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A simple blood culture bacterial pellet preparation for faster accurate direct bacterial identification and antibiotic susceptibility testing with the VITEK 2 system

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20 # Abstract

21 An ammonium chloride procedure to prepare bacterial pellet from positive blood cultures was
22 used for direct inoculation of VITEK. Correct identification reached 99% for
23 *Enterobacteriaceae* and 74% for staphylococci. For susceptibility testing, very major and
24 major errors were 0.1% and 0.3% for *Enterobacteriaceae*, and 0.7% and 0.1% for
25 staphylococci.
26 Bacterial pellets prepared with ammonium chloride allow direct inoculation of VITEK cards
27 with excellent accuracy for *Enterobacteriaceae* and lower accuracy for staphylococci.
28

29 **Main manuscript**

30 Blood cultures are the best approach to establish the etiology of bloodstream infections.
31 Direct automated identification and antibiotic susceptibility testing (AST) using blood-culture
32 bacterial pellets were applied for Gram negative bacteria (Bruins *et al.*, 2004; de Cueto *et al.*,
33 2004; Kerremans *et al.*, 2004) but remains unsatisfactory for the identification of Gram
34 positive cocci (de Cueto *et al.*, 2004; Kerremans *et al.*, 2004). We developed a simple
35 procedure to prepare pellets for bacterial identification using MALDI-TOF (Prod'hom *et al.*,
36 2010). Here, we applied this procedure to directly inoculate VITEK cards (bioMérieux, Marcy
37 l'Etoile, France) for bacterial identification (ID) and for AST of Gram-positive cocci in cluster
38 (GPC) and Gram-negative bacteria (GNB) present in blood culture.

39 During 26 consecutive weeks, all positive blood culture having GNB (1 per patient) or GPC
40 (1 per site of puncture/per patient) on Gram-stained slides of positive vials were included.
41 Mixed blood cultures were excluded. Bacterial pellets from positive blood culture vials (Plus
42 aerobic/F, Lytic anaerobic/F and Peds/F) detected by the automated blood culture system
43 BACTEC 9240 (Becton Dickinson, Sparks, USA) were prepared with an ammonium
44 chloride-driven hemolysis (Prod'hom *et al.*, 2010). Briefly, five ml of positive medium was

45 mixed to 40 ml of sterile water and centrifuged at 1'000xg for 10 min. Supernatant and blood
46 cells layer were removed. The remaining blood cells were lysed mixing 1 ml of ammonium
47 chloride (0.15 M NH_4Cl, 1 mM $KHCO_3$; pH 7.31) to the bacterial pellet and a second
48 centrifugation step at 140xg for 10 min was done. Supernatant was discarded. When the pellet
49 remained hemorrhagic, the lysing step was repeated with 2 ml of water (Prod'hom *et al.*,
50 2010).

51 Bacterial pellets ($\geq 10^8$/ml) were used to directly inoculate (McFarland 0.6-0.8) VITEK cards,
52 GP and AST 580 cards for GPC and GN and GN26 cards for GNB. Positive blood culture vial
53 were subcultured on blood agar (GPC & GNB) and McConkey agar plates (GNB) to obtain a
54 pure culture with isolated colonies. ID and AST using the same VITEK cards from colonies
55 obtained by subculture on agar were used as the gold standard. Quality control of VITEK2
56 was performed weekly by testing *Escherichia coli* ATCC 25922 and *Staphylococcus aureus*
57 ATCC 29213 for both identification and AST.

58 For the interpretation of ID results, the following criteria were used i) correct identification
59 when direct ID and ID from colony gave the same identification, ii) misidentified, when
60 discordant results were observed between direct ID and ID from colony, iii) not identified
61 when direct ID gave no identification with the VITEK.

62 For the interpretation of AST, only cases with correct identification results were analyzed.
63 VITEK MIC data and interpretation were used for comparison for direct AST and AST from
64 colonies. Essential agreement (EA), categorical agreement (CA), minor discrepancy (md),
65 major errors (ME) and very major errors (VME) were used according to definition of
66 Guidance document of FDA (FDA, 2009). EA was the overall agreement within plus or
67 minus one two-fold dilution of direct versus colonies inoculation of VITEK cards. CA, the
68 agreement of interpretive results, susceptible, intermediate, resistant between direct versus

