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Review

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Advances in automated techniques to identify *Acinetobacter calcoaceticus–Acinetobacter baumannii* complex

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Abstract

Acinetobacter species, particularly those within *Acinetobacter calcoaceticus–A. baumannii* complex (ACB complex), have emerged as clinically relevant pathogens in hospital environments worldwide. Early and quick detection and identification of *Acinetobacter* infections is challenging, and traditional culture and biochemical methods may not achieve adequate levels of speciation. Moreover, currently available techniques to identify and differentiate closely related *Acinetobacter* species are insufficient. The objective of this review is to recapitulate the current evolution in phenotypic and automated techniques used to identify the ACB complex. Compared with other automated or semiautomated systems of bacterial identification, matrix-assisted laser desorption–ionization time-of-flight mass spectrometry (MALDI-TOF MS) demonstrates a high level of *Acinetobacter* species identification and discrimination, including newly discovered species *A. seifertii* and *A. dijkshoorniae*.

Keywords: *Acinetobacter*; automation, laboratory; bacterial typing techniques; bacteriological techniques; spectrometry, mass, matrix-assisted laser desorption-ionization

Currently, the genus *Acinetobacter* comprises about 62 species, 51 with valid species names and 11 unnamed species, most of which are nonclinical isolates, with more species likely to be discovered [1, 2]. *Acinetobacter calcoaceticus–Acinetobacter baumannii* (ACB) complex is the most worrisome in the genus, being responsible for over 80% of hospital-acquired infections in immunocompromised patients [3, 4]. The ACB complex comprises of *Acinetobacter baumannii* (genospecies 2), *A. calcoaceticus, A. nosocomialis* (genospecies 13TU), and *A. pittii* (genospecies 3) along with the newly identified species, *A. seifertii* and *A. dijkshoorniae* [5]. The phenotypic similarity among the members of the ACB complex makes them difficult to distinguish from other species by routine detection methods. Despite their close phenotypic relatedness, there exist differences in their biological characteristics [6], which necessitates accurate identification and differentiation of individual members of the ACB complex [7, 8]. The commonly used techniques used to identify ACB complexes include biochemical tests, gene sequencing (16S rRNA, *rpo*B gene) [9], polymerase chain reaction [10, 11], and matrix-assisted laser desorption–ionization time-of-flight mass spectrometry (MALDI-TOF MS) [12]. Despite advances in molecular identification techniques for the ACB complex, the techniques are not suitable for routine diagnosis [13, 14] and are applied mainly in research settings [15]. In the present review, we aim to provide an insight into the available

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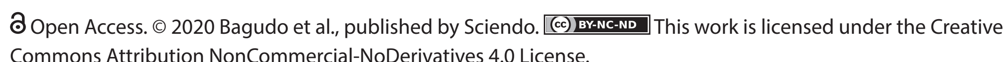
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phenotypic methods and recent developments in automated systems for rapid and reliable identification of the pathogen species belonging to the ACB complex.

Search methodology

The information in the present review article was synthesized from Google Scholar, PubMed, and Wiley online library. We explored English-language sources using the following keywords: "Automated or semi-automated identification method of *Acinetobacter baumannii* complex"; "Biolog system," "BD Phoenix system," "MicroScan WalkAway," "Accelerate Pheno system," "VITEK system," and "MALDI-TOF MS." Abstracts, theses, or unreviewed work were not included. Emphasis was given to the sources published within the past 7 years.

