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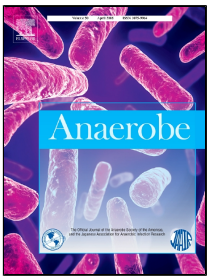

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Sample preparation method influences direct identification of anaerobic bacteria from positive blood culture bottles using MALDI-TOF MS

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Abstract

Rapid detection and identification of anaerobic bacteria from blood is important to adjust antimicrobial therapy by including antibiotics with activity against anaerobic bacteria. Limited data is available about direct identification of anaerobes from positive blood culture bottles using MALDI-TOF mass spectrometry (MS). In this study, we evaluated the performance of two sample preparation protocols for direct identification of anaerobes from positive blood culture bottles, the MALDI Sepsityper kit (Sepsityper) and the in-house saponin (saponin) method. Additionally, we compared two blood culture bottle types designed to support the growth of anaerobic bacteria, the BacT/ALERT-FN Plus (FN Plus) and the BACTEC-Lytic (Lytic), and their influence on direct identification. A selection of 30 anaerobe strains belonging to 22 different anaerobic species (11 reference strains and 19 clinical isolates) were inoculated to 2 blood culture bottle types in duplicate. In total, 120 bottles were inoculated and 99.2% (n=119) signalled growth within 5 days of incubation. The Sepsityper method correctly identified 56.3% (n=67) of anaerobes, while the saponin method correctly identified 84.9% (n=101) of anaerobes with at least log(score) ≥1.6 (low confidence correct identification), (p<0.001). Gram negative anaerobes were better identified with the saponin method (100% vs. 46.5%; p<0.001), while Gram positive anaerobes were better identified with the Sepsityper method (70.8% vs. 62.5%; p=0.454). Average log(score) values among only those isolates that were correctly identified simultaneously by both sample preparation methods were 2.119 and 2.029 in favour of the Sepsityper method, (p=0.019). The inoculated bottle type didn't influence the performance of the two sample preparation methods. We confirmed that direct identification from positive blood culture bottles with MALDI-TOF MS is reliable for anaerobic bacteria. However, the results are influenced by the sample preparation method used.

Keywords: anaerobes, blood culture system, direct identification, MALDI-TOF MS, MALDI Sepsityper kit

Introduction

Anaerobes can cause a variety of human infections including bloodstream infections [1,2]. They represent less than 10% of all bacterial isolates from blood, as detected by positive blood culture (BC), however, significant mortality rate is associated with anaerobic bacteraemia that can be as high as 30%, depending on different clinical settings [1-3]. Early detection of the causative agent of anaerobic bacteraemia, rapid initiation of anti-anaerobic antimicrobial therapy and adequate source control are associated with decreased mortality among patients with anaerobic bacteraemia [3-5]. Isolation of anaerobic bacteria from blood is a well-known challenge for clinical microbiology laboratories. This is because of fastidious nature of anaerobic bacteria and special growth requirements, but also because of their slow growth [1,2]. The detection of bacteria from blood has been improved in the last 2-3 decades, mainly following the development of the automated continuously monitoring BC systems [6]. Several BC systems are available worldwide, however, two of them are predominantly used, the BacT/ALERT (bioMérieux, Marcy l'Étoile, France) and BACTEC (Becton Dickinson, Sparks, USA). Both of them include dedicated anaerobic bottles which contain complex and enriched liquid culture media for the recovery of anaerobes. Furthermore, they may include different types of neutralizing agents against antibiotics possibly present in the blood. BacT/ALERT-FN Plus (FN Plus) BC bottles contain a specialised adsorbent polymer which has replaced charcoal as a neutralizing agent due to its influence on direct identification from positive blood cultures with matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). On the other hand, BACTEC-Lytic/10 Anaerobic/F (Lytic) BC bottles do not contain any neutralizing substances, but instead contain a detergent, saponin for possible lysis of leucocytes and release of phagocytosed intracellular bacteria [7,8].