69 colony inoculation of VITEK cards. AST discordance results were classified as: VME (false
70 susceptible); ME (false resistant) and md (all others). In case of misidentification, strains were
71 retested using MALDI-TOF MS directly from colonies. The identification of these isolates by
72 MALDI-TOF was performed on a Microflex LT instrument (Bruker Daltonics, Leipzig,
73 Germany) with FlexControl software (version 3.0) (Bizzini *et al.*, 2010). Discrepancies of
74 AST (VME and ME) were solved by testing isolates using Etest system (BioMérieux). For
75 each antibiotic Wilcoxon signed rank test were performed to evaluate the MIC values after
76 log conversion. P value <0.05 were considered significant.
77 During the study period, 278 positive blood culture where included in the study. Table 1
78 shows the results of the VITEK identification obtained directly from bacterial pellet compared
79 to final identification. Overall 226/278 (81%) gave a correct identification at the species level
80 when VITEK was directly inoculated with bacterial pellets. The proportion of correct
81 identification for *Enterobacteriaceae*, non-fermentative GNB, staphylococci and other Gram
82 positive cocci were of 87/88 (99%), 5/7 (71%), 133/180 (74%) and 1/3 (33%), respectively
83 (Table 1). Misidentifications were observed for 31/278 (11%) bacterial pellets. For 21/278
84 (8%) bacterial pellets, VITEK gave no-identification. Noteworthy, all bacterial pellets
85 identified as *S. aureus* by the VITEK system were correct. However, as many as 16/77 *S.*
86 *aureus* (21%) were misidentified (Table 1).

87 Direct AST results from the blood-culture bacterial pellet were analyzed for 220 of the 226
88 isolates with congruent identification at the species level: 87 *Enterobacteriaceae* and 133
89 staphylococci (Table 2). AST GN26 and AST 580 are not appropriate for non-fermentative
90 bacteria (n=5) and *S. pyogenes* (n=1). For *Enterobacteriaceae*, the AST from one case was
91 excluded since VITEK gave no results due to insufficient growth. For 3 additional cases,
92 result from 1 antibiotic was excluded since VITEK gave no result. The majority of
93 discrepancy tests (27/41) confirmed categorical results obtained from colonies.

94 For the other cases, the EA and CA was overall of 98.5% and 97.7%, respectively. The
95 number of VME, ME and md were 2 (0.1%), 5 (0.3%) and 30 (1.9%), respectively. For 133
96 staphylococci, the EA and CA was 96% and 96.2%, respectively. The number of VME, ME
97 and md were 18 (0.7%), 2 (0.1%) and 76 (3.1%), respectively. The majority of VME was
98 observed for TMP-SMX (15/18; 83%).

99 In this study we applied a simple blood pellet procedure using ammonium chloride to
100 inoculate VITEK cards for both identification and AST. This procedure has several
101 advantages. First, we do not use additional device such as "serum separator tube" for
102 preparation of the bacterial pellet (Bruins *et al.*, 2004; de Cueto *et al.*, 2004; Kerremans *et al.*,
103 2004). Second, the method could be used for both bacterial identification and AST for
104 *Enterobacteriaceae* and staphylococci.

105 For staphylococci AST, very major errors predominate for TMP-SMX. Similar results have
106 already observed using serum separator tube for bacterial pellet preparation (Kerremans *et al.*,
107 2004) or saponin as detergent(Lupetti *et al.*, 2010). For staphylococci, the MICs for several
108 antimicrobial agents was one or more dilutions lower using the direct inoculum method
109 ($P<0.05$).

110 The performance of the direct AST fulfilled performance criteria considered as acceptable by
111 the FDA administration(FDA, 2009). Thus, we obtained a categorical agreement >90% for all
112 antibiotics (except fosfomycin (87%) and TMP-SMX (86%) for staphylococci), an essential
113 agreement >90% for all antibiotics (except teicoplanin (73%) for staphylococci), ≤ 1.5% of
114 very major errors (0.1% for *Enterobacteriaceae*, 0.7% for staphylococci) and < 3% of major
115 errors (0.3% for *Enterobacteriaceae*, 0.1% for staphylococci).

116 Two hypothetical factors may explain differences between the tested and the reference
117 method, i) the presence of residual blood proteins, of blood cells and of blood culture

118 medium ii) low homogeneity of bacteria in the pellet. The first factors may modify
119 standardized conditions necessary for identification and AST with VITEK card's and possibly
120 may increase the bacterial growth with a significant impact on the biochemical results. The
121 likely less homogeneous viability of bacteria present in the pellet than that of bacteria
122 obtained from a subculture may explain altered growth rate and modified MIC's
123 determination. Polymicrobial blood cultures may cause errors in antibiotic susceptibility
124 testing and should be excluded. In most hospitals, the rate of polymicrobial blood cultures is
125 relatively low (< 10%) allowing the successful application of this method to more than 90%
126 of all positive blood cultures.

