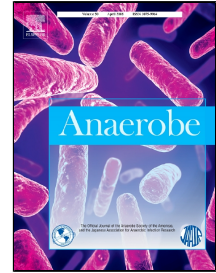# Accepted Manuscript

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

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1   **Sample preparation method influences direct identification of anaerobic bacteria from positive blood**

2   **culture bottles using MALDI-TOF MS**

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1

13 **Abstract**

14 Rapid detection and identification of anaerobic bacteria from blood is important to adjust antimicrobial therapy

15 by including antibiotics with activity against anaerobic bacteria. Limited data is available about direct

16 identification of anaerobes from positive blood culture bottles using MALDI-TOF mass spectrometry (MS). In

17 this study, we evaluated the performance of two sample preparation protocols for direct identification of anaerobes

18 from positive blood culture bottles, the MALDI Sepsityper kit (Sepsityper) and the in-house saponin (saponin)

19 method. Additionally, we compared two blood culture bottle types designed to support the growth of anaerobic

20 bacteria, the BacT/ALERT-FN Plus (FN Plus) and the BACTEC-Lytic (Lytic), and their influence on direct

21 identification. A selection of 30 anaerobe strains belonging to 22 different anaerobic species (11 reference strains

22 and 19 clinical isolates) were inoculated to 2 blood culture bottle types in duplicate. In total, 120 bottles were

23 inoculated and 99.2% (n=119) signalled growth within 5 days of incubation. The Sepsityper method correctly

24 identified 56.3% (n=67) of anaerobes, while the saponin method correctly identified 84.9% (n=101) of anaerobes

25 with at least log(score) ≥1.6 (low confidence correct identification), (p<0.001). Gram negative anaerobes were

26 better identified with the saponin method (100% vs. 46.5%; p<0.001), while Gram positive anaerobes were better

27 identified with the Sepsityper method (70.8% vs. 62.5%; p=0.454). Average log(score) values among only those

28 isolates that were correctly identified simultaneously by both sample preparation methods were 2.119 and 2.029

29 in favour of the Sepsityper method, (p=0.019). The inoculated bottle type didn't influence the performance of the

30 two sample preparation methods. We confirmed that direct identification from positive blood culture bottles with

31 MALDI-TOF MS is reliable for anaerobic bacteria. However, the results are influenced by the sample preparation

32 method used.

34 **Introduction**

35 Anaerobes can cause a variety of human infections including bloodstream infections [1,2]. They represent less

36 than 10% of all bacterial isolates from blood, as detected by positive blood culture (BC), however, significant

37 mortality rate is associated with anaerobic bacteraemia that can be as high as 30%, depending on different clinical

38 settings [1-3]. Early detection of the causative agent of anaerobic bacteraemia, rapid initiation of anti-anaerobic

39 antimicrobial therapy and adequate source control are associated with decreased mortality among patients with

40 anaerobic bacteraemia [3-5]. Isolation of anaerobic bacteria from blood is a well-known challenge for clinical

41 microbiology laboratories. This is because of fastidious nature of anaerobic bacteria and special growth

42 requirements, but also because of their slow growth [1,2]. The detection of bacteria from blood has been improved

43 in the last 2-3 decades, mainly following the development of the automated continuously monitoring BC systems

44 [6]. Several BC systems are available worldwide, however, two of them are predominantly used, the

45 BacT/ALERT (bioMérieux, Marcy l'Étoile, France) and BACTEC (Becton Dickinson, Sparks, USA). Both of

46 them include dedicated anaerobic bottles which contain complex and enriched liquid culture media for the

47 recovery of anaerobes. Furthermore, they may include different types of neutralizing agents against antibiotics

48 possibly present in the blood. BacT/ALERT-FN Plus (FN Plus) BC bottles contain a specialised adsorbent

49 polymer which has replaced charcoal as a neutralizing agent due to its influence on direct identification from

50 positive blood cultures with matrix-assisted laser desorption ionization time-of-flight mass spectrometry

51 (MALDI-TOF MS). On the other hand, BACTEC-Lytic/10 Anaerobic/F (Lytic) BC bottles do not contain any

52 neutralizing substances, but instead contain a detergent, saponin for possible lysis of leucocytes and release of

53 phagocytosed intracellular bacteria [7,8].

