. glabrata* can be quite different, with *C. parapsilosis* more frequently resistant to echinocandins and *C. glabrata* to azoles (Silva et al., 2012). Furthermore, invasive trichosporonosis is characterized by resistance to amphotericin and echinocandins, and poor prognosis (Miceli et al., 2011). Therefore, the ability to rapidly identify these yeasts may be useful to promptly streamline empirical antimicrobial therapy. However, the emergence and spread of MDR fungal pathogens (Lamoth et al., 2018) have posed a pressing need for rapid AFST. In general, echinocandins are widely used as empirical antifungal therapy for patients with candidemia, at least until AFST results become available; when resistance is detected, treatment with echinocandins needs to be immediately streamlined. Therefore, a rapid method for AFST may be of vital importance to achieve a favorable outcome, especially in critically ill patients. To this purpose, the application of MALDI-TOF MS to AFST has been attempted in the last few years (Vella et al., 2013; Saracli et al., 2015; Vatanshenassan et al., 2018). In this context, two methods have been proposed, which revealed potential for the development of rapid assays based on MALDI-TOF MS. The first is based on the comparative analysis of mass spectra of fungal isolates exposed to different concentrations of antifungal drugs (Vella et al., 2013, 2017; Saracli et al., 2015). The second relies on the application and optimization of the MALDI Biotyper antibiotic susceptibility test rapid assay (MBT ASTRA), originally developed for rapid antibiotic susceptibility testing in bacteria (Sparbier et al., 2016), to AFST (Vatanshenassan et al., 2018).

The present review provides a synthetic, updated overview of the proposed methods based on MALDI-TOF MS and aimed at

yielding rapid and accurate information regarding antimicrobial resistance of clinically relevant fungi.

ANTIFUNGAL SUSCEPTIBILITY TESTING BY COMPARISON OF COMPOSITE CORRELATION INDEXES

A method has been proposed for the detection of resistance to specific antifungal agents that relies on the comparison of MALDI-TOF MS spectra of yeast strains after incubation with high, intermediate or null antifungal concentrations (De Carolis et al., 2012; Vella et al., 2013, 2017; Saracli et al., 2015; Sanguinetti and Posteraro, 2016). In particular, MALDI-TOF MS spectra of yeast isolates after exposure to high, intermediate or null drug concentrations are used to generate a composite correlation index (CCI). The intermediate drug concentration is the minimal concentration of the compound able to induce a detectable proteome change in the mass spectrum of a susceptible strain. When a susceptible strain is tested, the mass spectrum at the intermediate drug concentration is more similar to that at the high concentration; therefore, the CCI of the intermediate/null concentration is lower than that of the intermediate/high concentration. This method has been used to test the resistance of *C. albicans*, *C. tropicalis*, and *C. glabrata* to triazole drugs. The results showed essential agreement ranging between 54 and 97% for the MALDI-TOF method in comparison to conventional AFST. The wide variability in essential agreement was dependent on both the triazole drug and *Candida* species tested (Saracli et al., 2015). A limit of this method was that the time saving over the conventional AFST method was of modest entity (overnight versus 24 h).

In another study based on the same approach (Vella et al., 2013), resistance to caspofungin could be detected in 10/11 (90.6%) *C. albicans* clinical isolates after an incubation of only 3 h, and 51/51 (100%) isolates were correctly classified as susceptible. The single very major error (false susceptibility) related to an isolate with a low level of caspofungin resistance. Caspofungin selectively targets the fungal cell wall by inhibiting 1,3-β-glucan synthase. Due to its high efficacy and reduced cases of side effects and adverse events in comparison with other antifungal agents, caspofungin has become a front-line agent in the treatment of candidemia and other invasive fungal infections (Patil and Majumdar, 2017). However, resistance to caspofungin can arise because of mutations in the *FSK1* gene in *C. albicans* (Balashov et al., 2006), and *FSK1* and *FSK2* genes in *C. glabrata* (Pham et al., 2014). Due to the emergence of drug resistant yeast strains (Chen et al., 2011), rapid detection of caspofungin resistance in *Candida* spp. clinical isolates is essential for timely and appropriate treatment of systemic fungal infections.

Recently, the same method based on detection of changes in the protein spectrum after a 3-h incubation with antifungal drugs was used to test a panel of 80 *C. glabrata* clinical isolates against anidulafungin or fluconazole (Vella et al., 2017). In comparison to the reference method recommended by the Clinical and Laboratory Standards Institute [CLSI], 2012), 58/58 (100%) susceptible isolates were classified as susceptible, and 11/22 (50.0%) isolates in the resistant category were classified as resistant to anidulafungin. For fluconazole, 40/41 (97.6%) susceptible isolates were classified as susceptible, and 37/39 (94.9%) isolates in the resistant category were classified as resistant. Of interest, when the assay was repeated for the 11 resistant isolates giving very major error with longer incubation times (6, 9, and 12 h), only two errors for anidulafungin remained unresolved; with a 15-h incubation, 100% essential agreement was reached with both the antifungal drugs. Therefore, incubation time was a critical factor to achieve maximum accuracy and reliability of this assay, especially for anidulafungin.

In a recent study (Paul et al., 2018), an assay based on CCI analysis was used to evaluate fluconazole resistance in 15 fluconazole resistant (MICs ranging from 16 to 128 μg/mL) and 19 fluconazole susceptible *C. tropicalis* isolates (MIC $\leq$ 1 μg/mL) in comparison with the reference CLSI microdilution method (Clinical and Laboratory Standards Institute [CLSI], 2012). In India, *C. tropicalis* is the commonest agent causing candidemia, and fluconazole resistant strains have been increasingly isolated (Chakrabarti et al., 2015). Therefore, a rapid method allowing the detection of fluconazole resistance in *C. tropicalis* strains grown in BCs may be very helpful to promptly streamline antifungal therapy. The authors reported that spectral changes were detectable by visual inspection soon after 4-h exposure to high (128 μg/mL), intermediate (1 μg/mL) or null concentrations of fluconazole for all *C. tropicalis* isolates. For software-based analysis, the incubation time was optimized at 5 h with 4 μg/mL as intermediate drug concentration. Among the 34 isolates tested, the minimal profile change concentrations (MPCCs), i.e., the minimum drug concentration at which an alteration of mass spectra can be detected, coincided with the MICs for 16 isolates (4 resistant, 12 susceptible), whereas the MPCC was one twofold dilution lower than the corresponding MIC in the remaining 18 isolates (11 resistant, 7 susceptible). Categorical and essential agreement were observed for all 34 isolates, indicating that CCI analysis might be used as a rapid screening method for fluconazole resistance in *C. tropicalis* clinical isolates.

DETECTION OF CASPOFUNGIN RESISTANCE IN *C. albicans* AND *C. glabrata* BY MBT ASTRA

In a recent proof of concept study (Vatanshenassan et al., 2018) the detection of *C. albicans* and *C. glabrata* strains resistant to caspofungin was evaluated by MBT ASTRA. This is a MALDI-TOF-based semi-quantitative technique, which was originally designed for rapid antibiotic susceptibility testing in bacteria. MBT ASTRA is a phenotypic assay comparing growth of microorganisms after incubation in the absence or in the presence of different concentrations of antimicrobial drugs. Cell growth is inferred from the comparison of the area under the curve (AUC) of the MALDI-TOF MS spectra for the different incubation setup (with or without antimicrobial drug). The relative growth (RG) is calculated for each concentration of antimicrobial agent as the ratio of the AUC observed with or without drug exposure, and a RG cutoff discriminating resistance from susceptibility

is determined experimentally: strains showing RG above this cutoff are considered resistant, whereas those below the cutoff are considered susceptible. The MBT ASTRA results were compared with those obtained by the CLSI reference microdilution method (Clinical and Laboratory Standards Institute [CLSI], 2017) on 58 *C. albicans* and 57 *C. glabrata* clinical isolates. A categorical agreement of 100% was observed for 29 susceptible and 22 resistant strains of *C. albicans*. For seven *C. albicans* strains, insufficient growth was observed in the control setup (no caspofungin) and, therefore, these strains were excluded from further evaluation, resulting in a validity of the MBT ASTRA assay of 88%. For *C. glabrata*, 31 out of 33 strains categorized as resistant by the microdilution method resulted resistant also by MBT ASTRA, and four out of five susceptible strains were correctly detected. Eighteen out of 19 strains categorized as intermediate by the microdilution method resulted resistant by MBT ASTRA, while only one intermediate strain resulted susceptible. Therefore, a sensitivity of 94% and a specificity of 80% were observed using MBT ASTRA on *C. glabrata* strains. These results were obtained after a 6-h incubation of yeast cells with or without caspofungin. Time saving compared to the reference microdilution method was approximately 18 and 42 h for *C. albicans* and *C. glabrata*, respectively. The RG cutoff was set at 0.6 for both *C. albicans* and *C. glabrata*; breakpoint concentrations of 1 and 0.5 μg/mL caspofungin, discriminating susceptible from resistant strains, were determined for *C. albicans* and *C. glabrata*, respectively.

Overall, this approach seems promising, and it would be interesting to evaluate its performance in clinical settings, and the possibility to extend its application to the detection of resistance to other antifungal agents. In addition, it would be of great usefulness if it could be efficiently applied to yeasts directly recovered from positive BCs by lysis/centrifugation in Serum Separator Tubes (BD Vacutainer system) or using other suitable methods (Florio et al., 2018a). To this purpose, further studies might be necessary to optimize the analysis software and experimental conditions.

CONCLUSION

In this review, we summarized recently proposed methods and significant advances for rapid detection of antifungal resistance by MALDI-TOF MS with a particular focus on methods applicable to positive BCs, as schematically represented in **Figure 1**. The diagnosis of fungal BSIs by conventional methods is particularly time consuming, while in case of severe, life-threatening infections, such as systemic mycoses, timeliness and accuracy of test results are crucial factors for clinicians to decide and promptly administer an effective and targeted antifungal therapy. Conventional microdilution methods for AFST are generally very accurate but require long incubation times. Since mortality rates in systemic infections are strictly correlated with time between onset of symptoms and administration of effective therapy, rapid methods for AFST are strongly needed. Therefore, major research efforts have to be made to refine and optimize MALDI-TOF MS-based assays in order to obtain timely, accurate and reliable results. Because of the increasing diffusion of MDR fungal pathogens, methods that allow simultaneous susceptibility testing to different classes of antifungals are expected to represent a major focus of research in this field. Differently from bacteria, the existence of specific peaks associated with antimicrobial resistance in mass spectra of fungal pathogens has not been reported so far, leaving this possibility an open issue. In addition, the development of new analytical algorithms, automation of procedures, and optimization of assays are

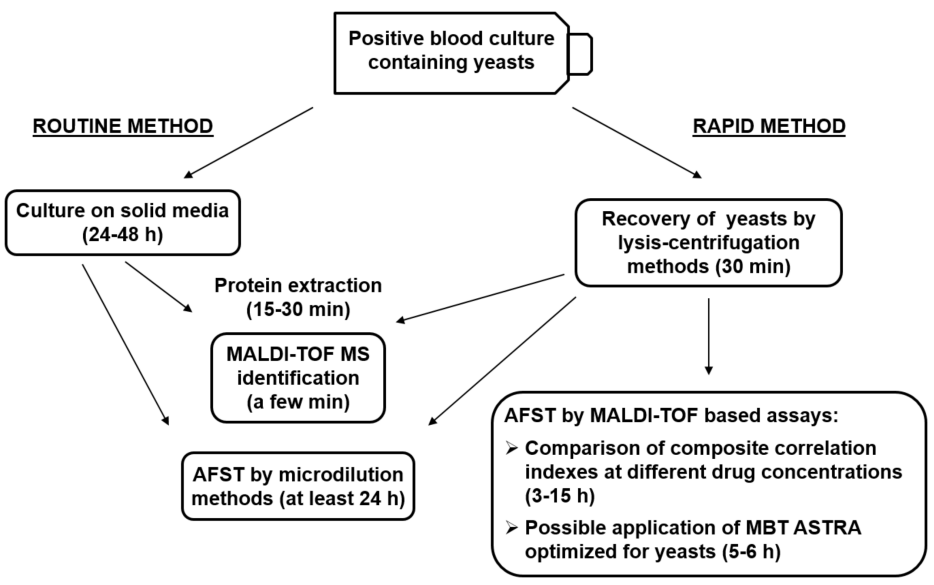


FIGURE 1 | Schematic representation of the routine and rapid methods for identification and antifungal susceptibility testing (AFST) of yeasts grown in monomicrobial blood cultures, and possible applications of newly described methods. MBT ASTRA, MALDI Biotyper antibiotic susceptibility test rapid assay.

expected to expand and refine the clinical applications of MALDI-TOF MS technology to AFST in the near future.

AUTHOR CONTRIBUTIONS

WF and AL contributed to the conception and design of the study. WF wrote the first draft of the manuscript. WF, AT, EG, and AL contributed to manuscript revision and read and approved the submitted version.

FUNDING

Financial support was obtained from the University of Pisa (Grant No. PRA_2017_18 n°18).

REFERENCES

Balashov, S., Park, S., and Perlin, D. S. (2006). Assessing resistance to the echinocandin antifungal drug caspofungin in *Candida albicans* by profiling mutations in *FKS1*. *Antimicrob. Agents Chemother.* 50, 2058–2063.

Bao, J. R., Master, R. N., Azad, K. N., Schwab, D. A., Clark, R. B., Jones, R. S., et al. (2018). Rapid, accurate identification of *Candida auris* by using a novel matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) database (library). *J. Clin. Microbiol.* 56:e01700-17. doi: 10.1128/JCM.01700-17

Barchiesi, F., Orsetti, E., Gesuita, R., Skrami, E., Manso, E., and The Candidemia Study Group (2016). Epidemiology, clinical characteristics, and outcome of candidemia in a tertiary referral center in Italy from 2010 to 2014. *Infection* 44, 205–213. doi: 10.1007/s15010-015-0845-z

Barchiesi, F., Orsetti, E., Mazzanti, S., Trave, F., Salvi, A., Nitti, C., et al. (2017). Candidemia in the elderly: what does it change? *PLoS One* 12:e0176576. doi: 10.1371/journal.pone.0176576

Barnini, S., Ghelardi, E., Brucculeri, V., Morici, P., and Lupetti, A. (2015). Rapid and reliable identification of Gram-negative bacteria and Gram-positive cocci by deposition of bacteria harvested from blood cultures onto the MALDI-TOF plate. *BMC Microbiol.* 15:124. doi: 10.1186/s12866-015-0459-8

Benedict, K., Richardson, M., Vallabhaneni, S., Jackson, B. R., and Chiller, T. (2017). Emerging issues, challenges, and changing epidemiology of fungal disease outbreaks. *Lancet Infect. Dis.* 17, e403–e411. doi: 10.1016/S1473-3099(17)30443-7

Cantòn, R., Akòva, M., Carmeli, Y., Giske, C. G., Glupczynski, Y., Gniadkowski, M., et al. (2012). The European network on Carbapenemases. Rapid evolution and spread of carbapenemases among *Enterobacteriaceae* in Europe. *Clin. Microbiol. Infect.* 18, 413–431. doi: 10.1111/j.1469-0691.2012.03821.x

Chakrabarti, A., Sood, P., Rudramurthy, S. M., Chen, S., Kaur, H., Capoor, M., et al. (2015). Incidence, characteristics and outcome of ICU-acquired candidemia in India. *Intensive Care Med.* 41, 285–295. doi: 10.1007/s00134-014-3603-2

Chang, K.-C., Chung, C.-Y., Yeh, C.-H., Hsu, K.-H., Chin, Y.-C., Huang, S.-S., et al. (2018). Direct detection of *carbapenemase-associated* proteins of *Acinetobacter baumannii* using nanodiamonds coupled with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *J. Microbiol. Meth.* 147, 36–42. doi: 10.1016/j.mimet.2018.02.014

Chatterjee, S. S., Chen, L., Joo, H. S., Cheung, G. Y., Kreiswirth, B. N., and Otto, M. (2011). Distribution and regulation of the mobile genetic element-encoded phenol-soluble modulin PSM-mec in methicillin-resistant *Staphylococcus aureus*. *PLoS One* 6:e28781. doi: 10.1371/journal.pone.0028781

Chen, S., Slavin, M. A., and Sorrell, T. C. (2011). Echinocandin antifungal drugs in fungal infections: a comparison. *Drugs* 71, 11–41. doi: 10.2165/11585270-000000000-00000

Cleveland, A. A., Farley, M. M., Harrison, L. H., Stein, B., Hollick, R., Lockhart, S. R., et al. (2012). Changes in incidence and antifungal drug resistance in candidemia: results from population-based laboratory surveillance in Atlanta and Baltimore, 2008–2011. *Clin. Infect. Dis.* 55, 1352–1361. doi: 10.1093/cid/cis697

Clinical and Laboratory Standards Institute [CLSI] (2012). *Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts Informational Supplement*, 4th Edn. Wayne, PA: Clinical and Laboratory Standards Institue.

Clinical and Laboratory Standards Institute [CLSI] (2017). *Performance Standards for Antifungal Susceptibility Testing of Yeasts; Approved Standard, CLSI Document M60*, 1st Edn. Wayne, PA: Clinical and Laboratory Standards Institue.

Cornely, O. A., Lass-Flörl, C., Lagrou, K., Arsic-Arsenijevic, V., and Hoenigl, M. (2017). Improving outcome of fungal diseases - Guiding experts and patients towards excellence. *Mycoses* 60, 420–425. doi: 10.1111/myc.12628

Davies, G. E., and Thornton, C. R. (2014). Differentiation of the emerging human pathogens *Trichosporon asahii* and *Trichosporon asteroides* from other pathogenic yeasts and moulds by using species-specific monoclonal antibodies. *PLoS One* 9:e84789. doi: 10.1371/journal.pone.0084789

Davuodi, S., Manshadi, S. A., Salehi, M. R., Yazdi, F., Khazravi, M., and Fazli, J. T. (2015). Fatal cutaneous mucormycosis after kidney transplant. *Exp. Clin. Transplant.* 13, 82–85. doi: 10.6002/ect.2013.0216

De Carolis, E., Vella, A., Florio, A. R., Posteraro, P., Perlin, D. S., Sanguinetti, M., et al. (2012). Use of matrix-assisted laser desorption ionization-time of flight mass spectrometry for caspofungin susceptibility testing of *Candida* and *Aspergillus* species. *J. Clin. Microbiol.* 50, 2479–2483. doi: 10.1128/JCM.00224-12

Ferreira, L., Sanchez-Juanes, F., Porras-Guerra, I., Garcia-Garcia, M. I., Garcia-Sanchez, J. E., González-Buitrago, J. M., et al. (2011). Microorganisms direct identification from blood culture by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Clin. Microbiol. Infect.* 17, 546–551. doi: 10.1111/j.1469-0691.2010.03257.x

Florio, W., Morici, P., Ghelardi, E., Barnini, S., and Lupetti, A. (2018a). Recent advances in the microbiological diagnosis of bloodstream infections. *Crit. Rev. Microbiol.* 44, 351–370. doi: 10.1080/1040841X.2017.1407745

Florio, W., Tavanti, A., Barnini, S., Ghelardi, E., and Lupetti, A. (2018b). Recent advances and ongoing challenges in the diagnosis of microbial infections by MALDI-TOF mass spectrometry. *Front. Microbiol.* 9:1097. doi: 10.3389/fmicb.2018.01097

Gaibani, P., Galea, A., Fagioni, M., Ambretti, S., Sambri, V., and Landini, M. P. (2016). Evaluation of matrix-assisted laser desorption ionization–time of flight mass spectrometry for identification of KPC-producing *Klebsiella pneumoniae*. *J. Clin. Microbiol.* 54, 2609–2613. doi: 10.1128/JCM.01242-16

Garey, K. W., Rege, M., Pai, M. P., Mingo, D. E., Suda, K. J., Turpin, R. S., et al. (2006). Time to initiation of fluconazole therapy impacts mortality in patients with candidemia: a multi-institutional study. *Clint. Infect. Dis.* 43, 25–31.

Guimarães, T., Nucci, M., Mendonça, J. S., Martinez, R., Brito, L. R., Silva, N., et al. (2012). Epidemiology and predictors of a poor outcome in elderly patients with candidemia. *Int. J. Infect. Dis.* 16, e442–e447. doi: 10.1016/j.ijid.2012.02.005

Guo, F., Yang, Y., Kang, Y., Zang, B., Cui, W., Qin, B., et al. (2017). China-SCAN Team. Invasive candidiasis in intensive care units in China: a multicentre prospective observational study. *J. Antimicrob. Chemother.* 68, 1660–1668. doi: 10.1093/jac/dkt083

Hooff, G. P., van Kampen, J. J., Meesters, R. J., van Belkum, A., Goessens, W. H., and Luider, T. M. (2012). Characterization of β-lactamase enzyme activity in bacterial lysates using MALDI-mass spectrometry. *J. Proteome Res.* 11, 79–84. doi: 10.1021/pr200858r

Hrabák, J., Studentová, V., Walková, R., Zemlicková, H., Jakubu, V., Chudácková, E., et al. (2012). Detection of NDM-1, VIM-1, KPC, OXA-48, and OXA-162 carbapenemases by matrix-assisted laser desorption ionization-time of flight mass spectrometry. *J. Clin. Microbiol.* 50, 2441–2443. doi: 10.1128/JCM.01002-12

Idelevich, E. A., Grunewald, C. M., Wüllenweber, J., and Becker, K. (2014). Rapid identification and susceptibility testing of *Candida* spp. from positive blood cultures by combination of direct MALDI-TOF mass spectrometry and direct inoculation of Vitek 2. *PLoS One* 9:e114834. doi: 10.1371/journal.pone.0114834

Idelevich, E. A., Sparbier, K., Kostrzewa, M., and Becker, K. (2017). Rapid detection of antibiotic resistance by MALDI-TOF mass spectrometry using a novel direct-on-target microdroplet growth assay. *Clin. Microbiol. Infect.* 24, 738–743. doi: 10.1016/j.cmi.2017.10.016

Jung, J. S., Popp, C., Sparbier, K., Lange, C., Kostrzewa, M., and Schubert, S. (2014). Evaluation of matrix-assisted laser desorption ionization-time of flight mass spectrometry for rapid detection of β-lactam resistance in *Enterobacteriaceae* derived from blood cultures. *J. Clin. Microbiol.* 52, 924–930. doi: 10.1128/JCM.02691-13

Kett, D. H., Azoulay, E., Echeverria, P. M., and Vincent, J. L. (2011). *Candida* bloodstream infections in intensive care units: analysis of the extended prevalence of infection in intensive care unit study. *Crit. Care Med.* 39, 665–670. doi: 10.1097/CCM.0b013e318206c1ca

Kliem, M., and Sauer, S. (2012). The essence on mass spectrometry based microbial diagnostics. *Curr. Opin. Microbiol.* 15, 397–402. doi: 10.1016/j.mib.2012.02.006

Kohlenberg, A., Struelens, M. J., Monnet, D. L., Plachouras, D., and The Candida Auris Survey Collaborative Group (2018). *Candida auris*: epidemiological situation, laboratory capacity and preparedness in European Union and European Economic Area countries, 2013 to 2017. *Euro Surveill.* 23:18-00136. doi: 10.2807/1560-7917.ES.2018.23.13.18-00136

Kollef, M., Micek, S., Hampton, N., Doherty, J. A., and Kumar, A. (2012). Septic shock attributed to *Candida* infection: importance of empiric therapy and source control. *Clin. Infect. Dis.* 54, 1739–1746. doi: 10.1093/cid/cis305

Kordalewska, M., Lee, A., Park, S., Berrio, I., Chowdhary, A., Zhao, Y., et al. (2018). Understanding echinocandin resistance in the emerging pathogen *Candida auris*. *Antimicrob. Agents Chemother.* 62:e00238-18. doi: 10.1128/AAC.00238-18

Lamoth, F., Lockhart, S. R., Berkow, E. L., and Calandra, T. (2018). Changes in the epidemiological landscape of invasive candidiasis. *J. Antimicrob. Chemother.* 73, i4–i13. doi: 10.1093/jac/dkx444

Lau, A. F., Wang, H., Weingarten, R. A., Drake, S. K., Suffredini, A. F., Garfield, M. K., et al. (2014). 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.* 52, 2804–2812. doi: 10.1128/JCM.00694-14

Lee, C. R., Lee, J. H., Park, K. S., Kim, Y. B., Jeong, B. C., and Lee, S. H. (2016). Global dissemination of carbapenemase-producing *Klebsiella pneumoniae*: epidemiology, genetic context, treatment options, and detection methods. *Front. Microbiol.* 7:895. doi: 10.3389/fmicb.2016.00895

Miceli, M. H., Diaz, J. A., and Lee, S. A. (2011). Emerging opportunistic yeast infections. *Lancet Infect. Dis.* 11, 142–151. doi: 10.1016/S1473-3099(10)70218-8

Nordmann, P., Naas, T., and Poirel, L. (2011). Global spread of carbapenemase-producing *Enterobacteriaceae*. *Emerg. Infect. Dis.* 17, 1791–1798. doi: 10.3201/eid1710.110655

Oviano, M., Sparbier, K., Barba, M. J., Kostrzewa, M., and Bou, G. (2016). Universal protocol for the rapid automated detection of carbapenem-resistant Gram-negative bacilli directly from blood cultures by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF/MS). *Int. J. Antimicrob. Agents* 48, 655–660. doi: 10.1016/j.ijantimicag.2016.08.024

Pana, Z. D., Roilides, E., Warris, A., Groll, A. H., and Zaoutis, T. (2017). Epidemiology of invasive fungal disease in children. *J. Pediatric Infect. Dis. Soc.* 6(Suppl._1), S3–S11. doi: 10.1093/jpids/pix046

Patel, G., and Bonomo, R. A. (2013). "Stormy waters ahead": global emergence of carbapenemases. *Front. Microbiol.* 4:48. doi: 10.3389/fmicb.2013.00048

Patil, A., and Majumdar, S. (2017). Echinocandins in antifungal pharmacotherapy. *J. Pharm. Pharmacol.* 69, 1635–1660. doi: 10.1111/jphp.12780

Paul, S., Singh, P., Shamanth, A. S., Rudramurthy, S. M., Chakrabarti, A., and Ghosh, A. K. (2018). Rapid detection of fluconazole resistance in *Candida tropicalis* by MALDI-TOF MS. *Med. Mycol.* 56, 234–241. doi: 10.1093/mmy/myx042

Pfaller, M. A. (2012). Antifungal drug resistance: mechanisms, epidemiology, and consequences for treatment. *Am. J. Med.* 125, S3–S13. doi: 10.1016/j.amjmed.2011.11.001

Pfaller, M. A., Andes, D. R., Diekema, D. J., Horn, D. L., Reboli, A. C., Rotstein, C., et al. (2014). Epidemiology and outcomes of invasive candidiasis due to non-*albicans* species of *Candida* in 2,496 patients: data from the Prospective Antifungal Therapy (PATH) registry 2004–2008. *PLoS One* 9:e101510. doi: 10.1371/journal.pone.0101510

Pham, C. D., Iqbal, N., Bolden, C. B., Kuykendall, R. J., Harrison, L. H., Farley, M. M., et al. (2014). Role of FKS mutations in *Candida glabrata*: MIC values, echinocandin resistance, and multidrug resistance. *Antimicrob. Agents Chemother.* 58, 4690–4696. doi: 10.1128/AAC.03255-14

Pierce, C. G., and Lopez-Ribot, J. L. (2013). Candidiasis drug discovery and development: new approaches targeting virulence for discovering and identifying new drugs. *Expert Opin. Drug Discov.* 8, 1117–1126. doi: 10.1517/17460441.2013.807245

Rhoads, D. D., Wang, H., Karichu, J., and Richter, S. S. (2016). The presence of a single MALDI-TOF mass spectral peak predicts methicillin resistance in staphylococci. *Diagn. Microbiol. Infect. Dis.* 86, 257–261. doi: 10.1016/j.diagmicrobio.2016.08.001

Richter, C., Hollstein, S., Woloszyn, J., Kaase, M., Gatermann, S. G., and Szabados, F. (2012). Evaluation of species-specific score cut-off values for various *Staphylococcus* species using a MALDI Biotyper-based identification. *J. Med. Microbiol.* 61, 1409–1416. doi: 10.1099/jmm.0.042606-0

Rodríguez-Baño, J., Gutiérrez-Gutiérrez, B., Machuca, I., and Pascual, A. (2018). Treatment of infections caused by extended-spectrum-beta-lactamase-, AmpC-, and carbapenemase-producing *Enterobacteriaceae*. *Clin. Microbiol. Rev.* 31:e00079-17. doi: 10.1128/CMR.00079-17

Sanguinetti, M., and Posteraro, B. (2016). Mass spectrometry applications in microbiology beyond microbe identification: progress and potential. *Expert Rev. Proteomics* 13, 965–977. doi: 10.1080/14789450.2016.1231578

Saracli, M. A., Fothergill, A. W., Sutton, D. A., and Wiederhold, N. P. (2015). Detection of triazole resistance among *Candida* species by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS). *Med. Mycol.* 53, 736–742. doi: 10.1093/mmy/myv046

Silva, S., Negri, M., Henriques, M., Oliveira, R., Williams, D. W., and Azeredo, J. (2012). *Candida glabrata*, *Candida parapsilosis* and *Candida tropicalis*: biology, epidemiology, pathogenicity and antifungal resistance. *FEMS Microbiol. Rev.* 36, 288–305. doi: 10.1111/j.1574-6976.2011.00278.x

Spanu, T., Posteraro, B., Fiori, B., D'Inzeo, T., Campoli, S., Ruggeri, A., et al. (2012). Direct MALDI-TOF mass spectrometry assay of blood culture broths for rapid identification of *Candida* species causing bloodstream infections: an observational study in two large microbiology laboratories. *J. Clin. Microbiol.* 50, 176–179. doi: 10.1128/JCM.05742-11

Sparbier, K., Schubert, S., and Kostrzewa, M. (2016). MBT-ASTRA: a suitable tool for fast antibiotic susceptibility testing? *Methods* 104, 48–54. doi: 10.1016/j.ymeth.2016.01.008

Szabados, F., Tix, H., Anders, A., Kaase, M., Gatermann, S. G., and Geis, G. (2012). Evaluation of species-specific score cutoff values of routinely isolated clinically relevant bacteria using a direct smear preparation for matrix-assisted laser desorption/ionization time-of flight mass spectrometry-based bacterial identification. *Eur. J. Clin. Microbiol. Infect. Dis.* 31, 1109–1119. doi: 10.1007/s10096-011-1415-7

Vatanshenassan, M., Boekhout, T., Lass-Flörl, C., Lackner, M., Schubert, S., Kostrzewa, M., et al. (2018). Proof of concept for MBT ASTRA, a rapid matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS)-based method to detect caspofungin resistance in *Candida albicans* and *Candida glabrata*. *J. Clin. Microbiol.* 56:e00420-18. doi: 10.1128/JCM.00420-18

Vecchione, A., Florio, W., Celandroni, F., Barnini, S., Lupetti, A., and Ghelardi, E. (2018). A rapid procedure for identification and antifungal susceptibility testing of yeasts from positive blood cultures. *Front. Microbiol.* 9:2400. doi: 10.3389/fmicb.2018.02400

Vella, A., De Carolis, E., Mello, E., Perlin, D. S., Sanglard, D., Sanguinetti, M., et al. (2017). Potential use of MALDI-TOF mass spectrometry for rapid detection of antifungal resistance in the human pathogen *Candida glabrata*. *Sci. Rep.* 7:9099. doi: 10.1038/s41598-017-09329-4

Vella, A., De Carolis, E., Vaccaro, L., Posteraro, P., Perlin, D. S., Kostrzewa, M., et al. (2013). Rapid antifungal susceptibility testing by matrix-assisted laser desorption ionization time-of- flight mass spectrometry analysis. *J. Clin. Microbiol.* 51, 2964–2969. doi: 10.1128/JCM.00903-13

Walsh, T. J., and Gamaletsou, M. N. (2013). Treatment of fungal disease in the setting of neutropenia. *Hematology Am. Soc. Hematol. Educ. Program* 2013, 423–427. doi: 10.1182/asheducation-2013.1.423

Walsh, T. R. (2010). Emerging carbapenemases: a global perspective. *Int. J. Antimicrob. Agents* 36, S8–S14. doi: 10.1016/S0924-8579(10)70004-2

Wang, H., Liu, N., Yin, M., Han, H., Yue, J., Zhang, F., et al. (2014). The epidemiology, antifungal use and risk factors of death in elderly patients with candidemia: a multicentre retrospective study. *BMC Infect. Dis.* 14:609. doi: 10.1186/s12879-014-0609-x

Yan, Y., He, Y., Maier, T., Quinn, C., Shi, G., Li, H., et al. (2011). Improved identification of yeast species directly from positive blood culture media by combining Sepsityper specimen processing and Microflex analysis with the matrix-assisted laser desorption ionization biotyper system. *J. Clin. Microbiol.* 49, 2528–2532. doi: 10.1128/JCM.00339-11

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 23 November 2018
doi: 10.3389/fmicb.2018.02854

A Full MALDI-Based Approach to Detect Plasmid-Encoded KPC-Producing *Klebsiella pneumoniae*

Miriam Cordovana[1], Markus Kostrzewa[2], Jörg Glandorf[2], Michael Bienia[3], Simone Ambretti[1] and Arthur B. Pranada[3]*

[1] Laboratory of Bacteriology, Operative Unit of Microbiology, University Hospital of Bologna Policlinico Sant'Orsola-Malpighi, Bologna, Italy, [2] Bruker Daltonik GmbH, Bremen, Germany, [3] Department of Medical Microbiology, MVZ Dr. Eberhard & Partner Dortmund, Dortmund, Germany

KPC-producing *Klebsiella pneumoniae* represents a severe public health concern worldwide. The rapid detection of these isolates is of fundamental importance for the adoption of proper antibiotic treatment and infection control measures, and new applications of MALDI-TOF MS technology fit this purpose. In this study, we present a full MALDI-based approach to detect plasmid-encoded KPC-producing strains, accomplished by the automated detection of a KPC-specific peak (at 11,109 m/z) by a specific algorithm integrated into the MALDI Biotyper system (Bruker Daltonik), and the confirmation of carbapenemase activity by STAR-Carba imipenem hydrolysis assay. A total of 6209 *K. pneumoniae* isolates from Italy and Germany were investigated for the presence of the KPC-related peak, and a subset of them (n = 243) underwent confirmation of carbapenemase activity by STAR-Carba assay. The novel approach was further applied directly to positive blood culture bottles (n = 204), using the bacterial pellet obtained with Sepsityper kit (Bruker Daltonik). The novel approach enabled a reliable and very fast detection of KPC-producing *K. pneumoniae* strains, from colonies as well as directly from positive blood cultures. The automated peak detection enabled the instant detection of KPC-producing *K. pneumoniae* during the routine identification process, with excellent specificity (100%) and a good sensitivity (85.1%). The sensitivity is likely mainly related to the prevalence of the specific plasmid harboring clones among all the KPC-producing circulating strains. STAR-Carba carbapenemase confirmation showed 100% sensitivity and specificity, both from colonies and from positive blood cultures.

Keywords: MALDI-TOF MS, KPC, *Klebsiella pneumoniae*, carbapenemase, multidrug resistance, KPC-related peak, pKpQIL plasmid

OPEN ACCESS

Edited by:
Rustam Aminov, University of Aberdeen, United Kingdom

Reviewed by:
Jaroslav Hrabak, Charles University, Czechia
Stefan Zimmermann, Heidelberg University Hospital, Germany

****Correspondence:***
Miriam Cordovana
miri-78@live.it

Specialty section:
This article was submitted to Antimicrobials, Resistance and Chemotherapy, a section of the journal Frontiers in Microbiology

Received: *05 July 2018*
Accepted: *06 November 2018*
Published: *23 November 2018*

Citation:
Cordovana M, Kostrzewa M, Glandorf J, Bienia M, Ambretti S and Pranada AB (2018) A Full MALDI-Based Approach to Detect Plasmid-Encoded KPC-Producing Klebsiella pneumoniae. Front. Microbiol. 9:2854. doi: 10.3389/fmicb.2018.02854

INTRODUCTION

Carbapenem-resistant *Enterobacteriaceae* (CRE) have recently emerged as a class of bacterial pathogens that threaten the effectiveness of last resort treatment, posing a serious threat to global public health (Tzouvelekis et al., 2012). Even though resistance to carbapenems may involve several combined mechanisms (Nordmann et al., 2012a), from the public health point of view

attention is focused on the isolates that produce specific carbapenem-hydrolyzing β-lactamases (carbapenemases). Such resistance determinants can be transferred between bacteria of the same or of different species by mobile genetic elements (Kumarasamy et al., 2010; Nordmann et al., 2012b). The carbapenemase transmission often is linked to other non-β-lactam resistance determinants, leading to the rise and the rapid dissemination of multi-drug or pan-drug resistant (MDR, PDR) organisms (Kumarasamy et al., 2010).

