Original Article

Direct Bacterial Identification and Antimicrobial Drug Susceptibility from Hemoculture at Faculty of Medicine Vajira Hospital

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Background: Bloodstream infections are the most serious infectious diseases and one of the leading causes of morbidity and mortality worldwide. Rapid bacterial identification (ID) and antimicrobial susceptibility test (AST) could lead to quicker microbiological diagnosis and appropriate antimicrobial therapy.

Objective: To compare the performance of ID and AST between bacteria directly from positive blood culture broths by using differential centrifugation method (direct method) and conventional culture-based methods.

Materials and Methods: The prospective descriptive study on 349 positive monobacterial blood culture bottles was conducted between November 15, 2016 and January 15, 2017 at the Vajira Hospital, Bangkok, Thailand. The differential centrifugation method was used to isolate and concentrate bacterial cells directly from positive blood culture broths. This sample preparation process takes only 15 to 20 minutes, and the bacteria cell pellet was used as a sample for ID by MALDI Biotyper system (direct ID method) and AST by disc diffusion assay (direct AST method). Results were then compared with those obtained from isolated colonies grown overnight on agar plates, as conventional methods.

Results: For direct ID, correct species ID was obtained for 297 (85.10%) isolates and 49 isolates (14.04%) were not identified. Two isolates (0.57%) were identified to genus only, and only one isolate (0.29%) was misidentified. There were limitations in the MALDI-TOF technology. A chi-square test showed that there was no statistically significant difference between the two methods in the ID of Gram-negative isolates, *Staphylococcus aureus*, and Enterococci. For direct AST, overall categorical agreement was 98.20% with no false resistance or susceptibility.

Conclusion: The present data suggest that the direct ID was useful for preliminary ID of bacteria in positive blood culture, especially for Gram-negative bacteria, *S. aureus*, and Enterococci. Results can be reported within 30 to 45 minutes after the positive signal. The direct AST provided excellent susceptibility testing results with both Gram-positive and Gram-negative bacteria, and yielded results 18 to 24 hours earlier than conventional AST method. These direct ID and AST methods were simple, rapid, and inexpensive.

Keywords: Identification, Antimicrobial susceptibility testing, Matrix-assisted laser desorption ionization-time of flight mass spectrometry, MALDI-TOF MS, Direct identification method, Conventional culture-based method

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Bloodstream infections (BSIs) are the most serious infectious disease and one of the leading causes of morbidity and mortality worldwide[1]. Blood culture testing is the "gold standard" for diagnosis of BSIs and is based on the detection of viable microorganisms circulating in the bloodstream of the patients[2]. Rapid detection, identification (ID), and antimicrobial susceptibility testing (AST) of the pathogens are crucial for guiding clinicians to appropriate antimicrobial therapy, leading to lower mortality rates, providing antimicrobial stewardship, shortened length of stay, and decreased medical care costs[3]. ID and AST by conventional methods are still based on cultivating microorganisms and require

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incubation for extended periods of time, which usually takes about 48 to 72 hours after being flagged positive by automated systems.

Recently, matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) is one approach that has been used for the ID of microorganisms in routine clinical laboratories worldwide and has been given much attention as an alternative method to replace conventional phenotypic methods[4]. This technique enables ID of causative pathogens within six minutes, directly from colonies grown on culture plates, which can provide results about 5 to 48 hours earlier than conventional methods[5]. Moreover, several researchers have used MALDI-TOF MS to identify microorganism directly from positive blood culture, with results available within about 10 to 45 minutes of flagging positive[6-16]. However, this technique requires sample preparation protocols for isolation of microbial cells from blood cells, human proteins, and culture media prior to analyses. A variety of sample preparation methods have been reported, including lysis-centrifugation methods, lysis-filtration method, differential (stepwise) centrifugation method, separator gel tube-based method, and a commercial kit (Bruker Sepsityper)[6,11-17]. The differential centrifugation method has been shown to be non-toxic to many microbes, non-chemical, inexpensive, simple, and rapid[6,10,18]. The resulting microbial pellet can be used directly for AST with automated systems or Kirby-Bauer disk diffusion method, thus reducing turnaround time to less than 24 hours[12,13,19,20].

In the present study, the authors evaluated the performance of ID and AST of bacteria directly from positive blood culture broths by using differential centrifugation combined with MALDI-TOF MS (direct ID method) and the Kirby-Bauer disk diffusion method (direct AST method), and compared them with the conventional culture-based method.

Materials and Methods

Ethics statement

The present study was reviewed and approved by the Research Ethics Committee at the Faculty of Medicine Vajira Hospital, Navamindradhiraj University, Bangkok, Thailand, on October 17, 2016 (COA No.108/2559), which waived informed consent from the patients, because all specimens used in the study were leftover specimens from routine standard clinical testing that would normally be discarded and their clinical data were anonymized and de-identified prior to analysis.

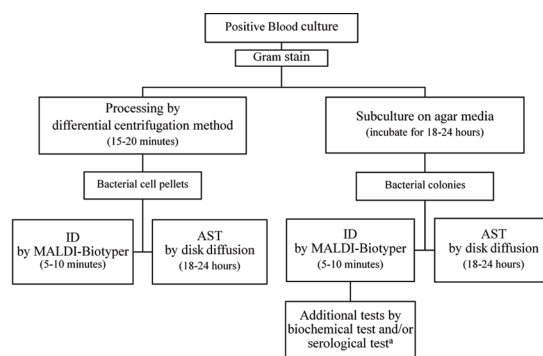

Figure 1. Flow chart showing the study design.

