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Direct antimicrobial susceptibility tests of bacteria and yeasts from positive blood cultures by using serum separator gel tubes and MALDI–TOF MS

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ABSTRACT

Early and appropriate antimicrobial treatment can effectively reduce the mortality rate caused by bloodstream infections (BSIs) and is critical for favorable patient outcomes. In general, > 90% of positive blood cultures will show positive results within 48 h after incubation in the BACTECTM FX system. However, an additional 6–8 h are required to obtain clones of the bacterium and another 10–24 h to obtain antimicrobial susceptibility test (AST) results. In this study, direct ASTs of bacteria and yeasts from positive blood cultures were performed by using serum separator gel tubes and matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS). 153 BSI cases were caused by a single pathogen. The coincidence rates of genus and species identification between the direct method (from positive blood cultures) and reference method (from subcultured clones) were 86.9% and 83%, respectively. On average, 98.6% of the direct ASTs in 88 Gram-negative bacteria tested had an accurate result compared to the reference method. In Gram- positive bacteria and yeasts, the accuracy rates were 99.2% and 100%, respectively. MALDI-TOF MS combined with serum separator gel tubes can be used for rapidly identifying and performing ASTs on positive blood cultures.

1. Introduction

With the application of invasive procedures and a considerable increase in the number of immunocompromised patients, the incidence of bloodstream infections (BSIs) has also been increasing (Zha et al., 2016; Li et al., 2013). Sepsis is one of the major causes of mortality in hospitalized patients worldwide. Mortality rates caused by sepsis range from 30 to 70% (National Center for Health Statistics. Health, United States, 2017). BSIs have been among the top 10 diseases with a high mortality rate in the USA for many years (National Center for Health Statistics. Health, United States, 2017).

Diagnosis of BSI is achieved by continuously monitoring clinical symptoms and vital signs of the patient, as well as by conducting laboratory tests including leukocyte counts, C reactive protein (CRP) level and procalcitonin concentration. While blood culture is the most important evidence for diagnosis of bacterial BSI, blood cultures can also provide sequential ASTs for clinical anti-infection treatment. In general, > 90% of positive blood cultures will show positive results within 48 h after incubating in the BACTECTM FX system (Park et al., 2010). Six to 8 h are needed to obtain clones of the pathogen, and at least an additional 10–24 h are required to obtain AST results. Early and appropriate antimicrobial treatment should effectively reduce the mortality rate of BSIs and is critical towards a favorable patient outcome (Funk and Kumar, 2011). Researchers have made numerous attempts to explore ways to quickly obtain pathogen identification (ID) and AST results (Yonetani et al., 2016; Hou et al., 2017). In recent years, matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) has become a powerful tool in microbial diagnosis, which provides an alternate method for rapid bacterial and fungal pathogen identification directly from positive blood culture (Stevenson et al., 2017; Chen et al., 2013).

MALDI-TOF MS is a fast and reliable method for the identification of clinical bacterial infections (van Veen et al., 2010; Bille et al., 2012). It has been shown to shorten bacterial identification by at least 10 h when it was used on positive blood culture (Patel et al., 2017). However, due to a reliance of subcultured clones, ASTs are still generally not readily available. Clinicians can only provide empirical treatment for patients but not targeted treatment. In this study, cell pellets were obtained from positive blood cultures by SST-II Vacutainer serum separator gel tubes (Becton Dickinson, USA). After gathering the cell pellets, the IDs and ASTs were analyzed by the VITEK-2 Compact automated microbiology system (bioMérieux, France). Results from this method were compared

Abbreviations: MALDI–TOF MS, matrix-assisted laser desorption ionization–time of flight mass spectrometry; BSI, bloodstream infection; AST, antimicrobial susceptibility test; CRP, C reactive protein; ID, identification; Rpm, revolutions per minute

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to IDs and ASTs that were determined based on subcultured clones.