127 In our hospital, direct identification using VITEK's cards are used when identification using
128 MALDI-TOF analysis of bacterial pellet failed. Direct AST on the blood-culture pellet is
129 applied on both *Staphylococci* and *Enterobacteriaceae*. For staphylococci, TMP-SMX result
130 is not provided to the physician. Implementation of such method in another laboratory may
131 need an independent validation since the method is an adaptation of CE/FDA approved tool
132 for off-label purposes.

133 In conclusion, bacterial pellets from positive blood cultures prepared with an ammonium
134 chloride-driven hemolysis allow direct inoculation of VITEK cards used for identification and
135 for antimicrobial susceptibility testing with an excellent accuracy for *Enterobacteriaceae* and
136 lower accuracy for staphylococci. To circumvent the lower accuracy of bacterial identification
137 for staphylococci, we perform a MALDI-TOF identification from bacterial pellet (Prod'hom
138 *et al.*, 2010).

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141

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165

166 **Legends**

167 Table 1 :

168 Direct VITEK identification obtained from bacterial pellet compared to reference VITEK
169 identification obtained after subcultured colonies. Please not that misidentification were only
170 observed for Gram positive bacteria

171

172 Table 2:

173 Comparison of MIC's determined using the VITEK 2 method obtained from direct
174 inoculation of the blood-culture bacterial pellet with MIC's determined using the VITEK 2
175 method obtained from subcultured colonies (reference method) and analysis of categorical
176 errors.

177

178 Table 1

Species	Total	Correct identification (%)	Misidentified*	Not identified
Enterobacteriaceae	**88**	**87 (99)**		**1**
Escherichia coli	50	50		
Klebsiella pneumoniae	16	16		
Enterobacter cloacae	9	8		1
Klebsiella oxytoca	5	5		
Other *Enterobacteriaceae* †	8	8		
Non-fermentative Gram-negative bacteria	**7**	**5 (71)**		**2**
P. aeruginosa	4	4		
Other non-fermentative Gram-negative bacteria ‡	3	1		2
Staphylococci	**180**	**133 (74)**	**30**	**17**
S. epidermidis	85	66	10	9
S. aureus	77	55	16 §	6
S. hominis	9	5	2	2
Other staphylococci ǁ	9	7	2	
Other Gram-positive cocci ¶	**3**	**1**	**1**	**1**
Total	**278**	**226 (81)**	**31**	**21**

179

180 * In case of discordance, identification was confirmed by MALDI-TOF or other reference
181 methods.

182 † *Serratia marcescens* (3), *Proteus mirabilis* (2), *Citrobacter freundii* (1), *Citrobacter koseri*
183 (1), *Enterobacter aerogenes* (1).

184 ‡ *Achromobacter xylosoxidans* (1), *Pseudomonas fluorescens* (1), *Stenotrophomonas*
185 *maltophilia* (1).

186 § *S. aureus* misidentification: *S. intermedius* (13), *S. chromogenes* (1), *Streptococcus*
187 *pyogenes* (1), *Kocuria rosae* (1).

188 ǁ *S. capitis* (3), *S. schleiferi* (2), *S. auricularis* (1), *S. lugdunensis* (1), *S. warneri* (1), *S.*
189 *xylosus* (1).

190 ¶ *Micrococcus luteus* (1), *Peptinophilus sp.* (1), *Streptococcus pyogenes* (1)