Phenotypic methods for identification of *Acinetobacter* species

Bacterial pathogens are traditionally identified in clinical laboratories based on the morphological and biochemical characteristics displayed on the growth media [16]. *Acinetobacter* species produce morphological characteristics similar to *Enterobacteriaceae* [17], with some species of *Acinetobacter* producing tiny, glowing colonies with no hemolysis on blood agar. However, a few species produce hemolysis on 5% sheep blood agar. Bouvet and Grimont developed the use of 28 phenotypic tests to identify *Acinetobacter* species, which focused on the physiological, nutritional, and enzymatic characteristics of *Acinetobacter* spp. [18]. This method successfully identifies 12 *Acinetobacter* genospecies, but is unable to identify *Acinetobacter iwoffii*. Bouvet and Grimont later in 1987 improved their phenotypic tests to comprise growth at temperatures of 37 °C, 41 °C, and 44 °C, and in addition included the utilization of 14 carbon sources [17]. *Acinetobacter* species' ability to grow at different temperatures was used to discriminate *A. baumannii*, *A. nosocomialis, A. pittii*, and *A. calcoaceticus* [17, 19]. A further upgrade of the Bouvet and Grimont identification scheme for *Acinetobacter* species identification to include growth at temperatures of 32 °C, 35 °C, 41 °C, and 44 °C, and additional biochemical tests such as acid production from glucose, hemolysis, gelatinase production, and utilization of 36 different carbon compounds as energy sources, has improved the scheme [17]. **Table 1** shows the biochemical test for the identification of the ACB complex. Although the current phenotypic system appears to be more robust than previously, it still falls short in terms of ease, rapidity, reliability, and consistency. Hence, the emergence of automated systems targeted to solve the challenges of accurate and rapid identification and differentiation of the ACB complex.

Commercial automated systems for the identification of *Acinetobacter* species

Recent advances in miniaturization and automation provide promising solutions for rapid bacterial identification and antibiotic susceptibility profiling, which will potentially make a significant impact on the clinical management of infectious diseases [20]. Automated and semiautomated commercial identification systems for the identification of bacteria are in high demand and widely used in clinical microbiology laboratories [21, 22]. Some systems require preisolation of bacteria from the sample and a high concentration of isolates in suspension [23]. Automated bacterial identification systems can measure the differences in protein expression within the bacterial genus or among the bacterial species, providing a particular protein expression with a relatively high degree of accuracy [24]. The commonly used automated or semiautomated bacterial identification systems include the following.

API 20NE system

The Analytical Profile Index (API) 20NE system is a standardized system for the quick identification of nonfastidious, nonenteric gram-negative rods. It combines 8 conventional tests, 12 assimilation tests, and a database. The API 20NE BioMérieux system demonstrated 92% accuracy when compared with conventional biochemical tests in the identification of 198 clinical isolates [19, 25]. Bernards et al. [28] achieved 87% API 20NE accuracy of identification using an updated database (version 5.1). However, the technique was not sufficient to correctly identify and discriminate between *Acinetobacter* species. Another study that compared API 20NE with 16s rRNA gene sequencing in the identification of gram-negative bacteria, including *Acinetobacter*, reported successful identification of 58 of 107 isolates. However, some members of the ACB complex could not be accurately identified or discriminated at the species level [26].

Table 1. Phenotypic tests for identification of *Acinetobacter baumannii* (ACB) complex

	A. calcoaceticus	***A. baumannii***	***A. pittii***	***A. nosocomialis***	***A. seifertii***	***A. dijkshoorniae***
Acid production from glucose	–	+	V (+)	+	+	+
Growth temperature						
37 °C	(+)	+	+	+		
41 °C	(–)	+	V	+		
44 °C	–	+	+	V		
Carbon source utilization test						
Citroconitate	–	V (+)	–	–	–	–
β-Alanine	V (–)	+	V (+)	V (+)	V (+)	+
Malate	V (–)	V (+)	V (+)	+	V (+)	
Ribose	V (–)	V (+)	V (–)	–		
Leucine	V (–)	V (+)	V (+)	V (+)	V (+)	+
Ornithine	+	V (+)	V (+)	V (+)	V (+)	V (+)
Gentisate	–	V (–)	V (–)	V (+)	V (+)	+
Azelate	+	V (+)	+	V (+)		+
L-Arabinose	V (–)	V (+)	V (+)	+	–	+
Adipate	+	V (+)	+	V (+)	V (+)	+
Putrescine	+					
Tartarate	V (–)	V (–)	V (+)	–	V (+)	V (+)
Trigonrlline	V (–)	V (+)	V (+)	V (–)	–	V (+)
Sorbinic acid	(–)	V (+)	(–)	(+)		
Glycerate	V (+)	(–)	V (–)	(–)		
Acetylglutamate	(–)	V (–)	V (–)	V (–)		
Asparagine	+	–	–	(–)		
L-Hydroxylproline	(–)	(+)	(–)	V (–)		
Levulinate	V (–)	V (–)	–	V (–)		

V (+) variable, majority are positive; V (–) variable, majority are negative; (+), more than 80% are positive; (–), 20% or less are positive; +, all are positive; –, all are negative; variable means 20%–79% are positive.