2. Materials and methods

2.1. Positive blood culture samples

A total of 158 positive blood cultures from 137 patients were included in this study and were obtained from hospitalized patients between March 2016 and February 2017 in Hangzhou First People's Hospital. Of these, 153 samples were infected by a single bacterium, while five had mixed infections.

2.2. Patient-derived blood cultures

Bactec Plus Aerobic/F and Lytic/10 Anaerobic/F bottles (Becton Dickinson, USA) were removed within 4 h after being reported positive by the BACTEC™ FX automated incubation system (Becton Dickinson, USA). Aliquots of blood culture medium were Gram stained to confirm bacterial growth and to initiate subcultures to eliminate potential false positive results.

2.3. Sample preparation using serum separator gel tubes

Positive blood culture bottles were inverted several times to mix. Using a syringe, 8 ml of the blood culture medium was then transferred to an SST-II Vacutainer serum separator gel tube (Becton Dickinson, USA). The bacterial cells were separated after centrifugation for 10 min at 3000 revolutions per minute (rpm) at room temperature. The supernatant was discarded and 1 ml sterile deionized water was added. The bacterial suspension was transferred to a 1.5-ml microcentrifuge tube after mixing gently and centrifuged at 12,000 rpm for 5 min. The pellet was resuspended in 1 ml sterile deionized water and centrifuged again at 12,000 rpm for 5 min. The supernatant was discarded and the pellet was retained for further analysis.

2.4. MALDI-TOF analysis

A small amount of the pellet was spotted onto a steel target plate and overlaid with 1 μl of α-cyano-4-hydroxycinnamic acid matrix (Bruker Daltonics, Germany); yeast needed another 1 μl of 70% formic acid according to the instrument manufacturer's suggestion. After drying, samples were subjected to analysis using a Bruker Microflex LT system (Bruker Daltonics) according to the manufacturer's recommendations. The mass spectra were analyzed with the Bruker Biotyper 3.0 software (Bruker Daltonics). The result was considered valid at the species level when the identification score was 1.7 to 3.0 and the top ten results were from the same species. The result was considered valid at the genus level when the score was 1.4 to 1.7 and the top ten results were from the same genus. Scores < 1.4 were considered invalid (Kohlmann et al., 2015).

2.5. Direct AST method

A bacterial suspension at 0.5 McFarland equivalence turbidity was prepared from the remaining cell pellet by nephelometer. The requisite AST cards were inoculated with the bacterial suspension and placed into a VITEK-2 Compact instrument. ASTs were not performed when MALDI-TOF MS scores were below 1.4 or when Gram staining showed a polymicrobial result or Gram-positive bacilli. A minor error was defined as the AST reported intermediate susceptibility to antimicrobials by the direct method but was susceptible or resistance using the reference method. A major error was defined as when the direct AST suggested the strain was resistant to an antimicrobial, while the reference method reported it as susceptible. A critical error was defined as when the direct AST result reported the strain to be susceptible while the reference method reported it as resistant.

2.6. The reference methods of ID and AST

Blood culture was viewed after Gram staining for preliminary identification and inoculated on Columbia blood agar (bioMérieux, France). Chocolate agar and Sabouraud's agar (bioMérieux) were used as needed. Solid media plates were incubated at 37 °C with 5% CO_2 for 18–24 h. Anaerobic cultures were performed within anaerobic bottles. Reference identification for all microorganisms grown on agar was performed using the MALDI-TOF MS. Only results with concordance scores (higher than 2.0) above the manufacturer's proposed cutoff value for reliable species level identification were retained. ASTs were attempted for all isolates using the appropriate AST cards in a VITEK-2 Compact system as instructed by the manufacturer.

3. Results

A total of 158 blood cultures were confirmed positive by subculture. After subculturing, 153 of the blood samples were shown to be monomicrobial and 5 were polymicrobial cultures.