Conventional method like Gram stain from a positive BC bottle is not capable of differentiating between aerobic and anaerobic bacteria and additional 24 to 48 hours are required for subculture and identification of causative organisms. MALDI-TOF MS has revolutionized the way bacteria are identified in microbiology laboratories today. It is fast and reliable method for identification of bacteria [9] with huge influence on the way positive BC bottles are processed in the laboratory. Several in-house and one commercial procedure for direct identification of bacteria from positive BC bottles were described [10-13]. Studies have shown that direct identification from positive BC bottles with MALDI-TOF MS greatly reduces turn-around time of identification and positively influence the selection and optimization of antimicrobial therapy in patients with BC positive sepsis [14-16]. However, most of the studies have focused primarily on aerobic bacteria, as they are the predominant bacterial isolates from blood. Consequently, very limited data exists about the direct identification from positive

BC bottles for anaerobic bacterial species.

The aims of this study were to evaluate two sample preparation methods for direct identification of anaerobes from positive BC bottles with MALDI-TOF MS and to determine the influence of two different anaerobic BC bottle types on the direct identification following the two sample preparation methods used.

Methods

Study design and bacterial strains

The study was performed at the Institute of Microbiology and Immunology, Medical Faculty, University of Ljubljana, Ljubljana, Slovenia. Thirty challenging anaerobic isolates (11 reference strains and 19 clinical isolates) belonging to 22 different anaerobic species were selected for this study, based on the distribution of anaerobic species among positive BC isolates during the past 5 years in our institution and based on the growth of the isolates in the two tested BC bottle types [17]. The collection included isolates belonging to the following genera: *Bacteroides* (n=10), *Clostridium* (n=6), *Fusobacterium* (n=4), *Prevotella* (n=3), *Actinomyces* (n=2), *Veillonella* (n=1), *Lactobacillus* (n=1) and 3 species belonging to Gram positive anaerobic cocci (GPAC).

Inoculation and incubation of the BC bottles

Each bacterial strain was inoculated simultaneously and in duplicate into 2 BC bottle types, namely the FN Plus and the Lytic. Altogether, 120 BC bottles were inoculated and included 60 from the two bottle types. For inoculation, we used the procedure described in our previous study [17]. Briefly, fresh bacterial cultures were suspended in the brain heart infusion broth to reach 0.5 McFarland standard (≈10^8 CFU/mL). Following serial dilutions, a final concentration ≈10^4 CFU/mL was prepared, of which 0.1 mL inoculum (≈10^3 CFU) was added to FN Plus and Lytic BC bottles in duplicate. Both bottle types contain 40 mL of culture media to which 5 mL of defibrinated sterile horse blood (Becton Dickinson, Sparks, USA) was added to simulate real BC specimen's conditions. Final preincubation concentration of anaerobic bacteria in the BC bottle was ≈20-30 CFU/mL which is similar to the estimated real-time situation in the adult septic patient [18]. BacT/ALERT 3D and BACTEC 9000 BC systems were used for cultivation, according to the bottle type and waited for the machine to signal positivity. The incubation was set to 5 days as this is the predominant interval used in clinical settings. After signalled positivity, 0.1 mL of the BC media was inoculated on the Schaedler agar and incubated anaerobically for 48-96 hrs for evaluation of colony count and for identification confirmation with conventional MALDI-TOF MS.

Direct identification

MALDI-TOF MS identification was performed from all BC bottles that signalled positive within 5 days of incubation. Two different sample preparation methods were used from each BC bottle, namely the MALDI Sepsityper kit (Bruker Daltonik, Bremen, Germany) (Sepsityper) and the in-house saponin (saponin) method. The direct identification was performed with Microflex LT MALDI-TOF MS system (Bruker Daltonik, Bremen, Germany) using Bruker Biotyper software 3.1 at mass spectra ranging from 2,000 to 20,000 Daltons. For each direct identification, two positions on MALDI target plate were spotted and better log(score) of the two was used as the final result. The MALDI-TOF MS log(score)s of ≥1.8 and ≥1.6 were interpreted as high confidence and low confidence correct identification, respectively. Log(score)s lower than 1.6 or no peaks were detected following identification were interpreted as no identification (NI) [19].