Biolog system

The Biolog system can rapidly identify both gram-positive and gram-negative bacteria. The system works based on universal redox chemistry alone to detect bacterial cellular reactions. By inoculation of microorganism into diverse preselected carbon sources, the system usually produces a specific growth pattern based on the carbon source utilization profile of the bacteria. The resulting metabolic pattern is then compared with the Biolog in-built database to identify the bacteria. The Biolog system works based on the oxidation of 95 substrates from 8 different sets of carbon sources [27]. In a parallel study to compare the Biolog system and DNA–DNA hybridization to identify *Acinetobacter* species using cluster analysis, Bernards et al. [28] recorded an 84.5% accurate identification to the genus level by the Biolog system. Being a commercial system, frequent incorporation of new information regarding distinct bacterial strains into the system's database will increase the accuracy of the system in *Acinetobacter* species identification [28].

BD Phoenix system and MicroScan WalkAway

The BD Phoenix system works on the redox principle with an appropriate indicator and growth turbidity measurement. While the MicroScan WalkAway system uses a photometer or fluorometer to assess bacterial growth, it is based on fluorescent technology and provides results in an average of 4 h or 6–42 h

with slow-growing bacteria [29]. Synder et al. [30] compared the performance of the BD Phoenix and MicroScan WalkAway systems to identify nonfermentative gram-negative bacilli and reported a 100% agreement. The MicroScan WalkAway system was reported to have misidentified *A. baumannii* as *Ralstonia paucula* [31].

VITEK system

The VITEK system is an entirely automated device that produces quick results typically in less than 7 h, with same-day turnaround time. The system permits ad hoc or group processing of samples and possesses an integrated quality control component with a complete data management system [32]. The VITEK 2 system works on the principles of detection of microbial growth in small wells within plastic cards referred to as gram-negative card BioMérieux. A gram-negative card BioMérieux can identify *A. baumannii* with up to 99% accuracy, while the phenotypically related *A. nosocomialis* and *A. pittii* are identified as the *A. baumannii* complex. VITEK MS correctly identified *A. junnii, A. haemolyticus, and A. jonhsonii* [33]. However, VITEK MS failed to distinguish between species within the ACB complex, as members of the ACB complex species are not included on the VITEK MS database, and the substrate used in this system was not specifically designed to identify the *Acinetobacter* species [17]. VITEK 2 misidentified 4 *A. baumannii* isolates as *Alcaligenes faecalis* [31]. A study compared the efficacy of the VITEK 2 anaerobic gram-negative card (ANC) with 16s rRNA identification; the ANC card accurately identified 79.4% of 301 clinical isolates to genus level, including 100 isolates that were not in the proprietary database. However, the species-level identification of those 100 isolates was just 47% [34].

Accelerate Pheno system

The Accelerate Pheno system is a fully automated test system that accomplishes identification and antimicrobial susceptibility testing (AST) directly from positive blood cultures within approximately 7 h [35]. The Accelerate Pheno system is a collection of gel electrofiltration and a large panel of fluorescence in situ hybridization for bacterial identification and morphokinetic cellular analysis, which measures the activities of cells and colonies in the presence of antibiotics over time and employs time-lapse imaging for antimicrobial susceptibility testing. This technique demonstrated excellent sensitivity of 98.6% and specificity of 99.7% with the positive and negative predictive values of 100% for *A. baumannii* [36]. In another study involving 101 clinical isolates of *Acinetobacter* spp., this system recorded a sensitivity of 100% and 97.6% for identification of *A. baumannii* and *A. baumannii* complex, respectively, and a specificity of 86.6% for the *A. baumannii* complex. The study identified *A. baumannii*, *A. pittii*, and *A. nosocomialis* with 100% sensitivity, although one *A. radioresistens* and one *A. baylyi* were erroneously identified as *A. baumannii*. Commercial automated systems have contributed immensely to the clinical diagnosis of important diseases. However, the weakness of commercial automated methods in terms of accuracy, have been highlighted in some studies [31].