3.1. Direct identification of monomicrobial cultures

The results for the monomicrobial cultures are shown in Table 1. Overall, 86.9% and 83% of bacteria were directly identified at the genus and species level from positive blood culture using MALDI-TOF MS. No misidentifications were made by the direct method when compared to the reference method, however not all samples could be assigned a species or genus ID. There was a distinct difference between the ability to assign IDs among Gram-negative and Gram-positive bacteria, as 92.4% of the Gram-negative bacilli were correctly identified against 83% of Gram-positive bacteria. Among Gram-negative bacilli, the direct method only failed in the identification of three *Klebsiella pneumoniae* cultures, one *Enterobacter cloacae* culture and one *Burkholderia cepacia* culture. Among Gram-positive bacteria, inconclusive results were observed for *Staphylococcus aureus*, coagulase-negative staphylococci and *Enterococcus*, which had lower scores with the direct method. It should be noted that the correct identification of *Streptococcus* was 85.7% (6/7), indicating that this method may still be advantageous to clinicians.

3.2. Direct identification of polymicrobial cultures

For the 5 polymicrobial blood cultures, two correct direct identifications were made. Four of the polymicrobial cultures were identified as *Klebsiella pneumoniae* mixed with another microorganism (two were *Enterococcus faecium*, and two were *Enterococcus avium*) by routine method. Of these cultures, using the direct method, two were identified as *Klebsiella pneumoniae*, and two were identified as an error ID. The fifth sample was a mixture of two Gram-negative bacteria and also resulted in no ID. The results for polymicrobial cultures are shown in Table 2.

3.3. Direct AST results

In total, 128 isolates (88 Gram-negative bacteria, 38 Gram-positive cocci and 2 yeasts), and 1763 bacteria-antimicrobial agent combinations were analyzed by VITEK-2 Compact. Of the 93 Gram-negative bacteria, 88 cultures were identified at the genus level by the direct method and 1208 ASTs were performed. A comparison of the direct AST results with the AST by reference showed categorical agreement (1191, 98.6%), minor error (8, 0.7%), major error (3, 0.2%) and critical error (6, 0.5%). Of the 47 Gram-positive bacteria, 38 cultures were identified at the genus level using the direct method, and 547 ASTs were completed. When compared with the reference method, the direct method was largely in agreement 99.2% (543) of the time and showed a minor error 0.2% (1), major error 0.2% (1), and critical error 0.4% (2) of the

Table 1
Identification results of Biotyper using serum separator tube with modified cutoff values (n = 153).

Microorganism	No. of strains	Species level	Genus level only	Not identified	Error identified (%)
		≥1.7(%)	1.4–1.7(%)	< 1.4(%)	
Gram-negative bacteria	**93**	**86(92.4)**	**2(2.2)**	**5(5.4)**	**0(0.0)**
Klebsiella pneumoniae	41	36	2	3	0
Escherichia coli	27	27	0	0	0
Enterobacter aerogenes	5	5	0	0	0
Enterobacter cloacae	4	3	0	1	0
Morganella morganii	2	2	0	0	0
Proteus mirabilis	2	2	0	0	0
Serratia marcescens	2	2	0	0	0
Burkholderia cepacia	2	1	0	1	0
Escherichia fergusonii	1	1	0	0	0
Salmonella enteritidis	1	1	0	0	0
Pseudomonas aeruginosa	3	3	0	0	0
Shewanella putrefaciens	1	1	0	0	0
Acinetobacter baumannii	1	1	0	0	0
Aeromonas veronii	1	1	0	0	0
Gram-positive bacteria	**51**	**40(78.4)**	**2(3.9)**	**9(17.6)**	**0(0.0)**
Staphylococcus epidermidis	9	7	1	1	0
Staphylococcus aureus	7	5	0	2	0
Staphylococcus hominis	5	3	1	1	0
Staphylococcus capitis	3	2	0	1	0
Staphylococcus lugdunensis	4	4	0	0	0
Staphylococcus haemolyticus	2	2	0	0	0
Staphylococcus warneri	2	1	0	1	0
Enterococcus faecium	5	4	0	1	0
Enterococcus faecalis	3	2	0	1	0
Streptococcus agalactiae	3	2	0	1	0
Streptococcus pyogenes	1	1	0	0	0
Streptococcus dysgalactiae	1	1	0	0	0
Streptococcus pneumoniae	1	1	0	0	0
Streptococcus anginosus	1	1	0	0	0
Listeria monocytogenes	3	3	0	0	0
Lactobacillus jensenii	1	1	0	0	0
Yeast	**9**	**1(11.1)**	**2(22.2)**	**6(66.7)**	**0(0.0)**
Candida tropicalis	3	0	0	3	0
Candida albicans	2	0	1	1	0
Candida parapsilosis	2	0	0	2	0
Candida glabrata	1	0	1	0	0
Cryptococcus neoformans	1	1	0	0	0
Total	153	127(83.0)	6(3.9)	20(13.1)	0(0.0)