The Sepsityper method was performed following the recommendations from manufacturer. Briefly, 200 µL of lysis buffer was added to 1 mL of positive BC sample, vortexed for 10 seconds, centrifuged at 13,000 rpm for 2 minutes and supernatant removed. Pellet was re-suspended in 1 mL of washing buffer. Following gentle mixing, centrifugation (13,000 rpm for 1 minute) and removal of supernatant, the pellet was re-suspended in 70% ethanol. After another cycle of vortexing, centrifugation and ethanol removal, the pellet was resuspended in 30 µL of 70% formic acid and equal amount of 100% acetonitrile, mixed and centrifuged for the last time. Finally, 1 µL of the supernatant was placed on the MALDI target plate, allowed to air dry, covered with 1 µL of HCCA matrix solution and analysed with MALDI-TOF MS.

The saponin method was performed as previously described [10]. Briefly, 1 mL of the positive BC sample was added to 200 µL of a 5% saponin lysis solution. After vortexing and incubation for 5 min at room temperature, the tube was centrifuged for 1 min at 14,500 rpm and the supernatant was discarded. Finally, the pellet was washed with 1 mL of deionized water that was discarded after a second 1 min centrifugation at 14,500 rpm. The pellet was smeared on a MALDI target plate and allowed to air dry, covered with 1 µL of HCCA matrix solution and analysed with MALDI-TOF MS.

Three batches of direct identifications were performed on each day of the study period, at 8.00, 12.00 and 15.00, on those bottles which became positive until the time indicated before. The BC bottles that became positive after 15.00, were processed at 8.00 the next morning after further incubation at 37 ºC. Positive BC bottles were also subcultured on Schaedler agar at the same time as the direct identification was carried out and incubated anaerobically for 48 h for purity check, confirmation of bacterial concentration and identification with standard

MALDI-TOF MS identification procedure from isolated colonies on agar plates in accordance with the instructions from the manufacturer.

Statistical analysis

The correct identification rate between the two sample preparation methods for direct identification and the two different BC bottle types were compared using McNemar's test for comparison of two matched groups. The MALDI-TOF MS log(score)s between the two groups were compared using paired T-test for comparison of two matched group means. Statistical significance was set to *p*-value of <0.05.

Results

In total, 120 BC bottles were inoculated and 99.2% (n=119) became positive within 5 days from the beginning of incubation and were included in the final calculations of the performance of direct identification as a denominator. One FN Plus BC bottle inoculated with *Fusobacterium nucleatum* ATCC 25586 was not signalling positive after 5 days, so it was excluded. All positive BC bottles were confirmed to contain the inoculating anaerobic species with log(score) >2.0, growing in concentration >10^6 CFU/mL.

From each of the 119 positive BC bottles two sample preparation procedures were performed, meaning that altogether, 238 direct identifications were performed. MALDI-TOF MS results for each inoculated bottle are shown in Table 1. In total, 70.6% (n=168) of the inoculated anaerobes were correctly identified, 58.8% (n=140) with high confidence and 11.8% (n=28) with low confidence. The Sepsityper method correctly identified 56.3% (n=67) and the saponin method 84.9% (n=101) of positive BC bottles (p<0.001). Aggregated results of direct identification are show in Table 2.

In a subgroup of Gram negative anaerobes (n=71), correct identification was achieved in 46.5% (n=33) with the Sepsityper method compared to 100% (n=71) with the saponin method, (p<0.001). Among the inoculated *Bacteroides* spp. isolates (n=40), the Sepsityper method identified 30% (n=12) of the isolates, while 70% (n=28) were not identified due to the very low log(score)s or because no peaks were found. Among direct identifications from the subgroup of Gram positive anaerobes (n=48), correct identification was achieved in 70.8% (n=34) with the Sepsityper method and 62.5% (n=30) with the saponin method, (p=0.454). (Table 2)

With regard to different BC bottle type and irrespective of the sample preparation procedure used, correct identification was achieved in 71.2% from the FN Plus and in 70% from the Lytic bottle type (p=0.841). Average

log(score) values among only those isolates that were correctly identified simultaneously by both sample preparation methods were 2.119 and 2.029 in favour of the Sepsityper method, (p=0.019).

