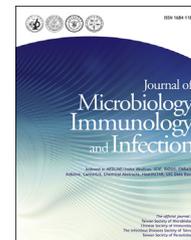




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Original Article

A simple method for rapid microbial identification from positive monomicrobial blood culture bottles through matrix-assisted laser desorption ionization time-of-flight mass spectrometry



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KEYWORDS

Blood culture;
Direct identification;
MALDI-TOF MS

Abstract *Background and purpose:* Rapid identification of microbes in the bloodstream is crucial in managing septicemia because of its high disease severity, and direct identification from positive blood culture bottles through matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) can shorten the turnaround time. Therefore, we developed a simple method for rapid microbiological identification from positive blood cultures by using MALDI-TOF MS.

Methods: We modified previously developed methods to propose a faster, simpler and more economical method, which includes centrifugation and hemolysis. Specifically, our method comprises two-stage centrifugation with gravitational acceleration (*g*) at 600*g* and 3000*g*, followed by the addition of a lysis buffer and another 3000*g* centrifugation.

Results: In total, 324 monomicrobial bacterial cultures were identified. The success rate of species identification was 81.8%, which is comparable with other complex methods.

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The identification success rate was the highest for Gram-negative aerobes (85%), followed by Gram-positive aerobes (78.2%) and anaerobes (67%). The proposed method requires less than 10 min, costs less than US\$0.2 per usage, and facilitates batch processing.

Conclusion: We conclude that this method is feasible for clinical use in microbiology laboratories, and can serve as a reference for treatments or further complementary diagnostic testing.

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Introduction

Patients who acquire bloodstream infections experience severe diseases. Orsi and Noah (2002)¹ reported a mortality rate of 35.2%–40.9% for hospital-acquired bloodstream infections. However, rapid microbial identification is helpful for treating septic patients; and also helps to minimize the length of hospital stays. As Beekmann (2003)² discovered in a study investigating the effect of positive blood culture identification turnaround time on hospital stay and cost, the average hospital stay was 18.1, 22.2, and 26.6 days when the turnaround time of positive blood culture identification was 24, 48, and 72 h, respectively. Shorter hospital stays are also more cost-effective.

Confirmation and treatment of bloodstream infections depend on blood culture results. New microbial identification techniques, such as matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), have been demonstrated to effectively reduce the turnaround time of positive blood cultures.³ In our hospital, determining whether a blood culture sample is infected takes an average of 17.1 h when using the BACTEC FX. Subsequently, 2.4 h are required for a Gram stain report, and then another 18 h is necessary for subcultures and identification to be made through MALDI-TOF MS. Although MALDI-TOF MS decreases the turnaround time by 24 h compared with traditional biochemical methods,⁴ it still requires 37.5 h for completion. By contrast, direct microbial identification from positive blood culture bottles can shorten the turnaround time to less than 24 h.

Direct identification from positive blood cultures must be simple, fast, inexpensive, and can be easily adopted by clinical microbiology laboratories. Numerous protocols for direct microbial identification from positive blood culture broths, including commercial kits, have been developed.^{5–11} However, these protocols are usually complex and entail either complicated preprocessing methods or subcultures to increase their accuracy,^{12,13} which render these methods time- and cost-intensive.^{5,6,8,7,14} In this study, we developed a simple, fast, economical and accurate method that is suitable for clinical microbiology laboratories. The identification results obtained using this simple method indicate that it is highly consistent with single colony identification. Moreover, Gram stain and preliminary strain identification can be reported within 24 h of placing the blood culture bottles in the culture system.

We suggest that the results of this study can serve as a treatment reference for clinicians.

Materials and methods

Blood cultures

Three hundred and twenty four monomicrobial blood cultures were selected for this study from Chang Gung Memorial Hospital in Linkou, a 3715-bed tertiary medical center in northern Taiwan, between August 2013 to May 2014. All blood culture bottles were incubated in BACTEC FX (Becton Dickinson, Heidelberg, Germany), an automated continuous blood culture monitoring system. The blood cultures were collected using standard procedures, and positive samples were processed further.