ID, identification; AST, antimicrobial susceptibility testing

[a] Additional tests for no species identification, no identification and some species currently cannot be reliably identified by MALDI-Biotyper technology

Study design, subjects, sample size calculation

The present prospective descriptive study was conducted at the Microbiology Laboratory, Department of Central Laboratory and Blood Bank, Faculty of Medicine Vajira Hospital, Navamindradhiraj University, Bangkok, Thailand between November 15, 2016 and January 15, 2017. Sample size was calculated using the formula of Cochran[21]: $n = P(1-P)Z^2/d^2$ (with n=minimum sample size required; P=proportion of successes from previous study which is 0.67[6]; Z=confidence level (z-score) at 95% is 1.96; d=acceptable p-value given as 0.05. The minimum sample size obtained was 340.

Leftover positive blood culture specimens (BD BACTEC™ Plus Aerobic and BD BACTEC™ Peds Plus™ blood culture bottles) were included in the present study. Positive blood culture with false positive, anaerobic bacteria, polymicrobial, and fungus were excluded from the study. All samples were stored at room temperature prior to processing and tested within 24 hours after being flagged positive by the blood culture monitoring system (BD BACTEC™ FX blood culture system). Positive blood culture broths were subjected to direct ID and AST in parallel with the conventional culture-based method of processing. In an experiment, the same specimen was analyzed within the same run by the same medical technologist to avoid interpersonal bias. A flow chart of the study is presented in Figure 1.

Direct ID and AST

Sample preparation by differential centrifugation method: All leftover positive blood culture specimens were examined by Gram-staining. Five milliliters

(ml) of blood culture broth were drawn from positive culture bottles by using a 21-gauge 1-inch needle on a 5 ml syringe and transferred to a sterile screw cap tube (12×100 mm). The sample was centrifuged in a Kokusan centrifuge (model H-11N, Tokyo, Japan) at 1,500 rpm for two minutes to remove human blood cell components. The supernatant was carefully collected with a sterile Pasteur pipette and placed into a new sterile tube and centrifuged again at 3,500 rpm for five minutes. Following centrifugation, the supernatant was discarded, and the pellet was resuspended in 5 ml of sterile distilled water to lyse any residual red blood cells and wash the pellet. The mixture was vortexed for 30 seconds prior to centrifugation at 3,500 rpm for five minutes, and then the supernatant was discarded. The resulting bacterial pellets were used for direct ID and AST.

Direct ID using Bruker's MALDI Biotyper system: One microliter (µl) of bacterial pellet was smeared in duplicate onto the MSP 96 polished steel target plate (Bruker Daltonics, GmbH, Bremen, Germany) using a 1 µl calibrated plastic disposable loop and allowed to air dry at room temperature. Then each spot was overlaid with 1 µl of 70% (v/v) formic acid (Sigma-Aldrich, St. Louis, MO, USA) and again allowed to air dry. The dried spots were overlaid with 1 µl of matrix solution consisting of 10 mg/ml of α-cyano-4-hydroxycinnamic acid (HCCA) (Bruker Daltonics, GmbH, Bremen, Germany) in standard solvent (acetonitrile 50%, water 47.5% and trifluoroacetic acid 2.5%) (Sigma-Fluka Sigma Aldrich No.19182-250 ML) and air dry completely prior to MALDI-TOF MS analysis. The identified organisms were analyzed using a Bruker's MALDI Biotyper system, comprising a Microflex LT mass spectrometer with FlexControl software and the MALDI Biotyper software (Bruker Daltonics, GmbH, Bremen, Germany). The spectrum of the unknown test organism was analyzed by using MALDI Biotyper software (version 3.0) (MALDI Biotyper Library version 3.1.1.0; Bruker Daltonik). Each mass spectrum was compared between samples and the references in the database. A log (score) value between 0.00 and 3.00 was calculated. The results were evaluated according to the standard Bruker interpretation criteria of the manufacturer similar to that described in the conventional methods. The log (score) value and result were recorded for further analysis. In each run of the experiment, Bacterial Test Standard (*Escherichia coli* DH5 alpha protein extract; Bruker Daltonics ref. 255343) was included to calibrate of the mass spectrometer, a laser setting check, evaluation of spectrum quality and positive control.

Direct AST: The remaining bacterial pellet after direct ID was suspended in sterile normal saline solution, adjusted to a turbidity of a 0.5 McFarland standard and used for AST by the Kirby-Bauer disc diffusion method according to Clinical Laboratory Standards Institute (CLSI) guidelines (M02-A12)[22]. The antimicrobial agents used in the present study, based on CLSI guideline recommendations combined with Vajira Hospital formulary availability, which distinguishes three important groups of antimicrobial agents depending on the direct ID result given by Bruker's MALDI Biotyper. For some isolates, for which ID could not be obtained, Gram-stain was used to guide the choice of antimicrobial agents. The following panel of antimicrobial disks, supplied by Oxoid Ltd (Basingstoke, Hampshire, England), was tested:

(i) Gram-negative bacilli: amikacin (AK) 30 µg, amoxicillin-clavulanic acid (AMC) 30 µg, ampicillin/sulbactam (SAM) 10/10 µg, ampicillin (AMP) 10 µg, cefoperazone/sulbactam (SCF) 75/30 µg, cefepime (FEP) 30 µg, cefotaxime (CTX) 30 µg, cefoxitin (FOX) 30 µg, ceftazidime (CAZ) 30 µg, ceftriaxone (CRO) 30 µg, ciprofloxacin (CIP) 5 µg, ertapenem (ETP) 10 µg, gentamicin (GM) 10 µg, imipenem (IPM) 10 µg, levofloxacin (LVX) 5 µg, meropenem (MEM) 10 µg, piperacillin/tazobactam (TZP) (100/10 µg), trimethoprim/sulfamethoxazole (SXT) 1.25/23.75 µg, tigecycline (TGC) 10 µg, chloramphenicol (C) 30 µg, tetracycline (TE) 30 µg, erythromycin (E) 15 µg.