Carbapenemase-producing *Enterobacteriaceae* (CPE) have become important causes of hospital acquired infections, associated with a significantly increased mortality, especially in critical wards (Vincent et al., 2009). In Europe, the highest prevalences have been reported from Greece, with 61.9% of carbapenem-resistant *K. pneumoniae* among invasive isolates, Italy with 33.5%, and from Romania (24.7%) (EARS-Net report 2016).

CPE show an epidemiology according to the enzyme type, with different prevalence in the different geographic areas. In Europe, KPC-producing *K. pneumoniae* is endemic in Italy and Greece, while in other countries class D (OXA-48 family) and class B (MβL - Metallo-β-Lactamases) carbapenemases are reported with overall lower prevalence. Nevertheless, the *Klebsiella pneumoniae* Carbapenemase (KPC) family has the most extensive global distribution of all carbapenemases associated with *Enterobacterales* (van Duin and Doi, 2017), and are the most clinically significant (Munoz-Price et al., 2013). KPCs are found in many Gram-negative species, including both *Enterobacterales* and non-fermenters, but *K. pneumoniae* is the most largely predominant species.

Actually, 20 variants of the *bla*KPC gene have been described, and the variants KPC-2 and KPC-3 are the most common (Doumith et al., 2017). The bla_{KPC} genes have been identified in a variety of plasmids (Chen et al., 2014), and reported in more than 100 different Sequence Types (ST) of *K. pneumoniae*. The transmission of bla_{KPC} genes can be mediated by different molecular mechanisms, from mobility of small genetic elements (i.e., Tn*4401* transposon) to horizontal transfer of plasmids, and via clonal spread (Munoz-Price and Quinn, 2009; Partridge, 2014). Nevertheless, the global diffusion of plasmid-borne bla_{KPC} has been linked to the clonal dissemination worldwide of a major multilocus sequence type (MLST or ST), namely ST258, and its related variants (Cuzon et al., 2010; Munoz-Price et al., 2013). KPC-Kp are considered endemic in several countries, including the North-eastern United States, Argentina, Brazil, Puerto Rico, Colombia, China, Israel, and, in Europe, Italy and Greece (Munoz-Price et al., 2013; Nordmann and Poirel, 2014). As treatment options are very limited and because of the epidemiological impact, rapid methods to detect CPE, and differentiate them from other CRE are desired. Several techniques have been developed, relying on different molecular or phenotypical principles. Although proven to be effective for the detection of KPC-producing isolates, overall these methods are either expensive and limited in the targets included, or slow with a time to report up to 24 h, and lack in sensitivity and/or specificity (Osei Sekyere et al., 2015; Bialvaei et al., 2016; Lutgring and Limbago, 2016; Rood and Li, 2017; Tamma et al., 2017).

Recently, it has been shown that the intrinsic speed of MALDI-TOF MS technology could be deployed and successfully applied to a prompt detection of carbapenemase-producing strains. First, it was proven that the hydrolytic activity of carbapenemases can be detected by a functional assay that relies on the evaluation of the mass spectra of the carbapenem molecule after a short incubation with the bacterial strains (Burckhardt and Zimmermann, 2011; Sparbier et al., 2012; Hrabák et al., 2013; Johansson et al., 2014; Rapp et al., 2018). This is even possible directly from positive blood cultures (Jung et al., 2014; Hoyos-Mallecot et al., 2014; Sakarikou et al., 2017).

Further, Lau et al. (2014) discovered a peak in MALDI-TOF mass spectra of KPC-producing *K. pneumoniae* related to a pKpQIL plasmid carrying bla_{KPC}. This specific peak at 11,109 m/z is clearly detectable in bacterial MALDI-TOF mass spectra. Different approaches to seek for this peak have been used and evaluated (Lau et al., 2014; Gaibani et al., 2016; Youn et al., 2016). Until recently, the detection of this peak required a manual operation, with visual analysis or additional software in a second step after the routine identification process, and therefore impeding a real-time detection of KPC strains (Lau et al., 2014; Gaibani et al., 2016; Youn et al., 2016).

In this study, we present a full MALDI-based approach to an instant detection of KPC-producing *K. pneumoniae* strains simultaneously during the standard routine species identification process. KPC-producers were indirectly identified by the automated detection of a specific peak related to a bla_{KPC}-carrying pKpQIL plasmid, using an algorithm that was integrated into the MALDI Biotyper software. A MALDI-TOF based imipenem hydrolysis assay was further used to confirm KPC enzyme activity.

This novel approach was applied to well characterized strains, to a large set of clinical routine strains, and finally directly to routine positive blood cultures.

MATERIALS AND METHODS

Optimization of the Algorithm for Automated KPC Detection With PCR Confirmed Strains

For automated detection of the pKpQIL related peak described by Lau et al. (2014), a software algorithm was developed. The peak detection was based on a manual analysis of spectra analyzed with the flexAnalysis software version 3.4 (Bruker Daltonik, Bremen, Germany) – **Figure 1**. Spectra were normalized and smoothed with standard settings. For precise detection the spectra were screened for potential peaks for an internal recalibration as described in Pranada et al. (2017). After internal recalibration, intensities 3 times higher than the surrounding noise were then counted as peaks. If such potential peak was detected in a window ± 5 m/z around the previously described mass of 11,109 m/z, the algorithm yielded the detection of the KPC related peak. Examples of these automated detections are shown in **Figure 2**.

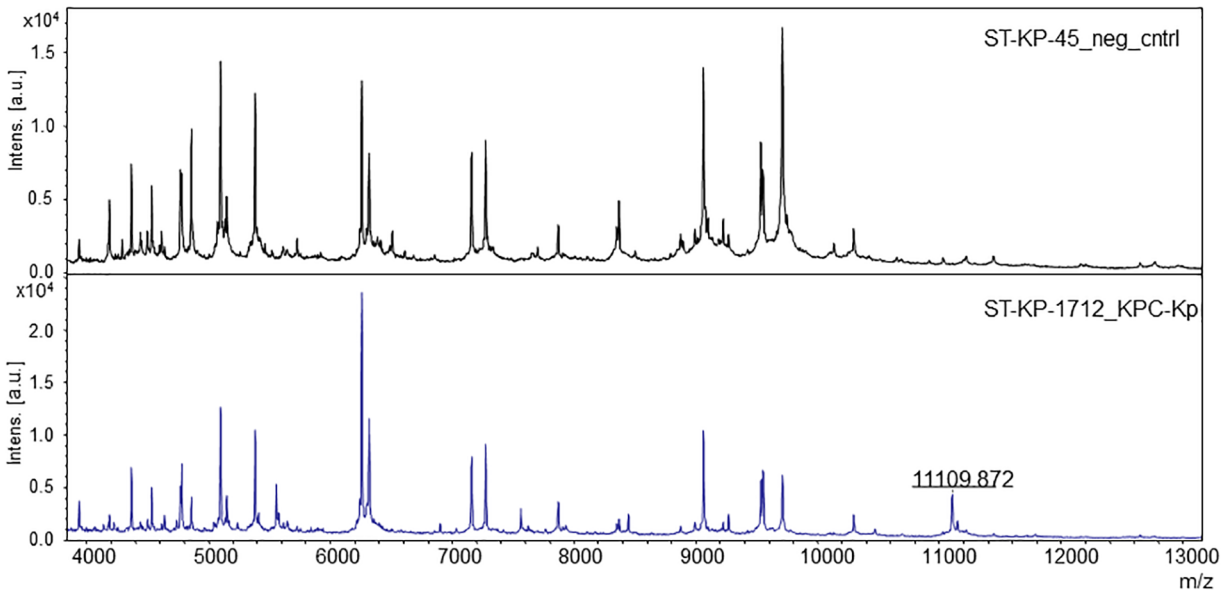


FIGURE 1 | The pKpQIL plasmid-related peak in the MALDI mass spectra of *K. pneumoniae.* The upper spectrum shows a negative control, without the specific peak. The lower spectrum shows a KPC-producing strain exhibiting the specific peak.

N = 266 spectra of *K. pneumoniae* previously characterized by PCR (Hyplex® SuperBug ID assay – Amplex Biosystems GmbH, Gießen, Germany), were used to test the algorithm. Among them, *n* = 152 were KPC-producers, and *n* = 114 were negative for the bla_{KPC} gene.

To assess the technical sensitivity of the automatic detection, all the spectra were also analyzed by visual inspection using the FlexAnalysis software (Bruker Daltonik GmbH) to seek for the presence of the KPC-specific peak.

Evaluation of the Automated KPC Detection With a Large Collection of Strains

The optimized algorithm was integrated into the commercial MALDI Biotyper RUO software, and the method was evaluated on a large set of clinical strains from Italy (in the Microbiology Unit of the University Hospital of Bologna Policlinico Sant'Orsola-Malpighi), as a country with a high prevalence of KPC-bearing strains, and from Germany (in the Department of Microbiology of the MVZ Dr. Eberhard & Partner Dortmund, Dortmund, Germany) as a low prevalence country.

A total of *n* = 6209 MALDI-TOF mass spectra of clinical and surveillance isolates of *K. pneumoniae* collected in a time frame between 2009 and 2017 were analyzed. The spectra were retrieved from the database of stored runs from routine identification in Dortmund. In Bologna, spectra for strains isolated between 2010 and September 2016 were recorded retrospectively from the collection of frozen strains as well as prospectively from October 2016 to December 2017.

Bacterial strains were cultured for 24–48 h on Tryptose Soy Agar with sheep blood in Bologna, and on Columbia Agar with sheep blood in Dortmund. Routine susceptibility testing was performed using VITEK2 XL systems (bioMérieux, Marcy l'Étoile, France).

All isolates with MICs greater than the EUCAST epidemiological cut-off for at least one of the carbapenems (ertapenem, meropenem, imipenem) underwent either detection of carbapenemase-encoding genes (in Dortmund by an in-house PCR) or confirmation of carbapenemase production, (in Bologna by disk diffusion synergy test with inhibitors (KPC+MBL Confirm ID Pack, ROSCO Diagnostika, Taastrup, Denmark), immunochromatographic assay (OXA-48 *K*-Set, CorisBioConcept, Gembloux, Belgium), and/or PCR (Hyplex® SuperBug ID assay –Amplex Biosystems GmbH, Gießen, Germany, GeneXpert® Carba-R –Cepheid, Sunnyvale, CA, United States).

Among these strains, at routine testing *n* = 2390 were KPC-producers (*n* = 2385 from Bologna, *n* = 5 from Dortmund), *n* = 179 MβL-producers (*n* = 119 from Bologna, *n* = 60 from Dortmund), *n* = 32 OXA-48 family producers (*n* = 19 from Bologna, *n* = 13 from Dortmund), *n* = 221 were negative for carbapenemase production but resistant to carbapenems (likely ESβL/AmpC producers associated with reduced membrane permeability – *n* = 207 from Bologna, *n* = 14 from Dortmund), and *n* = 3387 susceptible to carbapenems (β-lactamases producers or wild type – *n* = 105 from Bologna, *n* = 3282 from Dortmund) – **Table 1**.

Evaluation of Stability and Reliability of KPC-Peak Detection

Further, the sensitivity of the automated KPC detection by MALDI-TOF MS was assessed with regard to the type of culture medium and the age of cultures: spectra of *n* = 34 KPC-producing strains cultured on Sheep Blood Agar, CHR-KPC Agar,

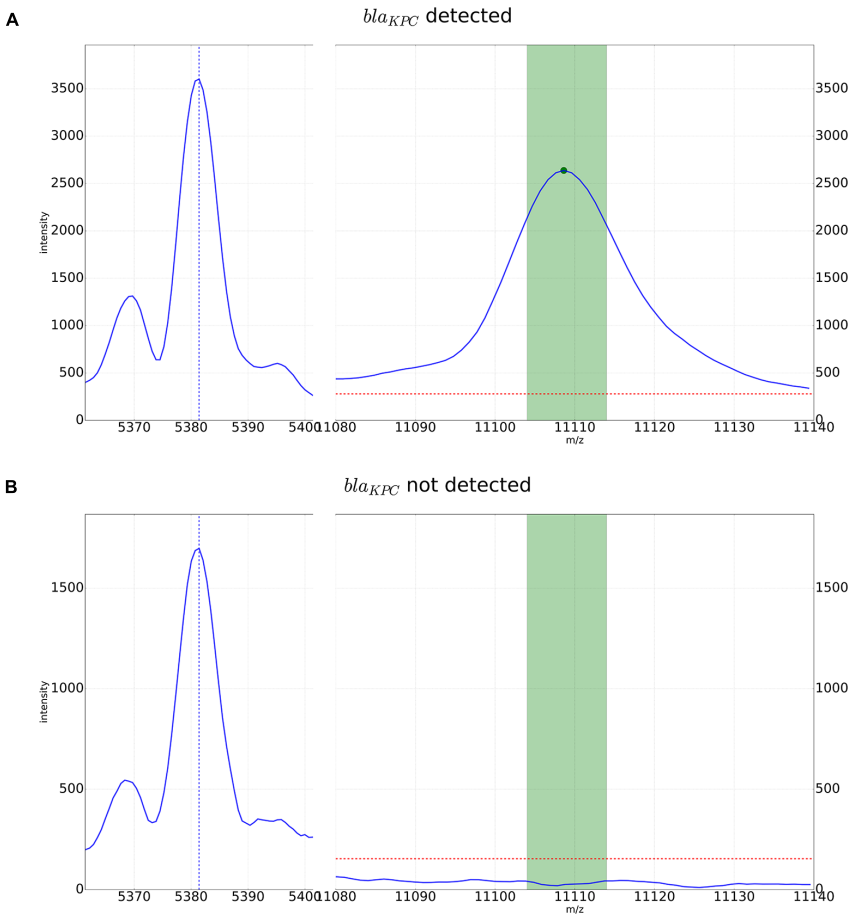


FIGURE 2 | Detection of the KPC-related peak by the automated algorithm. An internal calibration peak specific to *K. pneumoniae* is recognized and used for higher precision in peak detection in the window of m/z 11,109 +/−5. The dotted red line corresponds to a multiple of the average noise in the spectrum. It is used as a threshold for the intensity in peak detection. **(A)** KPC-positive *K. pneumoniae* strain with peak for the pKpQIL plasmid. **(B)** KPC-negative strain. In the detection window only noise below the detection threshold can be observed.

and Mueller-Hinton-Agar (MEUS, Piove di Sacco, Italy) were acquired after 24, 48, and 72 h of incubation and then compared to each other.

Since the intensity of the KPC related peak in some cases is low, we also investigated whether the sensitivity of the automated detection could be enhanced measuring the samples in duplicates. For all samples, spectra were recorded from two spots.

In case of discrepancies between results of the novel MALDI approach and results of routine methods, the samples underwent molecular investigation by GeneXpert® Carba-R (Cepheid, Sunnyvale, CA, United States).

TABLE 1 | Overview of *K. pneumoniae* isolates from Italy and Germany included in this study.

	Italy [n]	Germany [n]	Total [n]
KPC	2386	4	2390
MβL	119	60	179
OXA-48	13	19	32
Carbapenem-resistant non-carbapenemase producers	207	14	221
Carbapenem-susceptible	105	3282	3387
Total	2830	3379	6209

Application Directly to Positive Blood Cultures

The automated KPC detection was applied to 204 consecutive blood cultures positive for *K. pneumoniae* in routine diagnostics in Bologna. Among those, n = 90 were KPC-producers, n = 12 MβL-producers, n = 1 was NDM+OXA-48 producer, while n = 101 were susceptible to carbapenems (MIC of carbapenems lower than epidemiological cut-off). KPC-producers were identified by KPC *K*-Set, (CorisBioConcept, Gembloux, Belgium), MβL-producers were identified by KPC+MBL Confirm ID Pack, ROSCO Diagnostika, Taastrup, Denmark), while the strain NDM+OXA-48 was characterized by KPC+MBL Confirm ID Pack, ROSCO Diagnostika, Taastrup,

Denmark and GeneXpert® Carba-R (Cepheid, Sunnyvale, CA, United States).

The blood specimens were collected in BD BACTEC™ blood culture media (Plus Aerobic/F, Plus Anerobic/F, Lytic/10 Anaerobic/F and Peds Plus™/F), and incubated in a BACTEC™ FX blood culture system (Becton, Dickinson and Company, Sparks, MD 21152, Benex Limited Shannon, County Clare, Ireland). After the bottles were flagged as positive, the routine workflow at the time of the study provided the species identification by MALDI-TOF MS after a subculturing step on chocolate agar of the enriched pellet obtained from 8 ml of blood culture.

For this study, the blood culture flasks were directly processed with the Sepsityper kit according to instructions of the manufacturer (Bruker Daltonik GmbH, Bremen, Germany) and the extracted bacterial pellet was used to acquire the MALDI spectra.

The KPC subtyping was performed by the MALDI Biotyper software simultaneously with the species identification.

The same pellet was further used to perform STAR-Carba assay (Bruker Daltonik GmbH) for carbapenemase confirmation.

Confirmation of Carbapenemase Activity by STAR-Carba Assay

A subset of $n = 243$ randomly chosen (a representative proportion of strains for each year, selected at the discretion of the operator by manually choosing non-juxtaposed strains directly from the strains collection) KPC-producing strains, and all positive blood cultures containing *K. pneumoniae* ($n = 204$) underwent the MALDI-TOF MS based STAR-Carba imipenem hydrolysis assay (Bruker Daltonik GmbH, Bremen, Germany) to verify the carbapenemase activity. The test was performed according to the manufacturer's instructions. Briefly, 1 µl-loop of bacteria (samples and *E.coli* ATCC 25922 as negative control, and a PCR-confirmed KPC-producing *E. coli* as positive control) were resuspended in 50 µl of imipenem solution. After 30 min of incubation at 37°C under agitation, the bacteria were pelleted by centrifugation (2 min at 14000 rpm), and 1 µl of the supernatant was spotted in duplicates onto a MALDI target. Open air dried spots were overlaid with CHCA matrix containing an internal standard. The strains were detected as hydrolyzing or non-hydrolyzing by measuring the intensity of the peak corresponding to the intact form of imipenem (300 m/z), normalized on the intensities values of the imipenem peak measured in the negative and positive controls.

RESULTS

Optimization of the Algorithm for Automated KPC Detection With PCR Confirmed Strains

Internal calibration of the acquired MALDI-TOF mass spectra and optimization of the newly developed algorithm finally allowed narrowing down the detection window for the peak specific of the bla_{KPC}-carrying pKpQIL plasmid to a more specific range of m/z 11,109 ± 5 (from initially ± 15).

The novel algorithm detected the bla_{KPC}-carrying pKpQIL plasmid related peak in 99/152 PCR confirmed strains (65.1%).

The visual inspection of these spectra confirmed the presence of the peak in the 99 strains classified as positive by the algorithm (sensitivity 100%), while the remaining 53 strains didn't show the peak, suggesting that they likely harbor a different bla_{KPC}-carrying plasmid.

The KPC peak was not detected by the algorithm in the $n = 114$ KPC-PCR negative *K. pneumoniae* isolates (specificity 100%).

Evaluation of the Automated KPC Detection With a Large Collection of Strains

The pKpQIL plasmid-related peak was detected by Biotyper software in overall 2035 of the $n = 2390$ KPC-producing isolates (85.1%). It was detected in none of the strains resulted positive for the production of a class B or D carbapenemase ($n = 179$ MβL, $n = 32$ OXA-48-family), in none of the carbapenem-susceptible isolates ($n = 3387$), but in 5 out of the $n = 221$ strains resulted resistant to carbapenems but negative to routine testing for carbapenemase production. The positive predictive value and the negative predictive value resulted 100% and 91.5%, respectively.

In these 5 strains, the molecular test confirmed the presence of bla_{KPC}.

With regard to the proportion of KPC-producing strains showing the peak among all KPC-producing isolates, a constantly increasing trend from 63.2% in the years 2010–2011 to 94.1% in 2017 could be observed (**Figure 3**).

Evaluation of Stability and Reliability of KPC-Peak Detection

The evaluation of the KPC-detection with this huge set of spectra from clinical strains also comprised the investigation of culture conditions. No performance differences were observed in relation to culture medium used nor to time of incubation.

In 1798/2035 strains, and for 88.4% (1798/2035), the peak was detected in both spots. In 11.6% (237/2035) of the cases, the peak was detected only in one of the two spots.

Application Directly to Positive Blood Cultures

To assess the further clinical potential, the automated KPC-detection was also applied directly on blood cultures. Among the spectra recorded from the bacterial pellet extracted by Sepsityper, the KPC-specific peak was detected in 83/90 (92.2%) of the KPC-producing strains, corresponding to 83/84 (98.8%) of the strains in which the peak was present.

The peak was detected in none of the non-KPC strains (100% specificity).

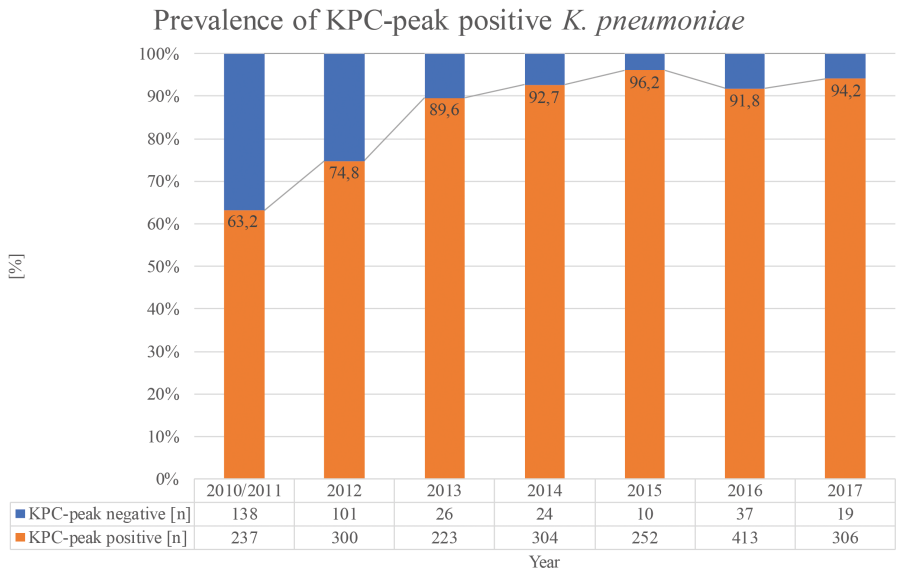


FIGURE 3 | Trend for the years 2010–2017 of KPC-producing *K. pneumoniae* strains isolated in Bologna showing the pKpQIL plasmid-related peak.

Confirmation of Carbapenemase Activity by STAR-Carba Assay

STAR-Carba hydrolysis assay resulted positive for all the randomly chosen KPC-producing strains ($n = 243$).

It also resulted positive for all the KPC-producing strains from positive blood cultures ($n = 90$), as well as for all the other carbapenemase-producing strains ($n = 12$ MβL-producers, and $n = 1$ NDM+OXA-48-producer), and negative for the $n = 101$ carbapenem-susceptible strains from the blood cultures (sensitivity and specificity 100%).

DISCUSSION

Carbapenem-resistant enterobacteria are continuously spreading worldwide, and pose a serious threat to public health (Tzouvelekis et al., 2012; Nordmann, 2014). Carbapenemase-producing *Enterobacteriaceae* (CPE) represent an important cause of acquired infections in hospital settings, burdened by a high mortality rate (Vincent et al., 2009). Although the carbapenemases associated with *Enterobacterales* show a different prevalence in the different geographic areas, the KPC family has the most extensive global distribution, and it is the most significant (Munoz-Price et al., 2013; van Duin and Doi, 2017).

Rapid methods to detect KPC-producing strains are highly demanded, both for therapeutic and infection control purposes. Several methods are commercially available, relying on different principles, but overall they are either expensive, or slow, with time to report up to 24 h (Lutgring and Limbago, 2016; Tamma et al., 2017). Recently, a specific peak at 11,109 m/z, related to a pKpQIL plasmid carrying bla_{KPC} was discovered in the MALDI-TOF mass spectra of KPC-producing *Klebsiella pneumoniae* (Lau et al., 2014). Different approaches to seek for this peak have been described (Lau et al., 2014; Gaibani et al., 2016; Youn et al., 2016). Nevertheless all of them required a second step after routine species identification process like visual inspection or an additional software analysis.

In this study, we developed a fully MALDI-TOF MS based approach to detect KPC-producing *K. pneumoniae* strains. This novel approach relies on the indirect identification of bla_{KPC}-carrying isolates by a now automated detection of the pKpQIL plasmid-related peak at 11,109 m/z during the standard routine identification process. The detection of the KPC-related peak is depicted in the MALDI Biotyper system as "presumptive KPC" in the subtyping column. Furthermore, we combined it with a subsequent verification of the carbapenemase activity by an imipenem hydrolysis assay, using the commercially available STAR-Carba kit, and according to the MALDI Biotyper software module (Bruker Daltonik, Bremen). The method was investigated on a large collection of strains from Italy and Germany – two countries with a very different epidemiology of CPE. The study aimed to evaluate this novel approach in a setting close to diagnostic routine, covering a broad period of time (2009–2017), in order to uncover pitfalls which can be missed by smaller dedicated study.

In addition, the integration of the algorithm for instant detection of the KPC-related peak into the MALDI Biotyper RUO software simplifies routine usage as no further special knowledge or additional tools are needed by the operator. The sensitivity of this automated method was found to be 85.1%. Our findings showed that it is mainly related to the prevalence of the KPC-producing strains harboring the pKpQIL plasmid among all the KPC-producing circulating strains. The visual inspection of the spectra included into the evaluation dataset of this study proved that the automated software detected the 11,109 m/z peak in all the strains in which it was present. For

all strains in which the peak at 11,109 m/z was not detected by the automated subtyping algorithm, visual inspection of spectra confirmed this result. Moreover, a permanently increasing trend of the KPC-producing strains showing the pKpQIL specific peak was observed (from 60.2% in 2010–2011 to 95.6% in 2017), suggesting that the spread of KPC in Italy seems to be related to the expansion of this specific clone over the years. Although only 2/4 (50%) of the German KPC-producing strains showed the 11,109 m/z peak, this number is too low to draw conclusions about the prevalence of KPC in German strains. The method showed excellent specificity and positive predictive value (100%), as the software correctly classified all non-KPC carbapenemase-producers (n = 179 MβL and n = 32 OXA-48 family), as well as all carbapenem-susceptible isolates as KPC-peak negative. Among the strains resistant to carbapenems but negative for carbapenemase production in routine testing, 216/221 were classified as KPC-peak negative. In n = 5 strains the peak was detected, and for all of them the molecular testing confirmed the presence of bla_{KPC}. Hence, interestingly, the method in study proved to be able to deliver an earlier detection of KPC carbapenemase in comparison with the other phenotypic methods applied. No differences were observed in the performance of the automated detection using different culture media (blood agar and chromogenic selective and non-selective media), or different incubation times (24–48–72 h), while the use of the spotting in duplicates enabled to achieve a better sensitivity in comparison to single spotting (85.1% vs. 80.2%). The failed detection of the peak is likely due to reasons linked to the sample preparation (operator technical handling, mucousity of the bacterial colonies). Therefore, spotting in duplicate might be advisable for increased robustness and sensitivity as minor biological or technical variations could be compensated.

Since the pKpQIL plasmid-related peak at 11,109 m/z is very well detectable in spectra of extractions, our study additionally investigated the performance of the method on positive blood cultures. Compared to colony material, the sensitivity of the peak detection on bacterial pellets extracted directly from positive blood culture using the Sepsityper protocol was 98.8% with an excellent specificity of 100%. This again underlines the value for clinical routine.

STAR-Carba imipenem hydrolysis assay was used with a subset of strains for confirmation of the carbapenemase activity, both in peak-positive and –negative KPC-producing isolates, and negative controls. It showed 100% sensitivity and specificity, from colonies as well as from the bacterial pellet extracted from positive blood cultures. In the approach we present here, this assay could play a fundamental role to detect carbapenemase activity, complementary to, and strengthening KPC peak detection. It can be used to detect carbapenemase activity in KPC-producing strains that don't exhibit the presence of the KPC-related peak at 11,109 m/z, and in all the other carbapenemases of other classes. Further, it can be used as a functional verification of the enzymatic activity predicted by the presence of the KPC-marker detected by subtyping.

Our study shows that the novel MALDI-based approach enabled a reliable and robust detection of KPC-producing *K. pneumoniae* isolates, using the same MALDI Biotyper platform as for classical bacterial species identification, with a turnaround time from 10-15 min to 1.5 h. This method, applicable to colony material as well as directly on positive blood cultures, is unique in performing real time detection of an antibiotic resistance marker in parallel to species identification. It might represent a very useful tool for an early warning for KPC-producing strains, helping to significantly accelerate the proper initiation or change of therapeutic and infection control measures in the future. In comparison to previously described methods based on the 11,109 m/z peak detection, the approach we developed presents several advantages. First, the peak detection here is implemented into a commercially available software, and totally automated. Thereby, it doesn't require any operator specific skills regarding spectra processing and analysis. The STAR Carba assay is easy to use, and requires a minimum handling time. Moreover, the interpretation of results is also automated, and hence free from any operator-depending factors, and results are automatically stored and available for eventual future investigation. Thus, this approach can fit with any setting, including high throughput routine laboratories.

The limitation of the presented approach is that the KPC detection by subtyping depends on the association of the peak to bla_{KPC} gene. Partridge (2014) pointed out that BLAST searches indicated that the underlying p019 protein has only been found in plasmids carrying bla_{KPC} but an independent genetic movement of the p019 gene might be possible in the future and then impair this indirect detection method. A further limitation is that the sensitivity of the subtyping approach depends on the regional epidemiology of KPC-producing strains, i.e., on the prevalence of the KPC-producing clones harboring the pKpQIL plasmid. Nevertheless, the excellent specificity, and the seamless integration into the commercial software already used for bacterial species identification, might make the implementation of this approach in routine valuable, regardless the current epidemiological context.

Further, as KPC-producing strains have started appearing also among other genera and species of enterobacteria, a future expansion of this approach to other species might be possible.

DATA AVAILABILITY

The authors state that datasets are available on request. The raw data supporting the conclusion of this manuscript, will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

MC, MK, and ABP designed the study. MC, MK, and ABP carried out the study. MB and JG worked on the computer algorithms and statistical analysis together with MC and ABP. MC, MK, ABP, and SA wrote the manuscript.

REFERENCES

Bialvaei, A. Z., Kafil, H. S., Asgharzadeh, M., Yousef Memar, M., and Yousefi, M. (2016). Current methods for the identification of carbapenemases. *J. Chemother.* 28, 1–19. doi: 10.1179/1973947815Y.0000000063

Burckhardt, I., and Zimmermann, S. (2011). Using matrix-assisted laser desorption ionization-time of flight mass spectrometry to detect carbapenem resistance within 1 to 2.5 h. *J. Clin. Microbiol.* 49, 3321–3324. doi: 10.1128/JCM.00287-11

Chen, L., Mathema, B., Chavda, K. D., DeLeo, F. R., Bonomo, R. A., and Kreiswirth, B. N. (2014). Carbapenemase-producing *Klebsiella pneumoniae*: molecular and genetic decoding. *Trends Microbiol.* 22, 686–696. doi: 10.1016/j.tim.2014.09.003

Cuzon, G., Naas, T., Truong, H., Villegas, M. V., Wisell, K. T., Carmeli, Y., et al. (2010). Worldwide diversity of *Klebsiella pneumoniae* that produce beta-lactamase blaKPC-2 gene. *Emerg. Infect. Dis.* 16, 1349–1356. doi: 10.3201/eid1609.091389

Doumith, M., Findlay, J., Hirani, H., Hopkins, K. L., Livermore, D. M., Dodgson, A., et al. (2017). Major role of pKpQIL-like plasmids in the early dissemination of KPC-type carbapenemases in the UK. *J. Antimicrob. Chemother.* 72, 2241–2248. doi: 10.1093/jac/dkx141

Gaibani, P., Galea, A., Fagioni, M., Ambretti, S., Sambri, V., and Landini, M. P. (2016). Evaluation of matrix-assisted laser desorption ionization-time of flight mass spectrometry for identification of KPC-Producing *Klebsiella pneumoniae*. *J. Clin. Microbiol.* 54, 2609–2613. doi: 10.1128/JCM.01242-16

Hoyos-Mallecot, Y., Riazzo, C., Miranda-Casas, C., Rojo-Martin, M. D., Gutiérrez-Fernández, J., and Navarro-Marí, J. M. (2014). Rapid detection and identification of strains carrying carbapenemases directly from positive blood cultures using MALDI-TOF MS. *J. Microbiol. Methods* 105, 98–101. doi: 10.1016/j.mimet.2014.07.016

Hrabák, J., Chudácková, E., and Walková, R. (2013). Matrix-assisted laser desorption ionization-time of flight (maldi-tof) mass spectrometry for detection of antibiotic resistance mechanisms: from research to routine diagnosis. *Clin. Microbiol. Rev.* 26, 103–114. doi: 10.1128/CMR.00058-12

Johansson, Å, Ekelöf, J., Giske, C. G., and Sundqvist, M. (2014). The detection and verification of carbapenemases using ertapenem and matrix-assisted laser desorption ionization-time of flight mass spectrometry. *BCM Microbiol.* 14:89. doi: 10.1186/1471-2180-14-89

Jung, J. S., Popp, C., Sparbier, K., Lange, C., Kostrzewa, M., and Schubert, S. (2014). Evaluation of matrix-assisted laser desorption ionization-time of flight mass spectrometry for rapid detection of b-lactam resistance in *Enterobacteriaceae* derived from blood cultures. *J. Clin. Microbiol.* 52, 924–930. doi: 10.1128/JCM.02691-13

Kumarasamy, K. K., Toleman, M. A., Walsh, T. R., Bagaria, J., Butt, F., Balakrishnan, R., et al. (2010). Emergence of a new antibiotic-resistance mechanism in India, Pakistan and the UK: a molecular, biological, and epidemiological study. *Lancet Infect. Dis.* 10, 597–602. doi: 10.1016/S1473-3099(10)70143-2

Lau, A. F., Wang, H., Weingarten, R. A., Drake, S. K., Suffredini, A. F., Garfield, M. K., et al. (2014). 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. Microb.* 52, 2804–2812. doi: 10.1128/JCM.00694-14

Lutgring, J. D., and Limbago, B. M. (2016). The problem of carbapenemase-producing-carbapenem-resistant-*Enterobacteriaceae* detection. *J. Clin. Microbiol.* 54, 529–534. doi: 10.1128/JCM.02771-15

Munoz-Price, L. S., Poirel, L., Bonomo, R. A., Schwaber, M. J., Daikos, G. L., Cormican, M., et al. (2013). Clinical epidemiology of the global expansion of *Klebsiella pneumoniae* carbapenemases. *Lancet Infect. Dis.* 13, 785–796. doi: 10.1016/S1473-3099(13)70190-7

Munoz-Price, L. S., and Quinn, J. P. (2009). The spread of *Klebsiella pneumoniae* carbapenemase: a tale of strains, plasmids, and transposons. *Clin. Infect. Dis.* 49, 1739–1741. doi: 10.1086/648078

Nordmann, P. (2014). Carbapenemase-producing *Enterobacteriaceae*: overview of a major public health challenge. *Med. Mal. Infect.* 44, 51–56. doi: 10.1016/j.medmal.2013.11.007

Nordmann, P., Dortet, L., and Poirel, L. (2012a). Carbapenem resistance in *Enterobacteriaceae*: here is the storm! *Trends Mol. Med.* 18, 263–272. doi: 10.1016/j.molmed.2012.03.003

Nordmann, P., Gniadkowski, M., Giske, C. G., Poirel, L., Woodford, N., Miriagou, V., et al. (2012b). Identification and screening of carbapenemase-producing *Enterobacteriaceae*. *Clin. Microbiol. Infect.* 18, 432–438. doi: 10.1111/j.1469-0691.2012.03815.x

Nordmann, P., and Poirel, L. (2014). The difficult-to-control spread of carbapenemase producers in *Enterobacteriaceae* worldwide. *Clin. Microbiol. Infect.* 20, 821–830. doi: 10.1111/1469-0691.12719

Osei Sekyere, J., Govinden, U., and Essack, S. Y. (2015). Review of established and innovative detection methods for carbapenemase-producing gram-negative bacteria. *J. Appl. Microbiol.* 119, 1219–1233. doi: 10.1111/jam.12918

Partridge, S. R. (2014). Tn4401 carrying blaKPC is inserted within another insertion in pKpQIL and related plasmids. *J. Clin. Microbiol.* 52, 4448–4449. doi: 10.1128/JCM.02426-14

Pranada, A. B., Witt, E., Bienia, M., Kostrzewa, M., and Timke, M. (2017). Accurate differentiation of mycobacterium chimaera from mycobacterium intracellulare by MALDI-TOF MS analysis. *J. Med. Microbiol.* 66, 670–677. doi: 10.1099/jmm.0.000469

Rapp, E., Samuelsen, Ø, and Sundqvist, M. (2018). Detection of carbapenemases with a newly developed commercial assay using matrix assisted laser desorption ionization-time of flight. *J. Microbiol. Methods* 146, 37–39. doi: 10.1016/j.mimet.2018.01.008

Rood, I. G. H., and Li, Q. (2017). Review: molecular detection of extended spectrum-β-lactamase- and carbapenemase-producing *Enterobacteriaceae* in a clinical setting. *Diagn. Microbiol. Infect. Dis.* 89, 245–250. doi: 10.1016/j.diagmicrobio.2017.07.013

Sakarikou, C., Ciotti, M., Dolfa, C., Angeletti, S., and Favalli, C. (2017). Rapid detection of carbapenemase-producing *Klebsiella pneumoniae* strains derived from blood cultures by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS). *BMC Microbiol.* 17:54. doi: 10.1186/s12866-017-0952-3

Sparbier, K., Schubert, S., Weller, U., Boogen, C., and Kostrzewa, M. (2012). Matrix-assisted laser desorption ionization-time of flight mass spectrometry-based functional assay for rapid detection of resistance against beta lactam antibiotics. *J. Clin. Microbiol.* 50, 927–937. doi: 10.1128/JCM.05737-11

Tamma, P. D., Opene, B. N., Gluck, A., Chambers, K. K., Carroll, K. C., and Simner, P. J. (2017). Comparison of 11 phenotypic assays for accurate detection of carbapenemase-producing *Enterobacteriaceae*. *J. Clin. Microbiol.* 55, 1046–1055. doi: 10.1128/JCM.02338-16

Tzouvelekis, L. S., Markogiannakis, A., Psichogiou, M., Tassios, P. T., and Daikos, G. L. (2012). Carbapenemases in *Klebsiella pneumoniae* and other *Enterobacteriaceae*: an evolving crisis of global dimensions. *Clin. Microbiol. Rev.* 25, 682–707. doi: 10.1128/CMR.05035-11

van Duin, D., and Doi, Y. (2017). The global epidemiology of carbapenemase-producing *Enterobacteriaceae*. *Virulence* 8, 460–469. doi: 10.1080/21505594.2016.1222343

Vincent, J. L., Rello, J., Marshall, J., Silva, E., Anzueto, A., Martin, C. D., et al. (2009). International study of the prevalence and outcomes of infections in intensive care units. *JAMA* 302, 2323–2329. doi: 10.1001/jama.2009.1754

Youn, J.-H., Drake, S. K., Weingarten, R., Frank, K. M., Dekker, J. P., and Lau, A. F. (2016). Clinical performance of a matrix-assisted laser desorption ionization-time of flight mass spectrometry method for detection of certain blaKPC-containing plasmids. *J. Clin. Microbiol.* 54, 35–42. doi: 10.1128/JCM.01643-15

Conflict of Interest Statement: MK and JG are employees of Bruker Daltonik GmbH, the manufacturer of the MALDI Biotyper system.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

METHODS
published: 24 January 2019
doi: 10.3389/fmicb.2019.00013

Rapid Detection of Extended-Spectrum β-Lactamases (ESBL) and AmpC β-Lactamases in *Enterobacterales*: Development of a Screening Panel Using the MALDI-TOF MS-Based Direct-on-Target Microdroplet Growth Assay

Carlos L. Correa-Martínez[1†], Evgeny A. Idelevich[1†], Katrin Sparbier[2], Markus Kostrzewa[2] and Karsten Becker[1]*

[1] *Institute of Medical Microbiology, University Hospital Münster, Münster, Germany,* [2] *Bruker Daltonik GmbH, Bremen, Germany*

OPEN ACCESS

Edited by:
Miklos Fuzi, Semmelweis University, Hungary

Reviewed by:
Armand Paauw, Netherlands Organisation for Applied Scientific Research (TNO), Netherlands
Filipa Grosso, Universidade do Porto, Portugal

***Correspondence:**
Karsten Becker
kbecker@uni-muenster.de

[†] These authors have contributed equally to this work

Specialty section:
This article was submitted to Antimicrobials, Resistance and Chemotherapy, a section of the journal Frontiers in Microbiology

Received: *27 September 2018*
Accepted: *07 January 2019*
Published: *24 January 2019*

Citation:
Correa-Martínez CL, Idelevich EA, Sparbier K, Kostrzewa M and Becker K (2019) Rapid Detection of Extended-Spectrum β-Lactamases (ESBL) and AmpC β-Lactamases in Enterobacterales: Development of a Screening Panel Using the MALDI-TOF MS-Based Direct-on-Target Microdroplet Growth Assay. Front. Microbiol. 10:13. doi: 10.3389/fmicb.2019.00013

Introduction: Antibiotic resistant bacteria are a growing concern worldwide. Extended-spectrum β-lactamases (ESBL) represent the most common resistance mechanism of Gram-negative bacteria against β-lactams, underlining the need for adequate diagnostic methods that provide reliable information in the shortest time possible. AmpC, a less prevalent but increasingly relevant class of β-lactamases, pose an additional challenge as their detection is complex. Here, we present an ESBL and AmpC screening panel employing the MALDI-TOF MS-based direct-on-target microdroplet growth assay (DOT-MGA).