Processing positive blood cultures using MALDI-TOF MS

By using MALDI-TOF MS, pathogens were identified from positive blood cultures through a two-part process that comprises direct and lytic steps (Fig. 1). In Step 1, positive blood culture bottles were first shaken vigorously to ensure homogeneous mixing. Subsequently, each 1.5-mL sample was dispensed into two Eppendorf tubes (Fig. 2A) and centrifuged at 600 gravitational acceleration (*g*) for 10 s using a 45° fixed-angle rotor (Eppendorf 5415D) (Fig. 2B). Approximately 1.5 mL of each supernatant was then aspirated into a new Eppendorf tube and centrifuged at 3000*g* for 60 s (Fig. 2C). Next, the supernatant was discarded and the white layer of one of the Eppendorf tubes was picked using a toothpick and placed on a 96-spot polished steel target plate (Bruker Daltonik GmbH, Leipzig, Germany).

In Step 2, the white layer of the other Eppendorf tube was added on 1.5 mL of a lysis buffer (ratio = 8.29 g of NH₄Cl:0.037 g of Na:EDTA:1 g of KHCO₃: 1 L of water) and mixed homogeneously. After incubation for 3 min at room temperature, the mixture was centrifuged at 3000*g* for 60 s. Then, the supernatant was discarded, and the white layer (Fig. 2D) was picked up using a toothpick and placed on a 96-spot polished steel target plate (Bruker Daltonik GmbH, Leipzig, Germany).

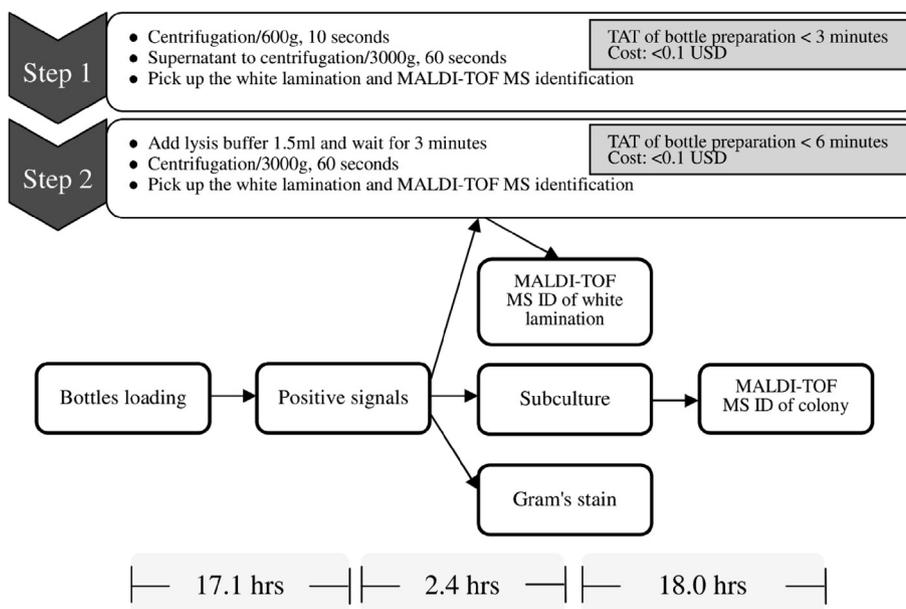


Fig. 1. Summary of estimated turnaround time for the rapid identification method (two-step process comprising direct and lytic methods) and standard colony identification through MALDI-TOF MS. g: gravitational acceleration; TAT: turnaround time; MALDI-TOF MS: matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; ID: identification.