time. The two yeasts tested with 8 AST combinations had the same results when compared with the reference method. The results are shown in Table 3.

Among the Gram-negative bacteria, the best direct AST results were seen for *Escherichia coli*, *Enterobacter aerogenes, Morganella morganii, Proteus mirabilis,* and *Serratia marcescens*. The results of tigecycline and nitrofurantoin for *Klebsiella pneumoniae*, ertapenem for *Enterobacter cloacae,* amoxicillin for *Salmonella enteritidis* were incongruously found to be susceptible by the direct method but were found to be resistant by the reference method. Among the Gram-positive bacteria, *Staphylococcus epidermidis, Enterococcus faecium,* and *Streptococcus agalactiae* gave erroneous AST results. The results of levofloxacin for *Staphylococcus epidermidis* and penicillin for *Streptococcus agalactiae* were found to be susceptible by the direct method but to be resistant by the reference method. These details are shown in Table 4.

4. Discussion

Sepsis is a severe clinical infection by pathogens invading the bloodstream, leading to a high morbidity and mortality rate (Forgacs et al., 1986; Laupland et al., 2002; Diekema et al., 2003). In recent years, MALDI-TOF MS has become a fast and reliable technique to enable rapid organism identification directly from positive blood cultures. This shortens identification by 10 h; however, since AST results could not be obtained immediately, this leads to patients being treated with broad-spectrum antimicrobial agents instead of a targeted therapy. Previous studies have shown that targeted therapy can improve patient outcomes with a shorter length of stay, lower mortality rate, and less overall hospital-associated cost (Patel et al., 2017; Martinez et al., 2014).

Previous studies have shown that the most common pathogens of BSIs from Class I hospitals (medical centers) in major cities of China are

Table 2
Specimens with polymicrobial identifications generated by the Biotyper using serum separator tube.

Mixed culture identified by routine methods	Mixed culture identified by the direct method
Klebsiella pneumoniae, Enterococcus faecium	*Klebsiella oxytoca*
Klebsiella pneumoniae, Enterococcus faecium	*Bacteroides fragile*
Klebsiella pneumoniae, Enterococcus avium	*Klebsiella pneumoniae*
Klebsiella pneumoniae, Enterococcus avium	*Klebsiella pneumoniae*
Proteus mirabilis, Proteus pennea	Not identified

Table 3
ASTs of bacteria from positive blood cultures by using serum separator tubes and the VITEK-2 Compact System.