Materials and Methods: Four reference strains recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) were used to develop the panel, which was further validated on 50 clinical *Enterobacterales* isolates resistant to third generation cephalosporins. The panel relies on the synergistic effect between ESBL and/or AmpC β-lactamase inhibitors and cephalosporins, which indicates β-lactamase production. Microdroplets containing the tested microorganism, cephalosporins in different concentrations and inhibitors were pipetted onto an MBT Biotarget and incubated for 3 or 4 h at 35 ± 1°C. Afterward, the liquid medium was removed and the material adhered to the spots was analyzed by MALDI-TOF MS. Synergy was detected by determining and comparing the minimum inhibitory concentrations of the tested cephalosporins with and without β-lactamase inhibitors. Data were interpreted following a diagnostic algorithm proposed by EUCAST in order to establish a final diagnosis. In comparison, PCR, broth microdilution (BMD) and combination disk tests (CDT) were performed.

Results: Compared to the PCR results, the following positive and negative percent agreement values (PPA/NPA) were obtained for each resistance mechanism: ESBL, 94.44/100%; AmpC, 94.44/93.75% and ESBL+AmpC, 100/100%. These results, obtained after 4 h of incubation, were comparable with those of BMD and showed a higher accuracy than CDT.

Discussion: We propose a novel phenotypic method for detection of ESBL and AmpC β-lactamases in *Enterobacterales* that provides reliable results in a short time, representing a promising alternative to the diagnostic techniques currently available. This easy-to-perform approach has potential for being implemented in routine laboratories, contributing to the further diversification of mass spectrometry technology into other fields such as antibiotic resistance testing.

Keywords: extended-spectrum beta-lactamase, AmpC beta-lactamase, MALDI-TOF mass spectrometry, rapid diagnostic assays, multiresistance, *Enterobacterales*, *Enterobacteriaceae*, minimum inhibitory concentration

INTRODUCTION

A new class of β-lactamases able to hydrolyze expanded-spectrum β-lactam antibiotics was first described in 1985 in a *Klebsiella pneumoniae* strain (Kliebe et al., 1985). By the end of the decade, a broad range of bacteria producing these enzymes could be found in healthcare facilities worldwide. Less than 20 years after their first identification, these microorganisms already represented one of the most important groups of nosocomial pathogens (Gniadkowski, 2001). Today, extended-spectrum β-lactamases (ESBL) are the most common resistance mechanism of Gram-negative bacteria against β-lactam antibiotics (Al-Bayssari et al., 2015) and have become a concern for public health, with growing infection and colonization rates worldwide (Karanika et al., 2016; McDanel et al., 2017). ESBL-producing bacteria have also been described to play an important role beyond the boundaries of the hospital setting, as indicated by the occurrence of community-associated infections in patients without discernible healthcare-associated risk factors (Coque et al., 2008; Doi et al., 2012). Moreover, high colonization rates among hospitalized and non-hospitalized individuals have been detected in several regions (Schaumburg et al., 2013; Köck et al., 2016), which brings the hidden burden of this problem into the light.

AmpC β-lactamases, which confer resistance against a broad range of substrates, are less prevalent than ESBL but still a growing issue, having been identified in several outbreaks (Roh et al., 2008; Mansouri et al., 2014; Uzunovic et al., 2014; Kameyama et al., 2015). Multiple factors contribute to the severity of this problem, including the fact that these enzymes confer resistance to carbapenems when combined with decreased outer membrane permeability (Philippon et al., 2002; Woodford et al., 2007) and that they are not neutralized by ESBL inhibitors, which limits the possible phenotypic diagnostic and therapeutic approaches. AmpC is chromosomally encoded in several common Gram-negative bacteria such as *Enterobacter* spp., *Citrobacter freundii,* or *Serratia marcescens*. Additionally, plasmid-encoded *ampC* genes can be horizontally transferred to other *Enterobacterales* with no chromosomally encoded AmpC such as *Klebsiella*, *Proteus*, and *Salmonella*, which represents a highly effective and dynamic mode of dissemination (Bauernfeind et al., 1998; Ingram et al., 2011). This underlines the importance of developing simple and valid detection methods for AmpC production, which are currently scarce (Reuland et al., 2015).

For the detection of ESBLs and AmpC β-lactamases, phenotypic and genotypic methods are employed. The latter, which include polymerase chain reaction (PCR) and next generation sequencing (NGS), have gained relevance in clinical laboratories in the last decades (Decousser et al., 2017). They allow for a highly accurate characterization of resistance mechanisms, being of great advantage in cases where phenotypic results are unclear. However, these methods must be performed by trained personnel and require facilities fully equipped with all necessary elements. This often translates into high costs, thus limiting the availability of such methods in routine laboratories. Moreover, unknown or not annotated variants will be missed.

Phenotypic approaches to detect ESBL and/or AmpC are based on the detection of synergy between β-lactam agents and specific substances that inhibit each enzyme type. In the broth microdilution (BMD), synergy is indicated by a substantial decrease of the minimum inhibitory concentration (MIC) of the β-lactam (Wiegand et al., 2008). Following this principle, several methods that employ disks containing β-lactams and inhibitors have been validated and are widely used (M'Zali et al., 2000; Nourrisson et al., 2015). Since these assays require overnight incubation, the turnaround time amounts to 18 h (Decousser et al., 2017). This delay is also the main disadvantage of other culture-based approaches such as the double-disk synergy test, three-dimensional tests, gradient diffusion tests as well as automated systems (Drieux et al., 2008). Rapid testing with disk diffusion has been described, however, being recommended only for preliminary susceptibility reports. It requires further standardization and adapted clinical breakpoints for a correct interpretation (Froding et al., 2017). Colorimetric methods represent a substantially faster alternative, although some of them display a low positive predictive value

in the presence of AmpC hyperproduction (Decousser et al., 2017).

Methods based on matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) can also be applied to identify β-lactam resistance. One of these approaches relies on the detection of products resulting from the hydrolysis of β-lactam antibiotics by β-lactamases (Sparbier et al., 2012; Oviano et al., 2017; Lee et al., 2018). Nonetheless, this depends on several factors that are specific to the enzyme-substrate interaction (such as enzyme availability), which could interfere with the results, as well as alternate mechanisms that also lead to misdetection of antibiotic hydrolysis. Moreover, a positive result indicates the presence of resistance, but does not provide an exact MIC value for the tested antibiotic (Decousser et al., 2017). Another strategy consists in detecting isotopically labeled amino acids, which requires a specific culture medium that can be supplemented with these substances (Sparbier et al., 2013; Jung et al., 2014). The applicability of techniques that analyze the amount of biomass resulting from bacterial incubation with antibiotic agents in order to establish susceptibility patterns has also been described (Sparbier et al., 2016).

Considering the need for rapid methods that are easy to standardize, we developed and validated a screening panel for detection of ESBL and AmpC β-lactamases in *Enterobacterales* adapting the principle of the MALDI-TOF MS-based direct-on-target microdroplet growth assay (DOT-MGA) previously described (Idelevich et al., 2018a,b). While this method does not rely on the detection of hydrolyzed β-lactam, it resembles the MIC determination by BMD. The panel's layout and the interpretation criteria follow the criteria of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (EUCAST, 2017). With this approach, we sought to establish a method able to overcome common obstacles in the detection of these resistance mechanisms, such as unclear results in isolates producing both types of β-lactamases as well as false negative-results due to inadequate AmpC identification. The assay was validated on clinical isolates of the order *Enterobacterales* including species of the *Enterobacteriaceae*, *Hafniaceae*, *Morganellaceae*, and *Yersiniaceae* families to further assess its practicability and accuracy.

MATERIALS AND METHODS

Bacterial Strains and Cultures

Enterobacterales strains were consecutively isolated from clinical samples processed in the routine diagnostic laboratory at the Institute of Medical Microbiology, University Hospital Münster, Germany. Species identification was performed by MALDI-TOF MS. Isolates displaying phenotypic resistance against third generation cephalosporins in the susceptibility testing performed routinely with Vitek 2® (bioMérieux, Marcy-l'Étoile, France) were consecutively collected. In total, 50 strains were tested, 25 belonging to each of the two groups defined by EUCAST depending on the mechanism of resistance most likely involved (EUCAST, 2017): group 1, ESBL (*Escherichia coli, Klebsiella pneumoniae, K. oxytoca, Raoultella ornithinolytica*); group 2, AmpC (*Hafnia alvei, C. freundii, C. koseri, S. marcescens, Enterobacter cloacae* complex*, E. aerogenes, Morganella morganii*). As a reference, three resistant control strains recommended by EUCAST for the detection of ESBL and AmpC production were tested (*K. pneumoniae* ATCC 700603, *E. coli* CCUG 62975, *E. coli* CCUG 58543), as well as one negative control strain (*E. coli* ATCC 25922) (EUCAST, 2017). Bacterial suspensions were prepared using colonies grown on blood agar. Density was adjusted to 0.5 McFarland employing a nephelometer (Densimat, bioMérieux, Marcy-l'Étoile, France). Subsequently, a dilution 1:100 was made with cation-adjusted Mueller-Hinton broth (CA-MHB).

DOT-MGA-Based Screening Panel

A screening panel was developed on a 96-spot format following the layout depicted on **Figure 1A**. The MIC of four cephalosporins was determined in absence and presence of an ESBL inhibitor (clavulanic acid, 4 μg/ml) and an AmpC inhibitor (cloxacillin, 512 μg/ml) in order to establish a result on the basis of the synergy observed. The layout was designed according to the detection algorithm suggested by EUCAST (2017). It comprises four zones: screening for resistance against third generation cephalosporins with cefpodoxim (blue zone), ESBL detection with cefotaxime and ceftazidime in presence and absence of ESBL inhibitor (yellow zone), AmpC detection with cefepime (gray zone), and detection of AmpC plus masked ESBL with cefepime plus ESBL inhibitor or cefotaxime plus ESBL inhibitor and AmpC inhibitor (purple zone).

Antibiotic Substances

Stock solutions were prepared following the guidelines of the Clinical and Laboratory Standards Institute (CLSI) (CLSI, 2018) by mixing the following antimicrobial agents in powder form with deionized distilled water: cefepime, cefpodoxime (TOKU-E, Bellingham, WA, United States), ceftazidime, potassium clavulanate (Sigma-Aldrich, Saint Louis, MO, United States), cefotaxime, cloxacillin (Tokyo Chemical Industry Co., LTD, Tokyo, Japan). Quality controls of the solutions were carried out in accordance with the specifications of the CLSI (2018) and EUCAST (2018).

MALDI-TOF MS

DOT-MGA was performed as previously described (Idelevich et al., 2018a). Briefly, microdroplets containing 3 μl of antibiotic solution and 3 μl of bacterial suspension (final inoculum approximately 5×10^5 CFU/ml) were pipetted onto an MBT Biotarget 96 (Bruker Daltonik, Bremen, Germany). Sterility and growth controls were spotted on a second target. Targets were incubated for 3 or 4 h at 35 ± 1°C using a plastic transport box (Bruker Daltonik) serving as a humidity chamber in order to prevent the microdroplets from evaporating. After incubation, the remaining liquid was removed from the spots using filter paper, (size 37 × 100 mm, GE Healthcare GmbH, Freiburg im Breisgau, Germany). After overlaying the spots with 1 μl of α-cyano-4-hydroxycinnamic acid matrix including internal standard (MBT MASTeR prototype kit, Bruker Daltonik), MALDI-TOF MS spectra were acquired on a microflex smart

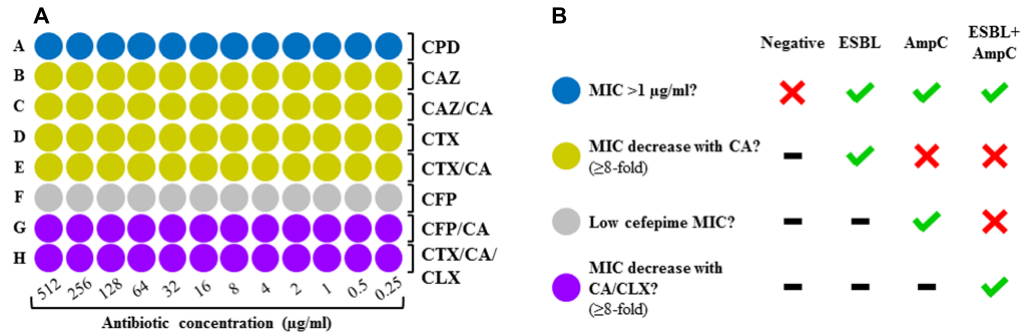


FIGURE 1 | (A) Layout of the DOT-MGA screening panel. Blue zone, resistance screening; yellow zone, detection of ESBL; gray zone, detection of AmpC; purple zone, detection of ESBL + AmpC. CPD, cefpodoxime; CAZ, ceftazidime; CTX, cefotaxime; CFP, cefepime; CA, clavulanic acid; CLX, cloxacillin. **(B)** Interpretation of results according to EUCAST's diagnostic criteria.

instrument (Bruker Daltonik). The method was performed in triplicate.

Interpretation of Panel Results

Minimum inhibitory concentration

The spectra obtained from the MALDI-TOF MS acquisitions were processed using the MALDI Biotyper Software 3.1 (Bruker Daltonik), resulting in an identification score for each spot analyzed. A score ≥2.0 was considered as an indicator of bacterial growth detection. For each antimicrobial substance or substance combination, the minimum antibiotic concentration showing no bacterial growth, equivalent to a score <2.0, was defined as the MIC. In case all spots of one dilution series displayed scores <2.0, the MIC was defined as ≤0.25. MICs were calculated in triplicate, as the assay was simultaneously performed on three identical targets for each strain. The median MIC (middle value in ascending order) was used for further analysis.

Screening and confirmation

The MICs of cephalosporins determined in absence and presence of ESBL and/or AmpC inhibitor were compared in order to detect synergy, which was considered to be present when a ≥eightfold reduction of the MICs was observed after addition of clavulanic acid and/or cloxacillin. The results obtained for each cephalosporin were interpreted using a computer-based algorithm following the EUCAST criteria (EUCAST, 2017), obtaining three possible results: ESBL, AmpC, ESBL+AmpC or negative (**Figure 1B**).

Detection of Resistance Genes

A genotypic characterization of all tested strains was performed with the PCR microarray Check-MDR CT103 XL (Check-Points, Wageningen, Netherlands). For an overview of the genes detected see **Supplementary Table S4**.

Additional Phenotypic Diagnostic Methods

Broth Microdilution

The screening panel was reproduced on microtiter plates by performing BMD according to the guidelines of CLSI (2018) and the International Organization for Standardization (ISO) (ISO, 2006). In short, bacterial suspension and antibiotic solutions employed in the DOT-MGA were pipetted onto a first plate (30 µl of each solution, total volume of 60 µl), using a second one for the growth and sterile controls. Both microtiter plates were incubated for 18 ± 2 h at 35 ± 1°C. The MIC was interpreted as the lowest antibiotic concentration at which complete growth inhibition was seen. All MIC determinations were performed in triplicate. Median values were calculated for further analysis.

Combination Disk Test (CDT)

The following combination disk tests (Mast Diagnostica GmbH, Reinfeld, Germany) were carried out following the procedure recommended by the manufacturer: D63C (cefpodoxime alone and combined with clavulanic acid), D67C (cefpodoxime, cefotaxime and ceftazidime alone and combined with clavulanic acid) and D69C (cefpodoxime alone and combined with AmpC inducer, clavulanic acid and cloxacillin). Briefly, bacterial suspensions with a density equivalent to 0.5 McFarland standard was spread on Mueller-Hinton agar plates (BD GmbH, Heidelberg, Germany). Disks were then placed onto the inoculated medium, leaving enough space for inhibition zones to be seen correctly. Plates were then incubated at 35–37°C for 18 h, after which the diameter of the zones of inhibition was measured and recorded according to the instructions of use. The results of the three tests were interpreted as shown in **Supplementary Table S1**.

Statistical Analysis

According to EUCAST's guidelines (EUCAST, 2017), genotypic testing is the conclusive method to identify resistance mechanisms in cases where phenotypic techniques do not provide clear results. Thus, we considered the PCR an imperfect reference standard (Valenstein, 1990) in order to determine the positive and negative percent agreements (PPA and NPA, respectively) of the DOT-MGA, BMD, and CDT, which were calculated according to the statistical guidance of the Food and Drug Administration (FDA, 2007).

RESULTS

In preliminary experiments, DOT-MGA was performed on four well-characterized reference strains recommended by EUCAST for detection of ESBL and AmpC production (EUCAST, 2017). After 3 h of incubation, the resistance mechanisms of two of three resistant microorganisms were correctly identified. A correct identification of all three resistant strains was possible after 4 h of incubation. At both time points, the assay yielded a negative result for a susceptible reference strain (**Table 1**).

A total of 50 clinical *Enterobacterales* isolates displaying resistance against third generation cephalosporins were tested in order to further evaluate the detection performance of DOT-MGA. The additional genotypic testing by PCR allowed the identification of an OXA-48-producing *K. pneumoniae* strain (**Supplementary Table S3**). For each strain, DOT-MGA was performed in two set-ups, with incubation times of three and 4 h, respectively. The results obtained were further analyzed and compared with those of the PCR (**Supplementary Table S2**). Here, it could be confirmed that 4 h of incubation are required for a reliable detection, as also indicated by the preliminary experiments on reference strains (**Supplementary Table S2**).

The different resistance mechanisms were detected by DOT-MGA (4-h incubation), BMD, CDT and PCR in the following number of isolates, respectively; ESBL, 17,19,18,18; AmpC, 19,22,15,18; ESBL+AmpC, 1,1,0,1; none, 13,8,17,13. A detailed overview of the results yielded by each method can be found in **Supplementary Table S3**.

The following positive and negative percent agreement values (PPA/NPA) were obtained for the detection of each resistance mechanism by DOT-MGA: ESBL, 94.44/100%; AmpC, 94.44/93.75% and ESBL+AmpC, 100/100%. Percent agreement values of BMD and CDT were also calculated and are presented in **Table 2**, which shows a comparative overview of the detection performance of all three methods.

TABLE 1 | Detection performance of the DOT-MGA screening panel on reference strains recommended by EUCAST.

Strain	Known resistance mechanism	DOT-MGA screening panel result	
		Incubation time	
		3 h	4 h
K. pneumoniae ATCC 700603	ESBL	ESBL	ESBL
E. coli CCUG 58543	AmpC	Negative	AmpC
E. coli CCUG 62975	ESBL + AmpC	ESBL + AmpC	ESBL + AmpC
E. coli ATCC 25922	None	Negative	Negative

TABLE 2 | Detection performance of DOT-MGA, BMD, and CDT on clinical isolates compared to PCR.

Resistance mechanism	Detection method (incubation time)					
	DOT-MGA (4 h)		BMD (18 h)		CDT (4 h)	
	PPA	NPA	PPA	NPA	PPA	NPA
ESBL	94.4%	100%	100%	96.9%	94.4%	96.9%
AmpC	94.4%	93.8%	100%	87.8%	61.1%	87.5%
ESBL + AmpC	100%	100%	100%	100%	0.0%	100%

DISCUSSION

The proposed approach offers an "all-in-one" screening method following the recommendations of EUCAST. It allows testing for different resistance mechanisms in a single step and displays higher PPA and NPA values than CDT, a well-established phenotypic test commonly used in routine laboratories. Furthermore, it yielded results comparable to those of BMD, while requiring an incubation period 14 h shorter. Since this method is not based on the detection of hydrolytic β-lactam products, it bypasses the challenges faced by other MALDI-TOF MS-based approaches that rely on this principle.

DOT-MGA was able to identify the production of ESBL in 17 of 18 isolates identified as positive by PCR. The remaining isolate displayed an indeterminate DOT-MGA result. This seems to be related to factors inherent to the strain in question, given that no significant growth was detected after 4 h of incubation when the assay was performed. Hence, it was not possible to identify any synergistic effects and, thus, any resistance mechanisms. The production of ESBL by this strain was confirmed by BMD after 18 h of incubation. The results of CDT were inconclusive as all three kits were positive. False negative results for slow growing strains shows to be one limitation of our method, since it relies on bacterial growth as do several other rapid phenotypic approaches.

AmpC-producing isolates pose a major diagnostic challenge, as ESBL inhibitors such as clavulanic acid have no effect on AmpC enzymes, thus interfering with the identification of ESBL production (Philippon et al., 2002). This leads to a wrong classification of such microorganisms as "non-ESBL-producing" and therefore as not multidrug resistant. In the case of strains displaying both resistance mechanisms, ESBL remains masked although AmpC can be successfully detected. For this reason, a method for detection of ESBL must necessarily also allow the proper identification of AmpC production. In order to tackle this problem, we designed a double identification strategy: (i) AmpC detection with cefepime, an AmpC-stable cephalosporin (**Figure 1A**, row F) (Thomson, 2001); (ii) additional detection of masked ESBL with either cefepime plus clavulanic acid (**Figure 1A**, row G) or cefotaxime plus clavulanic acid and cloxacillin as AmpC inhibitor (**Figure 1A**, row H). This confers our approach a higher accuracy of detection for strains producing solely AmpC as well as for those showing combined resistance mechanisms. The discrepancies observed between DOT-MGA and PCR correspond to strains showing no resistance genes, but a phenotypic resistance pattern compatible with AmpC production, confirmed by BMD, as is the case of one *E. aerogenes* isolate. Despite the high accuracy of the PCR, this was employed in our study as imperfect reference standard (Valenstein, 1990). Since such methods are based on the amplification of certain

target genes regardless of their phenotypic expression, they might yield positive results in strains showing no phenotypic correlation due to the non-expression of the resistance gene(s), as well as false negative results in strains with phenotypic signs of AmpC production, most likely mediated by genes not targeted and thus not detected.

The test has been designed as a complementary diagnostic tool that can be integrated to the routine laboratory workflow at different points, i.e., simultaneous to the standard susceptibility testing of *Enterobacterales* isolates on a regular basis, or as a confirmation once a resistance against third generation cephalosporines has been determined.

The proposed assay represents a promising alternative to the methods of detection of resistance mechanisms currently available. It yields reliable results in a short time, providing concrete evidence that could directly impact the decision-making process in the healthcare setting. This would have several implications such as a direct improvement of the clinical outcome, a more rational use of antibiotics and shorter reaction times in the context of hospital infection control.

DOT-MGA can potentially be adapted for commercial production. Possible strategies for the automation of the method include: (i) developing targets coated with lyophilized antibiotic substances according to the panel's layout, requiring only the addition of bacterial suspension to the spots; (ii) programming a new function within the existing Biotyper software in order to analyze the growth scores of each spot, programming it to follow an algorithm based on fix criteria (**Figure 1B**) in order to yield a final diagnosis.

AUTHOR CONTRIBUTIONS

CC-M, EI, and KB designed the experiments. CC-M and EI performed the experiments. CC-M, EI, KS, and MK designed and analyzed specific MALDI-TOF MS instrument settings for experiments. CC-M, EI, KS, and KB analyzed the data. CC-M and EI wrote the manuscript with input from KS, MK, and KB. All authors reviewed and edited the manuscript.

FUNDING

This study was funded in part by grants from the German Federal Ministry of Education and Research (BMBF) to EI and KB (16GW0150) and MK (16GW0149K).

ACKNOWLEDGMENTS

We thank Annkatrin Bibo and Damayanti Kaiser for their excellent technical assistance.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.00013/full#supplementary-material

REFERENCES

Al-Bayssari, C., Dabboussi, F., Hamze, M., and Rolain, J.-M. (2015). Detection of expanded-spectrum β-lactamases in Gram-negative bacteria in the 21st century. *Expert Rev. Anti Infect. Ther.* 13, 1139–1158. doi: 10.1586/14787210.2015.1066247

Bauernfeind, A., Chong, Y., and Lee, K. (1998). Plasmid-encoded AmpC beta-lactamases: how far have we gone 10 years after the discovery? *Yonsei Med. J.* 39, 520–525.

CLSI (2018). *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically. M07*, 11th Edn. Wayne, PA: Clinical Laboratory Standards Institute.

Coque, T., Baquero, F., and Canton, R. (2008). Increasing prevalence of ESBL-producing *Enterobacteriaceae* in Europe. *Euro Surveill.* 13:19044.

Decousser, J. W., Poirel, L., and Nordmann, P. (2017). Recent advances in biochemical and molecular diagnostics for the rapid detection of antibiotic-resistant *Enterobacteriaceae*: a focus on ss-lactam resistance. *Expert Rev. Mol. Diagn.* 17, 327–350. doi: 10.1080/14737159.2017.1289087

Doi, Y., Park, Y. S., Rivera, J. I., Adams-Haduch, J. M., Hingwe, A., Sordillo, E. M., et al. (2012). Community-associated extended-spectrum β-lactamase–producing *Escherichia coli* infection in the United States. *Clin. Infect. Dis.* 56, 641–648. doi: 10.1093/cid/cis942

Drieux, L., Brossier, F., Sougakoff, W., and Jarlier, V. (2008). Phenotypic detection of extended-spectrum beta-lactamase production in *Enterobacteriaceae*: review and bench guide. *Clin. Microbiol. Infect.* 14(Suppl. 1), 90–103. doi: 10.1111/j.1469-0691.2007.01846.x

EUCAST (2017). *Guidelines for Detection of Resistance Mechanisms and Specific Resistances of Clinical and/or Epidemiological Importance. The European Committee on Antimicrobial Susceptibility Testing. Version 2.0*. Available at: http://www.eucast.org

EUCAST (2018). *Routine and Extended Internal Quality Control for MIC Determination and Disk Diffusion as Recommended by EUCAST. The European Committee on Antimicrobial Susceptibility Testing. Version 8.0*. Available at: http://www.eucast.org

FDA (2007). *Guidance for Industry and FDA Staff–Statistical Guidance on Reporting Results from Studies Evaluating Diagnostic Tests*. Rockville, MD: Center for Devices and Radiologic Health Diagnostic Devices.

Froding, I., Vondracek, M., and Giske, C. G. (2017). Rapid EUCAST disc diffusion testing of MDR *Escherichia coli* and *Klebsiella pneumoniae*: inhibition zones for extended-spectrum cephalosporins can be reliably read after 6 h of incubation. *J. Antimicrob. Chemother.* 72, 1094–1102. doi: 10.1093/jac/dkw515

Gniadkowski, M. (2001). Evolution and epidemiology of extended-spectrum beta-lactamases (ESBLs) and ESBL-producing microorganisms. *Clin. Microbiol. Infect.* 7, 597–608. doi: 10.1046/j.1198-743x.2001.00330.x

Idelevich, E. A., Sparbier, K., Kostrzewa, M., and Becker, K. (2018a). Rapid detection of antibiotic resistance by MALDI-TOF mass spectrometry using a novel direct-on-target microdroplet growth assay. *Clin. Microbiol. Infect.* 24, 738–743. doi: 10.1016/j.cmi.2017.10.016

Idelevich, E. A., Storck, L. M., Sparbier, K., Drews, O., Kostrzewa, M., and Becker, K. (2018b). Rapid direct susceptibility testing from positive blood cultures by the matrix-assisted laser desorption ionization-time of flight mass spectrometry-based direct-on-target microdroplet growth assay. *J. Clin. Microbiol.* 56:e00913-18. doi: 10.1128/JCM.00913-18

Ingram, P. R., Inglis, T. J., Vanzetti, T. R., Henderson, B. A., Harnett, G. B., and Murray, R. J. (2011). Comparison of methods for AmpC β-lactamase detection in *Enterobacteriaceae*. *J. Med. Mirocbiol.* 60, 715–721. doi: 10.1099/jmm.0.029140-0

ISO (2006). *ISO 20776-1: 2006. Clinical Laboratory Testing and In Vitro Diagnostic Test Systems-Susceptibility Testing of Infectious Agents and Evaluation of Performance of Antimicrobial Susceptibility Test Devices-Part 1: Reference Method for Testing the In Vitro Activity of Antimicrobial Agents Against Rapidly*

Growing Aerobic Bacteria Involved in Infectious Diseases. Geneva: International Organization for Standardization.

Jung, J. S., Eberl, T., Sparbier, K., Lange, C., Kostrzewa, M., Schubert, S., et al. (2014). Rapid detection of antibiotic resistance based on mass spectrometry and stable isotopes. *Eur. J. Clin. Microbiol. Infect. Dis.* 33, 949–955. doi: 10.1007/s10096-013-2031-5

Kameyama, M., Yabata, J., Nomura, Y., and Tominaga, K. (2015). Detection of CMY-2 AmpC beta-lactamase-producing enterohemorrhagic *Escherichia coli* O157:H7 from outbreak strains in a nursery school in Japan. *J. Infect. Chemother.* 21, 544–546. doi: 10.1016/j.jiac.2015.03.004

Karanika, S., Karantanos, T., Arvanitis, M., Grigoras, C., and Mylonakis, E. (2016). Fecal colonization with extended-spectrum beta-lactamase-producing *Enterobacteriaceae* and risk factors among healthy individuals: a systematic review and metaanalysis. *Clin. Infect. Dis.* 63, 310–318. doi: 10.1093/cid/ciw283

Kliebe, C., Nies, B. A., Meyer, J. F., Tolxdorff-Neutzling, R. M., and Wiedemann, B. (1985). Evolution of plasmid-coded resistance to broad-spectrum cephalosporins. *Antimicrob. Agents Chemother.* 28, 302–307. doi: 10.1128/AAC.28.2.302

Köck, R., Werner, P., Friedrich, A. W., Fegeler, C., and Becker, K. (2016). Persistence of nasal colonization with human pathogenic bacteria and associated antimicrobial resistance in the German general population. *New Microbes New Infect.* 9, 24–34. doi: 10.1016/j.nmni.2015.11.004

Lee, A. W. T., Lam, J. K. S., Lam, R. K. W., Ng, W. H., Lee, E. N. L., Lee, V. T. Y., et al. (2018). Comprehensive evaluation of the MBT STAR-BL module for simultaneous bacterial identification and beta-lactamase-mediated resistance detection in gram-negative rods from cultured isolates and positive blood cultures. *Front. Microbiol.* 9:334. doi: 10.3389/fmicb.2018.00334

Mansouri, S., Kalantar Neyestanaki, D., Shokoohi, M., Halimi, S., Beigverdi, R., Rezagholezadeh, F., et al. (2014). Characterization of AmpC, CTX-M and MBLs types of beta-lactamases in clinical isolates of *Klebsiella pneumoniae* and *Escherichia coli* producing extended spectrum beta-lactamases in Kerman, Iran. *Jundishapur J. Microbiol.* 7:e8756. doi: 10.5812/jjm.8756

McDanel, J., Schweizer, M., Crabb, V., Nelson, R., Samore, M., Khader, K., et al. (2017). Incidence of extended-spectrum beta-lactamase (ESBL)-producing *Escherichia coli* and *Klebsiella* infections in the United States: a systematic literature review. *Infect. Control Hosp. Epidemiol.* 38, 1209–1215. doi: 10.1017/ice.2017.156

M'Zali, F. H., Chanawong, A., Kerr, K. G., Birkenhead, D., and Hawkey, P. M. (2000). Detection of extended-spectrum beta-lactamases in members of the family *enterobacteriaceae*: comparison of the MAST DD test, the double disc and the Etest ESBL. *J. Antimicrob. Chemother.* 45, 881–885. doi: 10.1093/jac/45.6.881

Nourrisson, C., Tan, R. N., Hennequin, C., Gibold, L., Bonnet, R., and Robin, F. (2015). The MAST D68C test: an interesting tool for detecting extended-spectrum β-lactamase (ESBL)-producing *Enterobacteriaceae*. *Eur. J. Clin. Microbiol. Infect. Dis.* 34, 975–983. doi: 10.1007/s10096-014-2305-6

Oviano, M., Gomara, M., Barba, M. J., Revillo, M. J., Barbeyto, L. P., and Bou, G. (2017). Towards the early detection of beta-lactamase-producing *Enterobacteriaceae* by MALDI-TOF MS analysis. *J. Antimicrob. Chemother.* 72, 2259–2262. doi: 10.1093/jac/dkx127

Philippon, A., Arlet, G., and Jacoby, G. A. (2002). Plasmid-determined AmpC-type beta-lactamases. *Antimicrob. Agents Chemother.* 46, 1–11. doi: 10.1128/AAC.46.1.1-11.2002

Reuland, E. A., Halaby, T., Hays, J. P., De Jongh, D. M., Snetselaar, H. D., Van Keulen, M., et al. (2015). Plasmid-mediated AmpC: prevalence in community-acquired isolates in Amsterdam, the Netherlands, and risk factors for carriage. *PLoS One* 10:e0113033. doi: 10.1371/journal.pone.0113033

Roh, K. H., Uh, Y., Kim, J. S., Kim, H. S., Shin, D. H., and Song, W. (2008). First outbreak of multidrug-resistant *Klebsiella pneumoniae* producing both SHV-12-type extended-spectrum beta-lactamase and DHA-1-type AmpC beta-lactamase at a Korean hospital. *Yonsei Med. J.* 49, 53–57. doi: 10.3349/ymj.2008.49.1.53

Schaumburg, F., Alabi, A., Kokou, C., Grobusch, M. P., Köck, R., Kaba, H., et al. (2013). High burden of extended-spectrum beta-lactamase-producing *Enterobacteriaceae* in Gabon. *J. Antimicrob. Chemother.* 68, 2140–2143. doi: 10.1093/jac/dkt164

Sparbier, K., Lange, C., Jung, J., Wieser, A., Schubert, S., and Kostrzewa, M. (2013). MALDI biotyper-based rapid resistance detection by stable-isotope labeling. *J. Clin. Microbiol.* 51, 3741–3748. doi: 10.1128/JCM.01536-13

Sparbier, K., Schubert, S., and Kostrzewa, M. (2016). MBT-ASTRA: a suitable tool for fast antibiotic susceptibility testing? *Methods* 104, 48–54. doi: 10.1016/j.ymeth.2016.01.008

Sparbier, K., Schubert, S., Weller, U., Boogen, C., and Kostrzewa, M. (2012). Matrix-assisted laser desorption ionization-time of flight mass spectrometry-based functional assay for rapid detection of resistance against beta-lactam antibiotics. *J. Clin. Microbiol.* 50, 927–937. doi: 10.1128/JCM.05737-11

Thomson, K. S. (2001). Controversies about extended-spectrum and AmpC beta-lactamases. *Emerg. Infect. Dis.* 7, 333–336. doi: 10.3201/eid0702.010238

Uzunovic, S., Bedenic, B., Budimir, A., Kamberovic, F., Ibrahimagic, A., Delic-Bikic, S., et al. (2014). Emergency (clonal spread) of methicillin-resistant *Staphylococcus aureus* (MRSA), extended spectrum (ESBL)–and AmpC beta-lactamase-producing Gram-negative bacteria infections at Pediatric Department, Bosnia and Herzegovina. *Wien. Klin. Wochenschr.* 126, 747–756. doi: 10.1007/s00508-014-0597-2

Valenstein, P. N. (1990). Evaluating diagnostic tests with imperfect standards. *Am. J. Clin. Pathol.* 93, 252–258. doi: 10.1093/ajcp/93.2.252

Wiegand, I., Hilpert, K., and Hancock, R. E. (2008). Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. *Nat. Protoc.* 3, 163–175. doi: 10.1038/nprot.2007.521

Woodford, N., Dallow, J. W., Hill, R. L., Palepou, M. F., Pike, R., Ward, M. E., et al. (2007). Ertapenem resistance among *Klebsiella* and *Enterobacter* submitted in the UK to a reference laboratory. *Int. J. Antimicrob. Agents* 29, 456–459. doi: 10.1016/j.ijantimicag.2006.11.020

Conflict of Interest Statement: EI and KB are inventors of a pending patent, which is owned by the University of Münster and licensed to Bruker. KS and MK are employees of Bruker Daltonik GmbH.