(ii) Gram-positive cocci in clusters (*Staphylococcus* species): cefoxitin (FOX) 30 µg, clindamycin (DA) 2 µg, erythromycin (E) 15 µg, gentamicin (GM) 10 µg, fusidic acid (FD) 10 µg, teicoplanin (TEC) 30 µg, trimethoprim/sulfamethoxazole (SXT) 1.25/23.75 µg.

(iii) Gram-positive cocci in pairs and chains (*Streptococcus* species/*Enterococcus* species): cefotaxime (CTX) 30 µg, chloramphenicol (C) 30 µg, clindamycin (DA) 2 µg, erythromycin (E) 15 µg, penicillin (P) 10 µg, vancomycin (VA) 30 µg, oxacillin (OX) 1 µg, trimethoprim/sulfamethoxazole (SXT) 1.25/23.75 µg, ampicillin (AMP) 10 µg, gentamicin (GM) 120 µg, tetracycline (TE) 30 µg, teicoplanin (TEC) 30 µg.

The zone diameters of each antimicrobial agent were interpreted using the recommendations of the CLSI guidance (M100-S25)[23].

Conventional ID and AST

Following Gram-staining, blood samples from

positive blood bottles were sub-cultured onto chocolate agar plates, 5% (v/v) sheep blood agar plates, and MacConkey agar (Oxoid) plates. The sheep blood agar plates and MacConkey agar plates were incubated in an atmosphere at 35°C for 18 to 24 hours, whereas chocolate agar plates were incubated under a 5% CO_2 atmosphere (in a candle jar or CO_2-incubator) at 35°C for 18 to 24 hours. When there was insufficient or no growth, the plates were further incubated for 48 hours. At the end of incubation, isolated colonies on sheep blood agar plate or chocolate agar plates (when some of the isolates failed to grow on sheep blood agar plates) were used for ID and AST.

In the authors' routine clinical microbiology laboratory, Bruker's MALDI Biotyper system (Bruker Daltonics, GmbH, Bremen, Germany) was used as a first-line method for ID of bacteria isolated from agar plates. Preparation of bacterial isolates for Bruker's MALDI Biotyper measurement used the direct transfer-formic acid method as previously described[24]. Finally, spectra were acquired and each mass spectrum of the samples compared with the references in the database. A log (score) value between 0.00 and 3.00 was calculated. The standard cut-off score for ID after culture on solid media was used as proposed by the manufacturer, a log (score) of 2.0 or more was reported as "high-confidence ID" (secure species ID). A log (score) of 1.7 to less than 2.0 was reported as "low-confidence ID" (secure genus ID and probable species ID). A log (score) of less than 1.7 was reported as "no reliable ID". Before each measurement, the mass spectrometer was calibrated using the Bruker Bacterial Test Standard according to the manufacturer's recommendation as described above. Each sample was measured in duplicate, the one with the best log (score). For susceptibility testing, the authors only analyzed bacterial isolates in the CLSI recommendations for Kirby-Bauer disk diffusion susceptibility method and interpretation. Pure colonies from overnight subculture on sheep blood agar plate were used to prepare a 0.5 McFarland suspension. The following procedures were the same as for the direct susceptibility testing as described previously.

Criteria for final ID of isolates

When identity log (score) was less than 2.0 or some bacterial species were not in the database of Bruker's MALDI Biotyper system[25], they were confirmed by additional standard biochemical tests and/or serotyping as described in Bergey's manual[26].

Quality control strain

The three American Type Culture Collection (ATCC) reference strains were used as controls for both AST and MALDI-TOF MS analyses: *E. coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213, *Pseudomonas aeruginosa* ATCC 27853. Stock cultures were maintained at –20°C in trypticase soy broth (TSB) containing 20% glycerol (v/v) (Sigma-Aldrich, MO, United States). For quality control (QC), the stock solution was inoculated into 5% sheep blood agar and incubated at 35°C overnight. This QC was done once a week.

Data analysis

The ID and AST obtained with direct methods were compared with conventional methods (reference method). The ID results from the direct method were reported as follows:

(i) "Correct ID of species (group or species complex) when the results obtained by direct ID methods and conventional methods were the same ID at the genus and species (group or species complex) level.

(ii) "Only the correct genus was identified" when direct ID methods could be identified to the genus only but not species level ID.

(iii) "No ID" when the direct ID methods were given a log (score) lower than 1.7 (no reliable ID), the isolates did not prove identifiable by this method or low-discrimination ID. When more than one ID of microorganism was displayed, thus requiring additional tests for confirmation of ID.

(iv) "Misidentification" when the results obtained by direct ID methods were different at the genus and/or species level from that identified by conventional methods.

The results of the direct AST were evaluated according to definitions given by the US Food and Drug Administration (FDA) guidance document as follows: categorical agreement (CA, results within the same category), very major error (false susceptibility), major error (false resistance), or minor error (susceptible/resistance versus intermediate susceptibility)[27].

