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Comparison among four proposed direct blood culture microbial identification methods using MALDI-TOF MS

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Summary Matrix-assisted laser desorption-ionization time-of-flight (MALDI-TOF) mass spectrometry facilitates rapid and accurate identification of pathogens, which is critical for sepsis patients.

In this study, we assessed the accuracy in identification of both Gram-negative and Gram-positive bacteria, except for *Streptococcus viridans*, using four rapid blood culture methods with Vitek MALDI-TOF-MS. We compared our proposed lysis centrifugation followed by washing and 30% acetic acid treatment method (method 2) with two other lysis centrifugation methods (washing and 30% formic acid treatment (method 1); 100% ethanol treatment (method 3)), and picking colonies from 90 to 180 min subculture plates (method 4). Methods 1 and 2 identified all organisms down to species level with 100% accuracy, except for *Streptococcus viridans*, *Streptococcus pyogenes*, *Enterobacter cloacae* and *Proteus vulgaris*. The latter two were identified to genus level with 100% accuracy. Each method exhibited excellent accuracy and precision in terms of identification to genus level with certain limitations.

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Introduction

Sepsis is one of the leading causes of death of both adults and children worldwide, imposing a heavy human and economic burden in both the developed and developing world [1—3]. In the United States, for example, incidence of sepsis is 0.5 per 1000 in children and 0.7—2.3 per 1000 in adults, with mortality rates being estimated at 10%—27% and 25%—50%, respectively [2,4]. Sepsis-related mortality in the United States is greater than that of stroke [5], and it is responsible for over five million pediatric deaths worldwide each year and has a particularly devastating impact in resource-poor countries [2,6]. Early recognition of causative infective agents is key to effective treatment of sepsis; time from triage and qualification to administration of appropriate antimicrobials are critical determinants of mortality [4,7]. The first 24 h has been identified as critical in terms of delivery of effective antimicrobial treatment. Each hour of delay in administration of appropriate antibiotics is associated with a decrease in survival of 7.6% over 6 h, while administration of inappropriate antibiotics has been associated with an approximately fivefold decreased survival rate [8,9]. As a result, clinicians initially assign patients with presumed bacterial infections to empiric broad-spectrum antibiotics. Due to increasing antibiotic resistance rates, however, approximately 20% of septic shock patients are initially assigned to inappropriate antimicrobials with serious consequences in terms of mortality rates [9—12]. Thus, techniques to decrease turnaround time in identification of causative bacterial agents are vital for the reduction of mortality due to sepsis.

Current standard protocols for microbial identification have a turnaround time of approximately 18—24 h from when signal-positive samples are identified and involve the overnight sub-culture of signal-positive samples on solid medium to obtain isolated colonies for identification and antibiotic susceptibility testing [13—15]. Use of more rapid identification technology, such as Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF-MS), has the potential to expedite identification of microbial species and help guide appropriate antibiotic treatment choices [15—17]. However, if MALDI-TOF-MS could be applied directly to blood culture, rather than to isolated colonies grown overnight, this application would further reduce the turnaround time. Various direct culture methods have been used with a degree of success in microbial identification to genus or species level using Vitek MALDI-TOF-MS (bioMérieux), including lysis filtration [16—18], Sepsityper (Bruker) or in-house saponin-based bacterial extraction [19,20] or serum separator [21]. However, in general, these methods have been more successful in identification of Gram-negative compared to Gram-positive bacteria. The methods have shown difficulty in differentiation of *Staphylococci* from each other [19—21]. The objective of this study was to compare our proposed direct method of lysis centrifugation followed by washing and 30% acetic acid treatment along with our modified lysis centrifugation followed by washing and 30% formic acid treatment method, lysis centrifugation followed by washing and 100% ethanol treatment method, and picking colonies from 90 to 180 min subculture plates in order to determine the accuracy and precision of each method. These methods were all compared to a gold standard method based on testing isolated colonies from overnight cultures in terms of accurate identification of both Gram-negative and Gram-positive bacteria using Vitek MALDI-TOF-MS.