The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 20 June 2019
doi: 10.3389/fmicb.2019.01413

Performance Evaluation of the MBT STAR®-Carba IVD Assay for the Detection of Carbapenemases With MALDI-TOF MS

Ahalieyah Anantharajah, Bastien Tossens, Nathalie Olive, Benoit Kabamba-Mukadi, Hector Rodriguez-Villalobos and Alexia Verroken*

Department of Microbiology, Cliniques Universitaires Saint-Luc, Université catholique de Louvain, Brussels, Belgium

Objectives: The increasing rate of carbapenem resistance in Gram-negative bacteria is a major public health problem and rapid detection is essential for infection management. We evaluated the performances of the MBT STAR®-Carba IVD assay (Bruker Daltonics) to detect carbapenemase-producing organisms (CPO) from bacterial colonies and directly from positive blood culture bottles with MALDI-TOF MS.

Methods: We analyzed 130 strains with a reduced susceptibility to at least one carbapenem including 109 CPO (6 KPC, 27 NDM, 21 VIM, 1 IMP, 41 OXA-48-like, 8 OXA-23, 2 OXA-24/-40, and 2 OXA-58) and 21 non-CPO. The assay on colonies was performed with all 130 strains while the assay on spiked blood cultures was performed with 45 strains. Samples were prepared with the MBT STAR®-CARBA IVD kit and imipenem hydrolysis by the potential carbapenemase was analyzed with the MBT STAR®-BL module (Bruker Daltonics) on MALDI-TOF MS.

Results: Performed on colonies, the assay detected all carbapenemase-producing Enterobacteriaceae (n = 78), *Pseudomonas* spp. (n = 19) and *Acinetobacter* spp. (n = 12). All 21 tested non-CPO remained negative resulting in sensitivity and specificity of 100%. Performed on positive blood cultures, the assay detected all carbapenemase-producing Enterobacteriaceae (n = 23) and *Pseudomonas* spp. (n = 4) but missed 9/12 carbapenemase-producing *Acinetobacter* spp. However, a prolonged imipenem-incubation time of the strain pellet improved carbapenemase detection. Non-CPO from positive blood culture bottles remained negative (n = 5) with the assay with the exception of one *Klebsiella pneumoniae* isolate.

Conclusion: The MBT STAR®-Carba IVD assay is a highly reliable method for the detection of carbapenemase activity in Gram-negative bacteria. However, time-consuming sample preparation steps and reagent costs need to be considered before implementation in a routine clinical microbiology laboratory.

Keywords: carbapenemase detection, hydrolysis assay, MALDI-TOF MS, Gram-negative bacteria, performances, positive blood cultures

OPEN ACCESS

Edited by:
Karsten Becker,
University of Münster, Germany

Reviewed by:
Laurent DORTET,
Bicêtre Hospital, France
João Nobrega De Almeida Júnior,
University of São Paulo, Brazil

****Correspondence:***
Ahalieyah Anantharajah
Ahalieyah.anantharajah@uclouvain.be

Specialty section:
This article was submitted to Antimicrobials, Resistance and Chemotherapy, a section of the journal Frontiers in Microbiology

Received: *10 February 2019*
Accepted: *05 June 2019*
Published: *20 June 2019*

Citation:
Anantharajah A, Tossens B, Olive N, Kabamba-Mukadi B, Rodriguez-Villalobos H and Verroken A (2019) Performance Evaluation of the MBT STAR®-Carba IVD Assay for the Detection of Carbapenemases With MALDI-TOF MS. Front. Microbiol. 10:1413. doi: 10.3389/fmicb.2019.01413

INTRODUCTION

Carbapenemase-producing organisms (CPO) are a worldwide threat for clinical patient care and public health. The mobile genetic elements bearing the carbapenemase genes are not only responsible for their rapid dissemination but also carry non-β-lactam resistance determinants hereby giving rise to extremely drug resistant isolates (Kumarasamy et al., 2010; Thomson, 2017).

Rapid and reliable detection methods allow appropriate antimicrobial therapy and early implementation of infection control measures to prevent subsequent dissemination of CPO. However, detection of carbapenemases has become a critical challenge in clinical microbiology. Nearly 20% of carbapenemase-producing isolates can be missed using interpretative criteria recommended by the EUCAST or the Clinical and Laboratory Standards Institute (CLSI) (Huang et al., 2014). Most carbapenemases hydrolyze the different carbapenems at variable levels hereby not systematically conferring full resistance. Besides, decreased carbapenem susceptibility may also be caused by reduced permeability due to porin down-regulation or over-expression of efflux pumps (Nordmann et al., 2012). Numerous phenotypic and DNA-based methods have been used in the laboratory aiming at the detection of CPO (Hrabak et al., 2014). Classically, results of culture-based phenotypic methods are available in 24–72 h after isolation of the bacteria from the infected samples, whereas PCR assay results are available within hours but at higher cost (Queenan and Bush, 2007). Moreover, PCR can only detect a predefined range of carbapenem-resistance genes and their presence does not guarantee their expression (Pellegrino et al., 2008). Recently rapid phenotypic methods have been developed such as hydrolysis methods allowing the recognition of all types of carbapenemases with optimal sensitivity and specificity (Dortet et al., 2014a; Tamma and Simner, 2018). These tests include biochemical tests and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) techniques. Following a short incubation of the Gram-negative strain with a defined carbapenem, MALDI-TOF MS monitors the distinct mass peaks of the hydrolysed and non-hydrolysed forms of the antibiotic in the bacterial suspension (Carvalhaes et al., 2013, 2014). Several arduous in-house hydrolysis MALDI-TOF MS assays have been described with different sets of antibiotic combinations, buffers, and variable incubation times from 15 min to 4 h (Hrabak et al., 2011; Mirande et al., 2015).

In the present study, we evaluated the commercial MBT STAR®-Carba IVD assay (Bruker Daltonik GmbH, Bremen, Germany) for MALDI-TOF MS carbapenemase detection. Testing was performed on culture isolates and directly on positive blood culture bottles.

MATERIALS AND METHODS

Strain Collection

A total of 130 Gram-negative isolates, including 89 Enterobacteriaceae (**Supplementary Table S1**), 29 *Pseudomonas* spp. and 12 *Acinetobacter baumannii* complex, intermediate or resistant to at least one carbapenem (imipenem, meropenem or ertapenem) were selected to evaluate the performances of the MBT STAR®-Carba assay on colonies (**Table 1A**) These strains included all carbapenem resistant isolates recovered from clinical and screening samples at the microbiology laboratory of the Cliniques universitaires Saint-Luc between January 2015 and March 2018. Duplicate isolates from the same patient and duplicate outbreak strains were excluded. They were characterized for antimicrobial susceptibility with the automated Phoenix system (Becton-Dickinson, Franklin Lakes, NJ, United States) and confirmed with the manual disk diffusion method (Bio-Rad, Marnes-la-Coquette, France) using the EUCAST 2018 clinical breakpoints. Ultimately a PCR, as described below, used as reference method in this study, was performed on each strain to detect the presence of carbapenemase resistance genes. Included isolates carried a carbapenemase resistance gene (n = 109) or expressed other resistance mechanisms (n = 21) mainly including extended-spectrum-β-lactamases (ESBL) (n = 7), AmpC-type cephalosporinases (n = 3) associated or not with uncharacterized carbapenem resistance mechanisms (porin loss, efflux pumps overexpression) (n = 11). For the evaluation of the MBT STAR®-Carba assay directly on positive blood cultures, 45 isolates were selected among this collection (**Table 1B**).

Detection of Genes Encoding Carbapenemases

The carbapenemase resistance genes ($bla_{\text{OXA}-48}$, bla_{KPC}, bla_{NDM}, bla_{VIM}, bla_{NDM}, and bla_{IMP}) were detected by an in-house multiplex PCR (Bogaerts et al., 2013). To differentiate between the OXA-48 variants, the $bla_{\text{OXA}-48}$-like genes were sequenced using the following primers: OXA-48F, 5′-ATGCGTGTATTAGCCTTATCG-3′ and OXA-48R, 5′-GAGCACTTCTTTTGTGATGGC-3′ (Bogaerts et al., 2013; Oueslati et al., 2015). The full sequence length was 774 bp. For carbapenemase-producing *A. baumannii* complex strains, detection of carbapenemase genes ($bla_{\text{OXA}-23}$, $bla_{\text{OXA}-24}$, and $bla_{\text{OXA}-58}$) was performed by the Belgian national reference center.

Blood Culture Preparation

Blood cultures were spiked by inoculating the bottles [Bactec Plus Aerobic, Lytic Anaerobic or Peds Plus media (Becton Dickinson)] with 10 mL of human blood and 20 μL of a 0.5 McFarland suspension from a fresh overnight culture isolate. Blood culture bottles were incubated directly in a Bactec FX blood culture system (Becton Dickinson) until they flagged positive. Bacterial material was isolated through an adapted Sepsityper (Bruker Daltonik GmbH) workflow: the amount of Lysis Buffer was reduced and no ethanol precipitation was performed to conserve the carbapenemase activity. Briefly, 1 mL of blood culture fluid was mixed with 100 μL Lysis Buffer. After vortexing, the mix was centrifuged at 14,000 rpm for 2 min, the supernatant was discarded, and the pellet was washed with 1 mL Washing Buffer.

TABLE 1 | Performances of the MBT STAR®-Carba IVD Assay for the detection of carbapenem hydrolysis in carbapenem resistant organisms.

	Carbapenemase	No. of isolates	Incubation time (min)	Assay results			
				Hydrolysed	Non-hydrolysed	Sensitivity (95% CI*)	Specificity (95% CI*)
A. Assay performed on bacterial colonies							
Enterobacteriaceae (*n* = 89)						100% (95.3–100.0)	100% (74.1–100.0)
K. pneumoniae (*n* = 49), *E. coli*	OXA-48-like	41	30	41	–		
(*n* = 22), *K. oxytoca* (*n* = 5)	NDM	27	30	27	–		
E. cloacae (*n* = 6), *C. freundii*	KPC	6	30	6	–		
(*n* = 4), *E. aerogenes* (*n* = 2),	VIM	4	30	4	–		
P. stuartii (*n* = 1)	Negative	11	30	–	11		
***Pseudomonas* spp. (*n* = 29)**						100% (82.4–100.0)	100% (74.1–100.0)
P. aeruginosa (*n* = 28),	VIM	17	30	17	–		
P. chlororaphis (*n* = 1)	GES (ESBL)	1	30	–	1		
	IMP	1	30	1	–		
	Negative	10	30	–	10		
***A. baumannii* complex (*n* = 12)**						100% (75.7–100.0)	NA#
	OXA-23	8	60	8	–		
	OXA-24/-40	2	60	2	–		
	OXA-58	2	60	2	–		
B. Assay performed on positive blood cultures							
Enterobacteriaceae (*n* = 26)						100% (85.7–100.0)	66.7% (20.8–93.8)
K. pneumoniae (*n* = 16),	OXA-48-like	12	60	12	–		
E. coli (*n* = 5), *E. cloacae*	NDM	5	60	5	–		
(*n* = 2), *C. freundii* (*n* = 2),	KPC	4	60	4	–		
E. aerogenes (*n* = 1)	VIM	2	60	2	–		
	Negative	3	60	1	2		
***Pseudomonas* spp. (*n* = 7)**						100% (51.0–100.0)	100% (43.8–100.0)
P. aeruginosa (*n* = 6),	VIM	3	60	3	–		
P. chlororaphis (*n* = 1)	IMP	1	60	1	–		
	Negative	3	60	–	3		
***A. baumannii* complex (*n* = 12)**						25%/58.3% (8.3–53.2)/ (31.9–80.7)	NA#
	OXA-23	8	60/120	2/4	6/4		
	OXA-24/-40	2	60/120	–/2	2/–		
	OXA-58	2	60/120	1/1	1/1		

*95% CI: 95% confidence interval. #NA: non-applicable.

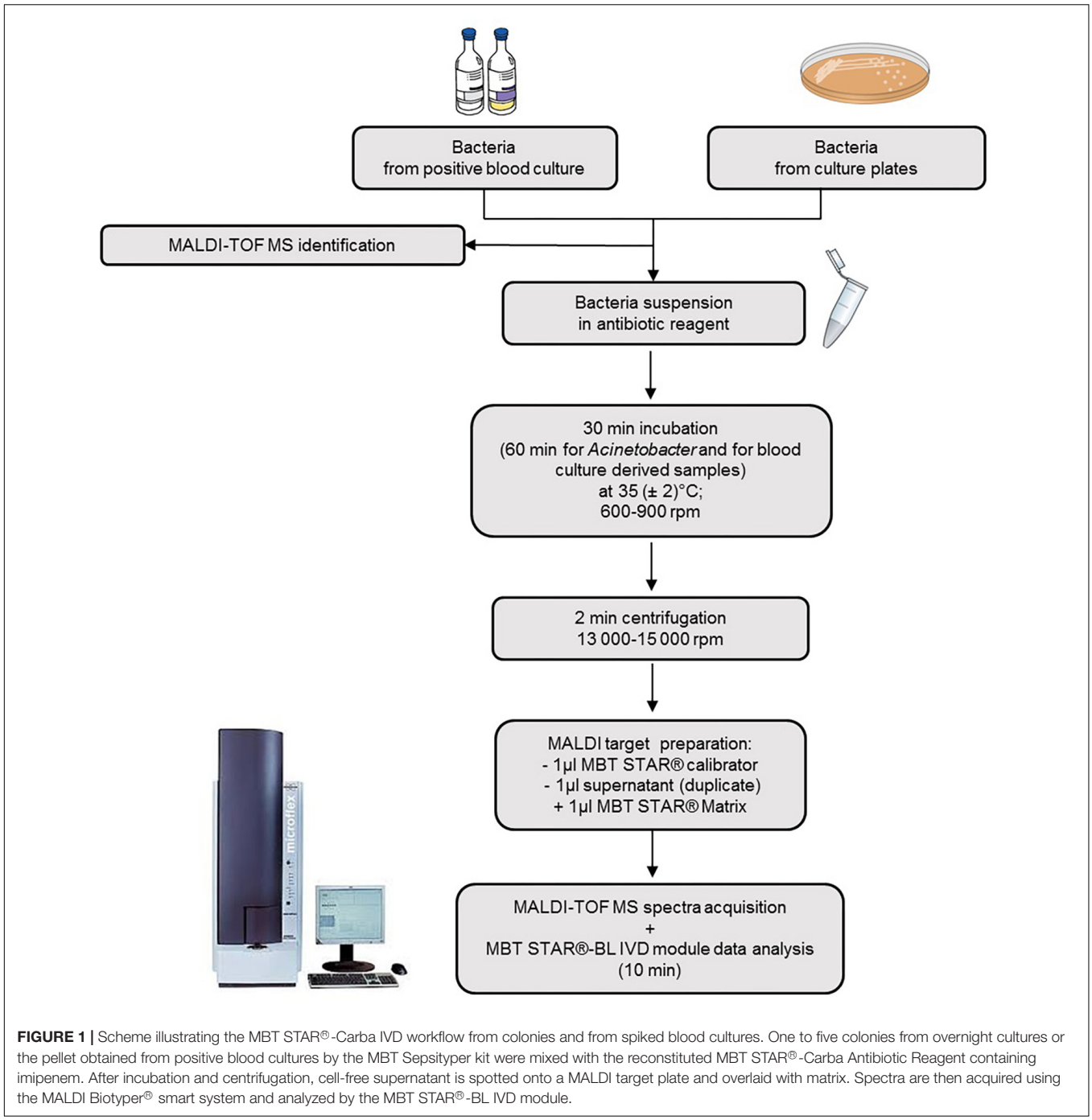


FIGURE 1 | Scheme illustrating the MBT STAR®-Carba IVD workflow from colonies and from spiked blood cultures. One to five colonies from overnight cultures or the pellet obtained from positive blood cultures by the MBT Sepsityper kit were mixed with the reconstituted MBT STAR®-Carba Antibiotic Reagent containing imipenem. After incubation and centrifugation, cell-free supernatant is spotted onto a MALDI target plate and overlaid with matrix. Spectra are then acquired using the MALDI Biotyper® smart system and analyzed by the MBT STAR®-BL IVD module.

The extracted bacterial pellets were used to perform MALDI-TOF MS identification and MBT STAR®-Carba assay.

MBT STAR®-Carba IVD Kit

The MBT STAR®-Carba IVD kit (Bruker Daltonik) was tested according to the manufacturer's instructions. Briefly one to five colonies from overnight cultures or the pellet obtained from positive blood cultures was mixed with the reconstituted MBT STAR®-Carba Antibiotic Reagent containing imipenem (0.25 mg/mL). After incubation at 35°C under agitation for 30 min (60 min for *Acinetobacter* spp. and for blood culture derived samples), the reaction mixture was centrifuged and 1 µL of the supernatant was spotted in duplicate onto the MALDI target. Dried spots were overlaid with MBT STAR® Matrix and were analyszed on the MALDI Biotyper smart system (Bruker Daltonik GmbH) with the MBT STAR®-BL IVD module (**Figure 1**). This software provides an interpretation of the carbapenemase activity of each tested isolate based on the imipenem hydrolysis intensity compared with a negative and positive control strain, respectively, *Escherichia coli* ATCC

25922 and a characterized clinical KPC-producing Klebsiella pneumoniae (**Figure 2**). Discordant results between the assay and the molecular resistance gene identification led to repetition of both methods.

Ethics Statement

Testing was performed in accordance with the ethical standards of the Cliniques universitaires Saint-Luc, in accordance with the ethical standards of the national research committee and in accordance with the 1964 Helsinki declaration and its later amendments. Information from microbiological and clinical files was anonymously analyzed and did not require patient's informed consent.

RESULTS

All MBT STAR®-Carba Assay results are detailed in **Table 1**. Evaluated on bacterial colonies (**Table 1A**) the assay showed 100% of specificity and sensitivity for the detection of carbapenemase activity in Enterobacteriaceae. In particular, all OXA-48-like producers including 37 OXA-48, 3 OXA-181 and one OXA-244 were correctly detected. No carbapenemase activity was detected among isolates being resistant to carbapenems but non-carbapenemase producers. Our evaluation of the assay on 41 non-fermentative Gram-negative bacilli also allowed optimal sensitivity and specificity results of both 100%. However, the absence in our study of non-carbapenemase-producing *A. baumannii* complex strains yet resistant to carbapenems, didn't allow the evaluation of the test's specificity for this group.

From positive blood cultures (**Table 1B**), the MBT STAR®-Carba Assay yielded positive results for the isolates harboring KPC (n = 4), metallo-β-lactamase (n = 11) or OXA-48-like (n = 12) genes. All carbapenemase-producing Enterobacteriaceae (n = 23) were successfully detected with the assay. One repeated false-positive result among the three non-carbapenemase Enterobacteriaceae was observed with a *K. pneumoniae* isolate expressing a SHV ESBL, conferring resistance to ertapenem but still susceptible to imipenem and meropenem. Regarding non-fermenters, the assay correctly assigned as carbapenemase-producers all blood cultures growing with *Pseudomonas* spp. (n = 4) but only 25% (3/12) of bottles containing *A. baumannii* complex isolates while all of them yielded positive results when tested from bacterial colonies. Extending imipenem incubation time to 2 h increased sensitivity to 58.3% (7/12) in this complex. Four OXA-23 and one OXA-58 producers remained negative. Non-specific imipenem hydrolysis related to prolonged incubation times was excluded by obtaining negative results with five wild-type *A. baumannii*.

DISCUSSION

There is an urgent need for rapid and accurate detection of CPO. The selection of a carbapenemase detection test is dependent on several factors, including local carbapenemase prevalence, organisms to be tested (i.e., Enterobacteriaceae and/or non-fermenters), labor intensity, necessary equipment, reagent preparation requirements, cost, performances, and turnaround time (TAT) of the test (Tamma and Simner, 2018). The TAT is especially important to prevent unnecessary treatments and rapid spread of CPO in hospital settings.

The MBT STAR®-Carba assay evaluated in our study demonstrated excellent performances for Enterobacteriaceae, *Pseudomonas* spp. and *A. baumannii* complex isolates from bacterial colonies. All tested carbapenemases were correctly detected and a negative result correctly excluded the presence of carbapenemase ultimately preventing further unnecessary testing on such isolates. Similarly, two recent studies reported 100% sensitivity and a specificity between 98.2 and 100% after evaluation of the assay on Enterobacteriaceae isolates from solid media (Dortet et al., 2018; Rapp et al., 2018). Rapp et al. further reported that the MBT STAR®-Carba correctly assigned 4/4 carbapenemase-positive *P. aeruginosa* tested isolates but only 4/8 carbapenemase-positive *A. baumannii* tested isolates. This assay's performances from bacterial colonies were similar to the performances of in-house developed MALDI-based methods particularly those developed with NH_4CO_3 increasing the catalytic efficiency of the OXA-48-like enzymes and therefore, their detection (Kempf et al., 2012; Papagiannitsis et al., 2015; Dortet et al., 2018; Miltgen et al., 2018; Oviano and Bou, 2019). OXA-48-like enzymes, known to possess a weak carbapenemase activity, are important to detect taking into account the large dissemination of OXA-48-like producers in Europe (Albiger et al., 2015). Moreover, the MBT STAR®-Carba assay showed superior sensitivity values than commercial colorimetric techniques for the detection of carbapenemases in Enterobacteriaceae and *A. baumannii* complex strains (ranging from 73 to 100% and from 21.0 to 86.0%, respectively). False negative results with these colorimetric assays occurred primarily with class D enzymes (Dortet et al., 2015; Noel et al., 2017; Simner et al., 2017; Tamma and Simner, 2018).

We additionally evaluated the applicability of the MBT STAR®-Carba assay performed directly on spiked blood cultures. Delays in appropriate antimicrobial treatment contribute to increased mortality of septic patients. Compared to phenotypic carbapenemase-detection approaches requiring cultured material, the assay performed directly on the blood bottles flagged positive drastically reduced time to results with more than 24 h. As observed from bacterial colonies, the assay detected 100% of the tested carbapenemase-producing Enterobacteriaceae and *Pseudomonas* spp. Our results stand out in comparison with colorimetric approaches reporting sensitivities between 64.0 and 91.3% for OXA-48-like CPO detection directly from positive blood cultures (Dortet et al., 2014b; Lima-Morales et al., 2018). One *K. pneumoniae* with a positive MALDI-TOF MS-based test and a negative PCR assay was observed in this study. However, precautions need to be taken when using the assay for the detection of carbapenemase-producing *A. baumannii* complex directly from positive blood cultures as the sensitivity after a 2 h incubation is only 58.3%. These results were in line with previous studies of in-house MALDI-TOF MS assays that demonstrated poor performances ranging from 27 to 63.2% for the detection of carbapenemase producing *A. baumannii* complex

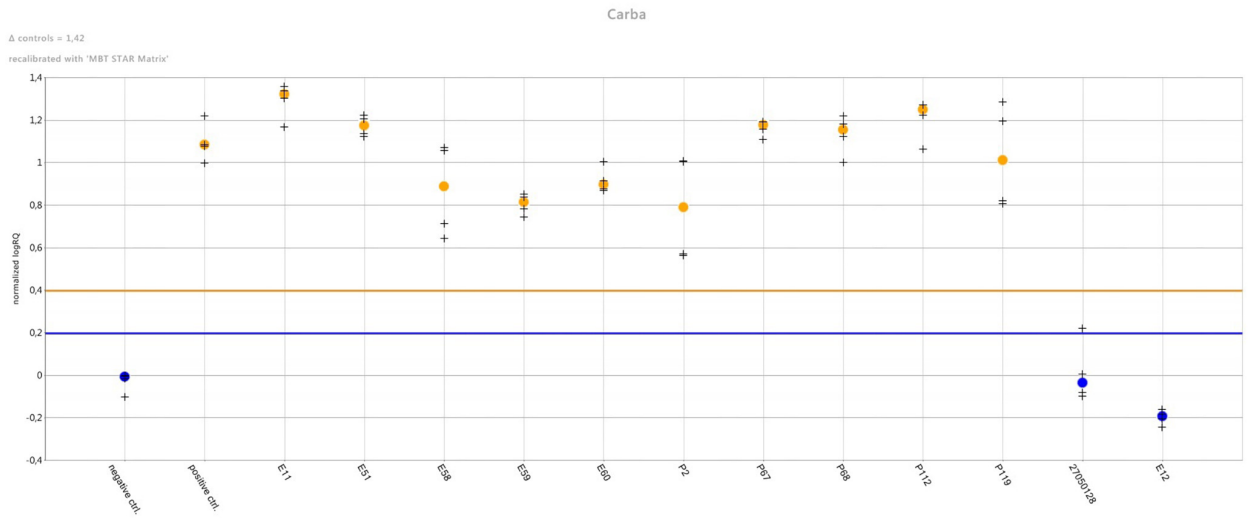


FIGURE 2 | Results overview in the MBT STAR®-BL Software Module. The software monitors the carbapenemase activity on acquired mass spectra (4 mass spectra per isolate) by automatic calculation of the intact Imipenem intensity and corresponding ratio hydrolysed / non-hydrolysed. Results are normalized to signal intensities obtained from positive and negative control strains (normalized logRQ value). Color-coded plot indicates presence (orange) or absence (blue) of carbapenemase activity in bacterial strains with the horizontal lines representing the limit for positive and negative results.

directly from positive blood cultures (Carvalhaes et al., 2014; Ghebremedhin et al., 2016). This could be explained by poor efficiency of Class D carbapenem-hydrolyzing β-lactamase (CHDL) (OXA-23, OXA-24/-40, and OXA-58) to hydrolyze the β-lactam ring of carbapenem antibiotics (Potron et al., 2015). Approaches to improve the assay for example increasing the bacterial inoculum size, increasing incubation time and modifying the Sepsityper bacterial extraction process might be valuable.

MALDI-TOF MS has become a well-established rapid identification tool and the simultaneous use of the system for carbapenemase detection seems convenient. It has the potential to detect carbapenemase activity regardless of the produced enzyme, including novel enzymes. Compared to the diversity of previous MALDI-TOF MS based in-house protocols (Mirande et al., 2015), the MBT STAR®-Carba IVD assay provides a fast and standardized method. Although the cost of the MBT STAR®-BL module needs to be considered, the automated interpretation with the software could be helpful for non-experienced users facing difficulties with spectrum analysis. The assay also overcomes the subjective visual interpretation of colorimetric assays, particularly in case of weak positive reactions leading to lower sensitivity for detection of OXA enzymes or more uncommon carbapenemases. The main drawback of the MBT STAR®-Carba assay is the hands-on time required for reagents and sample preparation during the pre-analytical procedure. Due to the relative instability of the imipenem solution, the MBT STAR®-Carba Antibiotic Reagent requires a reconstitution step before each use (Choquet et al., 2018; Oviano and Bou, 2019). Additionally, the laboratory has to possess a fresh culture of a carbapenemase-producing strain as positive control to perform the test.

The MALDI-TOF MS assay exclusively detects enzymatic carbapenem resistance but does not detect carbapenem resistance due to other mechanisms (i.e., efflux pump, porin loss) and extended phenotypic susceptibility testing is still required to define the antibiotic treatment options for the infected patient. Likewise, additional techniques are required for precise carbapenemase gene identification. The characterization of the carbapenemase type is of growing interest not only for epidemiology purposes but also for antimicrobial decisions. Indeed, some new β-lactamase inhibitor combinations in the pipeline as ceftazidime-avibactam or meropenem-vaborbactam are active on class A (KPC) and class D (OXA-48-like) carbapenemases but have no efficacy on metallo-β-lactamases (Liscio et al., 2015). Recent publications demonstrated the potential use of the MALDI-TOF MS platform for carbapenemase classification (Oviano et al., 2016; Cordovana et al., 2018) or for rapid AST independently of underlying resistance mechanisms (Idelevich et al., 2018). We would like to expect the development of a combined MALDI-TOF MS process allowing consecutive bacterial identification, AST, resistance detection, and classification of beta-lactamases.

The isolate collection tested in this study was a reflection of our local CPO epidemiology and was limited in the diversity of carbapenemase types. Therefore, additional testing of isolates carrying less common carbapenemase genes (bla_{OXA-51} with an upstream insertion of IS*Aba*, bla_{GES}, bla_{IMI}, bla_{NMC}, and bla_{SPM}) is warranted to confirm our analytical observations. In additional, a prospective evaluation of the MBT STAR®-Carba assay on consecutive clinical samples should be performed in future studies.

In conclusion, our results showed that the MBT STAR®-Carba IVD assay is a reliable approach for the detection of

carbapenemase-producing strains and may provide an answer within hours for antimicrobial therapy adjustment and early implementation of infection control measure.