Statistical analysis

Pearson's chi-square test was used for a comparison of the performance between conventional and direct ID method with the same specimens. p-values less than 0.05 were considered statistically significant. Statistical analyses were conducted using SPSS for Windows version 17.0 (SPSS Inc., Chicago,

Illinois) Kappa statistic was used to determine agreement in by both methods.

Results

Direct ID compared with final ID

Three hundred forty-nine monobacterial positive blood cultures were analyzed in the present study. These included 158 Gram-positive bacteria and 191 Gram-negative bacteria, representing 29 genera and 61 species. Overall, 297 (85.10%) isolates, 118/158 (74.68%) of Gram-positive bacteria and 179/191 (93.72%) of Gram-negative bacteria, were correctly identified to the species level. Of the remaining 52 (14.90%) isolates, two (0.57%) isolates were identified successfully to the genus level only, 49 (14.04%) isolates were not identified, and one (0.29%) isolate was misidentified. The details of these IDs are given in Table 1.

Of these 297 (85.10%) isolates, direct ID showed superior performance with Gram-negative bacteria than it did with Gram-positive bacteria (94.76% versus 74.68%; $p<0.05$). According to different groups of microorganisms, correct species ID was obtained for 97.06% (33/34) of *S. aureus*, 69.09% (38/55) of coagulase-negative staphylococci (CoNS), 83.33% (30/36) of streptococci, 100% (10/10) of enterococci, 30.43% (7/23) of other Gram-positive bacilli, 97.81% (134/137) of Enterobacteriaceae, 84.09% (37/44) of non-fermenting Gram-negative bacilli, and 80.00% (8/10) of other Gram-negative bacilli (Table 1).

Analysis of incomplete ID, misidentification, and no ID

Incomplete ID: Two (0.57% of 349) *Salmonella* species. Isolates, for which "high confidence scores" (score 2.019 and 2.051) were identified only for genus without giving species. By conventional serotyping method, both isolates were identified as *Salmonella* serogroup C_1 and C_2.

Misidentification: One (0.29% of 349) *Streptococcus mitis* isolate was misidentified as *Streptococcus pneumoniae*, although the Bruker's MALDI Biotyper system gave high confidence score (2.146).

No ID: Forty-nine isolates (14.04% of 349) with "no ID" results for Bruker's MALDI Biotyper system; 39 isolates (11.17% of 349) were Gram-positive bacteria, comprising *Staphylococcus haemolyticus* (n=7), *Staphylococcus capitis* (n=6), *Granulicatella adiacens* (n=6), *Staphylococcus epidermidis* (n=3), *Streptococcus gallolyticus* (n=2), *Streptococcus anginosus* (n=2), *Corynebacterium propinquum* (n=2), and one each of *S. aureus*, *Staphylococcus hominis*, *Streptococcus parasanguis*, *Kocuria varians*, *Kocuria marina*, *Micrococcus luteus*, *Corynebacterium afermentans*, *Corynebacterium singular*, *Corynebacterium striatum*, *Dermabacter hominis*, and *Dietzia cinnamea*. The remaining 10 isolates (2.87% of 349) were Gram-negative bacteria consisting of: *P. aeruginosa* (n=2), *Comamonas testosterone* (n=2), *Ralstonia pickettii* (n=2), and one each of *Stenotrophomonas maltophili*, *P. aeruginosa*, *Moraxella osloensis*, *Vibrio albensis*, and *Providencia stuartii*. The most common microorganism group with no ID was CoNS at 4.87% (17 of 349).

Direct ID versus conventional ID using Bruker's MALDI Biotyper system

Conventional ID by Bruker's MALDI Biotyper system, using pure colonies from subculture plates, identified 342 (97.99%) of 349 isolates correctly to species level. Two *Salmonella* isolates were identified to genus level only, without species ID. The remaining five (1.43%) isolates were not identified, three isolates (one *D. cinnamea* and two *Comamonas testosteroni*) had a log (score) less than 1.7, and two isolates (*Kocuria varians* and *Providencia rettgeri*) had low discrimination. The direct ID results were concordant with those of the conventional culture-based method in 87.11% (304/349) of the isolates, with a Cohen's kappa value (κ) of 0.21; $p<0.05$ indicating fair agreement. The percentage of correct species ID with the conventional method was significantly higher than that of the direct ID method [85.10% (297/349) versus 97.99% (342/349), $p<0.05$]. A comparison of performance in species-level ID for each group of bacteria between direct method and the conventional method is shown in Table 1. There was no significant difference between the two methods in the correct ID at the species level of *S. aureus*, Enterococci, Enterobacteriaceae, non-fermenting bacilli, and other Gram-negative bacilli ($p>0.05$). In contrast, the direct method achieved low rates of correct ID for CoNS, streptococci, other Gram-positive bacteria, and was, therefore, significantly less accurate than the conventional method ($p<0.05$).