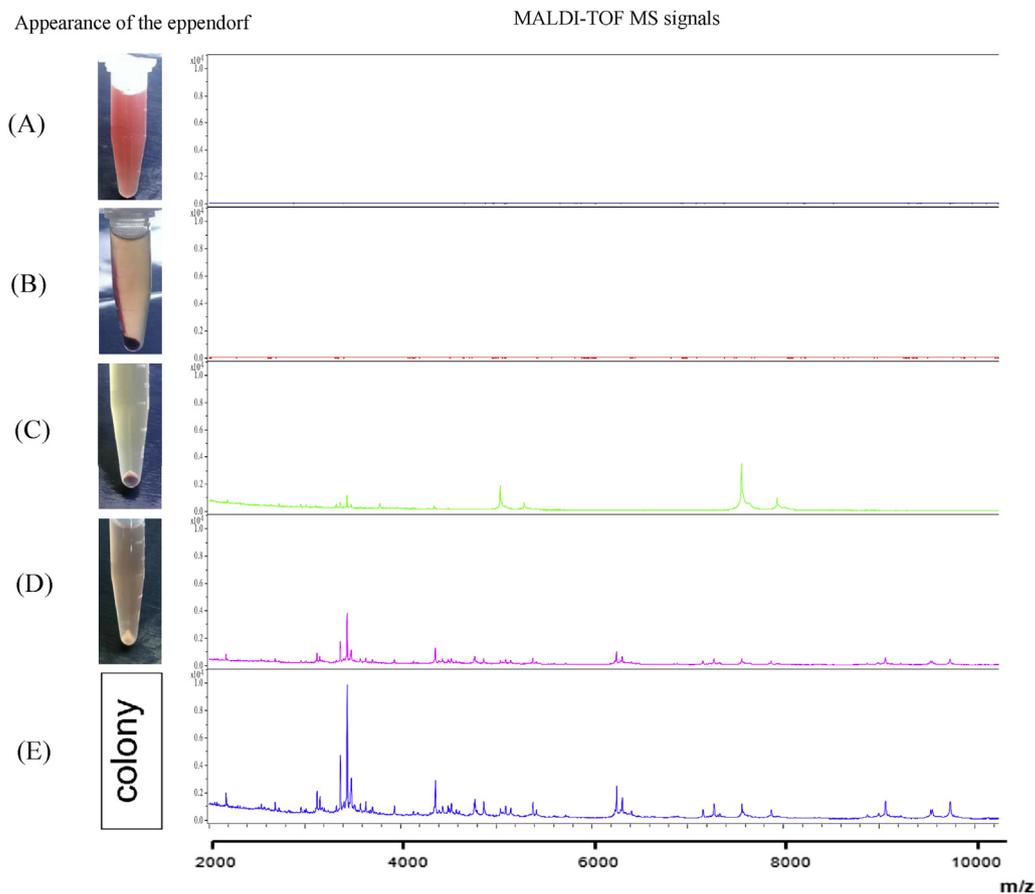


Fig. 2. Outward appearance of the Eppendorf tubes in the processing of positive blood cultures and the corresponding MALDI-TOF MS signals (demonstration: *E. coli*). (A) Dispensed liquid from vigorously shaken blood culture bottles. (B) ^aSupernatant from Panel A centrifuged at 600g. (C) ^aSupernatant from Panel B centrifuged at 3000g. (D) ^bWhite layer from Panel C mixed with a lysis buffer, incubated for 3 min at room temperature, and centrifuged at 3000g. (E) MALDI-TOF signals from a single subcultured colony. Superscript a: Fig. 2B and C are both from Step 1. Superscript b: Fig. 2D is from Step 2.

The entire procedure is summarized in Fig. 1. Three laboratory technicians were assigned for processing samples, and labeled as technicians A, B and C.

Microbial identification using MALDI-TOF MS

The microbial film was overlaid with 1 μ L of 70% formic acid. After the sample had dried, the film was overlaid with 1 μ L of the matrix solution (50% acetonitrile containing 1% α -cyano-4-hydroxycinnamic acid and 2.5% trifluoroacetic acid). After the sample had dried again, microorganism identification and data analysis were begun, for which Bruker LT Microflex MALDI-TOF MS and Bruker Biotyper 3.0 system software were adopted. All analyses were performed using standard methods. An identification was considered successful at the species level with high confidence when the score exceeded 2.0; if the score was between 2.0 and 1.7, the identification was considered successful at the genus level with adequate confidence.

Statistical analysis

Rapid identification success was defined as achieving the same result at species level with scores of high confidence through MALDI-TOF MS as was obtained using colony identification. The success rate was calculated by dividing the number of samples that could be identified at species level after Step 2 by the number of samples that could be identified at species level through single colony identification. Additionally, McNemar's test was used to analyze the difference in the success rate between the processed samples after Steps 1 and 2. Finally, the success rate of different laboratory technicians was compared using a Chi-square test. All statistical calculations were performed using Social Sciences software (SPSS) Version 18.0 (SPSS Inc., Chicago, IL, USA).

Results

Mass spectrometry signals

The mass spectrometry signals obtained at in each step are depicted in Fig. 2. Clear signals could not be detected through MALDI-TOF MS when the positive blood cultures were either unprocessed or were subjected to low-speed centrifugation only (Fig. 2A and B). Similarly, after high-speed centrifugation, the white layer provided only a weak signal (Fig. 2C). After Step 2, however, the signals became more intense (Fig. 2D) and comparable with that obtained from single colony identifications (Fig. 2E).