191 Table 2.

192

Bacteria/Drugs (no. of strains)	No. of VITEK 2 MICs that differed from reference MICs by the indicated dilution *								No. of errors after discrepancy analysis †			
	<-2	-2	-1	0	+1	+2	>+2	EA (%)	CA [](%)	md [] (%)	ME [] (%)	VME [] (%)
Enterobacteriaceae (87 ‡)												
Amikacin § (86)			1	78	6	1		85 (98.8)	86 (100)			
Amoxicillin/clavulanate (86)		1	9	70	5		1	84 (97.7)	84 (97.7)	1 (1.2)	1 (1.2)	
Ampicillin (86)	1		5	77	3			85 (98.8)	86 (100)			
Cefalotin (86)		1	9	68	8			85 (98.8)	79 (91.9)	7 (8.9)		
Cefepime (86)			1	84			1	85 (98.8)	85 (98.8)		1 (1.2)	
Cefotaxime (85 ‡)			1	84				85 (100)	85 (100)			
Cefoxitin (86)			3	80	3			86 (100)	85 (98.8)	1 (1.2)		
Cefpodoxime (86)		1	6	77	1	1		84 (97.7)	86 (100)			
Ceftazidime (86)				85			1	85 (98.8)	85 (98.8)		1 (1.2)	
Cefuroxime (86)			6	74	4	2		84 (97.7)	84 (97.7)	2 (2.4)		
Ciprofloxacin (86)				86				86 (100)	86 (100)			
Gentamicin (86)	1			84		1		84 (97.7)	85 (98.8)			1 (1.2)
Meropenem (86)				85			1	85 (98.8)	85 (98.8)		1 (1.2)	
Nitrofurantoin (86)		1	15	60	10			85 (98.8)	73 (84.9)	12 (16.4)		1 (1.4)
Norfloxacin (86)				85	1			86 (100)	85 (98.8)	1 (1.2)		
Piperacillin (85 ‡)		2	3	75	4		1	82 (96.5)	83 (97.6)	2 (2.4)		
Piperacillin/tazobactam (86)	3	2		80			1	80 (93)	81 [80] (94.2)	4 [3] (4.9)	1 (1.2)	0 [2]
TMP-SMX || (86)				86				86 (100)	86 (100)			
Tobramycin (85 ‡)				84	1			85 (100)	85 (100)			
Total (1631)	**5**	**8**	**59**	**1502**	**46**	**5**	**6**	**1607 (98.5)**	**1594 [1593] (97.7)**	**30 [29] (1.9)**	**5 (0.3)**	**2 [4] (0.1)**
Staphylococci (133)												
Clindamycin (133)	1	2		128		1	1	128 (96.2)	129 [128] (97)	4 (3.1)	0 [1]	
Erythromycin § (133)	8			123		1	1	123 (92.5)	131 (98.5)			2 (1.5)
Fosfomycin § (133)		4	1	128				129 (97)	133 [132] (100)			0 [1]
Fusidic Acid § (133)		8	16	107	1	1		124 (93.2)	116 (87.2)	17 (14.7)		
Gentamicin (133)	1		7	119	5	1		131 (98.5)	125 (94)	8 (6.4)		
Levofloxacin § (133)		1	32	94	5		1	131 (98.5)	124 (93.2)	9 (7.3)		
Linezolid § (133)		1	26	100	6			132 (99.2)	133 [132] (100)			0 [1]

Moxifloxacin § (133)			20	112	1			133 (100)	123 (92.5)	10 (8.1)		
Mupirocin (133)			1	132				133 (100)	133 (100)			
Nitrofurantoin § (133)			25	104	4			133 (100)	124 (93.2)	9 (7.3)		
Oxacillin § (133)		4	32	96	1			129 (97)	132 (99.2)		1 (0.8)	
Penicillin (133)		3	5	120	4	1		129 (97)	132 [131] (99.2)		1 (0.8)	0 [1]
Rifampicin (133)				133				133 (100)	133 (100)			
Teicoplanin § (133)	13	21	23	69	5	1	1	97 (72.9)	127 (95.5)	5 (3.9)		1 (0.8)
Tetracycline (133)			13	105	13	2		131 (98.5)	129 (97)	4 (3.1)		
Tigecycline (133)		2	11	116	3	1		130 (97.7)	133 (100)			
TMP-SMX § (133)	4	9	15	100	5			120 (90.2)	114 [111] (85.7)	4 (3.5)	0 [2]	15 [20] (13.2)
Tobramycin § (133)	1	1	8	119	4			131 (98.5)	127 [126] (95.5)	6 (4.7)		0 [1]
Vancomycin (133)		3	39	54	35	2		128 (96.2)	133 (100)			
Total (2527)	**28**	**59**	**274**	**2059**	**92**	**11**	**4**	**2425 (96)**	**2431 [2423] (96.2)**	**76 [72] (3.1)**	**2 [5] (0.1)**	**18 [27] (0.7)**

193

194 * Differences in MICs (<-2, -2, -1, 0, +1, +2, >+2) indicate log2 differences of VITEK 2 MICs obtained from direct inoculation versus colonies. EA (essential

195 agreement): no. of MICs concordant with reference VITEK MICs +/- 1 two-fold dilution.

196 † CA: number of categorical agreement (i.e. susceptible, intermediate, resistant). In bracket [ ]: no. of errors before discrepancy analysis. VME: no. of very

197 major error (falsely susceptible), ME: no. of major error (falsely resistant), md: no. of minor discrepancies (all other errors).

198 ‡ One inoculum by direct VITEK failed to grow for all antibiotic tested. In 3 cases, VITEK provided an alert for insufficient grow for 1 antibiotics.

199 § Significative difference of CMIs using Wilcoxon signed rank test (P<0.05)

200 || TMP-SXT: Trimethoprim-sulfamethoxazole

201