Direct AST

Three hundred forty-nine (158 Gram-positive bacteria, 191 Gram-negative bacteria) isolates, 16 Gram positive isolates (six *Corynebacterium* species, five *Bacillus* species, two *Kocuria* species, one *Micrococcus* species, one *D. hominis*, and one *D. cinnamea*) were excluded for susceptibility tests due to there being no interpretation criteria available

Table 1. Direct and conventional identification results of 349 isolates compared with final identification

Final identification	n	Conventional ID method, n (%)				Direct ID method, n (%)				p-value*
		Correct ID	Inc ID	No ID	Mis ID	Correct ID	Inc ID	No ID	Mis ID	
Staphylococcus aureus	34	34 (100)	0 (0.00)	0 (0.00)	0 (0.00)	33 (97.06)	0 (0.00)	1 (0.29)	0 (0.00)	NS
Coagulase-negative staphylococci	55	55 (100)	0 (0.00)	0 (0.00)	0 (0.00)	38 (69.09)	0 (0.00)	17 (30.91)	0 (0.00)	<0.05
Staphylococcus capitis	18	18				12		6		
Staphylococcus caprae	1	1				1				
Staphylococcus epidermidis	14	14				11		3		
Staphylococcus haemolyticus	12	12				5		7		
Staphylococcus hominis	5	5				4		1		
Staphylococcus nepalensis	1	1				1				
Staphylococcus pettenkoferi	1	1				1				
Staphylococcus warneri	3	3				3				
Streptococcus species	36	36 (100)	0 (0.00)	0 (0.00)	0 (0.00)	30 (83.33)	0 (0.00)	5 (13.89)	1 (2.78)	<0.05
Streptococcus agalactiae	8	8				8				
Streptococcus anginosus	3	3				1		2		
Streptococcus constellatus	2	2				2				
Streptococcus dysgalactiae	3	3				3				
Streptococcus gallolyticus	4	4				2		2		
Streptococcus mitis	1	1							1	
Streptococcus oralis	1	1				1				
Streptococcus parasanguis	1	1						1		
Streptococcus pneumoniae	5	5				5				
Streptococcus pyogenes	7	7				7				
Streptococcus salivarius	1	1				1				
Enterococcus species	10	10 (100)	0 (0.00)	0 (0.00)	0 (0.00)	10 (100)	0 (0.00)	0 (0.00)	0 (0.00)	NS
Enterococcus faecalis	4	4				4				
Enterococcus faecium	2	2				2				
Enterococcus gallinarum	4	4				4				
Other Gram-positive bacteria	23	21 (91.30)	0 (0.00)	2 (8.70)	0 (0.00)	7 (30.43)	0 (0.00)	16 (69.57)	0 (0.00)	<0.05
Kocuria varians	1			1				1		
Kocuria marina	1	1						1		
Micrococcus luteus	1	1						1		
Granulicatella adiacens	7	7				1		6		
Bacillus cereus	2	2				2				
Bacillus flexus	1	1				1				
Bacillus megaterium	1	1				1				
Bacillus pumilus	1	1				1				
Corynebacterium afermentans	1	1						1		
Corynebacterium propinquum	2	2						2		
Corynebacterium singulare	1	1						1		
Corynebacterium striatum	2	2				1		1		
Dermabacter hominis	1	1						1		
Dietzia cinnamea	1	0		1				1		
Total Gram-positive bacteria	158	156 (98.73)	0 (0.00)	2 (1.27)	0 (0.00)	118 (74.68)	0 (0.00)	39 (24.68)	1 (0.63)	<0.05

n=number of isolates tested; ID=identification; Inc ID=incomplete identification; No ID=no identification; Mis ID=misidentification; NS=not statistically significant

* p-values are calculated by comparison of direct identification with conventional identification (chi-square test)

Table 1. (continued)

Final identification	n	Conventional ID method, n (%)				Direct ID method, n (%)				p-value*
		Correct ID	Inc ID	No ID	Mis ID	Correct ID	Inc ID	No ID	Mis ID	
Enterobacteriaceae	137	134 (97.81)	2 (1.46)	1 (0.73)	0 (0.00)	134 (97.81)	2 (1.46)	1 (0.73)	0 (0.00)	NS
Escherichia coli	92	92				92				
Klebsiella pneumoniae	27	27				27				
Morganella morganii	2	2				2				
Proteus mirabilis	5	5				5				
Proteus vulgaris	1	1				1				
Providencia stuartii	1	0		1				1		
Salmonella group C1	1	0	1				1			
Salmonella group C2	1	0	1				1			
Serratia marcescens	7	7				7				
Non-fermenting bacilli	44	42 (95.45)	0 (0.00)	2 (4.55)	0 (0.00)	37 (84.09)	0 (0.00)	7 (15.91)	0 (0.00)	NS
Achromobacter xylosoxidans	2	2				2				
Acinetobacter baumannii complex	17	17				17				
Acinetobacter ursingii	1	1				1				
Capnocytophaga sputigena	1	1				1				
Chryseobacterium gleum	3	3				3				
Comamonas testosteroni	2	0		2				2		
Elizabethkingia meningosepticum	1	1				1				
Pseudomonas aeruginosa	14	14				12		2		
Ralstonia pickettii	2	2						2		
Stenotrophomonas maltophilia	1	1						1		
Other Gram-negative bacilli	10	10 (100)	0 (0.00)	0 (0.00)	0 (0.00)	8 (80.00)	0 (0.00)	2 (20.00)	0 (0.00)	NS
Chromobacterium violaceum	2	2				2				
Moraxella catarrhalis	3	3				3				
Moraxella osloensis	1	1						1		
Vibrio albensis	2	2				1		1		
Vibrio vulnificus	2	2				2				
Total Gram-negative bacteria	191	186 (97.38)	2 (1.05)	3 (1.57)	0 (0.00)	179 (93.72)	2 (1.05)	10 (5.24)	0 (0.00)	NS
Total	349	342 (97.99)	2 (0.57)	5 (1.43)	0 (0.00)	297 (85.10)	2 (0.57)	49 (14.04)	1 (0.29)	<0.05

n=number of isolates tested; ID=identification; Inc ID=incomplete identification; No ID=no identification; Mis ID=misidentification; NS=not statistically significant

* p-values are calculated by comparison of direct identification with conventional identification (chi-square test)

for disk diffusion results. The remaining 333 isolates (142 Gram-positive bacteria, 191 Gram-negative bacteria) were evaluated for comparison of direct AST and conventional AST, resulting in a total of 4,009 microorganism-antimicrobial combinations. Of these, the direct AST showed an overall categorical agreement of 98.20% (3,937/4,009) with 1.80% (72/4,009) minor errors (mEs), no major errors (MEs), and no very major errors (VMEs), as described in Table 2. For a detailed overview of all errors see Table 3.