AUTHOR CONTRIBUTIONS

AV and AA contributed to the conception and design of the study. AA, BT, and NO performed the experiments. AA and AV analyzed all the experiments. BK-M contributed to the design and analysis of DNA sequencing. AA wrote the manuscript. HR-V and AV provided critical feedback. AV contributed to the final version of the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.01413/full#supplementary-material

REFERENCES

Albiger, B., Glasner, C., Struelens, M. J., Grundmann, H., Monnet, D. L., and European Survey of Carbapenemase-Producing Enterobacteriaceae working, group (2015). Carbapenemase-producing *Enterobacteriaceae* in Europe: assessment by national experts from 38 countries, May 2015. *Euro. Surveill.* 20:30062. doi: 10.2807/1560-7917.ES.2015.20.45.30062

Bogaerts, P., Rezende de Castro, R., de Mendonca, R., Huang, T. D., Denis, O., and Glupczynski, Y. (2013). Validation of carbapenemase and extended-spectrum beta-lactamase multiplex endpoint PCR assays according to ISO 15189. *J. Antimicrob. Chemother.* 68, 1576–1582. doi: 10.1093/jac/dkt065

Carvalhaes, C. G., Cayo, R., Assis, D. M., Martins, E. R., Juliano, L., Juliano, M. A., et al. (2013). Detection of SPM-1-producing *Pseudomonas aeruginosa* and class D beta-lactamase-producing *Acinetobacter baumannii* isolates by use of liquid chromatography-mass spectrometry and matrix-assisted laser desorption ionization-time of flight mass spectrometry. *J. Clin. Microbiol.* 51, 287–290. doi: 10.1128/JCM.02365-12

Carvalhaes, C. G., Cayo, R., Visconde, M. F., Barone, T., Frigatto, E. A., Okamoto, D., et al. (2014). Detection of carbapenemase activity directly from blood culture vials using MALDI-TOF MS: a quick answer for the right decision. *J Antimicrob Chemother* 69, 2132–2136. doi: 10.1093/jac/dku094

Choquet, M., Guiheneuf, R., Castelain, S., Cattoir, V., Auzou, M., Pluquet, E., et al. (2018). Comparison of MALDI-ToF MS with the Rapidec Carba NP test for the detection of carbapenemase-producing *Enterobacteriaceae*. *Eur. J. Clin. Microbiol. Infect. Dis.* 37, 149–155. doi: 10.1007/s10096-017-3115-4

Cordovana, M., Kostrzewa, M., Glandorf, J., Bienia, M., Ambretti, S., and Pranada, A. B. (2018). A Full MALDI-Based approach to detect plasmid-encoded KPC-Producing *Klebsiella pneumoniae*. *Front. Microbiol.* 9:2854. doi: 10.3389/fmicb.2018.02854

Dortet, L., Agathine, A., Naas, T., Cuzon, G., Poirel, L., and Nordmann, P. (2015). Evaluation of the RAPIDEC(R) CARBA NP, the rapid CARB screen(R) and the Carba NP test for biochemical detection of carbapenemase-producing *Enterobacteriaceae*. *J. Antimicrob. Chemother.* 70, 3014–3022. doi: 10.1093/jac/dkv213

Dortet, L., Brechard, L., Cuzon, G., Poirel, L., and Nordmann, P. (2014a). Strategy for rapid detection of carbapenemase-producing *Enterobacteriaceae*. *Antimicrob. Agents Chemother.* 58, 2441–2445. doi: 10.1128/AAC.01239-13

Dortet, L., Brechard, L., Poirel, L., and Nordmann, P. (2014b). Rapid detection of carbapenemase-producing *Enterobacteriaceae* from blood cultures. *Clin. Microbiol. Infect.* 20, 340–344. doi: 10.1111/1469-0691.12318

Dortet, L., Tande, D., de Briel, D., Bernabeu, S., Lasserre, C., Gregorowicz, G., et al. (2018). MALDI-TOF for the rapid detection of carbapenemase-producing *Enterobacteriaceae*: comparison of the commercialized MBT STAR(R)-Carba IVD Kit with two in-house MALDI-TOF techniques and the RAPIDEC(R) CARBA NP. *J. Antimicrob. Chemother.* 73, 2352–2359. doi: 10.1093/jac/dky209

Ghebremedhin, B., Halstenbach, A., Smiljanic, M., Kaase, M., and Ahmad-Nejad, P. (2016). MALDI-TOF MS based carbapenemase detection from culture isolates and from positive blood culture vials. *Ann. Clin. Microbiol. Antimicrob.* 15:5. doi: 10.1186/s12941-016-0120-x

Hrabak, J., Chudackova, E., and Papagiannitsis, C. C. (2014). Detection of carbapenemases in *Enterobacteriaceae*: a challenge for diagnostic microbiological laboratories. *Clin. Microbiol. Infect.* 20, 839–853. doi: 10.1111/1469-0691.12678

Hrabak, J., Walkova, R., Studentova, V., Chudackova, E., and Bergerova, T. (2011). Carbapenemase activity detection by matrix-assisted laser desorption ionization-time of flight mass spectrometry. *J. Clin. Microbiol.* 49, 3222–3227. doi: 10.1128/JCM.00984-11

Huang, T. D., Poirel, L., Bogaerts, P., Berhin, C., Nordmann, P., and Glupczynski, Y. (2014). Temocillin and piperacillin/tazobactam resistance by disc diffusion as antimicrobial surrogate markers for the detection of carbapenemase-producing *Enterobacteriaceae* in geographical areas with a high prevalence of OXA-48 producers. *J. Antimicrob. Chemother.* 69, 445–450. doi: 10.1093/jac/dkt367

Idelevich, E. A., Sparbier, K., Kostrzewa, M., and Becker, K. (2018). Rapid detection of antibiotic resistance by MALDI-TOF mass spectrometry using a novel direct-on-target microdroplet growth assay. *Clin. Microbiol. Infect.* 24, 738–743. doi: 10.1016/j.cmi.2017.10.016

Kempf, M., Bakour, S., Flaudrops, C., Berrazeg, M., Brunel, J. M., Drissi, M., et al. (2012). Rapid detection of carbapenem resistance in *Acinetobacter baumannii* using matrix-assisted laser desorption ionization-time of flight mass spectrometry. *PLoS One* 7:e31676. doi: 10.1371/journal.pone.0031676

Kumarasamy, K. K., Toleman, M. A., Walsh, T. R., Bagaria, J., Butt, F., Balakrishnan, R., et al. (2010). Emergence of a new antibiotic resistance mechanism in India, Pakistan, and the UK: a molecular, biological, and epidemiological study. *Lancet Infect. Dis.* 10, 597–602. doi: 10.1016/S1473-3099(10)70143-2

Lima-Morales, D., Avila, H., Soldi, T., Dalmolin, T. V., Lutz, L., Aquino, V., et al. (2018). Rapid detection of carbapenemase production directly from blood culture by colorimetric methods: evaluation in a routine microbiology laboratory. *J. Clin. Microbiol.* 56:e00325-18. doi: 10.1128/JCM.00325-18

Liscio, J. L., Mahoney, M. V., and Hirsch, E. B. (2015). Ceftolozane/tazobactam and ceftazidime/avibactam: two novel beta-lactam/beta-lactamase inhibitor combination agents for the treatment of resistant Gram-negative bacterial infections. *Int. J. Antimicrob. Agents* 46, 266–271. doi: 10.1016/j.ijantimicag.2015.05.003

Miltgen, G., Plesiat, P., Mille, A., Chatelain, P., and Fournier, D. (2018). Detection of carbapenemase activity in *Pseudomonas aeruginosa* by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS). *J. Microbiol. Methods* 145, 66–68. doi: 10.1016/j.mimet.2017.12.011

Mirande, C., Canard, I., Buffet Croix Blanche, S., Charrier, J. P., van Belkum, A., Welker, M., et al. (2015). Rapid detection of carbapenemase activity: benefits and weaknesses of MALDI-TOF MS. *Eur. J. Clin. Microbiol. Infect. Dis.* 34, 2225–2234. doi: 10.1007/s10096-015-2473-z

Noel, A., Huang, T. D., Berhin, C., Hoebeke, M., Bouchahrouf, W., Yunus, S., et al. (2017). Comparative evaluation of four phenotypic tests for detection of carbapenemase-producing gram-negative bacteria. *J. Clin. Microbiol.* 55, 510–518. doi: 10.1128/JCM.01853-16

Nordmann, P., Gniadkowski, M., Giske, C. G., Poirel, L., Woodford, N., Miriagou, V., et al. (2012). Identification and screening of carbapenemase-producing *Enterobacteriaceae*. *Clin. Microbiol. Infect.* 18, 432–438. doi: 10.1111/j.1469-0691.2012.03815.x

Oueslati, S., Nordmann, P., and Poirel, L. (2015). Heterogeneous hydrolytic features for OXA-48-like beta-lactamases. *J. Antimicrob. Chemother.* 70, 1059–1063. doi: 10.1093/jac/dku524

Oviano, M., Barba, M. J., Fernandez, B., Ortega, A., Aracil, B., Oteo, J., et al. (2016). Rapid detection of OXA-48-producing *Enterobacteriaceae* by matrix-assisted

laser desorption ionization-time of flight mass spectrometry. *J. Clin. Microbiol.* 54, 754–759. doi: 10.1128/JCM.02496-15

Oviano, M., and Bou, G. (2019). Matrix-assisted laser desorption ionization-time of flight mass spectrometry for the rapid detection of antimicrobial resistance mechanisms and beyond. *Clin. Microbiol. Rev.* 32:e00037-18. doi: 10.1128/CMR.00037-18

Papagiannitsis, C. C., Studentova, V., Izdebski, R., Oikonomou, O., Pfeifer, Y., Petinaki, E., et al. (2015). Matrix-assisted laser desorption ionization-time of flight mass spectrometry meropenem hydrolysis assay with NH4HCO3, a reliable tool for direct detection of carbapenemase activity. *J. Clin. Microbiol.* 53, 1731–1735. doi: 10.1128/JCM.03094-14

Pellegrino, F. L., Casali, N., Nouer, S. A., Riley, L. W., and Moreira, B. M. (2008). A carbapenem-susceptible *Pseudomonas aeruginosa* strain carrying the bla(SPM) gene. *Diagn. Microbiol. Infect. Dis.* 61, 214–216. doi: 10.1016/j.diagmicrobio.2008.01.005

Potron, A., Poirel, L., and Nordmann, P. (2015). Emerging broad-spectrum resistance in *Pseudomonas aeruginosa* and *Acinetobacter baumannii*: mechanisms and epidemiology. *Int. J. Antimicrob. Agents* 45, 568–585. doi: 10.1016/j.ijantimicag.2015.03.001

Queenan, A. M., and Bush, K. (2007). Carbapenemases: the versatile beta-lactamases. *Clin. Microbiol. Rev.* 20, 440–458. doi: 10.1128/CMR.00001-07

Rapp, E., Samuelsen, O., and Sundqvist, M. (2018). Detection of carbapenemases with a newly developed commercial assay using Matrix assisted laser desorption ionization-time of flight. *J. Microbiol. Methods* 146, 37–39. doi: 10.1016/j.mimet.2018.01.008

Simner, P. J., Opene, B. N. A., Chambers, K. K., Naumann, M. E., Carroll, K. C., and Tamma, P. D. (2017). Carbapenemase detection among carbapenem-resistant glucose-nonfermenting gram-negative *Bacilli*. *J. Clin. Microbiol.* 55, 2858–2864. doi: 10.1128/JCM.00775-17

Tamma, P. D., and Simner, P. J. (2018). Phenotypic detection of carbapenemase-producing organisms from clinical isolates. *J. Clin. Microbiol.* 56:e01140-18. doi: 10.1128/JCM.01140-18

Thomson, K. S. (2017). The immaculate carbapenemase study. *J. Clin. Microbiol.* 55, 1608–1611. doi: 10.1128/JCM.00430-17

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 31 October 2019
doi: 10.3389/fmicb.2019.02504

Evaluation of *Staphylococcus aureus* Subtyping Module for Methicillin-Resistant *Staphylococcus aureus* Detection Based on Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry

*Yanyan Hu[1], Yonglu Huang[1], Yangzi Lizou[2], Jiaping Li[1] and Rong Zhang[1]**

[1]*Clinical Microbiology Laboratory, The Second Affiliated Hospital of Zhejiang University School of Medicine, Hangzhou, China,*
[2]*School of Laboratory Medicine and Life Science, Wenzhou Medical University, Wenzhou, China*

OPEN ACCESS

Edited by:
Karsten Becker,
University of Münster, Germany

Reviewed by:
Torsten Semmler,
Robert Koch Institute, Germany
Hong Du,
Soochow University, China

***Correspondence:**
Rong Zhang
zhang-rong@zju.edu.cn

Specialty section:
This article was submitted to Antimicrobials, Resistance and Chemotherapy, a section of the journal Frontiers in Microbiology

Received: *21 May 2019*
Accepted: *17 October 2019*
Published: *31 October 2019*

Citation:
Hu Y, Huang Y, Lizou Y, Li J and Zhang R (2019) Evaluation of Staphylococcus aureus Subtyping Module for Methicillin-Resistant Staphylococcus aureus Detection Based on Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry. Front. Microbiol. 10:2504. doi: 10.3389/fmicb.2019.02504

A recently developed *S. aureus* subtyping module for rapidly differentiate methicillin-resistant *Staphylococcus aureus* (MRSA) from methicillin-susceptible *S. aureus* (MSSA) had been introduced into China. The principle of this method was to identify the methicillin resistance through detection of a specific phenol soluble modulin-mec peak (PSM-mec) by matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS). A total of 347 non-duplicated *S. aureus* strains were collected from the Second Affiliated Hospital of Zhejiang University School of Medicine during January 2014 to February 2019. The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of the automated subtyping module in identifying MRSA were evaluated. The specificity and PPV of this method were both 100%, and the sensitivity was 60.2%. PSM-bearing MRSA was reported with different prevalence from different parts of the world, our sample collection has the highest percentage so far. The repeatability showed that 1.7% (6/347) and 18.4% (64/347) were reported differently in the intra- and inter-batch analysis, respectively, which demonstrated that the threshold of this method could be further optimized to increase the sensitivity of MRSA detection. Overall, Bruker™ MALDI Biotyper can detect *S. aureus* isolates with a quite high specificity and expedite the identification of MRSA isolates without using extra reagent, labor, or time. The reduced turnaround time of MRSA identification is essential for appropriate therapeutic management and timely intervention for infection control.

Keywords: matrix-assisted laser desorption ionization time-of-flight mass spectrometry, methicillin-resistant *Staphylococcus aureus*, sensitivity, specificity, resistant

INTRODUCTION

Staphylococcus aureus, one of the most important and prevalent nosocomial pathogens, often causes a wide range of infections involving skin, blood, endocarditis, or pneumonia (Tong et al., 2015). Of particular concern is the emergence of methicillin-resistant *S. aureus* (MRSA) strains. Infections caused by MRSA had a higher morbidity and mortality compared with those of

methicillin-susceptible *S.aureus* (MSSA) (Cosgrove et al., 2003). For MRSA, all the currently available beta-lactam antimicrobial agents with the exception of the new generation cephalosporins are limited for therapy, vancomycin, or the other antibiotics should be chose for treatment (Liu et al., 2011). Given the substantial morbidity and mortality associated with MRSA and the limitation scheme of treatments, it is necessary to develop new drugs as well as shorten the time for MRSA identification.

At present, there are mainly four methods for MRSA identification including traditional culture and susceptibility testing, chromogenic media, real-time polymerase chain reaction, and analysis of the mass spectra based on the ClinPro Tools software. Each has its own advantages and disadvantages (Palavecino, 2014; Wang et al., 2018). Rhoads et al. had developed another rapid detection method of MRSA based on the specific phenol soluble modulin-mec peak (PSM-mec) detection by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Rhoads et al., 2016). The PSM-mec peptide is encoded by staphylococcal cassette chromosome mec (SCCmec) types II, III, and VIII in the vicinity of *mecA* (Chatterjee et al., 2011). Whereas Timke et al. (2017) had developed a *S. aureus* subtyping module for MRSA identification based on the PSM-mec peak as an enhanced function to the Bruker MALDI Biotyper system without any extra manual operation, time, or reagent. Till now, only Germany, Canada, and the United States had reported the specificity and sensitivity data of this method in the identification of MRSA (Budvytiene et al., 2016; Rhoads et al., 2016; Lagacé-Wiens et al., 2017; Timke et al., 2017), and the detection rate of such *S.aureus* varies in different regions (approximately 10–30%). PSM data of such PSM-mec peak detection in China and in various specimens were limited. Thus, in the current study, we intended to evaluate the sensitivity, specificity, and repeatability of the automatic *S.aureus* subtyping module for the first time and to observe the proportion of such *S.aureus* in China.

MATERIALS AND METHODS

Bacterial Identification and Antimicrobial Susceptibility

About 347 non-duplicated *S. aureus* were collected from the Second Affiliated Hospital of Zhejiang University, School of Medicine during January 2014 to February 2019 including feces (n = 163), sputum (n = 94), blood (n = 15), and sterile body fluid (n = 75). Species identification and antimicrobial susceptibility were first performed using the VITEK-2 compact system (bioMérieux, Marcy-l'Étoile, France) and interpreted in accordance with the Clinical and Laboratory Standards Institute Guideline document M100. Then we identified the species by MALDI-Biotyper (Bruker Daltonics, Billerica, MA, USA) and tested antimicrobial susceptibility by disk diffusion method of cefoxitin (30ug). Cefoxitin is tested as a surrogate for oxacillin in disk diffusion method. Isolates that test resistance by cefoxitin disk should be reported as oxacillin resistant. *S. aureus* ATCC 25923 and *S. aureus* ATCC43300 (MRSA) were used as quality control. The results were interpreted according to the criteria of CLSI M100-S28 (CLSI, 2018).

Methicillin-Resistant *Staphylococcus aureus* Detection by Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry

All the isolates were recovered from the frozen storage onto the blood agar plates for 24 h at 35°C. Then the following steps were achieved the same as routine clinical operation. First, a fresh single bacterial colony was picked up and directly smeared onto a MALDI steel 96-spot target plate. Second, 1 μl of 70% formic acid was added to each target spot and dried at room temperature. Third, 1 μl of a-cyano-4-hydroxycinnamic acid (CHCA) matrix solution was introduced on the target spot again and air dried before analyzing on the MALDI-TOF MS. Mass spectrum was acquired and analyzed using a MALDI Biotyper standard system equipped with MBT Compass software (Bruker Daltonics GmbH, Bremen, Germany). For each sample, 240 laser shots were collected using the automatic mode, and *Escherichia coli* ATCC8739 was used as control in calibration of the instrument. Mass spectrum of each isolate was compared with those in the database and assigned scores. *S. aureus* strains with log (scores) ≥2.0 were further automatically identified by the subtyping module. When the peak of 2413 ± 2 m/z exists, the software alerts the *S. aureus* strain as "presumptive PSM positive MRSA", otherwise, only *S. aureus* was reported (**Supplementary Figure S1**). For the repeatability analysis, each strain was identified thrice on each individual clone to evaluate the inter-batch repeatability of this method. Simultaneously, mass spectra of the first batch samples were acquired twice on the same spot to evaluate the intra-batch repeatability.

Data Analysis

The Flex Analysis 3.3 program (Bruker Daltonics GmbH, Bremen, Germany) was used for the spectrum analysis. All the spectra were smoothed and baseline subtracted before comparing of the specific PSM-mec peak (2,413 ± 2 m/z). ClinProTools software (version 3.0, Bruker Daltonik GmbH, Bremen, Germany) was used to analyze the discrepancy between presumptive PSM positive MRSA (PSM-MRSA) and MSSA isolates, PSM-MRSA and MRSA isolates without the specific PSM-mec peak [MRSA(−)], and those strains with inconsistent results during the experiment. MBT Compass Explorer software (Bruker Daltonik GmbH, Bremen, Germany) was performed to show normalized spectra in gel view.

Statistics Analysis

To evaluate the performance of MRSA detection using MALDI-TOF MS *S.aureus* subtyping module, we use sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) as metrics. Pearson chi-square (χ^2) test or Fisher's exact test was used to compare the sensitivity discrepancy of various specimen types and different batches. $p < 0.05$ was considered to be significantly different. Statistic analysis was performed using the Statistic Package for Social Sciences Version 23.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Bacterial identification and cefoxitin antimicrobial susceptibility confirmed that 241 strains were MRSA and 106 were MSSA (**Table 1**). All the 347 isolates were identified as *S. aureus* with log (scores) ≥2.0. Among which, #121, #126, and #120 isolates were identified as "presumptive PSM positive MRSA" for batch #1, #2, and #3, respectively (**Table 1**). The sensitivity of each batch was 50.2, 52.3, and 45.6%, respectively. After combining the results of the three batches, the sensitivity of this method reached to 60.2%. Sensitivities of various specimen types were also different. However, the specificity was 100% despite of the batch and specimen type (**Table 1**). Meanwhile, there was no difference in sensitivity among the specimen type groups (χ^2 = 2.224, p = 0.329) or the batch groups (χ^2 = 3.778, p = 0.286).

The gel view representation of PSM-MRSA and MSSA classification models revealed a significant discrepancy at the peak of m/z 2,413 (**Figure 1A**). The area under curve (AUC) of the m/z 2,413 peak was 0.9997 (**Figure 1B**). The average intensity and single peak variance of the m/z 2,413 peak of the two classification models are further illustrated in **Figures 1C,D**. Similarly, if we compare the two classification models of PSM-MRSA and MRSA(−), the 2,413 m/z peak also demonstrated significant discrepancy between the two classes (**Figure 2**). The AUC of the specific peak was 0.992 (**Figure 2B**). These results demonstrated the specific characteristics and patterns of m/z 2,413 peak in the different classifications. Specifically, the classification of the PSM-MRSA and MSSA showed a better satisfactory separation at peak 2,413 than the classification of the PSM-MRSA and MRSA(−) isolates.

For the intra-batch repeatability, 1.7% (6/347) was identified differently. Three of the strains were chosen randomly for normalized spectra analysis. We can obviously found that all the strains had a peak at 2413 m/z (**Figure 3A**); however, when the peaks were normalized, only those with relatively high intensities were identified as PSM-MRSA (**Figure 3B**). Likewise, the normalized spectra analysis of the 18.4% (64/347) isolates with inconsistent results during the inter-batch repeatability analysis were in accordance with the above strains (data not shown).

DISCUSSION

MALDI-TOF MS had been mainly used in many clinical microbiology laboratories for effective and rapid identification of bacterial species (Ge et al., 2017). It also provides an alternative solution for molecular typing (Spinali et al., 2015) and antibiotic resistant study (Oviano and Bou, 2019). It was first applied to differentiate MRSA from MSSA by intact cell mass spectrometry in the year of 2000 (Edwards-Jones et al., 2000). In the current study, a recently developed *S. aureus* subtyping module was evaluated for MRSA detection based on MALDI-TOF MS. Unlike the previously reported parallel method (Rhoads et al., 2016), this subtyping module do not need any extra operations or a third party software assistance. It could identify MRSA automatically through the bacteria identification process.

Our results showed a quite high specificity of this method, which is accordant with researches in Germany, Canada, and the United States, and no false positive strains were detected among the *S. aureus* subtyping module evaluations (Budvytiene et al., 2016; Rhoads et al., 2016; Lagacé-Wiens et al., 2017; Timke et al., 2017). The sensitivity (60.2%) of this study was much higher than previously reported (Budvytiene et al., 2016; Rhoads et al., 2016; Lagacé-Wiens et al., 2017; Timke et al., 2017) which might indicate the prevalence of the SCCmec types II, III, and VIII strains in China is higher than Europe and America. However, we have to point out that the SCCmec types of these clinical MRSA isolates were not evaluated in our study.

Clinpro Tools analysis demonstrated that the 2,413 m/z peak could commendably distinguish MRSA from MSSA, as well as PSM-MRSA from MRSA(−). However, for the 1.7 and 18.4% inconsistent results in the intra- and inter-batch repeatability analysis, we speculate that some strains of the MRSA(−) group might possess the 2,413 m/z peak with relatively low intensity in view of the slight difference of the above two classification groups in AUC (0.9997 vs. 0.9992) and the very weak peak of 2,413 m/z in the MRSA(−) group rather than the MSSA group. As expected, the results of **Figure 3** proved this. Only the relatively high intensity of the normalized spectra was reported as PSM-MRSA, nevertheless, those possess the 2,413 m/z peak but with relatively low intensity strains were not reported. The inconsistent results demonstrated that the threshold of this method might be a little high and could be further optimized

TABLE 1 | Statistics analysis of different groups of *S. aureus* strains.

	S. aureus (No.)	MRSA (No.)	MSSA (No.)	PSM-MRSA (No.)	PPV* (%)	NPV# (%)	Sensitivity (%)	Specificity (%)
Batch 1	347	241	106	121	100.0	46.9	50.2	100.0
Batch 2	347	241	106	126	100.0	48.0	52.3	100.0
Batch 3	347	241	106	110	100.0	44.7	45.6	100.0
Total[a]	347	241	106	145	100.0	52.5	60.2	100.0
Feces	163	153	10	88	100.0	13.3	57.5	100.0
Sputum	94	46	48	27	100.0	71.6	58.7	100.0
Sterile body fluid	75	34	41	23	100.0	78.9	67.7	100.0
Blood	15	8	7	7	100.0	87.5	87.5	100.0

PPV, positive predictive value; #NPV, negative predictive value; [a]The data listed in the row "total," "feces," "sputum," "sterile body fluid," and "blood" combined the three batches of "PSM-MRSA" isolates. The isolate were judged as "PSM-MRSA" as long as one of the batches was identified as "PSM-MRSA" by MALDI Biotyper.

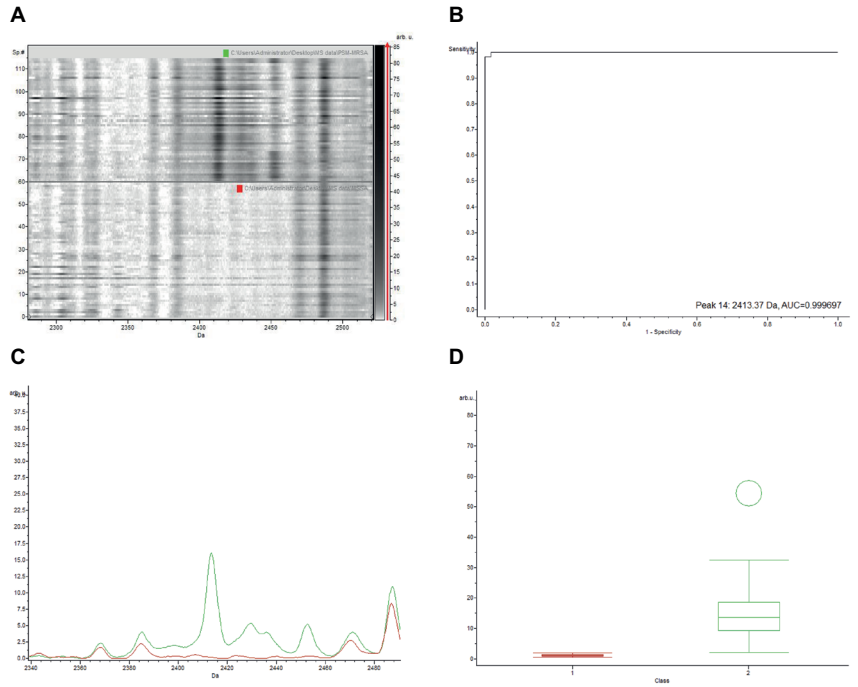


FIGURE 1 | Discrepancy of the characteristic 2,413 m/z peak between PSM-MRSA and MSSA classification models. **(A)** The gel view of the respective section. The red arrow indicates the characteristic peak of 2,413 m/z. Intensities of peaks were expressed in arbitrary units (arb.u.). **(B)** The AUC of the 2,413 m/z peak. **(C)** Average spectra of 2,413 m/z peak. Green line and red line represent for PSM-MRSA and MSSA group, respectively. **(D)** Display of single peak variance of 2,413 m/z.

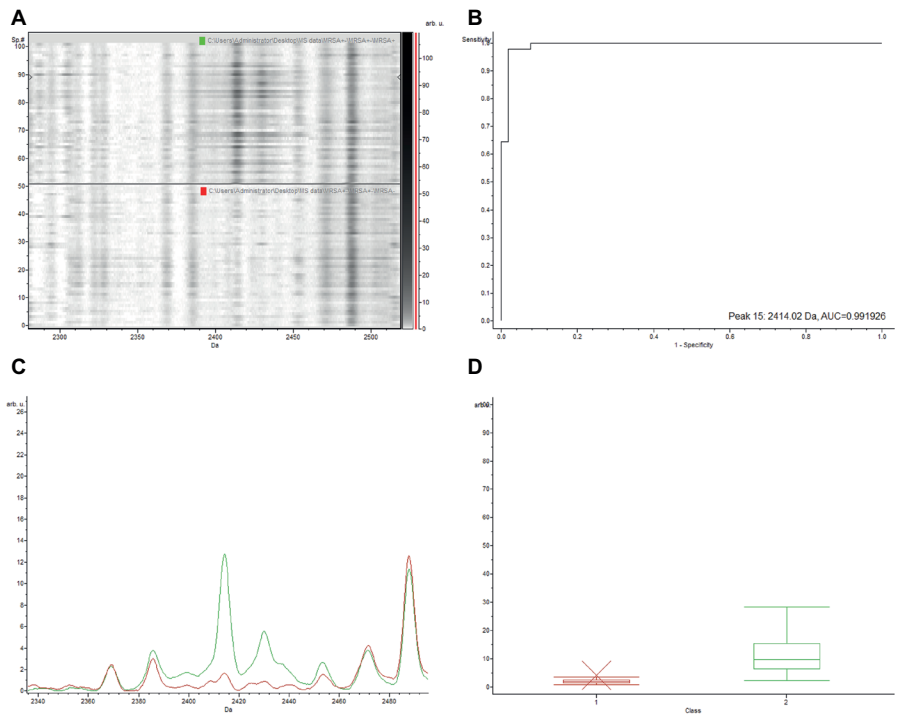


FIGURE 2 | Discrepancy of the characteristic 2,413 m/z peak between PSM-MRSA and MRSA(–) classification models. MRSA(–) represents for MRSA isolates without the specific PSM-mec peak. **(A)** The gel view of the respective section. The red arrow indicates the characteristic peak of 2,413 m/z. Intensities of peaks were expressed in arbitrary units (arb.u.). **(B)** The AUC of the 2,413 m/z peak. **(C)** Average spectra of 2,413 m/z peak. Green line and red line represent for PSM-MRSA and MRSA(–) group, respectively. **(D)** Display of single peak variance of 2,413 m/z.

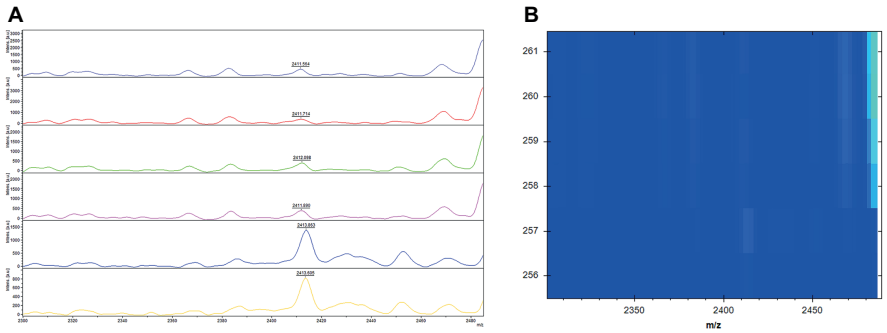


FIGURE 3 | Mass spectra of the characteristic 2,413 m/z peak in the intra batch repeatability and their corresponding normalized spectra in gel view. **(A)** Mass spectra of the characteristic 2,413 m/z peak analyzed by flexanalysis. The red rectangle displayed peaks at 2,413 2 m/z of the three isolates. (+) and (–) represents for PSM-MRSA and MRSA(–), respectively. x axis, mass per charge in daltons (m/z, Da); y axis, absolute intensity of signal. **(B)** Normalized spectra in gel view. The red rectangle displayed the corresponding peaks at 2,413 2 m/z of the three isolates.

to increase the sensitivity of MRSA detection. Meanwhile, in order to achieve optimization, the specificity should be monitored synchronously when adjusting parameters. However, it might be better to have 100% specificity than dealing with false positives.

To our knowledge, this present study is the first using a MALDI-TOF MS-based *S. aureus* subtyping module to differentiate MRSA from MSSA in China. In addition, previous studies did not distinguish between specimen types. Our study evaluated the sensitivity discrepancy of various specimen types using the *S. aureus* subtyping module for the first time. The result revealed that this subtyping module might be suitable for *S. aureus* strains collected from all kinds of specimens and the prevalence of the SCCmec types II, III, and VIII strains of different specimens might have no statistical difference in China. Meanwhile, this research also seems to be the first study to evaluate the repeatability of this method, which explains why we found that this method could be further optimized.

In conclusion, Bruker™ MALDI Biotyper can detect the *S. aureus* isolates with a high specificity and expedite the identification of MRSA isolates without adding any reagent, labor or time which is essential for appropriate therapeutic management and timely intervention for infection control.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The study was approved by the Ethics Committee of Second Affiliated Hospital of Zhejiang University, School of Medicine (2017-099). All subjects gave written informed consent in accordance with the Declaration of Helsinki.

BIOSAFETY STATEMENT

All concerns related to the safe and appropriate use of human-derived materials, infectious agents, or genetically modified organisms were approved by the Institutional Biosafety Committee of Second Affiliated Hospital of Zhejiang University, School of Medicine. All experiments were conducted under the guidelines from the Biological Agent Reference Sheet.

AUTHOR CONTRIBUTIONS

RZ and YaH designed the study. YL and YoH did the experiment. YaH and JL analyzed and interpreted the data. YaH and RZ wrote the manuscript. All authors read and approved the final manuscript.

ACKNOWLEDGMENTS

We sincerely thank Kun Zhu of Bruker (Beijing) Scientific Technology Co. Ltd. for the technical support.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this articles can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.02504/full#supplementary-material

REFERENCES

Budvytiene, I., Moon, H. W., Shi, G., and Banaei, N. (2016). Matrix-assisted laser desorption ionization time-of-flight mass spectrometry-based identification of methicillin-resistant *Staphylococcus aureus* through detection of a single peak. ASM Meeting 2016, Boston, Poster Presentation.

Chatterjee, S. S., Chen, L., Joo, H. S., Cheung, G. Y., Kreiswirth, B. N., and Otto, M. (2011). Distribution and regulation of the mobile genetic element-

encoded phenol-soluble modulin PSM-mec in methicillin-resistant *Staphylococcus aureus*. *PLoS One* 6:e28781. doi: 10.1371/journal.pone.0028781

CLSI (2018). *Performance standards for antimicrobial susceptibility testing; twenty-eighth informational supplement. CLSI document M100-S28*. Wayne, PA: Clinical and Laboratory Standards Institute.

Cosgrove, S. E., Sakoulas, G., Perencevich, E. N., Schwaber, M. J., Karchmer, A. W., and Carmeli, Y. (2003). Comparison of mortality associated with methicillin-resistant and methicillin-susceptible *Staphylococcus aureus* bacteremia: a meta-analysis. *Clin. Infect. Dis.* 36, 53–59. doi: 10.1086/345476

Edwards-Jones, V., Claydon, M. A., Evason, D. J., Walker, J., Fox, A. J., and Gordon, D. B. (2000). Rapid discrimination between methicillin-sensitive and methicillin-resistant *Staphylococcus aureus* by intact cell mass spectrometry. *J. Med. Microbiol.* 49, 295–300. doi: 10.1099/0022-1317-49-3-295

Ge, M. C., Kuo, A. J., Liu, K. L., Wen, Y. H., Chia, J. H., Chang, P. Y., et al. (2017). Routine identification of microorganisms by matrix-assisted laser desorption ionization time-of-flight mass spectrometry: success rate, economic analysis, and clinical outcome. *J. Microbiol. Immunol. Infect.* 50, 662–668. doi: 10.1016/j.jmii.2016.06.002

Lagacé-Wiens, P., Tran, V., Mulvey, M., Karlowsky, J., Adam, H., Baxter, M., et al. (2017). Rapid detection of methicillin-resistant *Staphylococcus aureus* (MRSA) by detection of phenol-soluble modulin (PSM) using MALDI-TOF mass spectrometry by extended direct transfer (eDT) and direct transfer (DT) methods. ECCMID Meeting 2017, Austria, Poster Presentation.

Liu, C., Bayer, A., Cosgrove, S. E., Daum, R. S., Fridkin, S. K., Gorwitz, R. J., et al. (2011). Clinical practice guidelines by the infectious diseases society of america for the treatment of methicillin-resistant *Staphylococcus aureus* infections in adults and children: executive summary. *Clin. Infect. Dis.* 52, 285–292. doi: 10.1093/cid/cir034

Oviano, M., and Bou, G. (2019). Matrix-assisted laser desorption ionization-time of flight mass spectrometry for the rapid detection of antimicrobial resistance mechanisms and beyond. *Clin. Microbiol. Rev.* 32. doi: 10.1128/CMR.00037-18

Palavecino, E. L. (2014). Rapid methods for detection of MRSA in clinical specimens. *Methods Mol. Biol.* 1085, 71–83. doi: 10.1007/978-1-62703-664-1_3

Rhoads, D. D., Wang, H., Karichu, J., and Richter, S. S. (2016). The presence of a single MALDI-TOF mass spectral peak predicts methicillin resistance in staphylococci. *Diagn. Microbiol. Infect. Dis.* 86, 257–261. doi: 10.1016/j.diagmicrobio.2016.08.001

Spinali, S., van Belkum, A., Goering, R. V., Girard, V., Welker, M., Van Nuenen, M., et al. (2015). Microbial typing by matrix-assisted laser desorption ionization-time of flight mass spectrometry: do we need guidance for data interpretation? *J. Clin. Microbiol.* 53, 760–765. doi: 10.1128/JCM.01635-14

Timke, M., Trojahn, M., Kostrzewa, M., and Wasner, M. (2017). Instant detection of PSM positive MRSA in MALDI biotyper workflow. ECCMID Meeting 2017, Austria, Poster Presentation.

Tong, S. Y., Davis, J. S., Eichenberger, E., Holland, T. L., and Fowler, V. G. Jr. (2015). *Staphylococcus aureus* infections: epidemiology, pathophysiology, clinical manifestations, and management. *Clin. Microbiol. Rev.* 28, 603–661. doi: 10.1128/CMR.00134-14

Wang, H. Y., Lien, F., Liu, T. P., Chen, C. H., Chen, C. J., and Lu, J. J. (2018). Application of a MALDI-TOF analysis platform (ClinProTools) for rapid and preliminary report of MRSA sequence types in Taiwan. *PeerJ* 6:e5784. doi: 10.7717/peerj.5784

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 14 February 2020
doi: 10.3389/fmicb.2020.00232

Detection of Methicillin Resistance in *Staphylococcus aureus* From Agar Cultures and Directly From Positive Blood Cultures Using MALDI-TOF Mass Spectrometry-Based Direct-on-Target Microdroplet Growth Assay

*Ilka D. Nix[1], Evgeny A. Idelevich[1], Luise M. Storck[1], Katrin Sparbier[2], Oliver Drews[2], Markus Kostrzewa[2] and Karsten Becker[1,3]**

[1] Institute of Medical Microbiology, University Hospital Münster, Münster, Germany, [2] Bruker Daltonik GmbH, Bremen, Germany, [3] Friedrich Loeffler-Institute of Medical Microbiology, University Medicine Greifswald, Greifswald, Germany

OPEN ACCESS

Edited by:
David Christopher Coleman, Dublin Dental University Hospital, Ireland

Reviewed by:
Kunyan Zhang, University of Calgary, Canada
Brenda A. McManus, Dublin Dental University Hospital, Ireland

***Correspondence:**
Karsten Becker
karsten.becker@med.uni-greifswald.de

Specialty section:
This article was submitted to Antimicrobials, Resistance and Chemotherapy, a section of the journal Frontiers in Microbiology

Received: 07 October 2019
Accepted: 31 January 2020
Published: 14 February 2020

Citation:
Nix ID, Idelevich EA, Storck LM, Sparbier K, Drews O, Kostrzewa M and Becker K (2020) Detection of Methicillin Resistance in Staphylococcus aureus From Agar Cultures and Directly From Positive Blood Cultures Using MALDI-TOF Mass Spectrometry-Based Direct-on-Target Microdroplet Growth Assay. Front. Microbiol. 11:232. doi: 10.3389/fmicb.2020.00232

Matrix-assisted laser desorption/ionization time-of-flight-mass spectrometry (MALDI-TOF MS)-based direct-on-target microdroplet growth assay (DOT-MGA) was recently described as a novel method of phenotypic antimicrobial susceptibility testing (AST). Here, we developed the application of MALDI-TOF MS-based DOT-MGA for Gram-positive bacteria including AST from agar cultures and directly from positive blood cultures (BCs) using the detection of methicillin resistance as example. Consecutively collected, a total of 14 methicillin-resistant *Staphylococcus aureus* (MRSA) and 14 methicillin-susceptible *S. aureus* (MSSA) clinical isolates were included. Furthermore, a collection of MRSA challenge strains comprising different *SCCmec* types, *mec* genes, and *spa* types was tested. Blood samples were spiked with MRSA and MSSA and positive BC broth processed by three different methods: serial dilution of BC broth, lysis/centrifugation, and differential centrifugation. Processed BC broth was directly used for rapid AST using DOT-MGA. Droplets of 6 μl with and without cefoxitin at the EUCAST breakpoint concentration were spotted in triplicates onto the surface of a MALDI target. Targets were incubated in a humidity chamber, followed by medium removal and on-target protein extraction with formic acid before adding matrix with an internal standard as a quality control (QC). Spectra were acquired and evaluated using MALDI Biotyper software. First, tests were considered as valid, if the growth control achieved an identification score of ≥1.7. For valid tests, same score criterion was used for resistant isolates when incubated with cefoxitin. An identification score <1.7 after incubation with cefoxitin defined susceptible isolates. On-target protein extraction using formic acid considerably improved detection of methicillin resistance in *S. aureus* and DOT-MGA showed feasible results for AST from agar cultures after 4 h incubation time. Comparing the different processing methods of positive BC broth, lysis/centrifugation

method with a final dilution step 10^{-1} of the 0.5 McFarland suspension resulted in best test performance after 4 h incubation time. Overall, 96.4% test validity, 100% sensitivity, and 100% specificity were achieved for detection of methicillin resistance in clinical isolates. All strains of the MRSA challenge collection were successfully tested as methicillin-resistant. This first study on Gram-positive organisms showed feasibility and accuracy of MALDI-TOF MS-based DOT-MGA for rapid AST of *S. aureus* from agar cultures and directly from positive BCs.