Proteomics-based methods of *Acinetobacter* species identification

Molecular methods are not optimal despite strenuous efforts at standardization; it is difficult to compare the results obtained from different laboratories, and the methods are labor-intensive and time-consuming. The search for unique and representative protein biomarkers on intact bacterial cell envelopes to rapidly identify bacteria has been increasing for decades [37, 38]. MALDI-TOF MS is a technique that provides rapid and less cumbersome identification of microorganisms in diagnostic laboratories [39, 40]. MALDI-TOF MS also demonstrates reliability and applicability for the epidemiological typing of bacteria during the outbreak of diseases [41]. It can process up to 384 specimens on a single plate. Its turnaround time is about 5–7 min for each sample, allowing results to be ready within 24 h of receiving a sample. MALDI-TOF MS requires as low as 10^4–10^6 colony forming units (CFU) of cells, and theoretically, identification can be performed using a single colony, obtained in a few hours, from the culture of an infected sample [20].

In a study to optimize MALDI-TOF MS for identification of common bacteria directly from blood samples, of 829 positive blood cultures, MALDI-TOF MS accurately identified 91.5% as gram-negative bacteria, 88.3% as gram-positive bacteria, 84.8% as fungi, 80% as anaerobic bacteria, and 66.67% as other rare bacteria, demonstrating that routinely encountered bacteria in positive blood cultures can be identified directly within 1 h using this method [42]. MALDI-TOF MS has high sensitivity, acceptable specificity, and a short detection time; thus, it can be regarded as an efficient technique for the rapid identification of pathogenic microorganisms in endophthalmitis. However, MALDI-TOF MS could not identify polymicrobial infection [43]. Being a highly selective analytical technique based on relative molecular mass, the mass-spectrometric component of MALDI-TOF MS provides

an added advantage [40]. For the MALDI-TOF MS process, a matrix with energy-absorbing capacity is used to coat the samples. Exposure of the matrix-coated samples to a laser beam causes desorption and ionization of microbial analytes present in the sample. Ions from the sample move upward into a time-of-flight chamber based on their mass-to-charge ratio (*m*/*z*). Charge is detected and recorded using the charge detector at the top of the time-of-flight chamber; and this charge is unique for each analyte [44–46].

Using a laser pulse, MALDI-TOF MS works based on the rapid ionization of the bacterial ribosomal proteins directly from cultured colonies or cell pellets from the clinical sample (**Figure 1**). Bacterial colonies treated with ethanol-formic acid, as well as pure bacterial colonies, have been used for identification [47, 48]. The direct smearing method of sample to prepare for MALDI-TOF MS analysis demonstrated higher accuracy of identification (99.85%) compared with bacterial extraction pretreatment (99.73%) when identifying *Acinetobacter* species. Any incorrect identification was attributed to the absence of reference mass spectra in the MALDI-TOF MS database rather than sample preparation procedures [48]. In a study to access the ability of MALDI-TOF MS (Bruker Biotyper) to identify *Acinetobacter* species accurately in 286 blood isolates belonging to ACB complex and 39 other *Acinetobacter* spp., 85.3% of ACB complex and non-*baumannii* members were correctly identified by MALDI-TOF MS [49]. MALDI-TOF MS (Bruker Biotyper) correctly identified *A. baumannii* (98.6%), *A. nosocomialis* (72.4%), and *A. pittii* (97.6%). Moreover, MALDI-TOF MS Bruker Biotyper correctly identified all *A. ursingii*, *A. radioresistens*, *A. junnii*, and *A. jonhsonii* isolates [49].