54 Conventional method like Gram stain from a positive BC bottle is not capable of differentiating between

55 aerobic and anaerobic bacteria and additional 24 to 48 hours are required for subculture and identification of

56 causative organisms. MALDI-TOF MS has revolutionized the way bacteria are identified in microbiology

57 laboratories today. It is fast and reliable method for identification of bacteria [9] with huge influence on the way

58 positive BC bottles are processed in the laboratory. Several in-house and one commercial procedure for direct

59 identification of bacteria from positive BC bottles were described [10-13]. Studies have shown that direct

60 identification from positive BC bottles with MALDI-TOF MS greatly reduces turn-around time of identification

61 and positively influence the selection and optimization of antimicrobial therapy in patients with BC positive sepsis

62 [14-16]. However, most of the studies have focused primarily on aerobic bacteria, as they are the predominant

63 bacterial isolates from blood. Consequently, very limited data exists about the direct identification from positive

3

64 BC bottles for anaerobic bacterial species.

65 The aims of this study were to evaluate two sample preparation methods for direct identification of

66 anaerobes from positive BC bottles with MALDI-TOF MS and to determine the influence of two different

67 anaerobic BC bottle types on the direct identification following the two sample preparation methods used.

68 **Methods**

69 *Study design and bacterial strains*

70 The study was performed at the Institute of Microbiology and Immunology, Medical Faculty, University of

71 Ljubljana, Ljubljana, Slovenia. Thirty challenging anaerobic isolates (11 reference strains and 19 clinical isolates)

72 belonging to 22 different anaerobic species were selected for this study, based on the distribution of anaerobic

73 species among positive BC isolates during the past 5 years in our institution and based on the growth of the isolates

74 in the two tested BC bottle types [17]. The collection included isolates belonging to the following genera:

75 *Bacteroides* (n=10), *Clostridium* (n=6), *Fusobacterium* (n=4), *Prevotella* (n=3), *Actinomyces* (n=2), *Veillonella*

76 (n=1), *Lactobacillus* (n=1) and 3 species belonging to Gram positive anaerobic cocci (GPAC).

77 *Inoculation and incubation of the BC bottles*

78 Each bacterial strain was inoculated simultaneously and in duplicate into 2 BC bottle types, namely the FN Plus

79 and the Lytic. Altogether, 120 BC bottles were inoculated and included 60 from the two bottle types. For

80 inoculation, we used the procedure described in our previous study [17]. Briefly, fresh bacterial cultures were

81 suspended in the brain heart infusion broth to reach 0.5 McFarland standard ($\approx 10^8$ CFU/mL). Following serial

82 dilutions, a final concentration $\approx 10^4$ CFU/mL was prepared, of which 0.1 mL inoculum ($\approx 10^3$ CFU) was added to

83 FN Plus and Lytic BC bottles in duplicate. Both bottle types contain 40 mL of culture media to which 5 mL of

84 defibrinated sterile horse blood (Becton Dickinson, Sparks, USA) was added to simulate real BC specimen's

85 conditions. Final preincubation concentration of anaerobic bacteria in the BC bottle was $\approx$20-30 CFU/mL which

86 is similar to the estimated real-time situation in the adult septic patient [18]. BacT/ALERT 3D and BACTEC 9000

87 BC systems were used for cultivation, according to the bottle type and waited for the machine to signal positivity.

88 The incubation was set to 5 days as this is the predominant interval used in clinical settings. After signalled

89 positivity, 0.1 mL of the BC media was inoculated on the Schaedler agar and incubated anaerobically for 48-96

90 hrs for evaluation of colony count and for identification confirmation with conventional MALDI-TOF MS.