The reproducibility of direct identification was higher with the Saponin method where 93% (55/59) of duplicate inoculations (A and B) gave qualitatively identical result, as compared to 80% (47/59) for the Sepsityper method. The reproducibility was the lowest with the combination of the Sepsityper method and the FN Plus BC bottles, 69% (20/29). (Table 1)

Altogether, 59% (n=70) of isolates were signaled positive between 15.00 and 6.00; i.e. after the last daily batch was processed for direct ID. Those isolates were waiting for processing and direct ID on average 7 hours and 44 minutes. During that time, they were incubating at 37 °C, meaning that the number of bacteria increased.

Discussion

Rapid detection and identification of a causative organisms in patients with sepsis may critically influence the selection of antibiotic therapy and consequently patients' survival [5,20]. Even though anaerobic bacteria are rare isolates from BCs, their rapid identification is important for adjusting the antibiotic coverage to include antimicrobial agents effective against anaerobes. In this study, we have used a selected panel of anaerobic bacteria comprised of reference and clinical strains, to evaluate the performance of two sample preparation methods for the rapid identification of anaerobes directly from positive BC bottles. They were compared in combination with two of the most commonly used anaerobic BC bottle media, the FN Plus with neutralizing agents for antibiotics and the Lytic without one. We provide evidence that direct identification from positive blood culture bottles with MALDI-TOF MS is reliable for anaerobes, however, it is influenced by the sample preparation method used. The saponin method was more reliable than the Sepsityper method for overall identification with correct identification rates of 84.9% and 56.3%, respectively. The difference was mostly attributed to the poorer identification of Gram negative anaerobes with the Sepsityper method, while Gram positive anaerobes were better identified with the Sepsityper method.

Controversy exists regarding the trend in incidence of anaerobic bacteraemia and the routine use of anaerobic BC bottles for all patients for the diagnosis of sepsis [21-23]. However, evidence exist that the incidence of anaerobic bacteraemia is most probably stable over several past decades if properly searched for [3]. In our institution, we have a long tradition of using both aerobic and anaerobic BC bottles as a part of diagnostic evaluation of patients with sepsis. Consequently, the selection of challenging anaerobic isolates reflected the most

common anaerobic isolates from BCs in our tertiary care hospital facility, with *Bacteroides fragilis* being the most prevalent among them [17]. However, the selection also included several Gram positive anaerobes since they are also frequently isolated from blood, namely *Clostridium* spp., GPAC and *Actinomyces* spp..

MALDI-TOF MS has revolutionised the way we approach and perform identification in clinical bacteriology, diminishing its complexity and hands-on-time while simultaneously increasing the reliability of results [24]. That is especially true in the case of anaerobic bacteria because of their slow growth, relative low metabolic activity and subsequent difficulties with the identification by conventional methods. Furthermore, direct identification of bacteria from positive BC bottles, i.e. before colonies even appear on agar plates, has additionally shortened turn-around time for BC processing and administration of appropriate antimicrobial therapy [15,16]. Several studies describing identification of bacteria directly from positive BC bottles using MALDI-TOF MS have been performed [10-13], however, the majority of them focusing primarily on aerobic bacteria with no or very limited number of anaerobes tested, mostly from *Bacteroides* and *Clostridium* genera [7,8].