Materials and methods

Samples

A mixture of 120 monomicrobial positive blood culture vials (60 Gram-negative samples that included 35 patient and 25 seeded vials, and 60 Gram-positive samples that included 42 patients and 18 seeded vials, containing various microorganisms that have been already identified using Vitek 2), including aerobic Bactec plus, anaerobic Bactec lytic, and Bactec pediatric plus, were assessed between October 2015 and December 2015. The aim of using the seeded vials was to make the number of most common blood culture isolates equal to five for each. This sample size was chosen to conform to FDA/CLSI guidelines laid down by the American Society of Microbiology (ASM Cumitechs 31A) [22]. For Gram-negative: three *Enterobacter aerogenes* ATCC 15038; two *Enterobacter cloacae* subsp. *cloacae* ATCC® 49141; two *Pseudomonas aeroginosa* ATCC 27853; four *Stenotrophomonas maltophilia* ATCC® 51331; three *Acinetobacter baumannii* ATCC® 19606; two *Proteus mirabilis* ATCC® 7002; three *Proteus vulgaris* ATCC® 29905; three *Citrobacter koseri* ATCC® BAA-895; three *Bacteroides fragilis* ATCC® 25285. For Gram-positive: one *Staphylococcus aureus* ATCC 25923; three *Enterococcus faecium* ATCC® 51559™; two *Streptococcus pyogenes* ATCC 19615; four *Staphylococcus capitis* ATCC® 27840; two *Streptococcus agalactiae* ATCC® 51487; two *Staphylococcus lugdunensis*

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ATCC® 43809; and four *Clostridium perfringens* ATCC® 13124.

MALDI-TOF slide preparation

Vials were incubated for up to five days in an automated Bactec FX blood culture instrument (Becton Dickinson, Cockeysville, MD). For each positive blood culture vial (spiked with 150 bacterial organisms for the seeded vials), 6 ml of broth was collected and dispensed into 4 Eppendorf tubes (1.5 ml). Fifty μl Triton (10×) was added to each tube followed by gentle mixing and centrifugation for 1 min at 13 000 rpm and 4 °C. The supernatants were removed and the pellets were re-suspended in 1.5 ml saline (0.9%), then centrifuged for 1 min at 13 000 rpm and 4 °C. The supernatants were removed and 50 μl of formic acid (30%) (method 1), acetic acid (30%) (method 2) or ethanol (100%) (method 3) were added to the first, second, and third tube consecutively. One microliter, 2 μl or 3 μl from each tube were added to three different MALDI-TOF slide wells consecutively and air dried. Onemicroliter of matrix (VITEK MS CHCA) was added to each well and air dried. The pellets from the fourth tubes (method 4) were inoculated onto blood agar plates and incubated at 37 °C and 5% CO_2 for 90 min, with the exception of Diphtheroids, which required 6 h growth. Microorganisms recovered from the primary streaking area were first applied into MALDI-TOF slide wells using a wire loop. One microliter of matrix (VITEK MS CHCA) was added to each well and air dried. Slides were then run on the Vitek MALDI-TOF-MS instrument (bioMérieux. Marcy l'Etoile, France) to obtain an identification. If two out of three wells were identified at genus or species level at a high confidence level, without conflicting identification from replicate wells of the same sample, it was considered as an acceptable identification. Results were compared to those obtained for genus and species level identification by our routine 'gold standard' method, which entails 18 h sub-culturing of samples on blood agar to obtain isolated colonies for Vitek MALDI-TOF-MS identification, or in the case of anaerobic organisms 48 h sub-culturing under anaerobic incubation conditions. To conform to FDA/CLSI-approved guidelines laid down by the ASM (Cumitech) for validation of a new method compared to a gold standard method [22], the accuracy of each test method to either genus or species level was calculated by the comparison of the number of correct results generated by the test method divided by the number generated by the gold standard method, multiplied by 100%. To measure the precision of each method, ten positive vials that included five different Gram-positive strains and five different Gram-negative strains were tested for three consecutive days by three different technologists to measure the precision of each method, according to FDA/CLSI guidelines laid down by the ASM (Cumitechs 31A) for validation of a new method against a gold standard method [22].