91 *Direct identification*

92 MALDI-TOF MS identification was performed from all BC bottles that signalled positive within 5 days of

93 incubation. Two different sample preparation methods were used from each BC bottle, namely the MALDI

94 Sepsityper kit (Bruker Daltonik, Bremen, Germany) (Sepsityper) and the in-house saponin (saponin) method. The

95 direct identification was performed with Microflex LT MALDI-TOF MS system (Bruker Daltonik, Bremen,

96 Germany) using Bruker Biotyper software 3.1 at mass spectra ranging from 2,000 to 20,000 Daltons. For each

97 direct identification, two positions on MALDI target plate were spotted and better log(score) of the two was used

98 as the final result. The MALDI-TOF MS log(score)s of ≥1.8 and ≥1.6 were interpreted as high confidence and

99 low confidence correct identification, respectively. Log(score)s lower than 1.6 or no peaks were detected

100 following identification were interpreted as no identification (NI) [19].

101   The Sepsityper method was performed following the recommendations from manufacturer. Briefly, 200

102 μL of lysis buffer was added to 1 mL of positive BC sample, vortexed for 10 seconds, centrifuged at 13,000 rpm

103 for 2 minutes and supernatant removed. Pellet was re-suspended in 1 mL of washing buffer. Following gentle

104 mixing, centrifugation (13,000 rpm for 1 minute) and removal of supernatant, the pellet was re-suspended in 70%

105 ethanol. After another cycle of vortexing, centrifugation and ethanol removal, the pellet was resuspended in 30

106 μL of 70% formic acid and equal amount of 100% acetonitrile, mixed and centrifuged for the last time. Finally, 1

107 μL of the supernatant was placed on the MALDI target plate, allowed to air dry, covered with 1 μL of HCCA

108 matrix solution and analysed with MALDI-TOF MS.

109   The saponin method was performed as previously described [10]. Briefly, 1 mL of the positive BC

110 sample was added to 200 μL of a 5% saponin lysis solution. After vortexing and incubation for 5 min at room

111 temperature, the tube was centrifuged for 1 min at 14,500 rpm and the supernatant was discarded. Finally, the

112 pellet was washed with 1 mL of deionized water that was discarded after a second 1 min centrifugation at 14,500

113 rpm. The pellet was smeared on a MALDI target plate and allowed to air dry, covered with 1 μL of HCCA matrix

114 solution and analysed with MALDI-TOF MS.

115   Three batches of direct identifications were performed on each day of the study period, at 8.00, 12.00

116 and 15.00, on those bottles which became positive until the time indicated before. The BC bottles that became

117 positive after 15.00, were processed at 8.00 the next morning after further incubation at 37 ºC. Positive BC bottles

118 were also subcultured on Schaedler agar at the same time as the direct identification was carried out and incubated

119 anaerobically for 48 h for purity check, confirmation of bacterial concentration and identification with standard

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

121   instructions from the manufacturer.

122   *Statistical analysis*

123   The correct identification rate between the two sample preparation methods for direct identification and the two

124   different BC bottle types were compared using McNemar's test for comparison of two matched groups. The

125   MALDI-TOF MS log(score)s between the two groups were compared using paired T-test for comparison of two

126   matched group means. Statistical significance was set to *p*-value of <0.05.

127   **Results**

128   In total, 120 BC bottles were inoculated and 99.2% (n=119) became positive within 5 days from the beginning of

129   incubation and were included in the final calculations of the performance of direct identification as a denominator.

130   One FN Plus BC bottle inoculated with *Fusobacterium nucleatum* ATCC 25586 was not signalling positive after

131   5 days, so it was excluded. All positive BC bottles were confirmed to contain the inoculating anaerobic species

132   with log(score) >2.0, growing in concentration >10^6 CFU/mL.

133   From each of the 119 positive BC bottles two sample preparation procedures were performed, meaning

134   that altogether, 238 direct identifications were performed. MALDI-TOF MS results for each inoculated bottle are

135   shown in Table 1. In total, 70.6% (n=168) of the inoculated anaerobes were correctly identified, 58.8% (n=140)

136   with high confidence and 11.8% (n=28) with low confidence. The Sepsityper method correctly identified 56.3%

137   (n=67) and the saponin method 84.9% (n=101) of positive BC bottles (*p*<0.001). Aggregated results of direct

138   identification are show in Table 2.