The Sepsityper method is the only IVD marked sample preparation method available to our knowledge so far [19]. In a recent review of 21 studies evaluating its performance and using challenging panels predominantly composed of aerobic bacterial species, overall correct direct identification was achieved in 80% of cases, 90% with Gram negative and 76% with Gram positive organism [19]. Our study is one of the few that evaluated anaerobic bacteria only. Overall correct direct identification from positive BCs was achieved in 56.3%. The difference can primarily be explained with the selection of organisms tested. Our panel comprised 22 different and exclusively anaerobic species. Only very limited number of different anaerobes were included in the previous studies [19]. In our study, the Sepsityper method performed better with Gram positive anaerobes, while among Gram negative anaerobes, the performance was non-significantly better when cultured from the FN Plus bottles. The direct identification of *Bacteroides* spp., the most common anaerobic BC isolate, was suboptimal in our study. Among 10 different isolates cultured in 4 bottles (n=40), 70% were repeatedly not identified, 55% and 85% from the FN Plus and the Lytic media. In a majority of the implicated cases, it was impossible to achieve pellet formation after the first procedural step, the lysis of positive BC bottle content and centrifugation, even after repeating the procedure. On the other hand, the direct identification of Gram positive anaerobes with the Sepsityper method was high (70.8%), with little difference from the two bottle types. Overall, there was a tendency for the Sepsityper method to performed better in combination with the FN Plus bottles (61%) compared to the Lytic bottles (51.6%), (p=0.180).

For comparison, we have used the in-house saponin method described by Martiny at al. [10]. The decision for comparator was based on lower cost, fewer manual steps of the procedure and good laboratory experience with the method. The saponin is a detergent that lyses blood cells and is also integrated in the Lytic BC media for possible release of intracellular bacteria [25]. The saponin method performed better than the Sepsityper method in our study, with overall correct identification of 84.9% and no difference between the evaluated bottle types. This is similar to the results from Almuhayawi et al. in which correct direct identification of anaerobic bacteria, with an in-house protocol that did not include saponin step, was achieved in 75-79%, depending on the bottle type used [7]. However, the overwhelming majority of anaerobic strains in that study belonged to *B. fragilis* and *Clostridium perfringens*, while no GPAC was tested. On the other hand, our testing panel was much more equilibrated.

Our study has some limitations. Only a limited number of different anaerobic species, which may cause bacteraemia were included in this study, nevertheless, they represent a diverse collection of anaerobes and were inoculated in large number of BC bottles, 4 bottles each. Direct identification was not carried out immediately after the positive signal from the system for a majority of BC bottles, which means that the number of bacteria in 1 mL sample was higher in the bottles that were positive after 15.00. However, this is also the case in the real-time clinical laboratory situation and we believe that it did not adversely affect the results of direct identification in those BC bottles.

In conclusion, well balanced and diverse panel of anaerobic strains was tested for the direct identification from positive BC bottles with two sample preparation methods using MALDI-TOF MS method. Overall, 70.6% of the direct identifications were correct using both methods and two BC bottle types. Direct identification with the in-house saponin method performed better than with the Sepsityper method. BC bottle type did not influence direct identification results.

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Conflict of Interest

Nothing to declare.

Ethical approval and Informed consent

Our work did not include any experiments on humans or animals.

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Table 1: Log(score) values for direct identification of selected anaerobic bacteria from positive blood culture bottles with MALDI-TOF MS, following two sample preparation methods and cultured from two blood culture bottle types in duplicate (A and B).