Mass spectra generation

A Vitek MS Axima Assurance mass spectrometer (bioMérieux) was used to generate mass spectra ranging from 2000 to 20 000 Da in positive linear mode using a laser frequency of 50 Hz and an acceleration voltage of 20 kV with extraction delay time of 200 ns [23]. Automatic mode was used for each spectrum, i.e., 500 shots were taken from different target shot positions for each spectrum in five-shot steps. The mass spectrometer was used together with Vitek MS Version 2.0 Acquisition Station software.

Mass spectra identification

The compute engine and advanced spectrum classifier (ASC) algorithm of the Vitek MS system were used to process MS fingerprints. Organisms were later identified by comparisons of the generated spectra to spectra typical of each species represented in the database [23]. The ASC algorithm generated percent probabilities representing the similarity of the comparison to the typical spectrum, based on presence or absence of particular peaks. Scores between 60% and 99.9% were considered to be a good identification. If the probability score was ≥60%, the result was recorded as 'genus level only identification' if there was a choice of 2—4 organisms all within the same genus, but no valid identification (at either species or genus level) was considered to have been made if the organisms were distributed between multiple genera [23].

Results

Gram-negative bacteria

Table 1 shows the number and identities of Gram-negative isolates that were correctly identified to genus and species level by each of the four methods, and the accuracy of each method was compared to the gold standard method. For Gram-negative organisms, methods 1, 2 and 3 were all used successfully to accurately

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Table 1 Gram-negative isolates tested.

Organism	Number correctly identified genus/species Gold standard	Number correctly identified genus/species Method 1	Number correctly identified genus/species Method 2	Number correctly identified genus/species Method 3	Number correctly identified genus/species Method 4
Escherichia coli	5/5	5/5	5/5	5/5	5/5
Klebsiella pneumoniae	5/5	5/5	5/5	5/5	5/5
Pseudomonas aeruginosa	5/5	5/5	5/5	5/5	5/5
Acinetobacter baumannii.	5/5	5/5	5/5	5/5	5/5
Citrobacter koseri	5/5	5/5	5/5	5/5	5/5
Enterobacter aerogenes	5/5	5/5	5/5	5/5	5/5
Stenotrophomonas maltophilia	5/5	5/5	5/5	5/5	5/5
Salmonella enterica	5/5	5/5	5/5	5/5	5/5
Proteus mirabilis	5/5	5/5	5/5	5/5	5/5
Bacteroides fragilis	5/5	5/5	5/5	5/5	0/0
Enterobacter cloacae	5/3	5/0	5/0	3/0	5/0
Proteus vulgaris	5/2	5/0	5/0	4/0	5/0
Total identified to genus level	*60*	*60*	*60*	*57*	*55*
Accuracy of test methods (%)[a]		*100*	*100*	*95*	*91.7*
Total identified to species level	*55*	*50*	*50*	*50*	*45*
Accuracy of test methods (%)[b]		*90.9*	*90.9*	*90.9*	*81.8*

[a] Accuracy to genus level.
[b] Accuracy to species level; compared to number identified using gold standard method.

identify 50 isolates to species level, providing an accuracy of 90.9% compared to the 55 isolates identified using the gold standard method, while 45 isolates were identified to species level using method 4, providing an accuracy of 81.8% (Table 1). *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Citrobacter koseri*, *Enterobacter aerogenes*, *Stenotrophomonas maltophilia*, *Proteus mirabilis*, *Salmonella enterica* and *Bacteroides fragilis* were identified to both genus and species level with 100% accuracy with each of methods 1, 2 and 3 (Table 1). However, the plate method (method 4) was not suitable for strictly anaerobic organisms, such as *B. fragilis*, which was not identified using method 4 after 90 min incubation on the blood agar plate due to its slow growth characteristics. Ten organisms (five each of *Enterobacter cloacae* and *Proteus vulgaris*) were identified up to genus level only using methods 1, 2 and 4, while method 3 failed to identify two out of five *E. cloacae* and 1 out of 5 *P. vulgaris* (Table 1). Three out of five *E. cloacae* and two out of five *P. vulgaris* were successfully identified to species level using the gold standard method.