Jeong et al. [50] evaluated the ability of MALDI-TOF MS in combination with an improved database to identify various *Acinetobacter* species. They showed that the addition of 63 profiles for *Acinetobacter* strains to the default Bruker database increased the overall concordance rate between

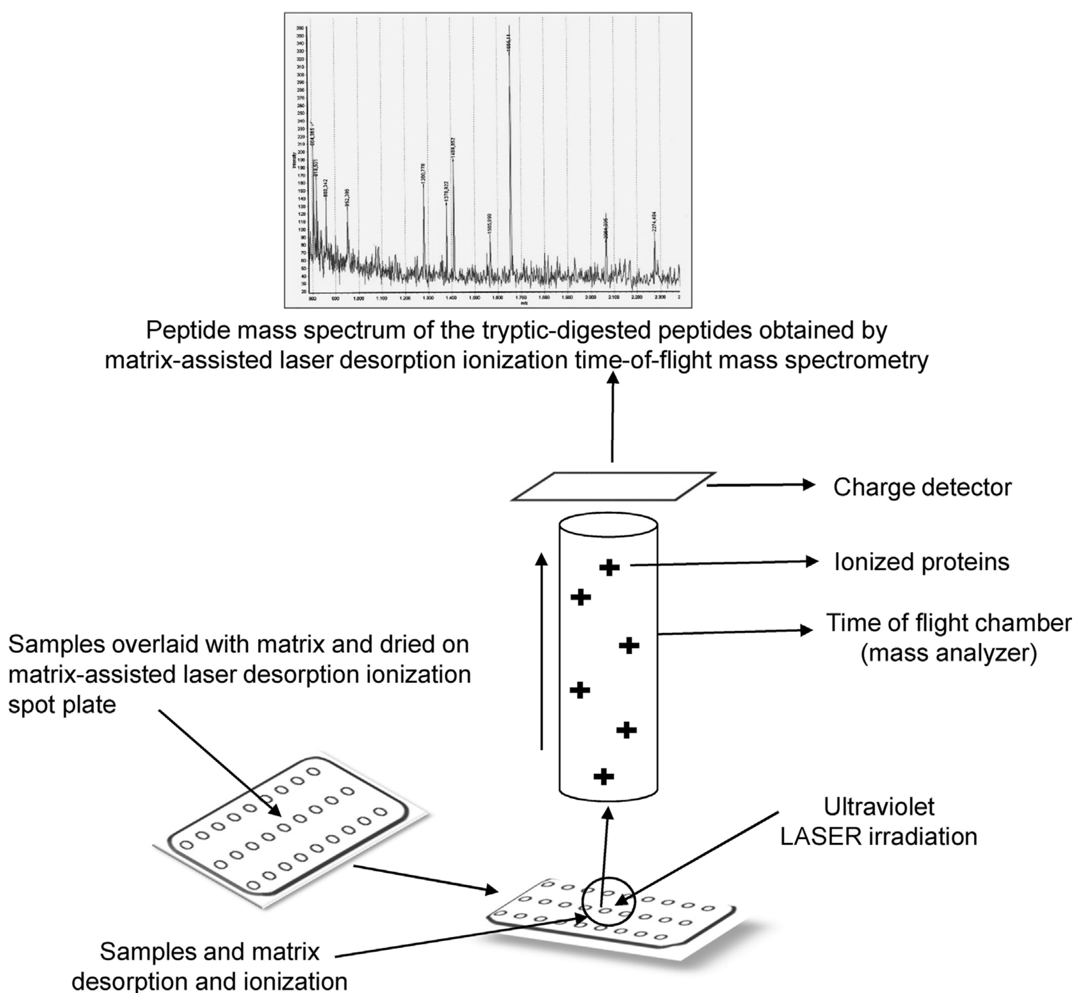

Figure 1. Diagrammatic representation of matrix-assisted laser desorption–ionization time-of-flight mass spectrometry work flow for microbial identification.

MALDI-TOF MS and *rpoB* sequencing from 69.8% to 100.0%. Additionally, after library modification, all 64 previously mismatched *Acinetobacter* strains were correctly identified. Sedo et al. [51] achieved accurate identification of Acinetobacter spp. by modification of the MALDI-TOF MS standard sample preparation procedure. The authors replaced α-cyano-4-hydroxycinnamic acid (saturated solution in water :acetonitrile:trifluoroacetic acid, 47.5:50:2.5, v/v) with ferulic acid (12.5 mg/mL solution in water:acetonitrile:formic acid 50:33:17, v/v), while other procedures remained unchanged, yielding more accurate identification and differentiation of *A. baumannii* and *A. nosocomialis*. However, a limitation is the automatic acquisition of mass spectra [51]. Toh et al. [52] analyzed the mass spectra of 73 *Acinetobacter* species, representing 10 different species, using an AB SCIEX 580 MALDI-TOF MS to differentiate members of the *Acinetobacter* genus, including the species of the ACB complex. They found that *A. pittii* or *A. calcoaceticus*, which could not be differentiated using 16S rRNA and *rpoB* gene sequencing, can be differentiated using *gyr*B multiplex PCR and MALDI-TOF MS [52].