Keywords: MALDI-TOF MS, direct-on-target microdroplet growth assay, antimicrobial susceptibility testing, rapid testing, blood culture, MRSA, *Staphylococcus aureus*

INTRODUCTION

In times of raising antimicrobial resistance, rapid microbiological diagnostics is important to improve patients' outcome (Trenholme et al., 1989; Barenfanger et al., 2008). If a timely effective antimicrobial therapy is missing, the mortality rate increases significantly (Kumar et al., 2006; Ferrer et al., 2014). Particularly in case of sepsis, a rapid and accurate detection of resistant pathogens is important (Banerjee et al., 2016; Dubourg et al., 2018; Idelevich et al., 2018a). Bloodstream infections are one of the most often cause of death (Singer et al., 2016). For routine sepsis diagnostics, several rapid molecular tests are available. However, these tests only detect the molecular resistance mechanism and do not give reliable information on the phenotypic resistance of the pathogen. Therefore, there is a great call for rapid phenotypic antimicrobial susceptibility testing (AST) in routine diagnostics (Doern, 2018; Dubourg et al., 2018; van Belkum et al., 2019). Implementation of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) for identification (Idelevich et al., 2014b) and AST (Idelevich et al., 2014a) from positive blood culture (BC) broth shortly incubated on solid medium leads to an adjustment of empiric antibiotic therapy (Köck et al., 2017) and has already reached the routine microbiological diagnostics (Idelevich et al., 2019).

A MALDI-TOF MS-based direct-on-target microdroplet growth assay (DOT-MGA) was recently developed which allows a universal rapid AST (Idelevich et al., 2018b). It could also be shown that rapid phenotypic detection of resistance directly from positive BCs using DOT-MGA is feasible and accurate (Idelevich et al., 2018c). To enable the best possible therapy for the patient, it should be a goal to provide the results of susceptibility testing on the same day as it is now possible for pathogen identification (Idelevich and Becker, 2019). MALDI-TOF MS method provides a solid basis for a method that can do both: identification and rapid AST in one diagnostic workflow. Direct susceptibility testing from positive BCs with Gram-negative species by the MALDI-TOF MS-based DOT-MGA was previously established (Idelevich et al., 2018c). In this study, we investigate different processing methods of positive BC broth to establish the detection of methicillin resistance in *Staphylococcus aureus* directly from positive BCs using MALDI-TOF MS-based DOT-MGA, in addition to the rapid detection of methicillin resistance in *S. aureus* from cultures grown on solid medium. Methicillin-susceptible *S. aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA) isolates were chosen here as an example for Gram-positive bacteria because of the high importance of this pathogen in general and due to the special therapeutic and infection prevention challenges of its methicillin- and multi-resistance phenotypes (Köck et al., 2014; World Health Organization [WHO], 2017).

MATERIALS AND METHODS

Bacterial Strains

For the study, 14 MRSA isolates were consecutively collected in the diagnostic laboratory of the Institute of Medical Microbiology, University Hospital Münster (Münster, Germany). Additionally, 14 consecutive MSSA isolates were collected and included into the study. To avoid copy isolates, only one isolate per patient was included. For genotypic characterization, proof of presence of *mecA* and *mecC* genes was done by GenoType MRSA (Hain Lifescience GmbH, Nehren, Germany) according to the manufacturer's instructions. MSSA and MRSA isolates were genotyped based on sequencing of the protein A gene's (*spa*) hypervariable region and *spa* types were assigned on the Ridom SpaServer[1] curated by the SeqNet.org initiative (Mellmann et al., 2006). An ethical review process was not required for this study, as patient data were neither collected nor used in this *in vitro* experimental study.

Additionally, a challenge strain collection of highly diverse MRSA strains including various *SCCmec* types and *mec* genes and diverse *spa* types was selected applying isolates from the studies of Kriegeskorte et al. (2012), Schaumburg et al. (2012), and Becker et al. (2018). Overall, 16 isolates were chosen, comprising each two of *mecA* gene-positive isolates with *SCCmec* types I, II, III, IV, IVa, and V, respectively (Schaumburg et al., 2012), three *mecC* gene-positive isolates with *SCCmec* type XI (Kriegeskorte et al., 2012), and one plasmid-encoded *mecB*-positive isolate (Becker et al., 2018) (see **Supplementary Table 2**).

Determination of Minimum Inhibitory Concentration

The minimum inhibitory concentration (MIC) of cefoxitin was determined for all isolates by broth microdilution reference

[1]http://spaserver.ridom.de

method according to ISO standard 20776-1 (ISO, 2006) corresponding to the European Committee on AST (EUCAST, 2019a) and the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2018). Using cation-adjusted Mueller–Hinton broth (CAMHB, BD Diagnostic, Heidelberg, Germany), a final inoculum size of approximately 5×10^5 cfu/ml was adjusted and confirmed by vital cell counting of serial dilutions onto tryptic soy agar (TSA) plates in triplicates after overnight incubation. Cefoxitin (TCI Deutschland, Eschborn, Germany) concentrations in a range of 0.25–128 mg/L were tested in double dilution steps. After 18 ± 2 h incubation at 35 ± 1°C the results were read. All tests were performed in triplicates and the median MIC was determined. MIC results were interpreted according to the EUCAST breakpoints (EUCAST, 2019a). MIC_{50}, MIC_{90}, and MIC ranges of consecutively collected clinical isolates were calculated for analysis. *S. aureus* ATCC 29213 reference strain was used as quality control (QC).

MALDI-TOF MS-Based Rapid Antimicrobial Susceptibility Testing From Cultures Grown on Solid Medium

MALDI-TOF MS-based DOT-MGA was performed according to Idelevich et al. (2018b). In brief, bacterial suspension with standard turbidity of 0.5 McFarland (approx. 10^8 cfu/ml) of an overnight culture on solid medium was prepared. The 0.5 McFarland suspension diluted 1:100 in CAMHB. A 96-well microtiter plate was prepared; 50 µl of the diluted bacterial suspension was added to 50 µl CAMHB as growth control. Additionally, 50 µl diluted bacterial suspension was added to 50 µl CAMHB containing cefoxitin, which results in a final inoculum size of approximately 5×10^5 cfu/ml, which is the recommended inoculum for broth microdilution by ISO/EUCAST (ISO, 2006; EUCAST, 2019a). Final inoculum size was confirmed by vital cell counting of serial dilutions onto TSA plates in triplicate and counting of colonies after overnight incubation. The final concentration of cefoxitin was 4 mg/L, which is the breakpoint concentration dividing between susceptible and resistant isolates, according to EUCAST (2019a). For each isolate a growth control and a set-up containing cefoxitin were done in triplicates, with tests performed simultaneously on the same target. Six microliters was transferred from each well of the microtiter plate directly onto disposable MALDI target (MBT Biotarget 96, Bruker Daltonik GmbH, Bremen, Germany). For each time point a separate target was prepared. The targets were incubated in a plastic transport box (Bruker Daltonik GmbH, Bremen, Germany) where 4 mL of water was added to generate a humid atmosphere to avoid evaporation of microdroplets. The boxes were incubated for 3, 4, and 5 h at 35 ± 1°C. After incubation, the liquid medium on top of the sedimented bacteria was removed to avoid interference with broth ingredients during MALDI-TOF MS measurement. While carefully touching the droplets in a row simultaneously sidewise at its bottom on the target surface using small filter papers (Whatman® Drying Block 556, Whatman GmbH, Dassel, Germany), the medium was removed due to capillary effects, as described in previous studies (Idelevich et al., 2018b), but using small filter papers instead of tissue wipes. The fully dried spots on the target were overlaid with 1 µl 70% formic acid to disrupt the cell membrane in order to achieve higher sensitivity and specificity for susceptibility detection. According to the standard protocol, the dried spots were overlaid with 1 µl MBT FAST Matrix (Bruker Daltonik GmbH, Bremen, Germany) solved in the standard solvent (Solution OS, LCH CHIMIE, Les Aires, France) according to the manufacturer's instructions.

MALDI-TOF MS-Based Rapid Antimicrobial Susceptibility Testing Directly From Positive Blood Cultures

Inoculation of Blood Culture Bottles

To simulate a bacteremia, 10 mL human blood (from KB, principal investigator, own blood donation for experimental *in vitro* purpose does not require this experiment to be reviewed or approved by the local ethics committee of the University of Münster) were spiked with a bacterial suspension to generate a bacteremia with approximately 10 cfu/ml. In brief, bacterial suspension with standard turbidity of 0.5 McFarland of an overnight culture on solid medium was prepared. The standardized bacterial suspension was diluted up to 10^{-5} in 1:10 dilution steps in CAMHB. 100 µl of dilution 10^{-5} were mixed with 10 ml blood. Disinfection of septum with 70% ethanol was done before and after injection. The spiked blood was injected into an aerobic plastic culture vial (BACTEC™ Plus Aerobic/F, BD Diagnostic, Heidelberg, Germany) and BC bottles were incubated in a BACTEC™ 9240 Automated BC System (BD Diagnostic, Heidelberg, Germany). Bacterial growth was detected using the fluorescent sensor technology and bottles were flagged positive when growth was detected. Time to positivity was automatically documented. The final inoculum size of approximately 10 cfu/ml was confirmed by vital cell counting of serial dilutions onto TSA plates in triplicate and counting of colonies after overnight incubation.

Processing of Positive Blood Cultures

Blood culture bottles were removed from the incubator when flagged positive within the personnel's working hours and processed immediately after removal. Preliminary experiments, done with six MRSA and six MSSA consecutively collected clinical isolates, used modified processing methods for isolation of bacteria from positive BCs for rapid AST, but could not reach satisfactory results. For preliminary experiments, each positive BC sample was processed by filtration/dilution, dilution, lysis/centrifugation, and differential centrifugation and DOT-MGA was incubated for 4, 5, and 6 h. Exact procedure of processing methods of preliminary experiments is described in **Supplementary Data 1**. To improve the results and processing methods of the preliminary experiments (**Supplementary Table 1**) and to analyze the best suitable method for isolation of bacteria from positive BCs for rapid AST in this present study, positive BC of 28 *S. aureus* consecutively collected clinical isolates and

additionally 16 isolates of MRSA challenge strain collection described above were finally processed with three different methods: dilution, lysis/centrifugation, and differential centrifugation (**Figure 1**).

Dilution

Positive BC broth was diluted in decimal dilution steps up to 10^{-4} in CAMHB. Dilution steps 10^{-2},10^{-3}, and 10^{-4} were used for DOT-MGA. Real bacterial concentration in positive BC broth was determined by vital cell counting of serial dilutions onto TSA plates in triplicate and counting of colonies after overnight incubation.

Lysis/centrifugation

For the lysis/centrifugation method, the MBT Sepsityper kit (Bruker Daltonik GmbH, Bremen, Germany) was used according to the manufacturer's instructions (version prior to introduction of rapid workflow and improved formulation), but instead of 200 µl lysis buffer only 100 µl lysis buffer was used and the obtained pellet was suspended in 1 ml 0.9% NaCl. A bacterial suspension with standard turbidity of 0.5 McFarland was prepared. The standardized bacterial suspension was diluted 1:10 and 1:100 in CAMHB and both dilutions were used for DOT-MGA. The real bacterial concentration in test was determined by vital cell counting as described above.

Differential centrifugation

Differential centrifugation was used as a third method. To separate the blood cells from the bacterial cells, a first low speed centrifugation step was performed. 1.5 ml positive BC broth was centrifuged at 2000 rpm for 2 min. 1 ml of supernatant was transferred into a new reaction tube and centrifuged at 13,000 rpm for 2 min. Supernatant was discarded and the obtained pellet was suspended in 1 ml 0.9% NaCl. A bacterial suspension with standard turbidity of 0.5 McFarland was prepared. The standardized bacterial suspension was diluted 1:10 and 1:100 in CAMHB and dilutions were used for DOT-MGA. Real bacterial concentration in test was determined by vital cell counting as described above.

MALDI-TOF MS Direct on-Target Microdroplet Growth Assay

Three different methods for processing of positive BC broth were performed and the resulting inoculum was used for rapid AST by DOT-MGA. Procedure of DOT-MGA was described above (see the section "MALDI-TOF MS-Based Rapid Antimicrobial Susceptibility Testing From Cultures Grown on Solid Medium"). In brief, 50 µl of cefoxitin solution in CAMHB was added to 50 µl of bacterial suspension in CAMHB in a well of a 96-well microtiter plate. The final concentration of cefoxitin was 4 mg/L. Additionally, 50 µl of bacterial suspension in CAMHB was added to 50 µl CAMHB as a growth control. For each sample, the growth control and the set-up containing cefoxitin were prepared in triplicate. Six microliters of droplets of each well of the microtiter plate was transferred directly on a hydrophilic spot of a disposable MBT Biotarget 96 (Bruker Daltonik GmbH, Bremen, Germany), resulting in three spots being inoculated with the sample with cefoxitin and three spots containing the growth control without cefoxitin for the corresponding sample. A separate target was prepared for each time point. The targets were incubated in a plastic transport box (Bruker Daltonik GmbH, Bremen, Germany) where 4 ml water was added to generate a humidity chamber. The boxes were incubated for 3, 4, 5, and 6 h at 35 ± 1°C. The microtiter plate was incubated for 18 ± 2 h at 35 ± 1°C too, as an additional control of each experiment. After incubation, the liquid medium was removed as described above and dried spots were treated with 1 µl 70% formic acid and overlaid with 1 µl MBT FAST Matrix (Bruker Daltonik GmbH, Bremen, Germany) solved in the standard solvent. MBT FAST Matrix contains an internal standard as a QC for spectra acquisition.

Spectrum Acquisition and Categorization

MALDI-TOF MS measurement was performed using the MALDI Biotyper smart instrument (Bruker Daltonik GmbH, Bremen, Germany) and the flexControl software (Version 3.4, Bruker Daltonik GmbH, Bremen, Germany). Spectra were acquired with optimized instrument settings for DOT-MGA assay. Random walk and adapted acceptance criteria of the intensity were selected. The acquired spectra were analyzed using the MALDI Biotyper Compass Explorer (Version 4.1; Bruker Daltonik GmbH, Bremen, Germany) and matched against the BDAL database [Update Version 4.1 with 7854 Main Spectra (MSP) entries]. Spectra were categorized and interpreted as previously described according to the identification (score of ≥1.7)/no-identification (score of <1.7) evaluation (Idelevich et al., 2018b,c). Briefly, if the growth control without cefoxitin achieved an identification score of ≥1.7 for the tested isolate, the test was considered as valid. The test was invalid, if the score of the growth control was <1.7. Valid samples with cefoxitin and a score ≥1.7 resulted in a successful species identification and were interpreted as resistant. Valid samples with cefoxitin and a score <1.7 resulted in a failed identification and were interpreted as susceptible. In analogy to current approach for MIC determination, median score results of three spots were calculated and used for analysis.

Data Evaluation

Detection of growth, i.e., score ≥ 1.7, as median result of three spots for the growth control was the criteria for a valid test, and test validity rate was calculated. Sensitivity of resistance detection was defined as the proportion of isolates correctly tested as resistant by DOT-MGA method among resistant isolates according to standard method. Specificity was defined as the proportion of isolates correctly tested among susceptible isolates determined by standard method. The relationship between two variables was expressed by the Pearson correlation coefficient (r) (Pearson, 1931, 1932). To test if calculated correlation is statistically significant (p), Student's

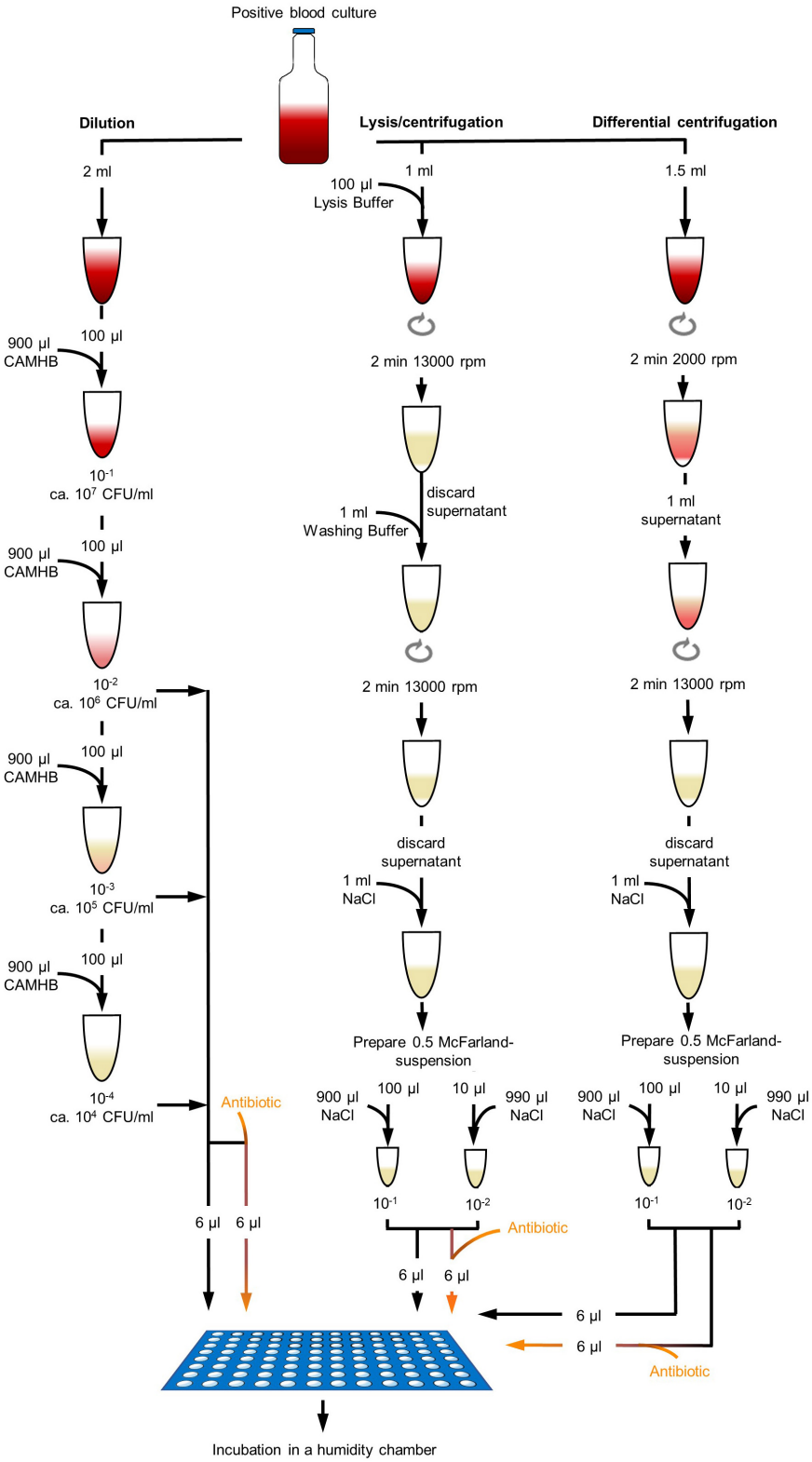


FIGURE 1 | Different processing methods of positive blood culture broth. Dilution method was done with dilution steps up to 10^{-4} in CAMHB. Dilutions were directly used for DOT-MGA. Lysis/centrifugation method was done according to the protocol of MBT Sepsityper kit (Bruker Daltonik GmbH, Bremen, Germany; version prior to introduction of rapid workflow and improved formulation), but only 100 µl lysis buffer was used. Differential centrifugation was performed with a first centrifugation step of 2 min at 2000 rpm and a second centrifugation step of 2 min at 13,000 rpm. Obtained pellets were resuspended and a standardized 0.5 McFarland suspension was prepared. Two different dilutions were used for DOT-MGA.

t-test (Student, 1908) was done and a significant level of $\alpha = 0.05$ was determined.

Spectrophotometric Measurement of Bacterial Growth by Optical Density

Bacterial growth of positive BC broth treated with different processing methods was analyzed by spectrophotometric measurement using a Multi-Mode Reader (Synergy HTX, BioTek, Bad Friedrichshall, Germany). Blood was spiked as described above and positive BC broth was processed (i) with differential centrifugation and a first centrifugation step of 2 min at 2000 rpm followed by a second centrifugation step of 2 min at 13,000 rpm as pictured above. (ii) The differential centrifugation method was modified with a first centrifugation step of 5 min at 2000 rpm. (iii) The MBT Sepsityper kit (Bruker Daltonik GmbH, Bremen, Germany; version prior to introduction of rapid workflow and improved formulation) was used according to the manufacturer's instructions and (iv) the MBT Sepsityper kit protocol was modified and instead of 200 µl lysis buffer only 100 µl lysis buffer was used. The standardized bacterial inoculum (McFarland 0.5) was diluted 1:200, while 10 µl was added to 1990 µl CAMHB. Dilution was directly transferred to a microtiter plate for analysis of bacterial growth. Microtiter plate was incubated at 37°C for 18 h in the Multi-Mode Reader, measurement of optical density was performed every 10 min and before measurement the plate was shaken. Three wells of a microtiter plate were read in parallel and mean of optical density at 578 nm was calculated.

RESULTS

Characterization of Bacterial Isolates With Standard Methods

Using reference AST method, the cefoxitin MIC_{50}, MIC_{90}, and MIC range of MSSA consecutively collected clinical isolates were 4, 4, and 2 to 4 mg/L, respectively. For consecutive clinical MRSA isolates, cefoxitin MIC_{50}, MIC_{90}, and MIC range were 128, >128, and 16 to >128 mg/L, respectively. The MIC of QC strain *S. aureus* ATCC 29213 was within the recommended range of 1–4 mg/L (EUCAST, 2019b). Phenotypic susceptibility results were confirmed by genotypic characterization. The *mecA* gene was detected in all consecutive clinical MRSA isolates while the *mecC* gene was not detected. Overall, 21 different *spa* types were detected. One isolate each within the 14 MSSA consecutively collected clinical isolates was *spa* type t002, t003, t012, t015, t021, t032, t084, t209, t315, t1043, t2078, or t2333. Two MSSA consecutively collected clinical isolates were *spa* type t091. One isolate each within the 14 consecutive clinical MRSA isolates was *spa* type t034 (livestock-associated MRSA), t045, t304, t437, t693, t4929, or t5857. Two MRSA each were *spa* type t003 or t011 (livestock-associated MRSA), respectively, and three MRSA isolates were *spa* type t032. MICs and *spa* type distribution for challenge strain collection isolates are demonstrated in **Supplementary Table 2**.

MALDI-TOF MS Based DOT-MGA From Cultures Grown on Solid Medium

The accuracy of rapid detection of methicillin resistance in consecutively collected clinical isolates of *S. aureus* using MALDI-TOF MS-based DOT-MGA from solid medium overnight cultures are shown in **Table 1**. The best performance of MALDI-TOF MS-based DOT-MGA was observed after 5 h of incubation showing each 100% for sensitivity, specificity, and test validity. The 4-h incubation provided equal percentages of sensitivity and specificity for detection of methicillin resistance, but only 85.7% test validity. Final inoculum size of approximately 5×10^5 cfu/ml was reached for all tested isolates (data not shown). MRSA challenge strain collection isolates were detected successfully after 5 or 6 h (**Supplementary Table 3**).

MALDI-TOF MS-Based DOT-MGA Directly From Positive Blood Cultures

The mean determined bacterial concentration in inoculated blood samples was 9 CFU/ml (range, 2–15 CFU/ml). The time to positivity of BC bottles inoculated with consecutively collected clinical MSSA isolates was 673 min on average. Bottles inoculated with consecutive clinical MRSA isolates were flagged as positive on average after 702 min. BC bottles were removed from the incubator when flagged positive during the daily working hours and processed immediately after removal. The average times between positivity signal and processing for MSSA was 233 and for MRSA was 217 min, respectively. The mean bacterial concentration in positive BC was 1.5×10^9 CFU/ml (range, 3.2×10^6–8.7×10^9 CFU/ml). It could be shown that shorter times to positivity were linked to higher bacterial concentrations in inoculated blood, see **Supplementary Figure 1**. Additionally, higher bacterial concentrations of positive BC broth were associated with shorter times to positivity. A relation between bacterial concentration of inoculated blood and bacterial concentration in positive BC broth could not be observed. Likewise, there was no connection between determined bacterial concentration in positive BC broth and time until processing after positivity signal, i.e., additional incubation after positivity (**Supplementary Figure 1**).

Using different processing methods for positive BC broth, the determined final bacterial concentration in test was different. Final real bacterial concentrations in test after processing positive BC broth with different methods are shown in **Table 2**. The mean inoculum size of lysis/centrifugation method

TABLE 1 | Rapid AST from cultures grown on solid medium.

Incubation time	Validity[a] (%)	Sensitivity[b] (%)	Specificity[b] (%)
3 h	28.6	92.9	100
4 h	85.7	100	100
5 h	100	100	100

Accuracy of detection of methicillin resistance in S. aureus (consecutively collected clinical isolates, n = 28) using MALDI-TOF MS-based DOT-MGA. [a]Valid test – the growth control was detected (identification score ≥1.7 for the tested isolate). [b]Calculated for valid tests.

TABLE 2 | Determined final bacterial concentrations in tests achieved with different processing methods of positive BCs (consecutively collected clinical isolates, *n* = 28).

Processing method	Dilution	Final concentration in test (CFU/ml)		
		Mean	Minimum	Maximum
Dilution	10^{-2}	7.2×10^6	1.6×10^4	4.4×10^7
	10^{-3}	7.6×10^5	1.6×10^3	4.4×10^6
	10^{-4}	7.6×10^4	1.6×10^2	4.4×10^5
Lysis/centrifugation	10^{-2}	3.8×10^4	4.8×10^3	3.6×10^5
	10^{-1}	3.8×10^5	4.8×10^4	3.6×10^6
Differential centrifugation	10^{-2}	3.0×10^4	2.3×10^1	1.2×10^5
	10^{-1}	3.0×10^5	2.3×10^2	1.2×10^6

(10^{-1} dilution) and differential centrifugation method (10^{-1} dilution) reached on average the recommended inoculum size of 5×10^5 CFU/ml (ISO, 2006; CLSI, 2018). Serial dilution of positive BC broth (10^{-3}) provided a mean inoculum size close to this recommended inoculum size as well. Performance of MALDI-TOF MS-based DOT-MGA for direct detection of methicillin resistance in *S. aureus* from positive BCs using different processing methods is shown in **Table 3**. After 4 h of incubation time, lysis/centrifugation method (10^{-1} dilution) showed best result: 100% accuracy of detection of methicillin resistance was achieved and test validity was 96.4%. After 6 h of incubation time, serial dilution of positive BC broth (10^{-4}) and lysis/centrifugation method (10^{-1} dilution) showed similar accurate results at a test validity of 96.4%. Comparing these results to results of preliminary experiments for MALDI-TOF MS-based DOT-MGA directly from positive BCs, that were shown in **Supplementary Table 1**, a considerably improvement was shown. Using the non-modified lysis/centrifugation method in these preliminary experiments, no valid tests could be observed. Results of the non-modified differential centrifugation method in preliminary experiments showed lower validity (41.7%) compared to validity of the modified differential centrifugation method (96.4%) after 6 h incubation time. However, even the dilution method showed distinct improvement. All MRSA challenge strain collection isolates were successfully detected after processing and performing MALDI-TOF MS-based DOT-MGA directly from positive BC (**Supplementary Table 4**).

TABLE 3 | Performance of MALDI-TOF MS DOT-MGA for direct detection of methicillin resistance in *Staphylococcus aureus* from positive BCs (consecutively collected clinical isolates, *n* = 28).

Processing method	Dilution	3 h			4 h			5 h			6 h		
		Validity[a] (%)	Sensitivity[b] (%)	Specificity[b] (%)	Validity[a] (%)	Sensitivity[b] (%)	Specificity[b] (%)	Validity[a] (%)	Sensitivity[b] (%)	Specificity[b] (%)	Validity[a] (%)	Sensitivity[b] (%)	Specificity[b] (%)
Dilution	10^{-2}	96.4	100	78.6	100	100	85.7	100	100	92.9	100	100	92.9
	10^{-3}	78.6	85.7	92.9	82.1	92.9	92.9	100	92.9	92.9	100	100	92.9
	10^{-4}	32.1	92.9	100	78.6	85.7	100	85.7	85.7	100	**96.4**	**100**	**100**
Lysis/ centrifugation	10^{-1}	75.0	85.7	92.9	**96.4**	**100**	**100**	**89.3**	**100**	**100**	**96.4**	**100**	**100**
	10^{-2}	7.1	85.7	–	46.4	100	100	71.4	100	100	89.3	100	100
Differential centrifugation	10^{-1}	60.7	92.9	100	82.1	92.9	100	85.7	100	92.9	96.4	100	92.9
	10^{-2}	3.6	100	–	50.0	100	100	53.6	100	100	67.9	92.9	100

[a]*Valid test – the growth control was detected (identification score ≥1.7 for the tested isolate).* [b]*Calculated for valid tests.* [c]*The values in bold indicate results with best test performance.*

DISCUSSION

For septic patients, a rapid AST directly from positive BCs would reduce time to beginning of an effective antimicrobial therapy and enhance patients' outcome in comparison to standard susceptibility testing (Kumar et al., 2006). MALDI-TOF MS' principal feasibility as a method for rapid AST could be shown in studies before (Lange et al., 2014). The recently developed DOT-MGA presents a universal phenotypic rapid AST based on MALDI-TOF MS (Idelevich et al., 2018b). MALDI-TOF MS-based DOT-MGA is able to perform identification and AST within few hours and could easily be implemented in

routine laboratory workflows. To accelerate in particular the diagnostics of sepsis, direct susceptibility testing from positive BCs with Gram-negative species by the MALDI-TOF MS-based DOT-MGA was recently established (Idelevich et al., 2018c). Idelevich et al. (2018c) could show that rapid detection of carbapenem non-susceptibility in *Enterobacterales* directly from positive BCs was possible within 4 h using the MALDI-TOF MS-based DOT-MGA.

In this study, we focused on the rapid detection of methicillin resistance in *S. aureus* using MALDI-TOF MS, that is the first study of using MALDI-TOF MS-based DOT-MGA for Gram-positive species, such as *S. aureus*. Previous studies investigated the use of MALDI-TOF MS for this purpose by comparing acquired spectra of MSSA strains to spectra of MRSA strains to identify unique mass peaks. They found different mass peaks that were applied to differentiate MSSA and MRSA obtaining different sensitivities and specificities of this procedure (Edwards-Jones et al., 2000; Bernardo et al., 2002; Du et al., 2002; Wang et al., 2013). Josten et al. (2014) identified a small peptide (PSM-*mec*) produced by *agr*-positive strains that is part of the genomes of health care-associated MRSA. This peptide can be detected in the MALDI-TOF MS spectra (2415 Da) and could be used for rapid identification of this subgroup of MRSA with high specificity and sensitivity. However, a universal approach to determine methicillin susceptibility is still missing. The newly introduced MALDI-TOF MS-based DOT-MGA is a universal phenotypic assay that offers the opportunity to distinguish between susceptible and non-susceptible isolates in the presence of an antibiotic agent. Here, to discriminate between MSSA and MRSA, isolates were incubated in the presence of cefoxitin and growth or no growth was analyzed.

To accelerate the detection of MRSA strains, we focused on the rapid detection of methicillin resistance in *S. aureus* directly from positive BCs in this study. To process positive BC broth, three different methods were used: dilution, lysis/centrifugation, and differential centrifugation. Preliminary experiments to this study applied the same processing approach that Idelevich et al. (2018c) used for detection of carbapenem non-susceptibility in *Enterobacterales*. Here, results showed (**Supplementary Table 1**) that dilution method was the best to detect methicillin resistance in *S. aureus* directly from positive BC broth using MALDI-TOF MS-based DOT-MGA. Lysis/centrifugation and differential centrifugation method revealed less validity for detection of methicillin resistance in *S. aureus*. Due to this, analysis of bacterial growth by spectrophotometric measurement of optical density for isolates of positive BC broth treated with different processing methods was done and results showed that there was a delayed beginning of bacterial growth (**Supplementary Figure 2**). When treating positive BC broth with lysis/centrifugation method using MBT Sepsityper (Bruker Daltonik GmbH, Bremen, Germany) standard protocol (version prior to introduction of rapid workflow and improved formulation) and adding 200 µl lysis buffer to 1 ml BC broth according to the manufacturer's instructions instead of adding only 100 µl lysis buffer to 1 ml BC broth, there was a delayed beginning of bacterial growth of up to 4 h. Idelevich et al. (2018c) demonstrated the performance of the lysis/centrifugation method for processing positive BC broth, when using the MBT Sepsityper standard protocol adding 200 µl lysis buffer to 1 ml blood for direct testing of carbapenem non-susceptibility in *Enterobacterales* using MALDI-TOF MS-based DOT-MGA. Gram-positive bacteria seem to be more sensitive against lysis buffer of MBT Sepsityper comparing Gram-negative bacteria. Due to the different cell wall structure *S. aureus* showed higher susceptibility compared to *Enterobacterales* isolates.

Additionally, we compared two different differential centrifugation methods (**Supplementary Figure 2**) one with a first centrifugation step of 2 min at 2000 rpm and the other with a first centrifugation step of 5 min at 2000 rpm to sediment the blood cells. After transferring the supernatant into a new centrifugation tube, bacteria were sedimented for 2 min at 13,000 rpm for both methods. Results showed that there was a delayed beginning of bacterial growth of 2 h when using a first centrifugation step of 5 min at 2000 rpm instead of 2 min. A probable explanation could be that Gram-positive cocci like to cluster and clustered cells become heavier, heavier cells were more sedimented when using a first centrifugation step of 5 min instead of 2 min at 2000 rpm. Lin et al. (2017) performed differential centrifugation method of positive BCs to identify species using MALDI-TOF MS and could show that identification of Gram-negative bacteria showed better results compared to Gram-positive species after differential centrifugation of positive BC broth.

Direct identification of species from positive BC broth using MALDI-TOF MS was introduced several years ago and different processing methods to treat the positive BC broth were tested (La Scola and Raoult, 2009; Christner et al., 2010; Moussaoui et al., 2010; Stevenson et al., 2010; Ferreira et al., 2011; Klein et al., 2012; Zhou et al., 2017). For choosing the best processing method of positive BC broth in our present study it is important that in contrast to classical MALDI-TOF MS identification, for DOT-MGA vital cells are needed for susceptibility testing. Regarding the results of previous experiments dilution method, lysis/centrifugation method with less lysis buffer compared to the manufacturer's protocol and differential centrifugation method were chosen to have less impact on the bacterial growth for susceptibility testing and were chosen for this study. Considering, that MALDI-TOF MS identification results of Gram-positive species achieved better results when performing an on-target lysis with formic acid before adding matrix (Haigh et al., 2011), this formic acid on-target lysis step was also performed in this present study.

The study has shown that the rapid phenotypic AST using MALDI-TOF MS-based DOT-MGA for detection of methicillin resistance in subcultivated *S. aureus* on solid medium is successful within 4 h with a validity of 85.7% (**Table 1**), even successful detection of resistance in all MRSA challenge strains could be shown (**Supplementary Table 3**). Further results for rapid detection of methicillin resistance in *S. aureus* directly from positive BCs using MALDI-TOF MS-based DOT-MGA could reduce time to results in comparison to the standard susceptibility testing method from subcultivated colonies on solid medium for effective and fast sepsis diagnostics. Results of this presented study showed best performance of MALDI-TOF MS-based DOT-MGA for detection of methicillin

resistance in *S. aureus* using modified lysis/centrifugation protocol and using a 10^{-1} dilution of McFarland 0.5 suspension to inoculate the assay (**Table 3**). Additionally, all MRSA challenge strains were detected successfully after processing the positive BC broth (**Supplementary Table 4**). Regarding the determined bacterial concentrations, the mean final inoculum was within the recommended inoculum according to EUCAST (5×10^5 CFU/ml) if the standardized inoculum was diluted 1:10 after processing the positive BC broth with lysis/centrifugation (3.8×10^5 CFU/ml) or differential centrifugation method (3.0×10^5 CFU/ml). After dilution of the positive BC broth, the recommended inoculum according to EUCAST was achieved with the 10^{-3} dilution (7.6×10^5 CFU/ml). Overall, best results of DOT-MGA in this presented study were achieved with the inoculum recommended by EUCAST (5×10^5 CFU/ml), which is a valid guideline for susceptibility testing. To give a general recommendation for inoculum density for DOT-MGA further analyses are needed.

