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Direct Identification of 80 Percent of Bacteria from Blood Culture Bottles by Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry Using a 10-Minute Extraction Protocol

Loïc Simon,[a] Estelle Ughetto,[a] Alice Gaudart,[a] Nicolas Degand,[a] Romain Lotte,[a,b,c] Raymond Ruimy[a,b,c]

[a]Laboratoire de Bactériologie, CHU de Nice, Hôpital de l'Archet 2, Nice, France
[b]Université Côte d'Azur, Nice, France
[c]INSERM U1065, C3M, Equipe 6 Virulence Microbienne et Signalisation Inflammatoire, Bâtiment Universitaire Archimed, Nice, France

ABSTRACT Matrix-assisted laser desorption ionization–time of flight mass spectrometry is not widely used to identify bacteria directly from positive blood culture bottles (BCBs) because of overlong protocols. The objective of this work was to develop and evaluate a simple extraction protocol for reliable identification from BCBs. The 10-min protocol was applied over a 5-month period. Direct identifications on day 0 were compared with those obtained from colonies on day 1 [log(score) of ≥2]. We evaluated a range of seven log(score) thresholds on day 0 from 1.4 to 2.0 to find the lower confidence score that provides the higher percentage of direct identifications without loss of accuracy. With a log(score) threshold of ≥1.5 at day 0, our protocol allowed us to identify 80% of bacteria in 632 BCBs (96% of *Enterobacteriaceae*, 95% of *Staphylococcus aureus*, 92% of enterococci, and 62% of streptococci). At least one bacterial species of the mixture was identified in 77% of the polymicrobial samples. The rapidity and reliability of the protocol were factors in its adoption for routine use, allowing us to save up to 24 h in identifying 80% of the bacteria in the BCBs and, thus, to supply useful information to adapt antibiotic therapy when necessary. We currently provide reliable daily direct identifications of staphylococci, enterococci, *Enterobacteriaceae*, *Pseudomonas aeruginosa,* and beta-hemolytic streptococci.

KEYWORDS MALDI-TOF, bacteremia, blood culture bottle, bloodstream infection, direct identification of bacteria, extraction protocol

Bloodstream infections are part of vital diagnoses made daily by microbiology laboratories (1–3). The pathogens responsible for these cases of bacteremia must be identified as fast as possible in order to provide prompt and appropriate treatment to ensure patient survival (4).

Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) is known to be a reliable technique for identifying bacteria from plate cultures using pathogen protein profiles (5, 6). However, the time it takes for the colonies to grow on an agar plate cannot be accelerated, which means that specific bacterial identification cannot be made until the next day.

To overcome this constraint, several bacterial identification protocols using MALDI-TOF MS have been developed in recent years, which essentially take one or the other of two different approaches. The first, identification after short-term incubation on an agar plate, has yielded good results but still requires 4 to 8 h to identify 80% of bacteria at the species level (7–9), resulting in evident workflow issues. The other approach is to identify bacteria directly from blood culture bottles (BCBs) (10–13), which we adopted

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Address correspondence to Romain Lotte, lotte.r@chu-nice.fr, or Raymond Ruimy, ruimy.r@chu-nice.fr.

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in our study because of its potential to significantly speed up bacterial identification (14).

However, the protocols described so far, such as the manufacturer's instructions for the Sepsityper kit (Bruker) (15), are still relatively time-consuming and arduous for routine use (12, 16–19), so there is still need for a quick, simple, reliable method of direct bacterial identification that would benefit patients.

The objective of this study was to compare our in-house 10-min protocol for direct bacterial identification from positive BCBs with the conventional method from plate cultures using the MALDI-TOF MS technique. Moreover, we wanted to integrate this technique into our laboratory routine in order to simplify and accelerate bacterial identification, which would allow us to promptly implement the best presumptive antibiotic treatment for the patient before the antimicrobial susceptibility results.