For 142 Gram-positive isolates, 1,016 microorganism-antimicrobial combinations were analyzed. There was categorical agreement of 99.51% (1,011/1,016) for the antimicrobials tested with 0.49% (5/1,016) mEs. The mEs were observed for chloramphenicol (2.38%), erythromycin (0.76%), gentamicin (2.06%) and trimethoprim/sulfamethoxazole (1.08%). These five mEs were detected as follows: with gentamicin in *S. epidermidis* and *S. hominis*, with trimethoprim/sulfamethoxazole in *S. epidermidis*, with erythromycin in *Staphylococcus nepalensis* and with chloramphenicol in *Streptococcus dysgalactiae* (Table 3).

For 191 Gram-negative isolates, 2,993 microorganism-antimicrobial combinations were analyzed.

Table 2. Antimicrobial susceptibility testing results between the direct AST and the conventional AST

Antimicrobial agent	Antimicrobial tested	Categorical agreement n (%)	Minor error n (%)	Major error n (%)	Very major error n (%)
Gram-positive bacteria	1,016	1,011 (99.51)	5 (0.49)	0 (0.00)	0 (0.00)
Cefoxitin (FOX)	89	89 (100)	0 (0.00)	0 (0.00)	0 (0.00)
Cefotaxime (CTX)	43	43 (100)	0 (0.00)	0 (0.00)	0 (0.00)
Chloramphenicol (C)	43	42 (97.62)	1 (2.38)	0 (0.00)	0 (0.00)
Clindamycin (DA)	132	132 (100)	0 (0.00)	0 (0.00)	0 (0.00)
Erythromycin (E)	132	131 (99.24)	1 (0.76)	0 (0.00)	0 (0.00)
Gentamicin (GM)	99	97 (97.94)	2 (2.06)	0 (0.00)	0 (0.00)
Fusidic acid (FD)	89	89 (100)	0 (0.00)	0 (0.00)	0 (0.00)
Penicillin (P)	38	38 (100)	0 (0.00)	0 (0.00)	0 (0.00)
Tetracycline (TE)	10	10 (100)	0 (0.00)	0 (0.00)	0 (0.00)
Teicoplanin (TEC)	99	99 (100)	0 (0.00)	0 (0.00)	0 (0.00)
Trimethoprim/sulfamethoxazole (SXT)	94	93 (98.92)	1 (1.08)	0 (0.00)	0 (0.00)
Vancomycin (VA)	138	138 (100)	0 (0.00)	0 (0.00)	0 (0.00)
Ampicillin (AMP)	10	10 (100)	0 (0.00)	0 (0.00)	0 (0.00)
Gram-negative bacteria	2,993	2926 (97.76)	67 (2.24)	0 (0.00)	0 (0.00)
Amikacin (AK)	186	186 (100)	0 (0.00)	0 (0.00)	0 (0.00)
Amoxicillin-clavulanic acid (AMC)	156	143 (91.67)	13 (8.33)	0 (0.00)	0 (0.00)
Ampicillin/sulbactam (SAM)	186	170 (91.40)	16 (8.60)	0 (0.00)	0 (0.00)
Ampicillin (AMP)	152	151 (99.34)	1 (0.66)	0 (0.00)	0 (0.00)
Cefoperazone/sulbactam (SCF)	34	32 (94.12)	2 (5.88)	0 (0.00)	0 (0.00)
Cefepime (FEP)	186	183 (98.39)	3 (1.61)	0 (0.00)	0 (0.00)
Cefotaxime (CTX)	152	147 (96.71)	5 (3.29)	0 (0.00)	0 (0.00)
Cefoxitin (FOX)	152	152 (100)	0 (0.00)	0 (0.00)	0 (0.00)
Ceftazidime (CAZ),	186	182 (97.85)	4 (2.15)	0 (0.00)	0 (0.00)
Ceftriaxone (CRO)	152	152 (100)	0 (0.00)	0 (0.00)	0 (0.00)
Ciprofloxacin (CIP)	186	184 (98.92)	2 (1.08)	0 (0.00)	0 (0.00)
Ertapenem (ETP)	152	151 (99.34)	1 (0.66)	0 (0.00)	0 (0.00)
Gentamicin (GM)	186	184 (98.92)	2 (1.08)	0 (0.00)	0 (0.00)
Imipenem (IPM)	186	186 (100)	0 (0.00)	0 (0.00)	0 (0.00)
Levofloxacin (LVX)	187	184 (98.40)	3 (1.60)	0 (0.00)	0 (0.00)
Meropenem (MEM)	186	186 (100)	0 (0.00)	0 (0.00)	0 (0.00)
Piperacillin/tazobactam (TZP)	186	177 (95.16)	9 (4.84)	0 (0.00)	0 (0.00)
Trimethoprim/sulfamethoxazole (SXT)	157	151 (96.18)	6 (3.82)	0 (0.00)	0 (0.00)
Tigecycline (TGC)	18	18 (100)	0 (0.00)	0 (0.00)	0 (0.00)
Erythromycin (E)	3	3 (100)	0 (0.00)	0 (0.00)	0 (0.00)
Tetracycline (TE)	4	4 (100)	0 (0.00)	0 (0.00)	0 (0.00)
Total	4,009	3,937 (98.20)	72 (1.80)	0 (0.00)	0 (0.00)