139   In a subgroup of Gram negative anaerobes (n=71), correct identification was achieved in 46.5% (n=33)

140   with the Sepsityper method compared to 100% (n=71) with the saponin method, (*p*<0.001). Among the inoculated

141   *Bacteroides* spp. isolates (n=40), the Sepsityper method identified 30% (n=12) of the isolates, while 70% (n=28)

142   were not identified due to the very low log(score)s or because no peaks were found. Among direct identifications

143   from the subgroup of Gram positive anaerobes (n=48), correct identification was achieved in 70.8% (n=34) with

144   the Sepsityper method and 62.5% (n=30) with the saponin method, (*p*=0.454). (Table 2)

145   With regard to different BC bottle type and irrespective of the sample preparation procedure used, correct

146   identification was achieved in 71.2% from the FN Plus and in 70% from the Lytic bottle type (*p*=0.841). Average

147 log(score) values among only those isolates that were correctly identified simultaneously by both sample

148 preparation methods were 2.119 and 2.029 in favour of the Sepsityper method, (*p*=0.019).

149   The reproducibility of direct identification was higher with the Saponin method where 93% (55/59) of

150 duplicate inoculations (A and B) gave qualitatively identical result, as compared to 80% (47/59) for the Sepsityper

151 method. The reproducibility was the lowest with the combination of the Sepsityper method and the FN Plus BC

152 bottles, 69% (20/29). (Table 1)

153   Altogether, 59% (n=70) of isolates were signaled positive between 15.00 and 6.00; i.e. after the last daily

154 batch was processed for direct ID. Those isolates were waiting for processing and direct ID on average 7 hours

155 and 44 minutes. During that time, they were incubating at 37 °C, meaning that the number of bacteria increased.

156 **Discussion**

157 Rapid detection and identification of a causative organisms in patients with sepsis may critically influence the

158 selection of antibiotic therapy and consequently patients' survival [5,20]. Even though anaerobic bacteria are rare

159 isolates from BCs, their rapid identification is important for adjusting the antibiotic coverage to include

160 antimicrobial agents effective against anaerobes. In this study, we have used a selected panel of anaerobic bacteria

161 comprised of reference and clinical strains, to evaluate the performance of two sample preparation methods for

162 the rapid identification of anaerobes directly from positive BC bottles. They were compared in combination with

163 two of the most commonly used anaerobic BC bottle media, the FN Plus with neutralizing agents for antibiotics

164 and the Lytic without one. We provide evidence that direct identification from positive blood culture bottles with

165 MALDI-TOF MS is reliable for anaerobes, however, it is influenced by the sample preparation method used. The

166 saponin method was more reliable than the Sepsityper method for overall identification with correct identification

167 rates of 84.9% and 56.3%, respectively. The difference was mostly attributed to the poorer identification of Gram

168 negative anaerobes with the Sepsityper method, while Gram positive anaerobes were better identified with the

169 Sepsityper method.

170   Controversy exists regarding the trend in incidence of anaerobic bacteraemia and the routine use of

171 anaerobic BC bottles for all patients for the diagnosis of sepsis [21-23]. However, evidence exist that the incidence

172 of anaerobic bacteraemia is most probably stable over several past decades if properly searched for [3]. In our

173 institution, we have a long tradition of using both aerobic and anaerobic BC bottles as a part of diagnostic

174 evaluation of patients with sepsis. Consequently, the selection of challenging anaerobic isolates reflected the most

175 common anaerobic isolates from BCs in our tertiary care hospital facility, with *Bacteroides fragilis* being the most

176 prevalent among them [17]. However, the selection also included several Gram positive anaerobes since they are

177 also frequently isolated from blood, namely *Clostridium* spp., GPAC and *Actinomyces* spp..

178   MALDI-TOF MS has revolutionised the way we approach and perform identification in clinical

179 bacteriology, diminishing its complexity and hands-on-time while simultaneously increasing the reliability of

180 results [24]. That is especially true in the case of anaerobic bacteria because of their slow growth, relative low

181 metabolic activity and subsequent difficulties with the identification by conventional methods. Furthermore, direct

182 identification of bacteria from positive BC bottles, i.e. before colonies even appear on agar plates, has additionally

183 shortened turn-around time for BC processing and administration of appropriate antimicrobial therapy [15,16].