Anaerobic bacterial strain	**Log(score)**							
	Sepsytiper				**Saponin**			
	FN Plus		**Lytic**		**FN Plus**		**Lytic**	
Gram negative	A	B	A	B	A	B	A	B
Bacteroides fragilis	2.119	NI	NI	NI	2.197	2.360	2.135	2.111
Bacteroides fragilis	1.938	1.918	1.837	NI	2.145	2.258	2.287	2.261
Bacteroides fragilis ATCC 23745	2.338	2.338	2.267	2.326	2.100	2.100	2.411	2.409
Bacteroides ovatus	NI	NI	NI	NI	2.118	2.093	2.137	2.098
Bacteroides ovatus BAA 1296	2.292	2.033	NI	NI	2.306	2.022	2.187	2.076
Bacteroides thetaiotaomicron	NI	NI	NI	NI	2.015	1.977	2.067	2.080
Bacteroides thetaiotaomicron	NI	1.747	NI	NI	1.933	1.775	2.129	1.989
Bacteroides thetaiotaomicron ATCC 29741	NI	2.182	NI	NI	2.382	2.485	2.353	2.364
Bacteroides uniformis	NI	NI	NI	NI	2.335	2.214	2.364	2.299
Bacteroides vulgatus	NI	NI	NI	NI	2.126	2.166	2.180	2.214
Fusobacterium necrophorum	2.177	2.123	2.148	2.170	1.873	2.144	2.135	1.970
Fusobacterium necrophorum ATCC 25286	2.111	NI	2.341	2.201	2.200	2.298	2.339	2.329
Fusobacterium nucleatum	NI	1.636	1.721	NI	1.700	1.908	2.018	1.727
Fusobacterium nucleatum ATCC 25586	/	2.275	2.195	2.100	/	1.849	2.052	1.973
Prevotella buccae	NI	1.963	2.244	2.250	2.125	2.140	2.200	2.207
Prevotella melaninogenica ATCC 25845	1.699	1.924	NI	NI	2.070	2.013	1.952	2.159
Prevotella nigrescens	2.017	2.227	2.212	2.133	2.144	2.190	2.171	2.171
Veillonella parvula	NI	NI	NI	NI	1.706	1.866	1.968	1.914
Gram positive								
Actinomyces odontolyticus	1.848	NI	1.814	NI	NI	NI	NI	NI
Actinomyces viscosus ATCC 15987	NI	1.655	1.709	1.726	NI	1.607	NI	NI
Clostridium innocuum	1.868	1.675	1.950	1.858	NI	NI	1.861	1.807
Clostridium perfringens	2.567	2.409	1.638	2.459	1.802	NI	2.020	1.694
Clostridium perfringens ATCC 13124	2.359	1.847	2.244	2.249	2.069	1.906	1.825	1.685
Clostridium septicum ATCC 12464	NI	2.046	NI	NI	1.696	1.828	1.730	1.788
Clostridium sordelii ATCC 9714	NI	NI	NI	NI	1.646	NI	1.687	1.830
Clostridium sporogenes ATCC 19404	2.467	2.521	2.511	2.427	1.978	1.747	2.123	2.190
Lactobacillus rhamnosus	NI	NI	NI	NI	NI	NI	NI	NI
Parvimonas (Micromonas) micra	2.142	2.218	2.458	2.475	2.045	2.078	2.174	1.889
Peptoniphilus asaccharolyticus	1.793	1.847	1.899	1.871	1.660	1.740	1.753	1.818
Peptoniphilus harei	1.944	2.101	1.726	2.105	NI	NI	NI	1.752

NI: no identification (log(score) ≤1.6) or no peaks on MALDI-TOF MS
/: Blood culture bottle did not signal positive within 5 days of incubation

319 **Table 2:** Direct identification of the selected anaerobic bacteria using two sample preparation methods.

Identification	**Sepsityper**		**Saponin**		**Total**		***p*-value***
	n	(%)	n	(%)	n	(%)	
All (n=119)							
≥1.6	67	(56.3)	101	(84.9)	168	(70.6)	**<0.001**
≥1.8	56	(47.1)	84	(70.6)	140	(58.8)	**<0.001**
No identification	52	(43.7)	18	(15.1)	70	(29.4)	
Gram negative (n=71)							
≥1.6	33	(46.5)	71	(100)	104	(73.2)	**<0.001**
≥1.8	29	(40.8)	67	(94.4)	96	(67.6)	**<0.001**
No identification	38	(53.5)	0	(0.0)	38	(26.8)	
Gram positive (n=48)							
≥1.6	34	(70.8)	30	(62.5)	64	(66.7)	0.454
≥1.8	27	(56.3)	17	(35.4)	44	(45.8)	**0.013**
No identification	14	(29.2)	18	(37.5)	32	(33.3)	

320 * *p*-value for the difference between the Sepsityper and the saponin method using McNemar's test

Highlights

- Direct identification from positive blood culture bottle is reliable for anaerobic bacteria
- It influenced by the sample preparation protocol / method
- It works equally well for BacT/ALERT-FN Plus and BACTEC-Lytic bottle type