To accelerate the diagnostic of sepsis, direct susceptibility testing from positive BCs needs to become a suitable method in routine laboratories. A simultaneous identification and susceptibility testing, after previous Gram classification, would reduce time, and an earlier start of an effective antimicrobial therapy could decrease mortality rate. MALDI-TOF MS-based DOT-MGA has the potential to further accelerate sepsis diagnostics, because it is characterized by minor hands-on-time and offers an easy and cost-efficient alternative way of rapid AST. A further advantage of this novel method is its ability to detect contaminations, because the correct identification of the growth control is a validity criterion along with the sufficient growth. An internal standard in the used MBT FAST matrix ensured a QC for spectra acquisition. As with other routine methods, identification and susceptibility testing by MALDI-TOF MS is hardly possible with mixed pathogens in the BC bottle. In this case, sub-culturing on solid medium is required to get a pure culture for identification and susceptibility testing. Since an increasing number of clinical microbiology laboratories apply MALDI-TOF MS instruments for rapid identification, rapid AST by MALDI-TOF MS could easily be integrated in daily diagnostic workflow. Our suggested workflow is shown in **Figure 2** with lysis/centrifugation method using the modified MBT Sepsityper protocol for species identification by MALDI-TOF. After identification, the MALDI-TOF MS-based AST is performed with the obtained pellet. Final susceptibility results will be available in <6 h, starting with the positive flagged BC and including total hands-on-time of half an hour. According to the standard workflow in the majority of routine diagnostics laboratories, positive BC would be subcultivated on solid medium and, after an overnight incubation, identification is done by MALDI-TOF MS. This is followed by standard AST, which also requires overnight incubation. Some laboratories integrated the identification by MALDI-TOF MS from shortly incubated cultures on solid medium according to Idelevich et al. (2014b) to reduce time to result. Standard workflow would take up to 2 days until susceptibility results are available, whereas results will be available within 24 h using brief cultures on solid medium. Rapid AST by MALDI-TOF MS DOT-MGA would provide susceptibility results the same day BC was flagged positive. MALDI-TOF MS identification before initiation of AST provides precise guide to the choice of appropriate antibiotic panels to be tested.

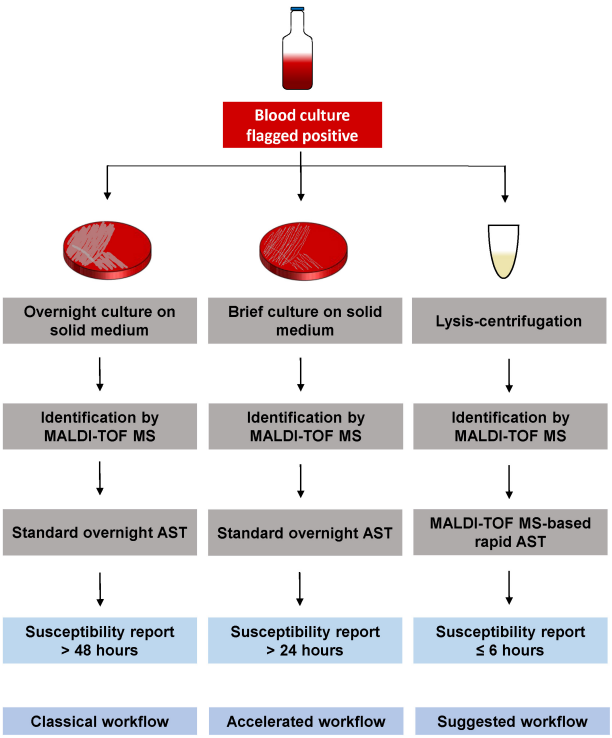


FIGURE 2 | Different workflows for susceptibility testing of positive BCs. Classical workflow: Overnight sub-cultivation of positive BC broth, followed by identification of the pathogen using MALDI-TOF MS. An overnight susceptibility testing is performed and susceptibility results are finalized approx. 2 days after BC was flagged positive. Accelerated workflow: Shortly incubated solid medium cultures (2–6 h) are used for MALDI-TOF MS identification, followed by an overnight susceptibility testing from the same shortly incubated cultures. Susceptibility results are available the day after BC was flagged positive. Suggested workflow: Positive BC broth is processed by the lysis/centrifugation method, using the modified MBT Sepsityper protocol, followed by the MALDI-TOF MS identification. The obtained pellet (without protein extraction) is used for MALDI-TOF MS-based rapid AST. Susceptibility results are available the same day BC was flagged positive within <6 h.

CONCLUSION

The first investigation of MALDI-TOF MS DOT-MGA for Gram-positive bacteria demonstrated the feasibility and accuracy of DOT-MGA from agar cultures and directly from positive BCs for detection of methicillin resistance in *S. aureus* after 4 h of incubation. To standardize and optimize test conditions and evaluation criteria, further research is needed. An optimized evaluation algorithm must be developed to guarantee the reliable performance of this new rapid AST assay. Furthermore, studies with larger amount of genetically diverse isolates from different locations elsewhere in the world should follow to develop a

standardized assay for clinical diagnostics. Additionally, using this universal phenotypic assay for simultaneous rapid AST of several antibiotics in parallel would be in focus of further studies.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

IN, EI, LS, and KB designed the experiments. LS performed the preliminary experiments. IN performed the experiments. IN, EI, KS, OD, and MK designed and analyzed specific MALDI-TOF MS instrument settings for experiments. IN, EI, KS, OD, and KB analyzed the data. IN wrote the manuscript with input from EI, LS, KS, OD, MK, and KB. All authors reviewed and edited the manuscript.

FUNDING

This study was funded in part by grants from the German Federal Ministry of Education and Research (BMBF) to EI and KB (Grant Number 16GW0150) and MK (Grant Number 16GW0149K).

ACKNOWLEDGMENTS

We thank Michaela Schreiner, Barbara Grünastel, and Damayanti Kaiser for their excellent technical assistance. This work was presented in part at the 29th European Congress of Clinical Microbiology and Infectious Diseases (ECCMID), Amsterdam, Netherlands, 13–16 April 2019 [P2812].

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.00232/full#supplementary-material

REFERENCES

Banerjee, R., Özenci, V., and Patel, R. (2016). Individualized approaches are needed for optimized blood cultures. *Clin. Infect. Dis.* 63, 1332–1339. doi: 10.1093/cid/ciw573

Barenfanger, J., Graham, D. R., Kolluri, L., Sangwan, G., Lawhorn, J., Drake, C. A., et al. (2008). Decreased mortality associated with prompt gram staining of blood cultures. *Am. J. Clin. Pathol.* 130, 870–876. doi: 10.1309/AJCPVMDQU2ZJDPBL

Becker, K., van Alen, S., Idelevich, E. A., Schleimer, N., Seggewiss, J., Mellmann, A., et al. (2018). Plasmid-encoded transferable *mecB*-mediated methicillin resistance in *Staphylococcus aureus*. *Emerg. Infect. Dis.* 24, 242–248. doi: 10.3201/eid2402.171074

Bernardo, K., Pakulat, N., Macht, M., Krut, O., Seifert, H., Fleer, S., et al. (2002). Identification and discrimination of *Staphylococcus aureus* strains using matrix-assisted laser desorption/ionization-time of flight mass spectrometry. *Proteomics* 2, 747–753. doi: 10.1002/1615-9861(200206)2:6<747:Aid-prot747<3.0.Co;2-v

Christner, M., Rohde, H., Wolters, M., Sobottka, I., Wegscheider, K., and Aepfelbacher, M. (2010). Rapid identification of bacteria from positive blood culture bottles by use of matrix-assisted laser desorption-ionization time of flight mass spectrometry fingerprinting. *J. Clin. Microbiol.* 48, 1584–1591. doi: 10.1128/JCM.01831-09

CLSI (2018). *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically. M07*, 11th Edn. Wayne, PA: Clinical Laboratory Standards Institute.

Doern, C. D. (2018). The slow march toward rapid phenotypic antimicrobial susceptibility testing: are we there yet? *J. Clin. Microbiol.* 56:e1999-17. doi: 10.1128/jcm.01999-17

Du, Z., Yang, R., Guo, Z., Song, Y., and Wang, J. (2002). Identification of *Staphylococcus aureus* and determination of its methicillin resistance by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Anal. Chem.* 74, 5487–5491. doi: 10.1021/ac020109k

Dubourg, G., Lamy, B., and Ruimy, R. (2018). Rapid phenotypic methods to improve the diagnosis of bacterial bloodstream infections: meeting the challenge to reduce the time to result. *Clin. Microbiol. Infect.* 24, 935–943. doi: 10.1016/j.cmi.2018.03.031

Edwards-Jones, V., Claydon, M. A., Evason, D. J., Walker, J., Fox, A. J., and Gordon, D. B. (2000). Rapid discrimination between methicillin-sensitive and methicillin-resistant *Staphylococcus aureus* by intact cell mass spectrometry. *J. Med. Microbiol.* 49, 295–300. doi: 10.1099/0022-1317-49-3-295

EUCAST (2019a). *Breakpoint Tables for Interpretation of MICs and Zone Diameters*. Denmark: The European Committee on Antimicrobial Susceptibility Testing.

EUCAST (2019b). *Routine and Extended Internal Quality Control for MIC Determination and Disk Diffusion as Recommended by EUCAST*. Denmark: The European Committee on Antimicrobial Susceptibility Testing.

Ferreira, L., Sánchez-Juanes, F., Porras-Guerra, I., García-García, M., García-Sánchez, J., González-Buitrago, J., et al. (2011). Microorganisms direct identification from blood culture by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Clin. Microbiol. Infect.* 17, 546–551. doi: 10.1111/j.1469-0691.2010.03257.x

Ferrer, R., Martin-Loeches, I., Phillips, G., Osborn, T. M., Townsend, S., Dellinger, R. P., et al. (2014). Empiric antibiotic treatment reduces mortality in severe sepsis and septic shock from the first hour: results from a guideline-based performance improvement program. *Crit. Care Med.* 42, 1749–1755. doi: 10.1097/ccm.0000000000000330

Haigh, J., Degun, A., Eydmann, M., Millar, M., and Wilks, M. (2011). Improved performance of bacterium and yeast identification by a commercial matrix-assisted laser desorption ionization–time of flight mass spectrometry system in the clinical microbiology laboratory. *J. Clin. Microbiol.* 49, 3441–3441. doi: 10.1128/jcm.00576-11

Idelevich, E. A., and Becker, K. (2019). How to accelerate antimicrobial susceptibility testing. *Clin. Microbiol. Infect.* 25, 1347–1355. doi: 10.1016/j.cmi.2019.04.025

Idelevich, E. A., Grunewald, C. M., Wüllenweber, J., and Becker, K. (2014a). Rapid identification and susceptibility testing of *Candida* spp. from positive blood cultures by combination of direct MALDI-TOF mass spectrometry and direct inoculation of Vitek 2. *PLoS One* 9:e114834. doi: 10.1371/journal.pone.0114834

Idelevich, E. A., Reischl, U., and Becker, K. (2018a). New microbiological techniques in the diagnosis of bloodstream infections. *Dtsch. Arztebl. Int.* 115, 822–832. doi: 10.3238/arztebl.2018.0822

Idelevich, E. A., Schüle, I., Grünastel, B., Wüllenweber, J., Peters, G., and Becker, K. (2014b). Rapid identification of microorganisms from positive blood cultures by MALDI-TOF mass spectrometry subsequent to very short-term incubation on solid medium. *Clin. Microbiol. Infect.* 20, 1001–1006. doi: 10.1111/1469-0691.12640

Idelevich, E. A., Seifert, H., Sundqvist, M., Scudeller, L., Amit, S., Balode, A., et al. (2019). Microbiological diagnostics of bloodstream infections in Europe - an

ESGBIES survey. *Clin. Microbiol. Infect.* 25, 1399–1407. doi: 10.1016/j.cmi.2019.03.024

Idelevich, E. A., Sparbier, K., Kostrzewa, M., and Becker, K. (2018b). Rapid detection of antibiotic resistance by MALDI-TOF mass spectrometry using a novel direct-on-target microdroplet growth assay. *Clin. Microbiol. Infect.* 24, 738–743. doi: 10.1016/j.cmi.2017.10.016

Idelevich, E. A., Storck, L. M., Sparbier, K., Drews, O., Kostrzewa, M., and Becker, K. (2018c). Rapid direct susceptibility testing from positive blood cultures by the MALDI-TOF MS-based direct-on-target microdroplet growth assay. *J. Clin. Microbiol.* 56, e913–e918. doi: 10.1128/jcm.00913-18

ISO (2006). ISO 20776-1: 2006. Clinical Laboratory Testing and In Vitro Diagnostic Test Systems - Susceptibility Testing of Infectious Agents and Evaluation of Performance of Antimicrobial Susceptibility Test Devices - Part 1: Reference Method for Testing the in Vitro Activity of Antimicrobial Agents Against Rapidly Growing Aerobic Bacteria Involved in Infectious Diseases. Geneva: International Organization for Standardization.

Josten, M., Dischinger, J., Szekat, C., Reif, M., Al-Sabti, N., Sahl, H.-G., et al. (2014). Identification of *agr*-positive methicillin-resistant *Staphylococcus aureus* harbouring the class A *mec* complex by MALDI-TOF mass spectrometry. *Int. J. Med. Microbiol.* 304, 1018–1023. doi: 10.1016/j.ijmm.2014.07.005

Klein, S., Zimmermann, S., Köhler, C., Mischnik, A., Alle, W., and Bode, K. A. (2012). Integration of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry in blood culture diagnostics: a fast and effective approach. *J. Med. Microbiol.* 61, 323–331. doi: 10.1099/jmm.0.035550-0

Köck, R., Becker, K., Cookson, B., Gemert-Pijnen, J. E. V., Harbarth, S., Kluytmans, J., et al. (2014). Systematic literature analysis and review of targeted preventive measures to limit healthcare-associated infections by meticillin-resistant *Staphylococcus aureus*. *Euro Surveill.* 19:20860. doi: 10.25646/1852

Köck, R., Wüllenweber, J., Horn, D., Lanckohr, C., Becker, K., and Idelevich, E. A. (2017). Implementation of short incubation MALDI-TOF MS identification from positive blood cultures in routine diagnostics and effects on empiric antimicrobial therapy. *Antimicrob. Resist. Infect. Control.* 6:12. doi: 10.1186/s13756-017-0173-4

Kriegeskorte, A., Ballhausen, B., Idelevich, E. A., Köck, R., Friedrich, A. W., Karch, H., et al. (2012). Human MRSA isolates with novel genetic homolog, Germany. *Emerg. Infect. Dis.* 18, 1016–1018. doi: 10.3201/eid1806.110910

Kumar, A., Roberts, D., Wood, K. E., Light, B., Parrillo, J. E., Sharma, S., et al. (2006). Duration of hypotension before initiation of effective antimicrobial therapy is the critical determinant of survival in human septic shock. *Crit. Care Med.* 34, 1589–1596. doi: 10.1097/01.Ccm.0000217961.75225.E9

La Scola, B., and Raoult, D. (2009). Direct identification of bacteria in positive blood culture bottles by matrix-assisted laser desorption ionisation time-of-flight mass spectrometry. *PLoS One* 4:e8041. doi: 10.1371/journal.pone.0008041

Lange, C., Schubert, S., Jung, J., Kostrzewa, M., and Sparbier, K. (2014). Quantitative matrix-assisted laser desorption ionization–time of flight mass spectrometry for rapid resistance detection. *J. Clin. Microbiol.* 52, 4155–4162. doi: 10.1128/JCM.01872-14

Lin, J.-F., Ge, M.-C., Liu, T.-P., Chang, S.-C., and Lu, J.-J. (2017). A simple method for rapid microbial identification from positive monomicrobial blood culture bottles through matrix-assisted laser desorption ionization time-of-flight mass spectrometry. *J. Microbiol. Immunol. Infect.* 51, 659–665. doi: 10.1016/j.jmii.2017.03.005

Mellmann, A., Friedrich, A. W., Rosenkötter, N., Rothgänger, J., Karch, H., Reintjes, R., et al. (2006). Automated DNA sequence-based early warning system for the detection of methicillin-resistant *Staphylococcus aureus* outbreaks. *PLoS Med.* 3:e33. doi: 10.1371/journal.pmed.0030033

Moussaoui, W., Jaulhac, B., Hoffmann, A. M., Ludes, B., Kostrzewa, M., Riegel, P., et al. (2010). Matrix-assisted laser desorption ionization time-of-flight mass spectrometry identifies 90% of bacteria directly from blood culture vials. *Clin. Microbiol. Infect.* 16, 1631–1638. doi: 10.1111/j.1469-0691.2010.03356.x

Pearson, E. S. (1931). The test of significance for the correlation coefficient. *J. Am. Stat. Assoc.* 26, 128–134. doi: 10.1080/01621459.1931.10503208

Pearson, E. S. (1932). The test of significance for the correlation coefficient: some further results. *J. Am. Stat. Assoc.* 27, 424–426. doi: 10.1080/01621459.1932.10502244

Schaumburg, F., Kock, R., Mellmann, A., Richter, L., Hasenberg, F., Kriegeskorte, A., et al. (2012). Population dynamics among methicillin-resistant *Staphylococcus aureus* isolates in Germany during a 6-year period. *J. Clin. Microbiol.* 50, 3186–3192. doi: 10.1128/jcm.01174-12

Singer, M., Deutschman, C. S., and Seymour, C. (2016). The third international consensus definitions for sepsis and septic shock (sepsis-3). *JAMA* 315, 801–810. doi: 10.1001/jama.2016.0287

Stevenson, L. G., Drake, S. K., and Murray, P. R. (2010). Rapid identification of bacteria in positive blood culture broths by matrix-assisted laser desorption ionization-time of flight mass spectrometry. *J. Clin. Microbiol.* 48, 444–447. doi: 10.1128/JCM.01541-09

Student, B. (1908). The probable error of a mean. *Biometrika* 6, 1–25. doi: 10.1093/biomet/6.1.1

Trenholme, G. M., Kaplan, R. L., Karakusis, P. H., Stine, T., Fuhrer, J., Landau, W., et al. (1989). Clinical impact of rapid identification and susceptibility testing of bacterial blood culture isolates. *J. Clin. Microbiol.* 27, 1342–1345. doi: 10.1128/jcm.27.6.1342-1345.1989

van Belkum, A., Bachmann, T. T., Lüdke, G., Lisby, J. G., Kahlmeter, G., Mohess, A., et al. (2019). Developmental roadmap for antimicrobial susceptibility testing systems. *Nat. Rev. Microbiol.* 17, 51–62. doi: 10.1038/s41579-018-0098-9

Wang, Y. R., Qian, C., Cui, S. H., and Li, F. Q. (2013). Characterization of *Staphylococcus aureus* isolated from clinical specimens by matrix assisted laser desorption/ionization time-of-flight mass spectrometry. *Biomed. Environ. Sci.* 26, 430–436. doi: 10.3967/0895-3988.2013.06.003

World Health Organization [WHO] (2017). *Global Priority list of Antibiotic-Resistant Bacteria to Guide Research, Discovery, and Development of New Antibiotics*. Geneva: World Health Organization.

Zhou, M., Yang, Q., Kudinha, T., Sun, L., Zhang, R., Liu, C., et al. (2017). An improved in-house MALDI-TOF MS protocol for direct cost-effective identification of pathogens from blood cultures. *Front. Microbiol.* 8:1824. doi: 10.3389/fmicb.2017.01824

Conflict of Interest: EI and KB are inventors of the patent application which is owned by the University of Münster and licensed to Bruker Daltonik GmbH, Bremen, Germany. KS, OD, and MK are employees of Bruker Daltonik GmbH, Bremen, Germany.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 28 February 2020
doi: 10.3389/fmicb.2020.00328

Rapid Detection of KPC-Producing Enterobacterales Susceptible to Imipenem/Relebactam by Using the MALDI-TOF MS MBT STAR-Carba IVD Assay

Marina Oviaño, Eva Gato and Germán Bou*

Servicio de Microbiología, Complejo Hospitalario Universitario A Coruña, A Coruña, Spain

KPC-producing Enterobacterales represent a serious public health concern. Limited therapeutic options are available for treatment, however, the novel combination of imipenem/relebactam represents a promising alternative. To preserve the activity of this new antibiotic combination, only targeted treatments will be recommended, and rapid tests to detect susceptible bacteria are therefore urgently needed. Here, we propose a MALDI-TOF-based method using the MBT STAR-Carba IVD assay, Bruker Daltonik, to detect KPC-producing Enterobacterales susceptible to imipenem/relebactam in a random selection of 143 clinical isolates previous molecular characterized, carrying 97 bla_{KPC}, 1 bla_{GES}, 12bla_{VIM}, 4bla_{IMP}, 3bla_{NDM}, and 26$bla_{OXA-48-like}$. Species identification was confirmed by MALDI-TOF MS. The molecular characterization of the isolates was performed by the Xpert Carba-R Assay and the results were used as gold standard. Besides, all isolates were submitted to imipenem and imipenem/relebactam microdilution susceptibility testing. The assay showed an overall sensitivity and specificity to detect class A-producing Enterobacterales susceptible to imipenem/relebactam of 98% (96/98) and 93% (42/45), respectively. This MALDI-TOF-based methodology, with a turnaround time of less than 1 h, is a reliable test for detecting imipenem/relebactam activity and its inclusion in routine laboratory screening would facilitate the correct use of this new combination of antimicrobials as a targeted treatment.

Keywords: MALDI-TOF, antimicrobial resistance, imipenem relebactam, carbapenemase resistance detection, clinical microbiology

OPEN ACCESS

Edited by:
Karsten Becker, University Medicine Greifswald, Germany

Reviewed by:
Miriam Cordovana, Sant'Orsola-Malpighi Polyclinic, Italy
Divakar Sharma, Indian Institute of Technology Delhi, India

****Correspondence:***
Marina Oviaño
marina.oviano.garcia@sergas.es

Specialty section:
This article was submitted to Antimicrobials, Resistance and Chemotherapy, a section of the journal Frontiers in Microbiology

Received: *12 November 2019*
Accepted: *14 February 2020*
Published: *28 February 2020*

Citation:
Oviaño M, Gato E and Bou G (2020) Rapid Detection of KPC-Producing Enterobacterales Susceptible to Imipenem/Relebactam by Using the MALDI-TOF MS MBT STAR-Carba IVD Assay. Front. Microbiol. 11:328. doi: 10.3389/fmicb.2020.00328

INTRODUCTION

The global rise in the incidence of carbapenemase-producing Enterobacterales (CPE) is alarming and has posed a challenge to health services worldwide (Nordmann and Poirel, 2014). The increase in the prevalence of *Klebsiella pneumoniae* is especially worrying as these are the most frequent carbapenemase producers worldwide (Glasner et al., 2013). Limited therapeutic options are available for infections caused by CPE. Thus, only "second line" drugs such as polymyxins, tigecycline, aminoglycosides and fosfomycin may be active, although double

carbapenem therapy can also be considered for use; finally, combination therapies are associated with better outcomes for high-risk patients (Rodríguez-Baño et al., 2018). Novel β-lactam inhibitors are being developed with the aim of restoring the activity of β-lactam antibiotics against CPE. Relebactam (MSD, EEUU) is an inhibitor of class A and C β-lactamases and is currently under clinical development in combination with imipenem-cilastatin. Imipenem/relebactam has recently undergone testing in phase 3 clinical trials for the treatment of patients with complicated intra-abdominal infections, complicated urinary tract infections and hospital-acquired/ventilator-associated bacterial pneumonia, with promising results. However, in order to reduce the spread of CPE and preserve the susceptibility to imipenem/relebactam, this treatment should only be used for infections that are proven or strongly suspected to be caused by susceptible bacteria to this antimicrobial combination. For new drugs, susceptibility testing is generally restricted to phenotypic tests like disc and/or gradient diffusion methods, until its inclusion in microdilution automated systems of susceptibility testing, all of which require at least 16 h to provide the results. MALDI-TOF MS has been used to detect carbapenems hydrolysis, as a surrogate test for susceptibility, and also to detect inhibition by β-lactam inhibitors (Oviaño and Bou, 2017, 2018; Carvalhaes et al., 2018; Anantharajah et al., 2019). EUCAST guidelines for carbapenemase detection also recommends the carbapenem hydrolysis assay by MALDI-TOF MS as a useful method for clinical practice. Here, we propose a rapid and simple MALDI-TOF MS-based assay for detecting class A-producing Enterobacterales, focused in KPC isolates due to their high prevalence, susceptible to imipenem/relebactam by using the commercial MBT STAR-Carba IVD assay.

MATERIALS AND METHODS

Bacterial Isolates

The proposed method was applied in a random selection of 143 clinical isolates previous molecular characterized by using the Xpert Carba-R Assay (Cepheid, Sunnyvale, United States), carrying 97 bla_{KPC}, 1 bla_{GES}, 12bla_{VIM}, 4bla_{IMP}, 3bla_{NDM}, and 26$bla_{\text{OXA-48-like}}$ (Traczewski et al., 2018). The isolates were collected during a 2 months period (December 2017-January 2018) from 31 hospitals from Spain. The isolates were multidrug resistant. The molecular results were used as gold standard. Species identification was confirmed by MALDI-TOF MS. The carbapenemase-producing Enterobacterales (CPE) comprised 104 *Klebsiella pneumoniae*, 3 *K. oxytoca*, 12 *Escherichia coli*, 16 *Enterobacter cloacae*, 6 *Citrobacter freundii* and 2 *Serratia marcescens*. All isolates were submitted to imipenem and imipenem/relebactam microdilution susceptibility testing following the EUCAST guidelines. A decrease of at least three double serial dilutions in the imipenem/relebactam minimum inhibitory concentration (MIC) with respect to the imipenem MIC was considered for defining a positive inhibition. *E. coli* ATCC 25922 was used as a negative control, and a PCR-confirmed KPC-producing *E. coli* was used as a positive control.

MALDI-TOF MS Assay

The proposed method was applied by using the MBT STAR-Carba IVD Kit in conjunction with the MBT STAR-BL IVD software (Bruker Daltonik, Germany). The test was performed according to the manufacturer's instructions, with slight modifications regarding the inclusion of relebactam in the medium. After an overnight incubation at 37°C in Trypticase Soy Agar (Beckton Dickinson, Heidelberg, Germany), a 1 μl loop of bacteria was resuspended in a tube of lyophilized imipenem dissolved in 50 μl relebactam (3 mg/ml in MBT STAR Buffer). After incubation for 30 min at 37°C under agitation, the bacteria were pelleted by centrifugation (2 min at 14000 rpm), and 1 μl of the supernatant was spotted (in duplicate) onto a MALDI target. Air-dried spots were overlaid with MBT STAR Matrix.

MALDI-TOF MS Analysis and Data Processing

The MBT STAR-Carba assay principle is the inactivation of imipenem by bacteria carrying carbapenemase enzymes due to the hydrolysis of the β-lactam ring. The hydrolysis reaction modifies the structure of the antibiotic that is associated with a mass shift that can be detected by MALDI-TOF. In cases of bacteria carrying carbapenemase enzymes inhibited by relebactam, no modification of imipenem could be observed. However, if the carbapenemase enzymes are not inhibited we could observe a proper hydrolysis of imipenem.

The mass spectrum was obtained using a MALDI Biotyper® Smart (Bruker Daltonik) instrument, with Flex Control 3.4 software, in the m/z range of 100–1.000 Da. The MBT STAR-BL Software module was used to evaluate the spectra. The software automatically calculates the LogRQ value (which indicates the rate of hydrolysis) for imipenem in the presence of relebactam. According to the manufacturer's instructions, normalized LogRQ values similar to or below 0.2 represent no proven imipenem hydrolysis. Normalized LogRQ values similar to or above 0.4 indicate proven imipenem hydrolysis. Normalized LogRQ values between 0.2 and 0.4 represent an ambiguous hydrolysis pattern which requires further testing or confirmation by other techniques.

RESULTS

In this study, MALDI-TOF MS showed good results for detection of class A-producing Enterobacterales compared to the results of molecular methods, with a sensitivity of 98% (96/98) and a specificity of 93% (42/45) (**Figure 1**). The average logRQ for class A was 0.08 (n = 98), with two isolates proving an intermediate logRQ (0.30 and 0.27) (**Supplementary Table S1**). These isolates were retested as recommended by the manufacturer proving again an intermediate result. In this case, the MALDI-TOF MS could not provide a clear result of susceptibility and confirmation techniques should be further used. The average logRQ for class B (n = 19) was 0.94, with all isolates proving logRQ values above 0.4. The average logRQ for class D (n = 19) was 0.79, with all isolates proving logRQ values above 0.4, except one isolate that had an intermediate result (logRQ = 0.22) and two isolates

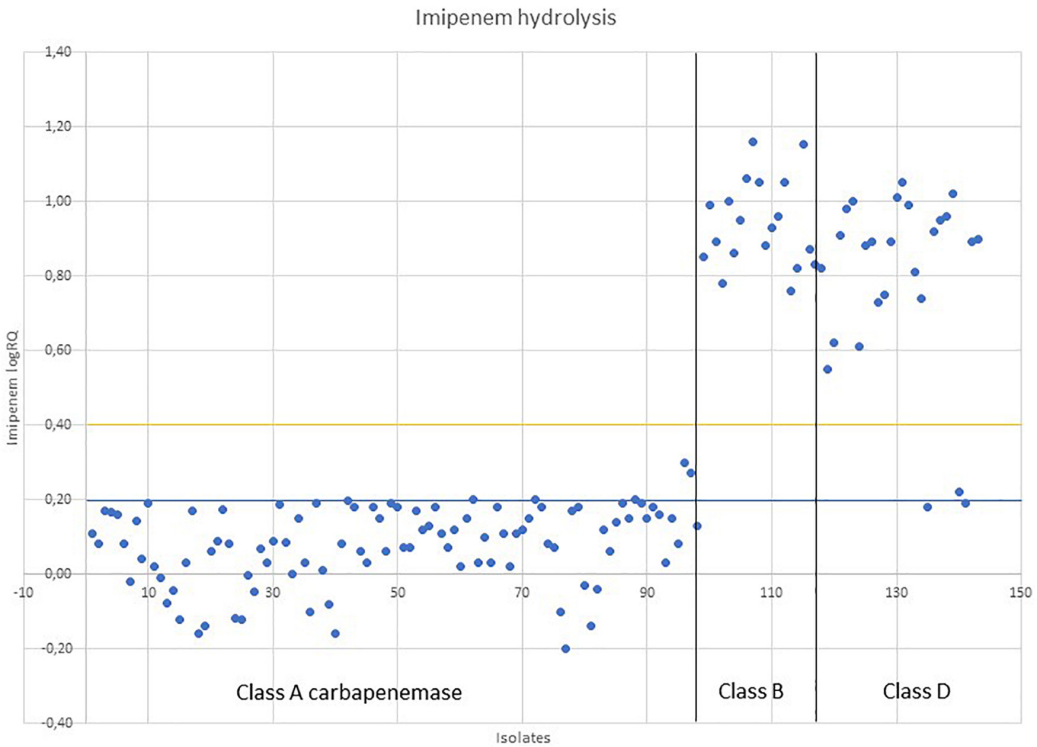


FIGURE 1 | Imipenem/relebactam susceptibility indicated by MALDI-TOF MS, based on the measurement of imipenem hydrolysis in the presence of relebactam.

that gave a negative result (logRQ = 0.18; 0.19). The isolate providing an intermediate result was also retested, providing the same value again.

Comparison with the results of imipenem/relebactam susceptibility testing was not possible as there are no clinical breakpoints established yet. Besides, as there has not been any correlation proven between the logRQ and the MIC in previous studies, we considered a criterion for relebactam inhibition an imipenem/relebactam MIC decrease in at least three double serial dilutions with respect to the imipenem MIC obtained by microdilution. Considering this criterion, we obtained a 100% sensitivity and specificity in detecting KPC-producing Enterobacterales susceptible to imipenem/relebactam (**Supplementary Table S1**), as all the isolates tested decreased in at least three double serial dilutions their MICs. For class B CPE the imipenem/relebactam MIC kept the same with respect to imipenem or in the minority of cases (2/19) decreased in one dilution. For class D CPE, the imipenem/relebactam MIC decreased in one dilution with respect to imipenem in the majority of isolates (21/26), whereas the minority of isolates (5/26) kept their MIC constant. There was no two or three double serial dilutions decrease observed for class B or D.

DISCUSSION

As there are few or no therapeutic alternatives available for treating infections caused by KPC-producing Enterobacterales, which are usually multidrug resistant, especially since the emergence of ceftazidime-avibactam resistance (Shields et al., 2017a,b) the early and accurate detection of imipenem/relebactam susceptibility in those isolates is extremely important in regard to prescribing this last resort antibiotic as a targeted treatment.

Our findings showed that the MALDI-TOF MS is an excellent tool for screening KPC-producing Enterobacterales susceptible to imipenem/relebactam before the microdilution results become available, providing results with a turnaround time of less than 1 h. Although, we only tested one GES-producing *K. oxytoca*, results seem consistent with those found in KPC-producing isolates. The sensitivity and specificity of the method is 98% (96/98) and 93% (42/45) respectively, compared to the molecular characterization of the isolates. When applying a second phenotypic criteria, as the decrease in at least three double serial dilutions of the imipenem/relebactam MIC with respect to the imipenem MIC obtained by microdilution, we obtained a 100% sensitivity and specificity. These results are in accordance to the imipenem/relebactam *in vitro* results found in previous studies that proved that the combination was active against class A CPE. Relebactam has not proved to be a good inhibitory substrate for class B and D CPE in previous studies (Lob et al., 2017; Karlowsky et al., 2018) as also found by MALDI-TOF MS. As expected, we did not detect relebactam inhibition in metallo-β-lactamase-producing isolates and in the majority of the class D CPE isolates. However, we

recommend to perform the imipenem/relebactam MIC to assure the susceptibility of a carbapenem resistant Enterobacteral as it is considered the gold standard.

The isolate collection tested in this study was a reflection of our national CPE epidemiology and did not include any imipenem/relebactam resistant KPC-producing Enterobacteral which represents a limitation. Besides, the MALDI-TOF MS assay exclusively detects enzymatic carbapenem resistance but does not detect carbapenem resistance due to other mechanisms that can confer resistance as the OmpK35 disruption and/or mutated OmpK36 that have been described as chromosomal resistance in these isolates (Galani et al., 2019).

This method has only been applied to colonies in agar plates but could probably also be applied to positive blood cultures. The STAR MBT-Carba IVD assay is easy to use, and the handling time is very short. Moreover, the automated interpretation of results minimizes the inter-test variability associated with different environmental conditions and operators. Detailed analysis of spectra is not required, which simplifies the procedure and enables application of the assay by personnel who are not trained in mass-spectrometry techniques and its inclusion in routine in high throughput screening in microbiology laboratories.

In conclusion, this method is the first one to our knowledge to provide rapid results for an imipenem/relebactam early administration in KPC-producing Enterobacterales. Our results showed that the application of MALDI-TOF MS with the STAR MBT-Carba IVD assay, proved to be an excellent tool for screening imipenem/relebactam activity, helping in the antimicrobial therapy adjustment and early implementation of infection control measures.

Normalized imipenem LogRQ values for 143 CPE after 30 min of incubation with imipenem/relebactam following the MBT STAR-Carba IVD assay. LogRQ values above 0.4 (yellow line) mean positive imipenem/relebactam hydrolysis, values below 0.2 (blue line) mean negative imipenem/relebactam hydrolysis and intermediate values (>0.2 and <0.4) requires further testing.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

MO and GB contributed to the conception and design of the study, and analyzed all the experiments. MO and EG performed the experiments. MO wrote the manuscript. GB contributed to the final version of the manuscript.