MATERIALS AND METHODS

This study was conducted at the laboratory of bacteriology at the University Hospital Centre of Nice, France, from December 2015 to May 2016. During this 5-month period, 2,371 BCBs were detected positive. For practical reasons, direct identifications had to be carried out during the morning, so we included in our study every BCB (1 per patient) detected positive from 7 p.m. (when the laboratory closed) to 12 a.m. A first MALDI-TOF MS analysis was performed at 8 a.m. for BCBs detected positive between 7 p.m. and 8 a.m., and a second was performed at 12 a.m. for BCBs detected positive between 8 a.m. and 12 a.m. A total of 713 BCBs were subjected to MALDI-TOF MS to compare the commonly used bacterial identification from agar plates with our in-house protocol of direct bacterial identification from positive BCBs. As the purpose of this study was to compare bacterial identification obtained from colonies with that obtained directly from BCBs, we excluded samples where no culture had grown on the agar plates, samples not identified by MALDI-TOF MS on agar plates and requiring 16S rRNA gene sequencing, and samples that were found to be yeast infections.

Blood culture processing. All aerobic (BacT/Alert FA plus), anaerobic (BacT/Alert FN plus), and pediatric/aerobic (BacT/Alert PF plus) BCBs were incubated in a BacT/Alert 3D automated device (bioMérieux, Marcy l'Etoile, France) for up to 5 days at 37°C until they were flagged as positive. Every positive BCB (day 0) was Gram stained using the PREVI color automated Gram staining system (bioMérieux), and various agar plates were inoculated with sheep blood agar (Becton Dickinson, Rungis, France), chocolate (Becton Dickinson), Drigalski agar (Bio-Rad, Marnes la Coquette, France), or Columbia CAP selective agar with sheep blood (Oxoid, Dardilly, France), depending on the results of the Gram staining. In addition, when the Gram-stained smear showed Gram-positive cocci in pairs and chains at day 0, an optochin disc (Mast Diagnostic, Amiens, France) was added to the panel of antimicrobial discs tested by the EUCAST disc diffusion method (http://www.eucast.org/ast_of_bacteria/disk_diffusion_methodology/) in order to differentiate *Streptococcus pneumoniae* from others isolates belonging to the *Streptococcus mitis/oralis* group. The disc was tested on Mueller-Hinton-F agar (Bio-Rad) inoculated with the isolate and incubated overnight under 5% CO_2. The isolate was categorized as optochin sensitive when the zone of inhibition was 14 mm or greater.

After 12 to 24 h of incubation, the conventional method of bacterial identification from agar plates was performed (day 1) using MALDI-TOF MS. For the *S. mitis/oralis* group, we further performed a bile solubility test using 2% sodium deoxycholate (bile salt) to discriminate soluble *S. pneumoniae* from other bile-insoluble streptococci of the *S. mitis/oralis* group. *S. pneumoniae* type strain CIP104340T (ATCC 49619) and *S. mitis* type strain CIP 103335T (ATCC 49546) were used as positive and negative controls, respectively.

In-house blood culture broth extraction method. In addition to the standard processing method, direct bacterial identification was carried out on the first positive BCB of each patient (day 0). First, 8 drops of blood culture broth (approximately 200 μl) were added to a 1-ml solution of Triton X-100 (Sigma-Aldrich, Lyon, France) at a concentration of 0.1%. The mix was vortexed for 5 s and then centrifuged at 13,000 rpm for 2 min. The supernatant was discarded, and then a further 1 ml of 0.1% Triton X-100 was added before a second cycle of vortexing and centrifugation. The supernatant was removed again, and the pellet was ready for identification using MALDI-TOF MS.

MALDI-TOF MS. Target plates were read in a Microflex LT (Bruker, Wissembourg, France) with MALDI Biotyper 3.1 software and Bruker database 5989. The mass spectrometer was calibrated using a Bruker BTS spot (bacterial test standard *Escherichia coli*) and two internal control spots: *Escherichia coli* CIP 7624 and *Pseudomonas aeruginosa* CIP 76110. The pellet obtained on day 0 was spread onto a target plate in duplicate and dried, and then 1.2 μl of formic acid was systematically added to each spot and dried. Lastly, every spot was overlaid with 1.2 μl of α-cyano-4-hydroxycinnamic acid matrix and then dried for MALDI-TOF analysis. For identification at day 1, an individual colony grown from an overnight culture of blood broth on an agar plate was selected using an inoculating loop. This was transferred to a target plate, dried, and overlaid with matrix for MALDI-TOF analysis. Formic acid is only added if the identification has failed (see below).