Gram-positive bacteria

Table 2 shows the number and identity of Gram-positive isolates that were correctly identified to genus and species level by each of the four methods and the accuracy of each method compared to the gold standard method. For Gram-positive organisms, 90% were accurately identified to species level using both methods 1 and 2, 76.7% by method 3 and 86.7% by method 4. Methods 1, 2 and 4 were 100% accurate in identification to species level of five *Staphylococci*, i.e., *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus haemolyticus*, *Staphylococcus lugdunensis* and *Staphylococcus capitis*, while method 3 failed to identify two out of five *Staphylococcus capitis* (Table 2). All four methods identified five out of five *Streptococcus agalactiae*, however while method 4 also identified *Streptococcus pyogenes* with 100% accuracy, methods 1 and 2 failed to identify two and method 3 failed to identify three *S. pyogenes*. Meanwhile *S. viridians* was identified at low accuracy by all methods (Table 2). If strictly anaerobic organisms (*Clostridium perfringens*) were excluded, method 4 showed higher

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Table 2 Gram-positive isolates tested.

Organism	Number correctly identified genus/species Gold standard	Number correctly identified genus/species Method 1	Number correctly identified genus/species Method 2	Number correctly identified genus/species Method 3	Number correctly identified genus/species Method 4
Staphylococcus aureus	5/5	5/5	5/5	5/5	5/5
Staphylococcus epidermidis	5/5	5/5	5/5	5/5	5/5
Staphylococcus haemolyticus	5/5	5/5	5/5	5/5	5/5
Staphylococcus lugdunensis	5/5	5/5	5/5	5/5	5/5
Staphylococcus capitis	5/5	5/5	5/5	3/3	5/5
Enterococcus faecalis	5/5	5/5	5/5	5/5	5/5
Enterococcus faecium	5/5	5/5	5/5	4/4	5/5
Streptococcus agalactiae	5/5	5/5	5/5	5/5	5/5
Streptococcus pyogenes	5/5	3/3	3/3	2/2	5/5
Streptococcus viridians	5/5	1/1	1/1	0/0	2/2
Clostridium perfringens	5/5	5/5	5/5	3/3	0/0
Diphteroid pseudo diphtericum	5/5	5/5	5/5	4/4	5/5[c]
Total identified to genus level	*60*	*54*	*54*	*46*	*52*
Accuracy of test methods (%)[a]		*90*	*90*	*76.7*	*86.7*
Total identified to species level	*60*	*54*	*54*	*46*	*52*
Accuracy of test methods (%)[b]		*90*	*90*	*76.7*	*86.7*
Total identified to species level excl. *Clostridium perfringens*	*55*	*49*	*49*	*42*	*52*
Accuracy of test methods (%) excl. *Clostridium perfringens*		*89*	*89*	*76.4*	*94.5*

[a] Accuracy to genus level.
[b] accuracy to species level; compared to number identified using gold standard method.
[c] Identified after 6 h instead of 3 h due to slow growth characteristics.

accuracy, with 94.5% of organisms (52/55) being correctly identified to species level compared to 89.1% (49/55) for methods 1 and 2 and 78.2% (43/55) for method 3 (Table 2). However, methods 1 and 2 both showed higher accuracy when five anaerobes were included (Table 2).

Precision of methods

To measure the precision of each method, ten positive vials that included five different Gram-negative strains (*E. coli*, *P. aeruginosa*, *K. pneumonia*, *A. baumannii* and *S. maltophilia*) and five different Gram-positive strains (*S. aureus*, *E. faecalis*, *S. agalactiae*, *S. lugdunensis* and *S. haemolyticus*) were tested for three consecutive days by three different technologists, for validation of the test methods against the gold standard method [22] (Table 3). All methods showed 100% precision (Table 3).