FUNDING

This work was supported by the Fondo de Investigación Sanitaria (grant numbers PI15/00860 and PI18/00860 to GB) integrated in the Plan Nacional de I+D and funded by the Instituto de Salud Carlos III (ISCIII). The results of this work have been funded by the Spanish Network for Research in Infectious Diseases (REIPI), N° RD16/0016/0006, integrated in the National Plan for Scientific Research, Development and Technological Innovation 2013–2016 and funded by the ISCIII – General Subdirection of Assessment and Promotion of the Research – European Regional Development Fund (FEDER) "A way of making Europe". GB has received a grant from the Investigator Initiated Studies Program economically supported by Merck Sharp and Dohme (MSD). This work was also supported by the Sociedad Española de Enfermedades Infecciosas y Microbiología Clínica (SEIMC) through their 2019 research projects grants to MO.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.00328/full#supplementary-material

REFERENCES

Anantharajah, A., Tossens, B., Olive, N., Kabamba-Mukadi, B., Rodriguez-Villalobos, H., and Verroken, A. (2019). Performance evaluation of the MBT STAR® -Carba IVD assay for the detection of carbapenemases with MALDI-TOF MS. *Front. Microbiol.* 10:1413. doi: 10.3389/fmicb.2019.01413

Carvalhaes, C. G., Ramos, A. C., Oliveira, L. C. G., Juliano, M. A., and Gales, A. C. (2018). Rapid detection of ceftazidime/avibactam resistance by MALDI-TOF MS. *J. Antimicrob. Chemother.* 73, 2579–2582. doi: 10.1093/jac/dky196

Galani, I., Souli, M., Nafplioti, K., Adamou, P., Karaiskos, I., Giamarellou, H., et al. (2019). In vitro activity of imipenem-relebactam against non-MBL carbapenemase-producing *Klebsiella pneumoniae* isolated in Greek hospitals in 2015-2016. *Eur. J. Clin. Microbiol. Infect. Dis.* 38, 1143–1150. doi: 10.1007/s10096-019-03517-y

Glasner, C., Albiger, B., Buist, G., Tambiæ Andraseviæ, A., Canton, R., Carmeli, Y., et al. (2013). European survey on carbapenemase-producing *Enterobacteriaceae* (EuSCAPE) working group. carbapenemase-producing enterobacteriaceae in europe: a survey among national experts from 39 countries. *Euro. Surveill.* 18:20525. doi: 10.2807/1560-7917.es2013.18.28.20525

Karlowsky, J. A., Lob, S. H., Kazmierczak, K. M., Hawser, S. P., Magnet, S., Young, K., et al. (2018). In vitro activity of imipenem/relebactam against gram-negative ESKAPE pathogens isolated in 17 European countries: 2015 SMART surveillance programme. *J. Antimicrob. Chemother.* 73, 1872–1879. doi: 10.1093/jac/dky107

Lob, S. H., Hackel, M. A., Kazmierczak, K. M., Young, K., Motyl, M. R., Karlowsky, J. A., et al. (2017). In vitro activity of imipenem-relebactam against gram-negative ESKAPE pathogens isolated by clinical laboratories in the United States in 2015 (Results from the SMART global surveillance program). *Antimicrob. Agents Chemother.* 61:e002209-16. doi: 10.1128/AAC.02209-16

Nordmann, P., and Poirel, L. (2014). The difficult-to-control spread of carbapenemase producers among *Enterobacteriaceae* worldwide. *Clin. Microbiol. Infect.* 20, 821–830. doi: 10.1111/1469-0691.12719

Oviaño, M., and Bou, G. (2017). Imipenem-avibactam: a novel combination for the rapid detection of carbapenemase activity in *Enterobacteriaceae* and *Acinetobacter baumannii* by matrix-assisted laser desorption ionization-time of flight mass spectrometry. *Diagn. Microbiol. Infect. Dis.* 87, 129–132. doi: 10.1016/j.diagmicrobio.2016.10.016

Oviaño, M., and Bou, G. (2018). Matrix-assisted laser desorption ionization-time of flight mass spectrometry for the rapid detection of antimicrobial resistance

mechanisms and beyond. *Clin. Microbiol. Rev.* 32:e0037-18. doi: 10.1128/CMR.00037-18

Rodríguez-Baño, J., Gutiérrez-Gutiérrez, B., Machuca, I., and Pascual, A. (2018). Treatment of infections caused by extended-spectrum-beta- lactamase-, AmpC-, and carbapenemase-producing *Enterobacteriaceae*. *Clin. Microbiol. Rev.* 31:e079-17. doi: 10.1128/CMR.00079-17

Shields, R. K., Chen, L., Cheng, S., Chavda, K. D., Press, E. G., Snyder, A., et al. (2017a). Emergence of ceftazidime-avibactam resistance due to plasmid-borne *bla* (KPC-3) mutations during treatment of carbapenem-resistant *Klebsiella pneumoniae* infections. *Antimicrob. Agents Chemother.* 61:e002097-16. doi: 10.1128/AAC.02097-16

Shields, R. K., Nguyen, M. H., Press, E. G., Chen, L., Kreiswirth, B. N., and Clancy, C. J. (2017b). Emergence of ceftazidime-avibactam resistance and restoration of carbapenem susceptibility in *Klebsiella pneumoniae* carbapenemase-producing *K. pneumoniae*: a case report and review of literature. *Open Forum Infect. Dis.* 4:ofx101. doi: 10.1093/ofid/ofx101

Traczewski, M. M., Carretto, E., Canton, R., Moore, N. M., and Carba-R Study Team, (2018). Multicenter evaluation of the xpert carba-R assay for detection of carbapenemase genes in gram-negative isolates. *J. Clin. Microbiol.* 56:e00272-18. doi: 10.1128/JCM.00272-18

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 03 June 2020
doi: 10.3389/fmicb.2020.01141

Detection of Colistin Resistance in *Salmonella enterica* Using MALDIxin Test on the Routine MALDI Biotyper Sirius Mass Spectrometer

*Laurent Dortet[1,2,3,4], Rémy A. Bonnin[2,3,4], Simon Le Hello[5], Laetitia Fabre[5], Richard Bonnet[4,6], Markus Kostrzewa[7], Alain Filloux[1] and Gerald Larrouy-Maumus[1]**

[1] MRC Centre for Molecular Bacteriology and Infection, Department of Life Sciences, Faculty of Natural Sciences, Imperial College London, London, United Kingdom, [2] Department of Bacteriology-Hygiene, Bicêtre Hospital, Assistance Publique – Hôpitaux de Paris, Le Kremlin-Bicêtre, France, [3] EA7361 "Structure, Dynamic, Function and Expression of Broad Spectrum ß-Lactamases", Paris-Sud University, LabEx Lermit, Faculty of Medecine, Le Kremlin-Bicêtre, France, [4] French National Reference Centre for Antibiotic Resistance, Le Kremlin-Bicêtre, France, [5] Institut Pasteur, French National Reference Centre for E. coli, Shigella and Salmonella, Paris, France, [6] Department of Bacteriology, University Hospital of Clermont-Ferrand, Clermont-Ferrand, France, [7] Bruker Daltonik GmbH, Bremen, Germany

OPEN ACCESS

Edited by:
Rustam Aminov, University of Aberdeen, United Kingdom

Reviewed by:
Guido Vincent Bloemberg, University of Zurich, Switzerland
Paola Bocanegra-Ibarias, Autonomous University of Nuevo León, Mexico

***Correspondence:**
Gerald Larrouy-Maumus
g.larrouy-maumus@imperial.ac.uk

Specialty section:
This article was submitted to Antimicrobials, Resistance and Chemotherapy, a section of the journal Frontiers in Microbiology

Received: *12 December 2019*
Accepted: *05 May 2020*
Published: *03 June 2020*

Citation:
Dortet L, Bonnin RA, Le Hello S, Fabre L, Bonnet R, Kostrzewa M, Filloux A and Larrouy-Maumus G (2020) Detection of Colistin Resistance in Salmonella enterica Using MALDIxin Test on the Routine MALDI Biotyper Sirius Mass Spectrometer. Front. Microbiol. 11:1141. doi: 10.3389/fmicb.2020.01141

Resistance to polymyxins in most Gram-negative bacteria arises from chemical modifications to the lipid A portion of their lipopolysaccharide (LPS) mediated by chromosomally encoded mutations or the recently discovered plasmid-encoded *mcr* genes that have further complicated the landscape of colistin resistance. Currently, minimal inhibitory concentration (MIC) determination by broth microdilution, the gold standard for the detection of polymyxin resistance, is time consuming (24 h) and challenging to perform in clinical and veterinary laboratories. Here we present the use of the MALDIxin to detect colistin resistant *Salmonella enterica* using the MALDxin test on the routine matrix-assisted laser desorption ionization (MALDI) Biotyper Sirius system.

Keywords: MALDI mass spectrometry, lipid A, colistin, *Salmonella enterica*, diagno stic

INTRODUCTION

Due to the limited pipeline of new antibiotics the increasing trend in antibiotic resistance is now threatening the global health. One of the most fearing issues is the dissemination of multidrug resistance (MDR) in Gram-negative bacteria. Indeed, these MDR bacteria may accumulate in a single strain resistance to the main classes of antimicrobial molecules, making colistin one of the last resort therapies for the treatment of infection caused by MDR Gram-negative bacteria.

In Gram-negative bacteria, acquired resistance to colistin results mostly from modifications of the drug target, i.e., the lipopolysaccharide (LPS). These modifications correspond to addition(s) of cationic groups such as 4-amino-L-arabinose (L-Ara4N) and/or phosphoethanolamine (pETN) on the lipid A, the anchor of the LPS (Olaitan et al., 2014; Zhang et al., 2019b). These modifications may result from chromosome-encoded mechanisms such as modification (basically mutations) of the two-component systems PmrA/PmrB and PhoP/PhoQ or alteration (mutation, disruption, down-regulation) of the global regulator MgrB. These chromosome-encoded mechanisms are mostly associated with addition of L-Ara4N on the lipid A. It has been reported that addition of pETN to lipid A can also occur through the expression of a plasmid-encoded pETN transferase, named mobilized colistin resistance (MCR) (Chew et al., 2017; Jayol et al., 2017; Jeannot et al., 2017;

Poirel et al., 2017; Zhang et al., 2019a). Despite, *mcr* genes have been described in many different enterobacteriales species, they are more prevalent in *Escherichia coli* and *Salmonella enterica* than in other Enterobacterales (Poirel et al., 2017). Of note, MCR-producing *S. enterica* have been often reported in livestock animals, mostly in chicken in which they are part of the normal gut flora. Overall, it is now accepted that the dissemination of *mcr* genes in animals was mostly driven by the intensive use of polymyxins during decades in livestock farming. With the soaring descriptions of MCR-producing isolates in animals during the 2 years following the original report of this mechanism, the use of colistin in veterinary medicine has been restricted in Europe (Catry et al., 2015). In addition, recommandations have been made for the reinforcement of the systematic monitoring of bacteria from food-producing animals for resistance to colistin (polymyxins). In 2013, monitoring and reporting of colistin resistance in commensal and zoonotic bacteria isolated from samples from certain food-producing animal populations and certain food became mandatory in Europe (European Union Commission, 2013). Accordingly, rapid detection of colistin resistance is therefore one of the key issues, not only in the human medicine to improve the treatment of patient infected with MDR bacteria, but also in the veterinary medicine where high-throughput methods would be of utmost interest. Unfortunately, detection of colistin resistance still relies on the determination of colistin susceptibility by performing minimal inhibitory concentration (MIC) using broth microdilution with 24 h of incubation time. Indeed, this susceptibility testing method remains the unique gold standard to identify colistin resistance. Therefore, there is an urgent need to develop a fast and robust assay to detect colistin-resistant Gram-negative bacteria including *S. enterica* (Carroll et al., 2019; Lima et al., 2019). Ultimately, this test should be a high-throughput assay to manage a large number of samples as it is the case in food industry.

Recently, we developed a rapid technique using matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) able to detect colistin resistance directly from whole bacteria in less than 15 min, the MALDIxin test (Dortet et al., 2018a,b). Here, we report on the application of the optimized MALDIxin, which has been applied to *E. coli*, *K. pneumoniae* and *Acinetobacter baumannii* (Dortet et al., 2018a,b, 2019; Furniss et al., 2019), using routine MALDI mass spectrometer and its application into the detection of colistin-resistant *S. enterica*.

MATERIALS AND METHODS

Bacterial Strains

A collection of 23 *S. enterica* clinical strains was used, which included 12 colistin resistant isolates and 11 colistin susceptible isolates. Among the resistant isolates,

TABLE 1 | GenBank accession number of colistin resistant and colistin susceptible *Salmonella enterica* isolates used in this study.

Strain	Name	Serotype	GenBank accession number	Colistin MIC (mg/L)	colistin resistance mechanism	Ref.
Colistin resistant strains						
Sal-R1	201607059	4,12:i:- (monophasic)	SAMN13531479	4	*mcr-1*	This study
Sal-R2	201606765	4,12:i:- (monophasic)	SAMN13531480	8	*mcr-1*	This study
Sal-R3	201609932	4,5,12:i:- (monophasic)	SAMN13531481	8	*mcr-1*	This study
Sal-R4	201610655	4,12:i:- (monophasic)	SAMN13531482	8	*mcr-1*	This study
Sal-R5	201610686	Paratyphi B d-tartrate + (biotype Java)	SAMN13531483	8	*mcr-1*	This study
Sal-R6	CNR 1776	Typhimurium	JAAOHZ000000000	8	*mcr-1*	This study
Sal-R7	13-SA01718	Paratyphi B d-tartrate + (biotype Java)	PRJNA396070	8	*mcr-5*	This study
Sal-R8	201600129	Dublin	SAMN13531484	4	Unknown	This study
Sal-R9	201607119	Enteritidis	SAMN13531485	4	mutated MgrB (K3T)	This study
Sal-R10	201606219	Typhimurium	SAMN13531486	4	mutated MgrB (Q30R)	This study
Sal-R11	201600169	Enteritidis	SAMN13531487	4	Unknown	This study
Sal-R12	R3445	4,12:i:- (monophasic)	MF543359	8	*mcr-4*	This study
Colistin susceptible strains						
Sal-S2	201604739	4,12:i:- (monophasic)	SAMN13531488	1	–	This study
Sal-S3	201604769	Enteritidis	SAMN13531489	2	–	This study
Sal-S4	201605339	4,12:i:- (monophasic)	SAMN13531490	1	–	This study
Sal-S5	201608919	Enteritidis	SAMN13531491	1	–	This study
Sal-S6	201606509	Typhimurium	SAMN13531492	1	–	This study
Sal-S7	201602769	Anatum	SAMN13531493	1	–	This study
Sal-S8	201606129	4,12:i:- (monophasic)	SAMN13531494	2	–	This study
Sal-S9	201607559	Enteritidis	SAMN13531495	0.5	–	This study
Sal-S10	201606439	4,12:i:- (monophasic)	SAMN13531496	1	–	This study
Sal-S11	201610299	Veneziana	SAMN13531497	0.5	–	This study
Sal-S12	201606239	Chester	SAMN13531498	2	–	This study

8 were MCR producers: 6 were MCR-1, 1 was MCR-4 and 1 was MCR-5 (**Table 1**). All the strains used in this study were part of the strain collections from the French National Reference Centre (NRC) for Antimicrobial Resistance, where all patient information are very partial and anonymized. The strains were not isolated for the purpose of our study, so ethical approval and consent were not required.

Susceptibility Testing

Minimal inhibitory concentrations were determined by broth microdilution (BMD) according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) joint subcommittee. Results were interpreted using EUCAST breakpoint as updated in 2019 (EUCAST, 2019).

Whole Genome Sequencing (WGS)

Whole Genome Sequencing was performed by the "Plateforme de Microbiologie Mutualisée (P2M)" at Institut Pasteur (Paris, France) for all colistin resistant isolates. Briefly, total DNA was isolated using the MagnaPure® microbial DNA isolation kit (Roche Laboratories) from overnight cultures on Mueller-Hinton agar (BioRad, Marnes-la-Coquette, France). Genomic DNA quantifications were performed using Qubit fluorometer (Life Technologies, Carlsbad, CA) and adjusted at 0.2 ng/µl. Library preparation was performed using NextEra® XT DNA sample preparation kit (Illumina, San Diego, CA, United States). Sequencing was performed on an Illumina NextSeq500 sequencer with v3 chemistry using 2×150-bp paired-end reads at a raw cluster density of˜1300,000 clusters/mm^2. Paired-end reads varied in read length depending on the sequencing

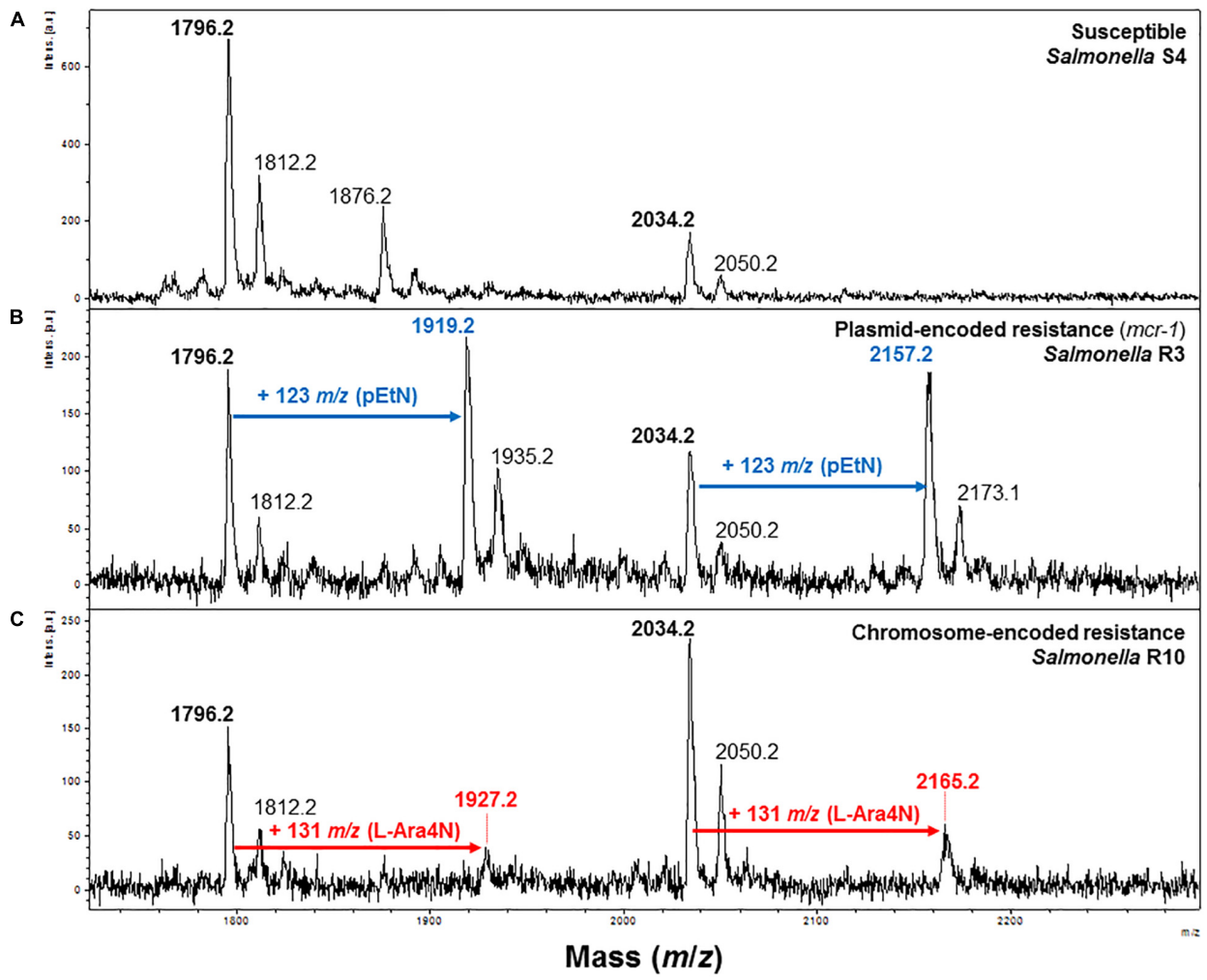


FIGURE 1 | Representative mass spectra of susceptible and modified *S. enterica* lipid A acquired using the linear negative-ion mode of a MALDI Biotyper Sirius system (Bruker Daltonics). **(A)** Susceptible *S. enterica* lipid A is detected as two major peaks at *m/z* 1796.2 and *m/z* 2034.2 (in bold). **(B)** Lipid A from *S. enterica* strains exhibiting MCR-mediated resistance to colistin exhibit additional peak at *m/z* 1919.2 and *m/z* 2157.2 (in blue) corresponding to pETN addition on the natural lipid A (peaks at *m/z* 1796.2 and *m/z* 2034.2). **(C)** Lipid A from colistin-resistant *S. enterica* isolates carrying chromosomal mutations is modified by L-Ara4N which are detected as additional peaks at *m/z* 1927.2 and *m/z* 2165.22 (in red).

platform/site, from 100 to 146 bp, yielding a minimum of 30-fold coverage per isolate.

Bioinformatic Analysis

Raw data were assembled into contigs using CLC Genomics Workbench v9.5.3[1]. To identify *mcr* genes, total raw data sequences of each isolate were subjected to ResFinder-2.1 Server[2] that is dedicated to the identification of acquired antimicrobial resistance genes. To identify mutation in PmrA, PmrB, PhoP, PhoQ and the master regulator MgrB (**Supplementary Figure S2**), sequences alignments were performed using ClustalW[3]. Sequence of *Salmonella enterica* subsp. *enterica* serovar Newport str. USMARC-S3124.1 (GenBank accession number CP006631) was used as reference sequence.

Nucleotide Sequence Accession Number

The whole genome sequences generated in the study have been submitted to the GenBank nucleotide sequence database under the accession number detailed in **Table 1**.

MALDIxin Test

A 10 µL inoculation loop of bacteria, grown on Mueller-Hinton agar for 18–24 h, was resuspended in 200 µL of water. Mild-acid hydrolysis was performed on 100 µL of this suspension, by adding 100 µl of acetic acid 2 % v/v and incubating the mixture at 98°C for 10 min. Hydrolyzed cells were centrifuged at 17,000 × *g* for 2 min, the supernatant was discarded and the pellet was resuspended in ultrapure water to a density of McFarland 10. A volume of 0.4 µL of this suspension was loaded onto the MALDI target plate and immediately overlaid with 1.2 µL of a matrix consisting of a 9:1 mixture of 2,5-dihydroxybenzoic acid and 2-hydroxy-5-methoxybenzoic acid (super-DHB, Sigma-Aldrich) solubilized in chloroform/methanol 90:10 v/v to a final concentration of 10 mg/mL.

The bacterial suspension and matrix were mixed directly on the target by pipetting and the mix dried gently under a stream of air. MALDI-TOF mass spectrometry analyses were performed with a MALDI Biotyper Sirius (Bruker Daltonics) using the linear negative-ion mode.

Data Analysis

The negative mass spectrum was scanned between *m/z* 1,600 and *m/z* 2,200 in the negative linear ion mode. Manual peak picking at masses relevant to colistin resistance was performed on the obtained mass spectra and the corresponding signal intensities at these defined masses was determined. The percentage of modified lipid A was calculated by dividing the sum of the intensities of the lipid A peaks attributed to addition of pETN (*m/z* 1919.2 and *m/z* 2157.2) and L-Ara4N (*m/z* 1927.2 and *m/z* 2165.2) by the intensity of the peaks corresponding to native lipid A (*m/z* 1796.2 and *m/z* 2034.2). All mass spectra were generated and analyzed in technical triplicate (i.e., measurements of each sample were repeated three times) and biological triplicate.

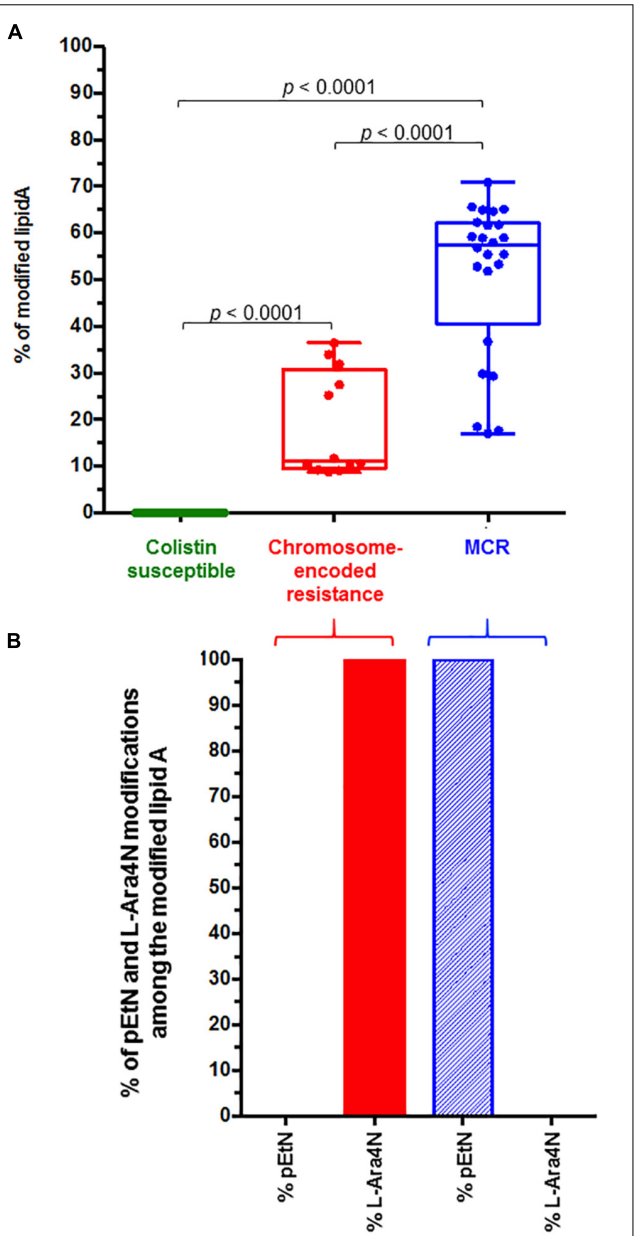


FIGURE 2 | (A) Representation of the percentage of the modified lipid A for colistin susceptible and colistin resistant *S. enterica* isolates. The global percentage of modified lipid A (L-Ara4N + pETN modified lipid A / native lipid A) is represented for colistin susceptible strains (*n*=11), colistin chromosome-encoded resistant *S. enterica* isolates (*n*=4) and MCR-producing *S. enterica* isolates (*n*=8). All experiments were performed in triplicate. **(B)** Representation of the percentage of L-Ara4N and pETN modified lipid A among the global modified lipid A for colistin resistant *S. enterica* isolates.

Statistical Analysis

All experiments were carried out on three independent bacterial cultures. Data were compared two-by-two using unpaired

[1]www.clcbio.com

[2]http://www.genomicepidemiology.org/

[3]https://www.genome.jp/tools-bin/clustalw

Mann-Whitney test. *P* values <0.05 were considered statistically different using GraphPad Prism 7.

RESULTS AND DISCUSSION

As shown in **Figure 1A** and **Supplementary Figure S1**, the mass spectrum of colistin susceptible *S. enterica* is dominated by a set of three of peaks assigned to bis-phosphorylated hexa-acyl lipid A, tri-phosphorylated hexa-acyl lipid A and bis-phosphorylated hepta-acyl lipid A. The major peaks at *m/z* 1796.2 and *m/z* 1876.2 correspond to hexa-acyl diphosphoryl and hexa-acyl triphosphoryl lipid A, respectively, containing four C14:0 3-OH, one C14:0 and one C12:0. The peak at *m/z* 2034.2 corresponds to hepta-acyl diphosphoryl lipid A four C14:0 3-OH, one C14:0, one C12:0 and one C16:0. Peaks at *m/z* 1812.2 and *m/z* 2050.2 are also observed in colistin susceptible *Salmonella enterica* and which can be tentatively be assigned to hexa-acyl diphosphoryl lipid A, containing five C14:0 3-OH and one C12:0, and hepta-acyl diphosphoryl lipid A five C14:0 3-OH, one C12:0 and one C16:0, respectively.

In MCR-producing *S. enterica* strains additional peaks at *m/z* 1919.2, *m/z* 1935.2, *m/z* 2157.2 and *m/z* 2173.2 were observed (**Figure 1B**). Those peaks correspond to the addition of one phosphoethanolamine (pETN) moiety to the phosphate group at position 1 of the native lipid A, leading to an increase of +123 mass units compared to the mass of the major peak of native lipid A at *m/z* 1796.2, *m/z* 1812.2, *m/z* 2034.2 and *m/z* 2050.2. In *mcr* negative colistin-resistant *S. enterica* strains, two additional peaks at *m/z* 1927.2 and *m/z* 2165.2 were observed. These signals correspond to the addition of 4-amino-L-arabinose (L-Ara4N) to the 4'-phosphate of the native lipid A, resulting in an increase of +131 *m/z* compared to the native lipid A peak at *m/z* 1927.2 and *m/z* 2034.2 (**Figure 1C**). As shown in **Figure 2A** and **Table 1**, the average percentage of modified lipid A remained significantly lower in chromosome-encoded colistin resistant isolates (18.25 ± 3.23%) compared to MCR-producers (51.12 ± 3.38%) (data not shown). The same observation was previously reported with *Klebsiella pneumoniae* and *E. coli* (Dortet et al., 2019; EUCAST, 2019). As expected, only pETN modification of the lipid A was observed in MCR-producers whereas only L-Ara4N addition could be detected in all chromosome-encoded colistin-resistant strains (**Figure 2B** and **Table 1**). Again, these results are in perfect agreement with those obtained in *K. pneumoniae* and *E. coli*.

Overall, the MALDIxin test is able to detect colistin resistance in *S. enterica* and to discriminate between chromosome-encoded resistance and MCR-producers. By targeting the modification of native lipid A, especially pETN addition, MALDIxin test is able to detect all variants of MCR. This is of the utmost importance since the increasing number of *mcr* variants (*mcr-1* to *mcr-9*, as of today) do not allow to perform a simple test (e.g., PCR) to detect all *mcr* variants. In addition, in some rare case, the *mcr* gene is not expressed, leading to colistin susceptibility but positive PCR, including in *S. enterica* isolates (Carroll et al., 2019). Finally, the MALDIXin test possesses high-throughput capacities that might be crucial in the context of large-scale screening. Accordingly, this test might be useful to answer the unmet need of a rapid, robust and high-throughput test for identification of colistin resistance that exist in veterinary medicine and food industry, where monitoring of colistin resistance in *S. enterica* is mandatory. However, one limitation of this study is the sample size used which might represent a fraction of the different types of colistin resistance mechanisms in *S. enterica*.

CONCLUSION

The MALDIxin test is now optimized on a routine machine, the MALDI Biotyper Sirius, for the detection of colistin resistance in *E. coli*, *K. pneumoniae*, *Acinetobacter baumannii*, and *S. enterica* (Dortet et al., 2018a,b, 2019; Furniss et al., 2019). The future development of automated algorithm and dedicated consumables (e.g., calibration standards, pre-portioned purified matrix) will also be able to standardize and simplify the assay, allowing its use in clinical and veterinary laboratories.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

GL-M and LD conceived the study, participated in its design and preformed the experiments. SL provided clinical isolates. LD, RB, SL, MK, AF, and GL-M wrote the manuscript. All authors discussed, reviewed, and approved the final manuscript.

FUNDING

This study was supported by the MRC Confidence in Concept Fund and the ISSF Wellcome Trust Grant 105603/Z/14/Z (GL-M).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.01141/full#supplementary-material

REFERENCES

Carroll, L. M., Gaballa, A., Guldimann, C., Sullivan, G., Henderson, L. O., and Wiedmann, M. (2019). Identification of novel mobilized colistin resistance gene mcr-9 in a multidrug-resistant, colistin-susceptible *Salmonella enterica* serotype typhimurium isolate. *mBio* 10:3. doi: 10.1128/mBio.00853-19

Catry, B., Cavaleri, M., Baptiste, K., Grave, K., Grein, K., Holm, A., et al. (2015). Use of colistin-containing products within the European Union and European Economic Area (EU/EEA): development of resistance in animals and possible impact on human and animal health. *Int. J. Antimicrob. Agents* 46, 297–306. doi: 10.1016/j.ijantimicag.2015.06.005

Chew, K. L., La, M. V., Lin, R. T. P., and Teo, J. W. P. (2017). Colistin and polymyxin B susceptibility testing for carbapenem-resistant and mcr-positive *Enterobacteriaceae*: comparison of sensititre, microscan, Vitek 2, and Etest with broth microdilution. *J. Clin. Microbiol.* 55, 2609–2616. doi: 10.1128/JCM.00268-17

European Union Commission (2013). *European Commission Commission Implementing Decision (2013/652/EU) on the Monitoring and Reporting of Antimicrobial Resistance in Zoonotic and Commensal Bacteria*. Brussels: European Union.

Dortet, L., Bonnin, R. A., Pennisi, I., Gauthier, L., Jousset, A. B., Dabos, L., et al. (2018a). Rapid detection and discrimination of chromosome- and MCR-plasmid-mediated resistance to polymyxins by MALDI-TOF MS in *Escherichia coli*: the MALDIxin test. *J. Antimicrob. Chemother.* 73, 3359–3367. doi: 10.1093/jac/dky330

Dortet, L., Broda, A., Bernabeu, S., Glupczynski, Y., Bogaerts, P., Bonnin, R., et al. (2019). Optimization of the MALDIxin test for the rapid identification of colistin resistance in *Klebsiella pneumoniae* using MALDI-TOF MS. *J. Antimicrob. Chemother.* 75, 110–116. doi: 10.1093/jac/dkz405

Dortet, L., Potron, A., Bonnin, R. A., Plesiat, P., Naas, T., Filloux, A., et al. (2018b). Rapid detection of colistin resistance in *Acinetobacter baumannii* using MALDI-TOF-based lipidomics on intact bacteria. *Sci. Rep.* 8:16910. doi: 10.1038/s41598-018-35041-y

EUCAST (2019). *The European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters. Version 9.0.*

Furniss, R. C. D., Dortet, L., Bolland, W., Drews, O., Sparbier, K., Bonnin, R. A., et al. (2019). Detection of colistin resistance in *Escherichia coli* using the MALDI Biotyper Sirius mass spectrometry system. *J. Clin. Microbiol.* 22:e01427-19. doi: 10.1128/JCM.01427-19

Jayol, A., Nordmann, P., Lehours, P., Poirel, L., and Dubois, V. (2017). Comparison of methods for detection of plasmid-mediated and chromosomally encoded colistin resistance in *Enterobacteriaceae*. *Clin. Microbiol. Infect.* 24, 175–179. doi: 10.1016/j.cmi.2017.06.002

Jeannot, K., Bolard, A., and Plesiat, P. (2017). Resistance to polymyxins in Gram-negative organisms. *Int. J. Antimicrob. Agents* 49, 526–535. doi: 10.1016/j.ijantimicag.2016.11.029

Lima, T., Domingues, S., and Da Silva, G. J. (2019). Plasmid-mediated colistin resistance in *Salmonella enterica*: a review. *Microorganisms* 7:55. doi: 10.3390/microorganisms7020055

Olaitan, A. O., Morand, S., and Rolain, J. M. (2014). Mechanisms of polymyxin resistance: acquired and intrinsic resistance in bacteria. *Front. Microbiol.* 5:643. doi: 10.3389/fmicb.2014.00643

Poirel, L., Jayol, A., and Nordmann, P. (2017). Polymyxins: antibacterial activity, susceptibility testing, and resistance mechanisms encoded by plasmids or chromosomes. *Clin. Microbiol. Rev.* 30, 557–596. doi: 10.1128/CMR.00064-16

Zhang, H., Hou, M., Xu, Y., Srinivas, S., Huang, M., Liu, L., et al. (2019a). Action and mechanism of the colistin resistance enzyme MCR-4. *Commun. Biol.* 2:36. doi: 10.1038/s42003-018-0278-1

Zhang, H., Srinivas, S., Xu, Y., Wei, W., and Feng, Y. (2019b). Genetic and biochemical mechanisms for bacterial lipid a modifiers associated with polymyxin resistance. *Trends Biochem. Sci.* 44, 973–988. doi: 10.1016/j.tibs.2019.06.002

Conflict of Interest: LD, AF, and GL-M are co-inventors of the MALDIxin test for which a patent has been filed by Imperial Innovations. MK is employee of Bruker, the manufacturer of the MALDI-TOF MS used in this study.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Advantages of publishing in Frontiers

OPEN ACCESS
Articles are free to read for greatest visibility and readership

FAST PUBLICATION
Around 90 days from submission to decision

HIGH QUALITY PEER-REVIEW
Rigorous, collaborative, and constructive peer-review

TRANSPARENT PEER-REVIEW
Editors and reviewers acknowledged by name on published articles

Frontiers
Avenue du Tribunal-Fédéral 34
1005 Lausanne | Switzerland

Visit us: www.frontiersin.org
Contact us: info@frontiersin.org | +41 21 510 17 00

REPRODUCIBILITY OF RESEARCH
Support open data and methods to enhance research reproducibility

DIGITAL PUBLISHING
Articles designed for optimal readership across devices

FOLLOW US
@frontiersin

IMPACT METRICS
Advanced article metrics track visibility across digital media

EXTENSIVE PROMOTION
Marketing and promotion of impactful research

LOOP RESEARCH NETWORK
Our network increases your article's readership