Analysis of identification scores. After analysis with Microflex LT, Biotyper software (Bruker) calculated a similarity score [log(score)] by comparing the protein spectra of each spot with the database spectra. In this way we obtained ten scores per spot, ranging from higher to lower probability of valid identification. We did not consider genus in analyzing our results but focused instead on identification at the species level, this being the information most useful for assessing adequate treatment.

In accordance with the manufacturer's recommendations, we established that, for conventional analysis from agar plates, identification at the species level was valid with a log(score) of ≥2. Where the identification score was ≤2 on day 1, the sample was spread again from the colony and analyzed a second time. If the log(score) was still ≤2, the sample was analyzed a third time by MADI-TOF MS after rapid extraction (1.2 μl of pure formic acid added onto the dried pellet and overlaid by the matrix) was performed on the colony. Finally, if the log(score) was still ≤2, the sample was identified by 16S rRNA gene sequencing and then excluded from our study.

Direct bacterial identifications (day 0) were compared to those obtained on day 1 (assumed to be correct results) using seven different log(score) cutoffs, ranging from 1.4 to 2.0, to determine which provided the greatest percentages of identification on at least one of the two spots.

Repeatability and reproducibility were assessed with two patient samples containing very common bacteria responsible for bacteremia, one Gram positive (*Staphylococcus aureus*) and one Gram negative (*Escherichia coli*). To assess repeatability, one operator performed the protocol 15 times over a very short period of time (15 identifications of 2 spots in a row) in each of the two samples. To assess reproducibility, 15 different operators performed identification (2 spots) in each of the two samples. In addition, we also ascertained interoperator variability among the 19 technicians who accomplished at least 25 direct identifications during the study period.

At the end of the study, we continued our investigations in order to validate the method on the less common bacteria for which we had had insufficient data during the 5-month study period.

RESULTS

After excluding 19 fungemia cases, 8 sterile blood cultures, and 5 bacteria requiring 16S rRNA gene sequencing for identification (one *Streptococcus australis*, two *Staphylococcus petrasii*, one *Propionimicrobium lymphophilum*, and one *Bacillus* species), 681 samples in which bacteria were identified by the conventional method on day 1 were included in the study.

Of these samples, 632 were monomicrobial, of which 422 (66.8%) contained Gram-positive organisms and 210 (33.2%) contained Gram-negative organisms, representing a total of 75 different bacterial species. The remaining 49 blood cultures were polymicrobial.

Including the polymicrobial cultures, a total of 736 bacteria were analyzed by MALDI-TOF MS on day 1, of which 88 (12%) required either a second pass or a rapid extraction to reach an identification score of 2 by the conventional method from colonies.

We compared concordance results on day 0 for each bacterial species identified on day 1 in monomicrobial cultures using seven different log(score) thresholds: 1.4/1.5/1.6/1.7/1.8/1.9/2.0 (Table 1). For each of them, the number of correct identifications with a score equal to or higher than the threshold has been divided by the total number of monomicrobial samples ($n = 632$). The percentages of total correct identifications at day 0 were 80.5%/80.5%/75.9%/68%/58.5%/45.9%/32.3%, respectively. In order to assess the reliability of the identifications for each log(score) threshold, we divided the number of correct identifications by the number of samples with identification scores equal to or higher than the threshold. The percentages of concordant results by log(score) were 96.2%/99.4%/99.8%/99.8%/99.7%/100%/100%, respectively. Concordance results based on log(score) thresholds showed that the 1.5 value provided the highest total number of identifications (80.5%) while keeping the rate of concordant identifications among the samples concerned above 99%. Accordingly, we chose the 1.5 threshold to assess our protocol throughout this study. Direct identifications from BCBs were considered valid when they were the same as those obtained by the conventional method with a log(score) of ≥1.5 and could be repeated three times (same identification for the top three scores; termed three-times repeatable) on at least one of the two spots. Polymicrobial blood cultures were analyzed separately according to the same criteria.