Evidence from another study comparing MALDI-TOF MS and *rpo*B sequencing of 123 *Acinetobacter* spp. isolated from blood showed 86.2% (106/123) correct identification to species level and 13% identification to genus level by MALDI-TOF MS. Although the *rpo*B sequence analysis was correctly confirmed in 84% (89) of the 106 isolates identified by MALDI-TOF MS as correct, 16% of isolates were incorrectly identified. The authors recommended adding reference spectra to the MALDI-TOF MS database to improve identification capability [53]. This was affirmed when a study in which a database updated to include reference signatures for *A. nosocomialis*, *A. seifertii*, and *A. dijkshoorniae*, permitted correct speciation of 78 isolates. The correctly identified isolates included *A. pittii, A. baumannii*, *A. dijkshoorniae*, *A. nosocomialis*, and *A. seifertii*, indicating that regular updating of the MALDI-TOF MS databases is pivotal to accurate identification [47]. Many studies emphasize the advantages of MALDI-TOF MS as a promising tool to identify species rapidly [54]. A comparison of the dendrogram function of MALDI-TOF MS with pulsed-field gel electrophoresis demonstrated that MALDI-TOF MS dendrograms have insufficient discriminatory power for multidrug-resistant *A. baumannii* clonality analysis [54]. An earlier study, in agreement with the findings described above, found 100% identification and differentiation of *A. baumannii, A. nosocomialis, A. pittii, A. calcoaceticus*, *A. dijkshoorniae*, and *A. seifertii* when chemometric tools were incorporated into the MALDI-TOF MS [55]. This combined approach used a partial least squares discriminant analysis and hierarchical cluster analysis to obtain 100% correct species identification based on mass spectra generated from intact cells. Sousa et al. [55] found that *A. dijkshoorniae* and *A. seifertii*, neither of which were captured in the MALDI-TOF MS Biotyper database, were successfully differentiated.

Successful identification of all 18 (100%) *A. baumannii* and 17 (94%) of 18 *A. pittii* isolates was achieved using the MALDI-TOF MS Bruker database to investigate 60 unrelated clinical isolates [56]. However, MALDI-TOF MS did not identify *A. nosocomialis* correctly until the signature profile for *A. nosocomialis* was included in the Bruker database. Variations in the protein signatures of *A. baumannii*, *A. pittii*, and *A. nosocomialis* are sufficient for accurate discrimination of the 3 species, based on the analysis performed on individual spectral peaks of representative strains [56]. A Korean study performed a comparative evaluation of the MALDI-TOF MS-based VITEK MS system versus the VITEK 2 and MicroScan automated systems to identify *Acinetobacter* species from blood cultures [57]. They found that the VITEK MS system is better than the VITEK 2 and MicroScan systems for the identification of *Acinetobacter* isolates, with fewer misidentifications and better discrimination between the *A. baumannii* and non-*A. baumannii* isolates [58] demonstrating a rapid and reliable identification of the 3 species of the ACB complex pathogenic for humans, namely, *A. baumannii*, *A. pittii*, and *A. nosocomialis*. The SARAMIS database of the Vitek MS was enriched and combined with a mass spectrometer to improve the identification of species of the ACB complex. For each species, reference spectra were obtained, and then a so-called "SuperSpectrum" was created based on the selection of 40 specific masses. In a second step, reference spectra and SuperSpectra with 100 isolates identified by *rpo*B gene sequencing were validated. The evolution of the MALDI-TOF MS technology to accommodate clinical applications might be in sight as more discoveries may arise concerning *Acinetobacter* species taxonomy.