Microorganism	No. of isolates	Correct ID	Antimicrobials Tested	Agreement	Minor Error[a]	Major Error[b]	Critical Error[c]
Gram-negative bacteria	**93**	**88**	**1208**	**1191(98.6%)**	**8(0.7%)**	**3(0.2%)**	**6(0.5%)**
Klebsiella pneumoniae	41	38	514	506	2	2	4
Escherichia coli	27	27	399	397	2	0	0
Enterobacter aerogenes	5	5	28	28	0	0	0
Enterobacter cloacae	4	3	42	41	0	0	1
Morganella morganii	2	2	22	22	0	0	0
Proteus mirabilis	2	2	34	34	0	0	0
Serratia marcescens	2	2	26	24	2	0	0
Burkholderia cepacia	2	1	18	17	1	0	0
Escherichia fergusonii	1	1	18	18	0	0	0
Salmonella enteritidis	1	1	18	17	0	0	1
Pseudomonas aeruginosa	3	3	43	41	1	1	0
Shewanella putrefaciens	1	1	18	18	0	0	0
Acinetobacter baumannii	1	1	11	11	0	0	0
Aeromonas veronii	1	1	17	17	0	0	0
Gram-positive bacteria	**47**	**38**	**547**	**543(99.2%)**	**1(0.2%)**	**1(0.2%)**	**2(0.4%)**
Staphylococcus epidermidis	9	8	136	134	0	1	1
Staphylococcus aureus	7	5	85	85	0	0	0
Staphylococcus hominis	5	4	70	70	0	0	0
Staphylococcus capitis	3	2	34	34	0	0	0
Staphylococcus lugdunensis	4	4	22	22	0	0	0
Staphylococcus haemolyticus	2	2	33	33	0	0	0
Staphylococcus warneri	2	1	17	17	0	0	0
Enterococcus faecium	5	4	42	41	1	0	0
Enterococcus faecalis	3	2	32	32	0	0	0
Streptococcus agalactiae	3	2	23	22	0	0	1
Streptococcus pyogenes	1	1	4	4	0	0	0
Streptococcus dysgalactiae	1	1	5	5	0	0	0
Streptococcus pneumoniae	1	1	6	6	0	0	0
Streptococcus anginosus	1	1	4	4	0	0	0
Yeast	**8**	**2**	**8**	**8(100%)**	**0(0.0%)**	**0(0.0%)**	**0(0.0%)**
Candida albicans	2	1	4	4	0	0	0
Candida glabrata	1	1	4	4	0	0	0
Candida tropicalis	3	0	0	0	0	0	0
Candida parapsilosis	2	0	0	0	0	0	0
Total	**148**	**128**	**1763**	**1742(98.8%)**	**9(0.5%)**	**4(0.2%)**	**8(0.5%)**

[a] Intermediate versus susceptible or resistant;
[b] False resistance;
[c] False susceptibility

Escherichia coli, coagulase-negative Staphylococci, *Staphylococcus aureus* and *Klebsiella pneumoniae* (Li et al., 2013; Li et al., 2014). In our research, Gram-negative bacteria (60.1%) were the main bacterial group isolated from patients with monomicrobial bloodstream infections in Hangzhou First People's Hospital from March 2016 to February 2017, followed by Gram-positive bacteria (30.7%). The most common isolates from BSIs were *Klebsiella pneumoniae* (26.8%), *Escherichia coli* (17.6%), *Staphylococcus epidermidis* (6.9%) and *Staphylococcus aureus* (4.6%), which is consistent with earlier studies (Zha et al., 2016; Li et al., 2013, 2014).

The aim of this study was to evaluate the efficacy of the direct identification of bacteria isolated from positive blood cultures with MALDI-TOF MS at the genus level using the serum separator gel tube-based method in a clinical microbiology laboratory. The high accuracy of identifying Gram-negative bacteria at the genus level (94.6%) was significantly higher than that of Gram-positive bacteria (82.3%) which is consistent with previously reported studies (Kok et al., 2011; Chen et al., 2015). The accuracy of identifying yeast at the genus level was low (33.3%). On the one hand, due to the small number of yeast samples, the accuracy of direct MALDI-TOF MS identification at the genus level is unclear. On the other hand, compared with the identification of bacteria, the accuracy of identification of yeast is much lower (Bille et al., 2012; Chen et al., 2015). For polymicrobial cultures, the ability of MALDI-TOF MS to identify multiple genera was poor, giving only one microorganism or no result.