n=number of isolates tested

There was agreement of 97.76% (2,926/2,993) for the antimicrobials tested with 2.24% (67/2,993) mEs. The mEs were observed for amoxicillin-clavulanic acid (8.33%), ampicillin/sulbactam (8.60%), ampicillin (0.66%), cefoperazone/sulbactam (5.88%), cefepime (1.61%), cefotaxime (3.29%), ceftazidime (2.15%), ciprofloxacin (1.08%), ertapenem (1.08%), gentamicin (1.08%), levofloxacin (1.60%),

Table 3. The discrepancy of direct susceptibility testing with conventional antimicrobial susceptibility testing

Microorganism	Minor error	Major error	Very major error
Gram-positive bacteria	5 (0.49%)	0	0
Staphylococcus epidermidis (n=2)	2; gentamicin (n=1), trimethoprim/sulfamethoxazole (n=1)	0	0
Staphylococcus hominis (n=1)	gentamicin (n=1)	0	0
Staphylococcus nepalensis (n=1)	erythromycin (n=1)	0	0
Streptococcus dysgalactiae (n=1)	chloramphenicol (n=1)	0	0
Gram-negative bacteria	67 (2.24%)	0	0
Escherichia coli (n=33)	40; amoxicillin-clavulanic acid (n=13), ampicillin/sulbactam (n=13), ampicillin(n=1), cefepime(n=2), ceftazidime(n=3), ciprofloxacin(n=1), gentamicin(n=1), levofloxacin(n=1), piperacillin/tazobactam(n=5), trimethoprim/sulfamethoxazole (n=1)	0	0
Klebsiella pneumoniae (n=4)	3; ampicillin/sulbactam (n=1), cefotaxime (n=1), piperacillin/tazobactam (n=2)	0	0
Morganella morganii (n=2)	ampicillin/sulbactam (n=2)	0	0
Proteus vulgaris (n=1)	ciprofloxacin (n=1)	0	0
Serratia marcescens (n=5)	7; cefotaxime (n=4), trimethoprim/sulfamethoxazole (n=3)	0	0
Achromobacter xylosoxidans (n=2)	3; levofloxacin (n=2), cefepime (n=1)	0	0
Acinetobacter baumannii complex (n=1)	gentamicin (n=1)	0	0
Acinetobacter ursingii (n=1)	piperacillin/tazobactam (n=1)	0	0
Chryseobacterium gleum (n=1)	trimethoprim/sulfamethoxazole (n=1)	0	0
Elizabethkingia meningosepticum (n=1)	trimethoprim/sulfamethoxazole (n=1)	0	0
Pseudomonas aeruginosa (n=2)	cefoperazone/sulbactam (n=2)	0	0
Ralstonia pickettii (n=1)	2; ceftazidime (n=1), piperacillin/tazobactam (n=1)	0	0
Chromobacterium violaceum (n=1)	ertapenem (n=1)	0	0
Total	72 (1.80%)	0	0

piperacillin/tazobactam (4.84%), and trimethoprim/sulfamethoxazole (3.82%). Among the 67 mEs, *E. coli* was the major cause of 1.34% of mEs (40/2,993); most of the mEs derived from results testing amoxicillin-clavulanic acid (16 errors) and ampicillin/sulbactam (13 errors) (Table 3).

Discussion

Rapid, accurate ID and AST are critically needed for the successful treatment of patients with BSIs. Direct separation of viable microbial cells from positive blood culture broths without subculture is one approach to reduce turnaround time for microbial ID and susceptibility testing results by approximately 18 to 24 hours[28-30]. In the present study, the authors evaluated the performance of ID and AST of bacteria directly from positive blood cultures by using differential centrifugation combined with MALDI-TOF MS and Kirby-Bauer disk diffusion method.

In the first phase of the present study, the overall correct ID to the species level by direct ID method (direct ID method) was achieved in 85.10% (297/349) of isolates, which was significantly lower than the conventional method [97.99% (342/349), $p<0.05$]. It was comparable to other studies with correct ID rates ranging from 82.9% and 85.5%[14-16]. Interestingly, the inferior performance of direct ID method was mainly as a result of non-reliable ID (log score of less than 1.7; 49 isolates) rather than incomplete ID (two isolates) and misidentification (one isolate). Of these 49 isolates, 43 were identified correctly to the species level with conventional ID method using colonies isolated by subculture. Therefore, these data suggest that non-reliable results may be related to the insufficient concentration of bacteria in the blood culture broth, cell wall composition and interference of the background signals, which has previously been described by various authors[17,31]. For the remaining six isolates, even the conventional ID method could not accurately identify any uncommon bacteria species. That may have been due to an absence of sufficient proteomic profiles in the Biotyper database[25]. In addition, there were 30 isolates, most commonly encountered as contaminants

in blood culture, for which the clinical impact of errors was very low: *S. epidermidis* group, viridans group streptococci, *Micrococcus* species, *Kocuria* species, *Corynebacterium* species, and various coryneforms. Furthermore, there was one incorrect ID of the *S. mitis* as *S. pneumoniae* as the MALDI-TOF MS system was unable to readily distinguish between *S. mitis* group and *S. pneumoniae*. Furthermore, two isolates of *Salmonella* were identified only at the genus level but limited to type or subtype *Salmonella* serovars. These problems were solved using the bile solubility test for the differentiation of *S. pneumoniae* from other mitis group streptococci and serological test for epidemiological typing of *Salmonella* species.