The identifications made directly from BCBs are reported by groups of bacteria in Table 2. In all, we were able to identify bacteria at the species level in 509 out of 632 samples (80.5%) with our 10-min protocol. More precisely, we identified 75.6% of Gram-positive bacteria and 90.5% of Gram-negative bacteria. Detailed analysis showed that 94.9% of *Staphylococcus aureus* organisms were identified, along with 92.1% of enterococci (*Enterococcus faecalis*, *Enterococcus faecium*, and *Enterococcus casseliflavus*)

TABLE 1 Concordant identifications from BCBs on day 0 of the bacterial species definitively identified on day 1 on the basis of the log(score) cutoff

Definitive identification	Total no.	No. (%) log(score) of:						
		≥1.4	≥1.5	≥1.6	≥1.7	≥1.8	≥1.9	≥2.0
Bacteroides coprocola	1	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)
Bacteroides fragilis	7	6 (85.7)	6 (85.7)	6 (85.7)	5 (71.4)	3 (42.9)	3 (42.9)	3 (42.9)
Bacteroides thetaiotaomicron	1	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)
Citrobacter koseri	1	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)
Clostridium clostridioforme	1	1 (100)	1 (100)	1 (100)	1 (100)	0 (0)	0 (0)	0 (0)
Enterobacter aerogenes	13	13 (100)	13 (100)	13 (100)	13 (100)	12 (92.3)	11 (84.6)	9 (69.2)
Enterobacter cloacae	5	5 (100)	5 (100)	5 (100)	4 (80)	2 (40)	1 (20)	0 (0)
Enterococcus casseliflavus	1	1 (100)	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Enterococcus faecalis	35	32 (91.4)	32 (91.4)	30 (85.7)	28 (80)	24 (68.6)	16 (45.7)	9 (25.7)
Enterococcus faecium	2	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	1 (50)	0 (0)
Escherichia coli	100	96 (96)	96 (96)	96 (96)	91 (91)	85 (85)	70 (70)	54 (54)
Granulicatella adiacens	3	1 (33.3)	1 (33.3)	1 (33.3)	1 (33.3)	1 (33.3)	1 (33.3)	0 (0)
Hafnia alvei	2	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	1 (50)
Klebsiella oxytoca	7	7 (100)	7 (100)	7 (100)	7 (100)	7 (100)	6 (85.7)	5 (71.4)
Klebsiella pneumoniae	29	28 (96.6)	28 (96.6)	28 (96.6)	25 (86.2)	22 (75.9)	19 (65.5)	15 (51.7)
Lactobacillus rhamnosus	3	1 (33.3)	1 (33.3)	1 (33.3)	1 (33.3)	0 (0)	0 (0)	0 (0)
Leuconostoc mesenteroides	4	3 (75)	3 (75)	3 (75)	1 (25)	1 (25)	0 (0)	0 (0)
Micrococcus luteus	2	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)
Moraxella catarrhalis	1	1 (100)	1 (100)	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)
Morganella morganii	1	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)
Paracoccus yeei	1	1 (100)	1 (100)	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)
Pasteurella multocida	2	2 (100)	2 (100)	1 (50)	0 (0)	0 (0)	0 (0)	0 (0)
Prevotella bivia	1	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)
Propionibacterium acnes	4	2 (50)	2 (50)	1 (25)	1 (25)	0 (0)	0 (0)	0 (0)
Proteus mirabilis	7	7 (100)	7 (100)	7 (100)	7 (100)	6 (85.7)	6 (85.7)	5 (71.4)
Pseudomonas aeruginosa	18	12 (66.7)	12 (66.7)	10 (55.6)	10 (55.6)	7 (38.9)	6 (33.3)	5 (27.8)
Pseudomonas mosselii	1	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	0 (0)	0 (0)