184 Several studies describing identification of bacteria directly from positive BC bottles using MALDI-TOF MS

185 have been performed [10-13], however, the majority of them focusing primarily on aerobic bacteria with no or

186 very limited number of anaerobes tested, mostly from *Bacteroides* and *Clostridium* genera [7,8].

187   The Sepsityper method is the only IVD marked sample preparation method available to our knowledge

188 so far [19]. In a recent review of 21 studies evaluating its performance and using challenging panels predominantly

189 composed of aerobic bacterial species, overall correct direct identification was achieved in 80% of cases, 90%

190 with Gram negative and 76% with Gram positive organism [19]. Our study is one of the few that evaluated

191 anaerobic bacteria only. Overall correct direct identification from positive BCs was achieved in 56.3%. The

192 difference can primarily be explained with the selection of organisms tested. Our panel comprised 22 different

193 and exclusively anaerobic species. Only very limited number of different anaerobes were included in the previous

194 studies [19]. In our study, the Sepsityper method performed better with Gram positive anaerobes, while among

195 Gram negative anaerobes, the performance was non-significantly better when cultured from the FN Plus bottles.

196 The direct identification of *Bacteroides* spp., the most common anaerobic BC isolate, was suboptimal in our study.

197 Among 10 different isolates cultured in 4 bottles (n=40), 70% were repeatedly not identified, 55% and 85% from

198 the FN Plus and the Lytic media. In a majority of the implicated cases, it was impossible to achieve pellet

199 formation after the first procedural step, the lysis of positive BC bottle content and centrifugation, even after

200 repeating the procedure. On the other hand, the direct identification of Gram positive anaerobes with the

201 Sepsityper method was high (70.8%), with little difference from the two bottle types. Overall, there was a tendency

202 for the Sepsityper method to performed better in combination with the FN Plus bottles (61%) compared to the

203 Lytic bottles (51.6%), (p=0.180).

204   For comparison, we have used the in-house saponin method described by Martiny at al. [10]. The

205   decision for comparator was based on lower cost, fewer manual steps of the procedure and good laboratory

206   experience with the method. The saponin is a detergent that lyses blood cells and is also integrated in the Lytic

207   BC media for possible release of intracellular bacteria [25]. The saponin method performed better than the

208   Sepsityper method in our study, with overall correct identification of 84.9% and no difference between the

209   evaluated bottle types. This is similar to the results from Almuhayawi et al. in which correct direct identification

210   of anaerobic bacteria, with an in-house protocol that did not include saponin step, was achieved in 75-79%,

211   depending on the bottle type used [7]. However, the overwhelming majority of anaerobic strains in that study

212   belonged to *B. fragilis* and *Clostridium perfringens*, while no GPAC was tested. On the other hand, our testing

213   panel was much more equilibrated.

214   Our study has some limitations. Only a limited number of different anaerobic species, which may cause

215   bacteraemia were included in this study, nevertheless, they represent a diverse collection of anaerobes and were

216   inoculated in large number of BC bottles, 4 bottles each. Direct identification was not carried out immediately

217   after the positive signal from the system for a majority of BC bottles, which means that the number of bacteria in

218   1 mL sample was higher in the bottles that were positive after 15.00. However, this is also the case in the real-

219   time clinical laboratory situation and we believe that it did not adversely affect the results of direct identification

220   in those BC bottles.

221   In conclusion, well balanced and diverse panel of anaerobic strains was tested for the direct identification

222   from positive BC bottles with two sample preparation methods using MALDI-TOF MS method. Overall, 70.6%

223   of the direct identifications were correct using both methods and two BC bottle types. Direct identification with

224   the in-house saponin method performed better than with the Sepsityper method. BC bottle type did not influence

225   direct identification results.

234   **Conflict of Interest**

235   Nothing to declare.

236   **Ethical approval and Informed consent**

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

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314   **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

315   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

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

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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

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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