Compared with reference method, the direct AST method shortens

Table 4
ASTs of bacteria that did not result in agreement with reference method.

Microorganism	Minor error	Major error	Critical error
Klebsiella pneumoniae	Cefepime	Amoxicillin, Aztreonam	Tigecycline, Nitrofurantoin
Escherichia coli	Nitrofurantoin, Tobramycin	–	–
Enterobacter cloacae	–	–	Ertapenem
Serratia marcescens	Imipenem, Ciprofloxacin	–	–
Burkholderia cepacia	Tobramycin	–	–
Salmonella enteritidis	–	–	Amoxicillin
Pseudomonas aeruginosa	Cefepime	Piperacillin/tazobactam	–
Staphylococcus epidermidis	–	Gentamicin	Levofloxacin
Enterococcus faecium	Erythromycin	–	–
Streptococcus agalactiae	–	–	Penicillin

the time by at least 10 h with improved therapeutic efficacy, reduction in morbidity and mortality due to BSIs (McElvania TeKippe, 2017). However, this method is based on the following two points: (1) the correct identification of the isolates, which is a decisive value for the AST; and (2) there are enough living bacteria for accurate testing. Hence, the process of bacterial purification from blood cultures is crucial. Many studies have explored the process of an initial bacterial purification, including the MALDI Sepsityper kit (Bruker,Germany) (Riederer et al., 2015), serum separator gel tube purification (Chen et al., 2015), adding saponin lysis and then centrifugal extraction (Febbraro et al., 2016), and short-term incubation (Kohlmann et al., 2015), which have resulted in satisfactory results. However, extra cost is added for the MALDI Sepsityper kit for bacterial purification. Adding saponin lysis followed by centrifugal purification is inefficient because of the many resin particles in the culture medium. Short-term incubation still requires at least another 4 h. Therefore, the serum separator gel tube-based method was selected for this study. AST results obtained with the direct method showed a high degree of concordance (99%) comparing when compared to that of the reference method. However, some major errors still occurred as the AST results of tigecycline showed resistance for *Klebsiella pneumoniae* in two cases with reference method, but susceptible with the direct method, which could lead to serious consequences. Unfortunately, the occurrence of these errors does not have an obvious pattern, as the literature has previously reported (Zhang and Huang, 2013). It was speculated that the errors may be related to the concentration and number of living bacteria after the purification process. Further studies may reduce errors by effectively monitoring the concentration and the number of living bacteria. But clinicians can be advised to avoid making/changing treatment decisions until definitive results are obtained based on the VITEK-2 Compact advanced expert system for detection of antimicrobial resistances (I et al., 2007).

In our study, the main advantage of the serum separator gel tube-based method for rapid organism identification and detection of AST directly from blood cultures is its suitability for busy laboratories, lack of subculturing, and reduced turn-around time. The ID and AST results were reported 24 h earlier on average and this timely diagnosis should reduce mortality rates among patients (Jiang et al., 2016). To increase the purity of the bacterial/yeast cells prepared, the process was modified with an additional wash step, although this step has some limitations: (1) fewer bacterial/yeast cells were recovered, which may reduce the accuracy of AST results or hinder the ID process. Therefore, adequate blood volume is necessary to avoid the poor ID and AST results; (2) hemolysis occurred in a few samples causing the supernatant fraction of the serum separator gel tube to retain red blood cell debris and resins, which could potentially influence the ID and AST results.

In summary, serum separator gel tubes combined with MALDI-TOF MS detection method is a fast and reliable technique to determine the IDs and AST results of bacteria within positive blood cultures, providing an excellent alternative to the reference protocol and effectively shortening the turn-around time. This should facilitate the earlier optimization of antibiotic treatments, improve the chosen therapy for critical patients, and in turn, reduce mortality. Furthermore, this method is cheaper, simpler and more efficient, making it suitable for application in clinical microbiological laboratories. For the low accuracy of the yeast ID results, the analysis of yeast should follow the reference protocol after Gram staining.

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Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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