Salmonella spp.	2	2 (100)	2 (100)	1 (50)	0 (0)	0 (0)	0 (0)	0 (0)
Serratia marcescens	2	2 (100)	2 (100)	2 (100)	2 (100)	1 (50)	1 (50)	0 (0)
Staphylococcus aureus	99	94 (94.9)	94 (94.9)	91 (91.9)	87 (87.9)	80 (80.8)	63 (63.6)	44 (44.4)
Staphylococcus capitis	16	13 (81.3)	13 (81.3)	13 (81.3)	11 (68.8)	9 (56.3)	8 (50)	5 (31.3)
Staphylococcus cohnii	1	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	0 (0)	0 (0)
Staphylococcus epidermidis	113	81 (71.7)	81 (71.7)	70 (61.9)	57 (50.4)	47 (41.6)	29 (25.7)	11 (9.7)
Staphylococcus equorum	1	1 (100)	1 (100)	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)
Staphylococcus haemolyticus	17	12 (70.6)	12 (70.6)	11 (64.7)	8 (47.1)	5 (29.4)	3 (17.6)	2 (11.8)
Staphylococcus hominis	46	39 (84.8)	39 (84.8)	38 (82.6)	35 (76.1)	33 (71.7)	31 (67.4)	25 (54.3)
Staphylococcus pasteuri	1	1 (100)	1 (100)	1 (100)	1 (100)	0 (0)	0 (0)	0 (0)
Staphylococcus spp.	2	1 (50)	1 (50)	1 (50)	1 (50)	0 (0)	0 (0)	0 (0)
Staphylococcus warneri	2	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	1 (50)	1 (50)
Stenotrophomonas maltophilia	1	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	0 (0)	0 (0)
Streptococcus agalactiae	1	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)
Streptococcus constellatus	4	1 (25)	1 (25)	1 (25)	0 (0)	0 (0)	0 (0)	0 (0)
Streptococcus dysgalactiae	2	1 (50)	1 (50)	1 (50)	1 (50)	1 (50)	1 (50)	1 (50)
Streptococcus gallolyticus	8	4 (50)	4 (50)	2 (25)	0 (0)	0 (0)	0 (0)	0 (0)
Streptococcus mutans	2	2 (100)	2 (100)	1 (50)	1 (50)	0 (0)	0 (0)	0 (0)
Streptococcus oralis	3	2 (66.7)	2 (66.7)	2 (66.7)	2 (66.7)	1 (33.3)	0 (0)	0 (0)
Streptococcus parasanguinis	3	1 (33.3)	1 (33.3)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Streptococcus pneumoniae	17	12 (70.6)	12 (70.6)	11 (64.7)	9 (52.9)	4 (23.5)	2 (11.8)	1 (5.9)
Streptococcus pyogenes	2	2 (100)	2 (100)	2 (100)	1 (50)	0 (0)	0 (0)	0 (0)
Streptococcus sanguinis	3	2 (66.7)	2 (66.7)	2 (66.7)	2 (66.7)	2 (66.7)	1 (33.3)	0 (0)
Log(score) of 0 or <1.4 on day 0[a]	31	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Total (75 species)	632	509 (80.5)	509 (80.5)	480 (75.9)	430 (68)	370 (58.5)	290 (45.9)	204 (32.3)
Total no. of samples by log(score)		529	512	481	431	371	290	204
Concordant results by log(score)		96.20%	99.40%	99.80%	99.80%	99.70%	100%	100%

[a]*Actinotignum* species (1), *Aggregatibacter* species (1), *Bacillus* species (1), *Bacteroides* species (1), *Brevibacterium* species (1), *Campylobacter* species (1), *Citrobacter* species (1), *Corynebacterium* species (3), *Dermabacter* species (1), *Dialister* species (1), *Gemella* species (1), *Haemophilus* species (1), *Lactobacillus* species (1), *Neisseria* species (1), *Rothia* species (1), *Staphylococcus* species (1), *Streptococcus* species (12), *Veillonella* species (1).

and 70.6% of *S. pneumoniae*. Among the Gram-negative bacteria, we identified 96.5% of *Enterobacteriaceae* and 72.7% of nonfermenting bacilli.