Discussion

In this study, the objective was to test our proposed direct method of lysis centrifugation followed by washing and 30% acetic acid treatment (method 2), our modified lysis centrifugation followed by washing and 30% formic acid treatment method (method 1), lysis centrifugation followed by

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Table 3 Precision of methods.

Organism	Method 1 Day			Method 2 Day			Method 3 Day			Method 4 Day		
	1	2	3	1	2	3	1	2	3	1	2	3
Gram −												
Escherichia coli	True	True	True	True	True	True	True	True	True	True	True	True
Pseudomonas aeruginosa	True	True	True	True	True	True	True	True	True	True	True	True
Klebsiella pneumonia	True	True	True	True	True	True	True	True	True	True	True	True
Acinetobacter baumannii	True	True	True	True	True	True	True	True	True	True	True	True
Stenotrophomonas maltophilia	True	True	True	True	True	True	True	True	True	True	True	True
Gram +												
Staphylococcus aureus	True	True	True	True	True	True	True	True	True	True	True	True
Enterococcus faecalis	True	True	True	True	True	True	True	True	True	True	True	True
Streptococcus agalactiae	True	True	True	True	True	True	True	True	True	True	True	True
Staphylococcus lugdunensis	True	True	True	True	True	True	True	True	True	True	True	True
Staphylococcus haemolyticus	True	True	True	True	True	True	True	True	True	True	True	True

washing and 100% ethanol treatment method (method 3), and picking colonies from 90 to 180 min subculture plates (method 4). These methods were employed in order to determine the effectiveness of each method in accurate identification of both Gram-negative and Gram-positive bacteria using Vitek MALDI-TOF-MS in comparison to our gold standard isolated colonies method. In general, each method showed excellent accuracy and precision in terms of identification to genus level. All four methods had similar or superior performance in identification of Gram-negative bacteria to both genus and species levels when compared to other published direct culture methods [16—21]. For example, the accuracy of our methods 1, 2 and 3 at species level compared to our gold standard for identification of Gram-negative bacteria was 90.9%, comparing favorably to the 81.8% and 80.7% identification levels achieved with Biotyper and Vitek MS with direct culture using the commercially available Sepsityper (Bruker) extraction in another study [19]. They also compared favorably overall to other direct culture methods such as Sepsityper (Bruker) or in-house methods in terms of identification of Gram-positive bacteria [19,20], including the distinguishing of *Staphylococci* species. For example, in one study in which either Sepsityper or a saponin-based in-house extraction method was used [20], species-level identifications of 58.21% and 52.24%, respectively, were achieved for 67 Gram-positive isolates compared to the 90% accuracy achieved for our methods 1 and 2, 76.6% for our method 3 and 86.7% for our method 4 [20]. Our methods overall gave comparable levels of identification of Gram-positive bacteria to another larger recent study in which a lysis centrifugation method was used to prepare samples for MALDI-TOF-MS identification, in which 95.7% and 90.1% of 161 Gram-positive isolates and 84.7% and 83.7% of 92 Gram-negative isolates, were identified to genus level and species level, respectively [18]. All four methods showed 100% precision over a three-day period.

Methods 1 and 2 both performed well in identifying both Gram-negative and Gram-positive isolates to genus only level, with the exception of *S. viridians*, which was correctly identified to genus and species level in only one out of five cases by each method. In general, *S. viridians* was identified with low accuracy by all four methods, with none of the five being identified by method 3 at either genus or species level. The viridians group streptococci (VGS) are acknowledged to be difficult to identify at species level [24]. Although MALDI-TOF has been identified as a promising method to overcome this difficulty, it may be affected for example by poor resolution of *Streptococcus mitis* from *Streptococcus pneumoniae*, similarly to16S rRNA gene sequence-based identification [24,25]. Methods 1 and 2 also failed to identify two out of five of another *Streptococcus* species, *S. pyogenes*. Identification of streptococci has also been problematic in other studies using MALDI-TOF [18,24] and it has been suggested that until MALDI-TOF databases are expanded to include more spectra for well-identified Streptococcal species, then this technique is not appropriate for identification of pneumococci [26].