Identification from a single colony through MALDI-TOF MS

There were 318 aerobes and 6 anaerobes identified in the 324 selected blood cultures (Table 1). Additionally, 180 Gram-negative and 138 Gram-positive bacteria were found in the aerobes, mostly comprising Enterobacteriaceae and staphylococci (89.4%, 161/180 and 73.9%, 102/138,

respectively). The anaerobes were composed of three genera evenly.

Success rate

The numbers of the rapid microbial identification at species and genus levels were 265 (261 aerobes and 4 anaerobes) and 300 (296 aerobes and 4 anaerobes). The success rate was 81.8% (265/324) at the species level and 92.6% (300/324) at the genus level.

As shown in Table 1, 85% (153/180) of Gram-negative aerobes were identified successfully at the species level, particularly Enterobacteriaceae (90.1%, 145/161). Lower success rates at the species or genus level for several non-fermenting Gram-negative aerobes, such as *Chryseobacterium* spp. and *Acinetobacter* spp., were also noted. Gram-positive aerobes appeared at a lower rate (78.3%, 108/138) than did Gram-negative aerobes, and coagulase-negative staphylococci had particularly low rates at the species level (44.1%, 15/34). Overall, anaerobes had a 67% (4/6) success rate. Single colony identification had the highest average score, followed by processing through both Step 1 and Step 2, and only Step 1. The average score was consistent with the MALDI-TOF signal quality presented in Fig. 2.

The number of operations and the success rate of each of the three laboratory technicians are listed in Table 2. All had a success rate exceeding 80% and there was no statistical significance ($p = 0.139$).

Comparison of success rate and identification score in Steps 1 and 2

The range of identification scores through single colony identification was 2.06–2.49 (Table 1), which was higher than the range of scores achieved through the rapid microbial identification method. For Gram-negative aerobes, the success rate of identification at the species level after Step 1 was 70% (126/180) and increased to 85% (153/180) after Step 2 (Table 1); by contrast, the rate for Gram-positive aerobes was lower (41.3%, 57/138) after Step 1 but increased to 78.3% (108/138) after Step 2 (Table 1). The overall success rate after Step 1 was 57.4% (186/324), which increased to 81.8% (265/324) after Step 2 with statistical significance ($p < 0.05$).

Discussion

In this study, we developed a simple method to directly identify pathogens from positive blood cultures using a two-part process, direct and lytic steps, through MALDI-TOF MS. Both steps entailed centrifugation. The species identification success rate was 81.8%. Gram-negative aerobes had the highest success rate, followed by Gram-positive aerobes and anaerobes. Among the Gram-positive aerobes, coagulase-negative staphylococci had the lowest success rate.

We used small centrifuges with 45° fixed angles, and small-volume samples (1.5 mL/tube), which profoundly shortened the centrifugation time to 70 s in Step 1 and 60 s in Step 2. Bernard (2009)⁵ proposed two identification

Table 1 MALDI-TOF MS score ranges and averages scores obtained using each identification method for various microorganisms.