If we look only at those identifications with a log(score) of ≥1.5 and that were three-times repeatable, excluding samples with no MALDI-TOF results, we find they are

TABLE 2 Direct identifications on day 0 by type of bacteria [log(score) of ≥1.5]

Group	Total no.	No. concordant	% Concordant	No result
Gram-positive bacteria	422	319	75.6	103
Gram-negative bacteria	210	190	90.5	20
Total	632	509	80.5	123
Aerobic (FA + PF)	433	357	82.4	76
Anaerobic (FN)	199	152	76.4	47
Total	632	509	80.5	123
Staphylococcus aureus	99	94	94.9	5
Coagulase-negative staphylococci	200	151	75.5	49
Total	299	245	81.9	54
Streptococcus pneumoniae	17	12	70.6	5
Streptococci	40	16	40.0	24
Enterococci	38	35	92.1	3
Other Gram-positive cocci[a]	12	7	58.3	5
Total	406	315	77.6	91
Enterobacteriaceae	170	164	96.5	6
Nonfermenting bacilli[b]	22	16	72.7	6
Other Gram-negative bacilli[c]	15	9	60.0	6
Total	207	189	91.3	18
Anaerobic Gram-positive bacilli[d]	1	1	100.0	0
Other Gram-positive bacilli	15	3	20.0	12
Total	16	4	25.0	12
Gram-negative cocci[e]	3	1	33.3	2
Anaerobic organisms[f]	14	10	71.4	4

[a]*Granulicatella* species, *Leuconostoc* species, *Micrococcus* species, *Paracoccus* species, *Rothia* species, *Gemella* species.
[b]*Pseudomonas* species, *Pasteurella* species, *Stenotrophomonas* species.
[c]*Aggregatibacter* species, *Bacteroides* species, *Haemophilus* species, *Dialister* species, *Prevotella* species, *Campylobacter* species.
[d]*Clostridium* species.
[e]*Moraxella* species, *Neisseria* species, *Veillonella* species.
[f]*Bacteroides* species, *Dialister* species, *Veillonella* species, *Prevotella* species, *Clostridium* species.

highly concordant with the conventional method: 99.4% for all bacteria (100% of Gram-negative bacteria and 99.1% of Gram-positive bacteria) (Table 1). Several important groups were identified at a rate of 100%: *Staphylococcus aureus*, coagulase-negative staphylococci, and enterococci; only streptococci had a lower concordance rate of 90.3%. Only 3 specimens yielded discordant results, all belonging to the genus *Streptococcus*: one that was differently identified on each spot (day 0, *S. pneumoniae*/*Streptococcus oralis*; day 1, *S. pneumoniae*) and two that were misidentified on one spot and unidentified on the other (day 0, *S. pneumoniae*/none; day 1, *S. mitis*; day 0, *S. oralis*/none; day 1, *S. mitis*).

Correct identification at the species level was obtained on both spots in 417 (66%) samples and on one of the two spots (with no result on the other) in 92 (14.5%) samples, while 43 (6.8%) samples yielded correct identifications but with a log(score) of <1.5, and only 3 (0.5%) yielded discordant results, as described above. No identification was obtained on either of the spots in 77 (12.2%) samples.

Regarding the 49 polymicrobial cultures, our technique allowed us to identify a single bacterial species in 73.5% of cases ($n = 36$) and two bacterial species in 4.1% of cases ($n = 2$). No result was obtained in 22.4% of cases ($n = 11$).

Repeatability and reproducibility assessments showed 100% correct identifications

TABLE 3 Repeatability and reproducibility [log(score) of ≥1.5]

Sample	Repeatability		Reproducibility	
	Method	No. concordant identifications/total (%)	Method	No. concordant identifications/total (%)
E. coli	15 identifications (30 spots) performed by 1 operator	15/15 (100)	1 identification (2 spots) performed by 15 different operators	15/15 (100)
S. aureus		15/15 (100)		15/15 (100)

for both *S. aureus* and *E. coli* (Table 3). Concerning interoperator variability, 19 of the 23 laboratory technicians analyzed at least 25 samples and made correct identifications in 63% to 92.6% of cases, with a mean of 80.3% and a standard deviation of 7.6%.