According to the sub-analyses for each group of bacteria (Table 1), the direct ID method performed as equally well as the conventional ID method for species ID of Gram-negative isolates (including Enterobacteriaceae, non-fermenting bacilli and other Gram-negative bacilli), *S. aureus* and Enterococci ($p>0.05$). On the other hand, in Streptococci, CoNS and other Gram-positive isolates the percentage identified to the species level with direct ID method was significantly lower than conventional ID method ($p<0.05$), especially for other Gram-positive isolates, at only 30.43%.

The authors propose that the direct ID method is performed as the preliminary species ID of all clinical isolates. If reliable ID is not achieved, the conventional ID method is used as the definitive ID, suggesting that testing from sub-culture plates is still required in some cases to validate test results before the final report is issued. Nevertheless, the direct ID method allows for rapid ID results within 30 to 45 minutes starting from the time of detection of a positive signal, and it is nearly the same time as the reporting of Gram-stain results. This method could provide important guidance regarding the clinical significance of isolates for clinicians that will lead to an appropriate choice of antibiotics even in the absence of susceptibility testing.

In the second phase of the present study, the authors compared the accuracy of direct AST with conventional AST, both using disk diffusion method. The overall categorical agreement was 98.20% for all antimicrobials tested and the overall error rates were 1.80% mEs and no MEs or VMEs, all of them were in agreement with the US FDA acceptance criteria for the accurate assessment of susceptibility test systems (categorical agreement 90% or more, with mEs of 10% or less, MEs of 3% or less, and VMEs of 1.5% or less). However, the overall error rates obtained in the present study were lower than previously reported by other authors[19,32-34].

For Gram-positive bacteria, categorical agreement was found in 99.51%, with only 0.49% minor errors. These minor errors were seen with gentamicin, trimethoprim/sulfamethoxazole, erythromycin in three isolates of CoNS, and with chloramphenicol in one isolate of *S. dysgalactiae* (Table 3). The frequencies of minor errors and clinical impact of these errors is very low. Importantly, all *S. aureus* and Enterococci showed complete categorical agreement with no errors for any drug tested. These results suggest that direct AST method is reliable for the detection of methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant enterococci (VRE).

Among Gram-negative bacteria, categorical agreement was found in 97.76%, with 2.24% minor errors. Ampicillin or sulbactam and amoxicillin-clavulanic acid caused high minor errors (Table 3) but were still within the FDA limits. In addition, 33 isolates of *E. coli* were found to be the major cause of the minor error, which may be due to the high number of *E. coli* isolates in the present study. Moreover, complete (100%) categorical agreement was detected for imipenem, meropenem and ertapenem for all Enterobacteriaceae and non-fermenting bacilli isolates tested, demonstrating that direct AST use is well suited for detection of carbapenem resistance in these isolates.

The present study suggests that direct AST method provided accuracy of susceptibility testing for wild-type and multidrug-resistant strains in both monomicrobial Gram-positive and Gram-negative bacteria, and reduced turnaround time by 18 to 24 hours, as compared to the conventional AST method. It may reduce inappropriate antibiotic use, morbidity, mortality, and health care costs.

Conclusion

In conclusion, differential centrifugation technique is used to isolate bacteria directly from positive blood culture broths for ID by MALDI-TOF MS (direct ID) and susceptibility testing by disk diffusion method (direct AST). It is a simple, rapid, and inexpensive method. Direct ID provided excellent results for Gram-negative isolates, *S. aureus* and Enterococci (except for CoNS, Streptococci, other Gram-positive cocci and Gram-positive bacilli), but direct AST provided excellent results in Gram-negative and Gram-positive isolates. Thus, the present study suggests that direct ID and AST with significant reduced turnaround time of 18 to 24 hours as compared to the conventional method could lead to improved

clinical outcomes and reduced inappropriate antibiotic use and health care costs.

What is already known on this topic?

Blood cultures are an important part of the evaluation of patients with suspected sepsis. Rapid ID and AST of the causative pathogens of BSIs is crucial for targeted antibiotic therapy and patient management. Currently, MALDI-TOF MS has also been evaluated as a useful tool for the rapid ID of pathogens in clinical microbiology laboratories. Although originally devised for ID of pathogens from isolated colonies, this technique has been recently applied and successfully used for ID of pathogens directly from positive blood cultures without subculture process on agar plates. However, there has been no previous study of ID of bacteria directly from positive blood cultures using MALDI-TOF MS in Thailand.

What this study adds?

The differential centrifugation technique is a preparation method that can be used for separating and concentrating bacterial cells directly from monomicrobial positive blood culture bottles. The bacterial cell pellets are then used for ID by MALDI-TOF MS and AST by Kirby-Bauer disk diffusion method in the same time period. The direct ID results can be reported within 30 to 45 minutes after the positive signal with a species-level accuracy of 85.10%. While the direct AST provides excellent susceptibility testing results at least 18 to 24 hours earlier than the conventional AST.

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Conflicts of interest

The authors declare no conflict of interest.

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