Microorganisms (no.)	The number in each MALDI-TOF score range after Step 1/Step 2			Average MALDI-TOF score of		
	Score < 1.7	1.7 ≤ score < 2	Score ≥ 2.0	Step 1	Step 2	Colony identification
Aerobes (318)	77/22	58/35	183/261			
Gram-negative bacteria (180)	30/11	24/16	126/153	1.93	2.14	2.35
Enterobacteriaceae (161)	19/4	21/12	121/145	1.98	2.18	2.36
<i>Citrobacter freundii</i>	0/0	0/0	3/3	2.19	2.24	2.36
(1)/ <i>koseri</i> (2)						
<i>Enterobacter cloacae</i>	3/1	4/3	7/10	1.77	2.00	2.25
(11)/ <i>asburiae</i> (3)						
<i>Escherichia coli</i> (78)	3/0	6/5	69/73	2.16	2.24	2.37
<i>Klebsiella pneumoniae</i> (43)	9/2	6/3	28/38	1.82	2.12	2.39
<i>Plesiomonas shigelloides</i> (1)	0/0	1/0	0/1	1.92	2.03	2.18
<i>Proteus mirabilis</i> (9)	3/0	1/0	5/9	1.44	2.24	2.29
<i>Serratia marcescens</i> (1)	1/0	0/1	0/0	0.00	1.93	2.21
<i>Salmonella</i> spp.(12)	0/1	3/0	9/11	2.13	2.17	2.40
<i>Aeromonas caviae</i> (1)/ <i>veronii</i> (2)	0/0	2/0	1/3	1.96	2.15	2.35
Non-fermenting Gram-negative bacilli (16)	11/7	1/4	4/5	1.57	1.72	2.24
<i>Acinetobacter baylyi</i>	4/3	1/1	1/2	1.52	1.76	2.15
(3)/ <i>nosocomialis</i> (2)/ <i>pitti</i> (1)						
<i>Burkholderia cepacia</i> (2)	0/0	0/0	2/2	2.20	2.19	2.22
<i>Chryseobacterium indologenes</i>	5/4	0/1	0/0	1.44	1.33	2.30
(1)/ <i>meningosepticum</i> (4)						
<i>Pseudomonas aeruginosa</i> (2)	1/0	0/1	1/1	1.73	2.04	2.42
<i>Stenotrophomonas maltophilia</i> (1)	1/0	0/1	0/0	1.01	1.92	2.15
Gram-positive bacteria (138)	47/11	34/19	57/108	1.75	2.07	2.32
<i>Enterococcus faecalis</i> (16)/ <i>faecium</i>	2/1	3/0	16/20	2.12	2.23	2.48
(4)/ <i>thailandicus</i> (1)						
<i>Staphylococcus epidermidis</i>	20/5	13/14	1/15	1.49	1.89	2.18
(15)/ <i>capitis</i> (8)/ <i>haemolyticus</i>						
(4)/ <i>hominis</i> (4)/ <i>warneri</i>						
(1)/ <i>saprophyticus</i> (1)/ <i>caprae</i> (1)						
<i>Staphylococcus aureus</i> (68)	20/3	14/5	34/60	1.80	2.13	2.35
<i>Streptococcus pyogenes</i>	2/1	4/0	5/10	1.84	2.01	2.37
(1)/ <i>dysgalactiae</i> (1)/ <i>constellatus</i>						
(1)/ <i>agalactiae</i> (6)/ <i>gallolyticus</i> (2)						
<i>Streptococcus pneumoniae</i> (4)	3/1	0/0	1/3 ^a	1.50 ^a	1.92 ^a	2.07
Anaerobes (6)	3/2	0/0	3/4	1.46	2.03	2.31
<i>Bacteroid fragilis</i> (1)/ <i>Bacteroid thetaiotaomicron</i> (1)	1/0	0/0	1/2	1.20	2.20	2.37
<i>Fusobacterium mortiferum</i> (2)	0/0	0/0	2/2	2.05	2.21	2.49
<i>Propionibacterium acnes</i> (2)	2/2	0/0	0/0	1.13	1.68	2.06

^a One of the *Streptococcus pneumoniae* had a high score after Step 1 (2.049) but very low score after Step 2 (1.299), and the average score of the MALDI-TOF MS in Step 1 and 2 did not match the success rate of MALDI-TOF score ≥ 2.

protocols, the first of which required 57 min and entailed different centrifugation speeds and the second of which required only 7 min. Most identification methods require complicated centrifugation processes,^{6,8,15} and all reported protocols need longer centrifugation times than does the method developed in this study (130 s). Moreover, this reduction in time did not affect our success rate. Quicker species identification can lead to faster clinical decisions, which renders our method more suitable than others for clinical applications.

Since the development of MALDI-TOF MS, the turnaround time for microbiological identification has shortened to approximately 25 h, with the subculture of specimens from blood being the most time-intensive process (approximately 24 h). Because our rapid identification method does not require subcultures, clinicians can be offered preliminary reports earlier, and appropriate antibiotics can be administered to patients more efficiently.

Although the centrifugation time of the MALDI Sep-sityper Kit is similar to that of our method, the workflow of

Table 2 Number of operations and the success rate of the three laboratory technicians.

	Technician A ^a	Technician B	Technician C ^b
The number of operations	64	156	104
The number of success	57	125	91
The number of failure	7	31	13
Success rate	89.1%	80.1%	87.5%
<i>p</i>	0.139		

^a Technician A is the most junior among the three operators.