In our investigations after the 5-month study period, we obtained 71.4% correct identifications of 35 *P. aeruginosa* isolates and 89.7% of 29 beta-hemolytic streptococci, including *Streptococcus agalactiae*, *Streptococcus dysgalactiae*, and *Streptococcus pyogenes*.

DISCUSSION

During our 5-month study at the University Hospital Centre of Nice, we developed a 10-min protocol capable of identifying at the species level 80.5% of the bacteria present in BCBs on day 0.

This study was carried out over a long period and included a large number of samples, which has not been common until now (20–22). We substantially shortened the duration of the protocol and reduced the amount of broth needed compared with that of other studies, which required 4- to 8-ml samples (13, 17, 19, 23, 24).

Like other protocols, ours provided better identifications of Gram-negative bacteria than of Gram-positive bacteria, as previously reported (25). Nevertheless, we correctly identified a higher rate of Gram-positive bacteria than those in other studies (18, 26) and obtained better results for *Streptococcus pneumoniae* (13, 24, 27), which may be due to use of the updated Bruker database. Overall, the performance of our technique was excellent for *Enterobacteriaceae*, enterococci, staphylococci, and nonfermenting bacilli. We failed to obtain a 100% identification rate only with streptococci, as did many other studies (10, 13, 24, 27), which means that a phenotypic identification method for agar plates appears to be necessary for these microorganisms, particularly the *S. mitis/oralis* group and *S. pneumoniae*. In our study, using MALDI-TOF MS combined with bile solubility test and optochin susceptibility test, we were able to correctly discriminate *S. pneumoniae* from other *S. mitis/oralis* group members. Indeed, 17 out of 17 isolates identified as *S. pneumoniae* based on MALDI-TOF MS at day 1 were also bile soluble and optochin susceptible. On the contrary, the 6 isolates belonging to the *S. mitis/oralis* group were all bile insoluble and optochin resistant.

Our results show that the acceptability score for direct bacterial identification from BCBs needs to be lower. In addition to bacterial proteins, BCB samples contain blood proteins, which modify the background noise in MALDI-TOF MS (28). This allowed us to lower the acceptable log(score) threshold without loss of specificity, as has been done in previous studies (15, 18–20, 27). With our modified log(score) threshold (≥1.5), our technique had a reliability of 100% for nearly all of the bacterial species and correctly identified 100% of the Gram-negative bacteria, distinguishing between *Enterobacteriaceae* and nonfermenting bacilli, between chromosomal *ampC*-producing *Enterobacteriaceae* and other species, and between anaerobic Gram-negative bacilli.

A log(score) threshold of ≥1.5 correctly identified 100% of the Gram-positive bacteria, except for streptococci, and distinguished between *S. aureus* and coagulase-negative staphylococci and between streptococci and enterococci.

It is important to emphasize the benefit of spreading samples on the target plate in duplicate, as correct identifications were obtained on both spots in only two-thirds of the samples, while the vast majority of the remaining samples yielded concordant identification on one spot and no result on the other. It would be instructive to see if

it is possible to increase the proportion of valid identifications by spreading a greater number of spots per sample. Gray et al. spread samples in triplicate in their study (19), but they did not assess the benefits obtained.

The benefit of the analysis in duplicate was also noted for the two polymicrobial cultures for which 2 bacterial species were correctly identified (*Klebsiella oxytoca* and *Enterobacter cloacae* for both samples). We considered these identifications reliable, because each of the 2 bacteria was identified on one of the two different MALDI-TOF spots (analysis in duplicate) with a log(score) of $>$1.5 and was three-times repeatable. These are interesting preliminary results for the identification at day 0 of polymicrobial samples that should be further explored in another study with a larger number of samples.