For Gram-negative *E. cloacae* and *P. vulgaris*, methods 1 and 2 both performed to 100% accuracy to genus only level, but, like methods 3 and 4, failed to distinguish either organism to species level. *E. cloacae* is one of the more difficult of the *Enterobacteriaceae* to identify to genus level, and our results are consistent with those of others

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who could not distinguish *E. cloacae* isolates from *Enterobacter asburiae* using the Vitek-MS system, while conventional phenotypic/genotypic systems were able to identify them to the species level [23]. We identified 3/5 of *E. cloacae* and 2/5 *P. vulgaris* isolates by our gold standard method. Databases may need to be expanded for both *E. cloacae* and *P. vulgaris* to facilitate accurate identification to species level by MALDI-TOF. Thus, with the exceptions of the Gram-positive *S. viridians* and *S. pyogenes* organisms and the Gram-negative *E. cloacae* and *P. vulgaris* organisms, both methods 1 and 2 identified both Gram-positive and Gram-negative organisms down to species level with 100% accuracy compared to our gold standard method. The latter two were identified to genus only level with 100% accuracy. Therefore, we have confirmed our proposed method 2 as an accurate, effective and rapid method in expediting identification of pathogens in blood cultures.

For method 3, the accuracy at species level exceeded 90%, equivalent to that achieved using methods 1 and 2. Again, *E. cloacae* and *P. vulgaris* were not identified to species-level. However, for Gram-positive bacteria the accuracy of method 3 was less than 90%, regardless of whether strictly anaerobic organisms (*C. perfringens)* were excluded, so this method was not validated for use for identification of Gram-positive bacteria in blood samples. As mentioned, none of the five *S. viridians* isolates were identified using method 3 at either genus or species level, while only two *S. pyogenes* were identified at genus and species levels, consistent with difficulties experienced by others in identification of streptococci using MALDI-TOF, including when using ethanol extraction [24].

Method 4, which relied on growth of isolates on blood agar before transfer to MALDI-TOF-MS slides, performed at a generally high accuracy for both Gram-positive and Gram-negative organisms. However, when used for anaerobic organisms such as the Gram-positive *C. perfringens* or the Gram-negative *B. fragilis*, method 4 was unfeasible due to the slow growth characteristics of the organisms and failed to identify either at genus or species level. The inability to accurately identify *E. cloacae*, *P. vulgaris* or *B. fragilis* to species level using method 4 resulted in the overall accuracy of this method for Gram-negative bacteria falling below the ASM-prescribed 90% accuracy levels required for validation of a proposed method against a gold standard method. Method 4 could, however, identify five out of five *S. pyogenes* organisms, so was more sensitive for this organism than methods 1, 2 or 3. Method 4 was used for identification of *Diphteroid pseudo diphtericum* after an extended incubation for 6 h. As Diphtheroids usually require 24—36 h for sufficient growth, this still represents a substantial reduction in turnaround time for identification. Moreover, methods 1 and 2 were able to identify *Diphteroid pseudo diphtericum* with 100% accuracy at both genus and species levels.

Rapid identification of causative infective agents is now recognized as being imperative in beginning early, appropriate antimicrobial treatment of sepsis, which is critical in reducing mortality [4,8,9]. We have confirmed that our rapid identification methods, particularly methods 1 and 2, which are based on extraction by lysis centrifugation followed by washing and 30% formic acid treatment method or 30% acetic acid treatment, respectively, are highly accurate and precise for a range of Gram-negative and Gram-positive bacteria, with the exceptions that we have noted. Adoption of these methods should be effective in reducing both hospital stay and health costs in treating patients with sepsis and in reducing mortality by facilitating early and appropriate antibiotic treatment.

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Competing interests

None declared.

Ethical approval

Not required.

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