Future perspectives in the identification of *Acinetobacter*

The criterion standard used for diagnosis of *A. baumannii* infection is the traditional culture-biochemical method, which usually takes 24–72 h of incubation to produce accurate results. Consequently, this technique cannot detect causative pathogens early, which is crucial for the proper use of antimicrobial agents. Recently, several molecular methods were developed to detect and identify *A. baumannii*. However, these methods are labor-intensive and time-consuming. Owing to the limitations posed by available phenotypic and genotypic

methods for bacterial identification, continuous efforts are being committed to the discovery and commercialization of new technologies that will make identification more straightforward and dramatically reduce the time required to obtain results. Coupling of analytical techniques and tests with new technologies, such as microfluidic devices is gaining more attention to achieve these goals. Some of these new technologies also exploit the use of ultrasensitive readout mechanisms and single-cell analysis [59, 60]. Miniaturized microfluidic devices and nanotechnologies are also beginning to find application in the area of antibiotic resistance detection and management, requiring only small samples, and less cumbersome, more cost-effective instruments, which together provide impressive high throughput analysis of single cells. The principle of microfluidic-based culture methods for bacteria detection is the monitoring of bacterial growth in small volumes housed within partitioned systems [21].

Some available identification tests depend on spectroscopic readings obtained from cell growth within a culture at numbers or levels sufficient for such monitoring. However, recent advances in this area now make it possible to monitor single cells for such identification and other observations rather than studying a group of cells together. More findings based on this principle are emerging. For instance, a rapid antibiotic susceptibility test system has been reported [61]. This microfluidic agarose channel coupled agarose in a microfluidic culture chamber to track single cells using microscopy to obtain images of the cell under varied antibiotic culture conditions, thus providing details of how the cell reacted to different antibiotic concentrations. Similar studies have also reported for several clinical bacterial pathogens [62–64]. Being small, these technologies and their like can be conveniently incorporated into automated, portable devices for use at points of care at a reduced cost compared with already existing detection methods [60].

Developments in miniaturized microfluidic detection systems are also advancing. These systems can significantly aid the automation of detection. An inertial microfluidic system was able to detect directly and separate bacteria pathogen in whole blood for direct identification by ribosomal RNA detection even when few pathogens (about 100/mL) are present [65]. The system does not require culturing of the cells or any form of enzymatic amplification. Coupling microfluidic cell separation with RNA-base detection, the system can shorten detection and antibiotic susceptibility test to approximately 8 h, representing a significant improvement in sample processing and therapeutic decision making. Another microfluidic system termed "Integrated Comprehensive Droplet Digital Detection" (IC 3D) with selective ability to detect single bacteria cells in a small quantity of blood without the need for culture or amplification has also been reported [66]. Similarly, Ismagilov et al. [67] reported a microfluidic system based on the separation of bacteria cells into nanovolume droplet plug for detection and screening for drug susceptibility.

New and more sensitive analytical methods with capacity for more rapid bacterial detection using mechanical and electrochemical transducers have been developed. Electrochemical sensors have been used in the identification of bacteria and resistance studies.

The developments mentioned above related to pathogen identification hold a promising future for *Acinetobacter* spp. differentiation, especially in clinical settings. Already, an optical biosensor has been developed, which used fluorescence to detect 57 pathogenic bacteria, including *A. baumannii*, in 10 h. The detection limit was as low as 10 CFU/assay and was successfully used to identify pathogens from blood specimens. A number of these technologies are still at the proof-of-concept stage, while some are still faced with the challenge of streamlining sample preparation, integration, and automation [60]. As these technologies continue to evolve and undergo improvements, they are likely to impact positively on available technologies for *Acinetobacter* spp. identification and differentiation, leading to improvements in patient care in cases arising from infections by clinically important members of the ACB complex.

Conclusions

Identification of *Acinetobacter* species among the phenotypically closely related ACB complex is fundamental for the administration of proper treatment. Significant advancement has been accomplished in the field of automated systems for bacterial identification, particularly for the *A. baumannii* complex leading to identification and differentiation of individual species among the members. The gains recorded through the use of MALDI-TOF MS has improved the process of identification of pathogens such as the ACB complex. Developments in microfluidic systems for pathogen detection appear to be advancing fast and hold great promise in reducing, if not solving, the ambiguity that exists with the differentiation of clinically important *Acinetobacter* spp.

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