Repeatability, reproducibility, and interoperator variability are important in validating the method, although these factors have rarely been addressed in the literature. Our repeatability and reproducibility results were perfect, reaching 100% correct identifications, whereas we found very high interoperator variability in this study, indicating the importance of the manual side of the technique for successful direct identification. Our protocol now has been routinely used for 2 years, and we noticed that the pellet obtained after the last centrifugation cycle has to be perfectly dried before MALDI-TOF analysis for better identification results. This precise manual movement, consisting of completely removing the supernatant, probably explains the interoperator variability between technologists in our study. It is therefore crucial to train technicians to a high level and to ensure their competence is maintained over time.

The turnaround time for positive blood culture identification was 12 to 24 h shorter with our method than with identification on agar plates. Several studies have assessed this important finding and shown it to have significant clinical impacts (29–32). Indeed, the technique makes it possible to select an appropriate antimicrobial agent earlier and helps locate the source of infection (30). It also reduces unnecessary antimicrobial therapy by identifying contaminated blood cultures (31) and even seems to reduce patient mortality (32).

As a result of this study, we have been able to successfully integrate direct identification of staphylococci, enterococci, and *Enterobacteriaceae* from BCBs into our laboratory's daily routine. Identification of these microorganisms from BCBs is now performed on day 0, and results are immediately transmitted to clinicians. Agar plates are still inoculated at day 0, at the same time of antimicrobial susceptibility testing (AST) performed directly from blood culture bottle by the EUCAST disc diffusion method, in order to detect potential polymicrobial cultures on day 1 and to obtain isolates for AST of each bacteria recovered from mixed culture. The speed of this technique means it can be perfectly integrated into the workflow of any laboratory. As the laboratory was closed from 7 p.m. to 8 a.m. at the time of the study, BCBs that flagged positive during this time period remained in the BacT/Alert 3D automated device and procedures were carried out at 8 a.m. This could have led to additional growth of bacteria, facilitating direct identification. However, the percentage of correct direct identification realized at 8 a.m. (BCBs incubated in the BacT/Alert all night) and at 12 a.m. were not significantly different. Furthermore, our laboratory was open 24 h a day just a few weeks after the end of inclusion, and since then, direct identifications are performed day and night as the BCB flags positive; we did not notice any loss of identification accuracy. In addition, since the end of this study, we have continued our research into bacteria for which we had insufficient samples to obtain significant results and have been able to add *P. aeruginosa* and beta-hemolytic streptococci to our direct identification routine.

The method using Triton X-100 has two major advantages over the Sepsityper kit, which are that it is faster and it is less expensive. The average handling time of the Sepsityper kit is around 30 min, depending on the number of samples being processed (25), but is only 10 min for the Triton X-100 method described here. Sepsityper kit consumables cost €8.90 per sample, against €0.40 for the Triton X-100 method. It is worth noting, moreover, that routine use of the Triton X-100 method has allowed us to

reduce the number of Xpert MRSA/SA BC assays (Cepheid, Maurens-Scopont, France) performed to detect methicillin resistance in *S. aureus*. Indeed, we used to perform the Xpert MRSA/SA BC assay for every Gram-stained smear with Gram-positive cocci in clusters before any identification. Now that we can differentiate *S. aureus* from coagulase-negative staphylococci, we chose to restrain the use of Xpert MRSA/SA BC for *S. aureus*, given the well-known high percentage of methicillin resistance in other coagulase-negative staphylococci and for Gram-positive cocci clusters not identified at day 0. In 6 months using the Triton X-100 method, the number of Xpert MRSA/SA BC assays has been reduced by 42%.

In summary, direct bacterial identification from BCBs by MALDI-TOF MS is now definitively recognized as a breakthrough in the management of patients with bloodstream infections and should be further developed in order to reduce mortality rates at end stages of infection. We reduced the duration of the extraction protocol to just 10 min while maintaining high reliability in the identification of bacterial species. To our knowledge, this is the fastest protocol described for direct identification from BCBs. The reliability, rapidity, and simplicity of our technique allow it to be easily adopted by any microbiology laboratory equipped with MALDI-TOF MS technology.

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