^b Technician C is the most senior among the three operators.

the MALDI Sepsityper Kit contains more complicated procedures for centrifugation and extraction^{15,16}; these procedures are also more time-consuming than our method (20 min vs. ≤ 10 min).¹⁶ In addition, each test using the MALDI Sepsityper Kit costs at least €4 Euros or US\$5.5,^{7,16} whereas each test using our method costs less than US\$0.2. Moreover, the success rate of the MALDI Sepsityper is not higher than that of our method.

The main difficulty with direct microbial identification from positive blood culture pellets is impurities, specifically, erythrocyte impurities. Erythrocytes affect identification results because red blood cells are the most abundant type of blood cells, and the proteins in these cells increase the background noise signals in MALDI-TOF MS readouts. Centrifugation cannot completely remove all red blood cells. However, many studies have improved the identification rate by using a modified extraction procedure rather than the routine method of MALDI-TOF MS (which is more time-consuming and complex).^{5,7,8} In the present study, we used a lysis buffer to dissolve erythrocytes and subjected the samples to centrifugation before proceeding with MALDI-TOF MS. Our method is simpler than the modified extraction method and yields similar results, making it more appropriate for clinical use.

The identification success rate was highest for Gram-negative aerobes (85%), especially Enterobacteriaceae. Gray⁸ reported similar results, but in our method, a higher percentage of Enterobacteriaceae scored ≥ 2.0 (90.1% vs. 76.5%). Non-fermenting Gram-negative bacilli had a much lower success rate (31.3%, 5/16) at the species level, which was again similar to Gray's results. Studies reporting high identification success rates for non-fermenting bacteria have often focused only on *Pseudomonas aeruginosa* and *Acinetobacter baumannii*.^{10,17} In the present study, non-fermenting Gram-negative bacilli that remained unidentified after the rapid method was used mainly consisted of *Acinetobacter* spp. addition to *A. baumannii*, *Chryseobacterium* spp., and *Stenotrophomonas maltophilia*. However, because these strains account for only a small number of blood cultures, their misidentification seldom affects clinical practice.

Among the Gram-positive aerobes, only 44.1% (15/34) of coagulase-negative staphylococci were identified at the species level, but 88.2% (60/68) of *Staphylococcus aureus*

were identified. Low success rates have been reported for Gram-positive aerobes in most studies^{14,18,19}; similar to this study, these low scores have been primarily attributed to the low identification score for coagulase-negative staphylococci. The threshold values of 2.0 for species identification of coagulase-negative staphylococci has been noted previously,^{20,21} with most studies recommending a threshold of ≥ 1.7 . We did not discuss thresholds in this study because the threshold of ≥ 1.7 still lacks consensus. Although some studies have reported higher success rates for Gram-positive aerobes at the species level than our study,^{14,22} their methods entail complicated and expensive centrifugation and extraction procedures. Moreover, the low identification success rate for coagulase-negative staphylococci at the species level has little impact on the clinical applicability of the method, because most coagulase-negative staphylococci bacteremia is considered contamination.

Overall, this simple method is very simple and easy to use. Three laboratory technicians performed the protocol, two of whom were inexperienced (Table 2). All of the technicians had success rates (i.e., MALDI-TOF score ≥ 2 in both single colony and rapid microbial identification) of more than 80%. These results confirm that our method can be easily employed by inexperienced laboratory staff.

Our study had some limitations. In particular, the number of anaerobes was too small to optimally represent the success rate of rapid identification (although the possibility of anaerobes causing bacteremia is relatively low).

In conclusion, the developed method involves a shorter centrifugation time and does not require a modified extraction step. The method takes less than 10 min to complete, costs less than US\$0.2 per usage, and allows 10 samples to be centrifuged simultaneously. Its identification success rate is also higher than that previously reported for non-modified extraction methods and is similar to that of more complicated protocols, including those using the Sepsityper kit. Finally, our method is suitable for clinical laboratory use with positive blood culture samples and can be applied appropriately by even inexperienced technicians. The developed method offers a preliminary report 18 h faster than conventional tests, thus facilitating early treatment by clinicians.

Conflicts of interest statement

All authors declare that they do not have any conflicts of interest.

Acknowledgments

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