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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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Abstract

An ammonium chloride procedure to prepare bacterial pellet from positive blood cultures was used for direct inoculation of VITEK. Correct identification reached 99% for *Enterobacteriaceae* and 74% for staphylococci. For susceptibility testing, very major and major errors were 0.1% and 0.3% for *Enterobacteriaceae*, and 0.7% and 0.1% for staphylococci.

Bacterial pellets prepared with ammonium chloride allow direct inoculation of VITEK cards with excellent accuracy for *Enterobacteriaceae* and lower accuracy for staphylococci.

Main manuscript

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

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

mixed to 40 ml of sterile water and centrifuged at 1'000xg for 10 min. Supernatant and blood cells layer were removed. The remaining blood cells were lysed mixing 1 ml of ammonium chloride (0.15 M NH4Cl, 1 mM KHCO3; pH 7.31) to the bacterial pellet and a second centrifugation step at 140xg for 10 min was done. Supernatant was discarded. When the pellet remained hemorrhagic, the lysing step was repeated with 2 ml of water (Prod'hom *et al.*, 2010).

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

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

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

colony inoculation of VITEK cards. AST discordance results were classified as: VME (false susceptible); ME (false resistant) and md (all others). In case of misidentification, strains were retested using MALDI-TOF MS directly from colonies. The identification of these isolates by MALDI-TOF was performed on a Microflex LT instrument (Bruker Daltonics, Leipzig, Germany) with FlexControl software (version 3.0) (Bizzini *et al.*, 2010). Discrepancies of AST (VME and ME) were solved by testing isolates using Etest system (BioMérieux). For each antibiotic Wilcoxon signed rank test were performed to evaluate the MIC values after log conversion. P value <0.05 were considered significant.

During the study period, 278 positive blood culture where included in the study. Table 1 shows the results of the VITEK identification obtained directly from bacterial pellet compared to final identification. Overall 226/278 (81%) gave a correct identification at the species level when VITEK was directly inoculated with bacterial pellets. The proportion of correct identification for *Enterobacteriaceae,* non-fermentative GNB, staphylococci and other Gram positive cocci were of 87/88 (99%), 5/7 (71%), 133/180 (74%) and 1/3 (33%), respectively (Table 1). Misidentifications were observed for 31/278 (11%) bacterial pellets. For 21/278 (8%) bacterial pellets, VITEK gave no-identification. Noteworthy, all bacterial pellets identified as *S. aureus* by the VITEK system were correct. However, as many as 16/77 *S. aureus* (21%) were misidentified (Table 1).

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

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

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

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

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

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

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

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

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

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Legends

Table 1 :

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

Table 2:

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

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 ||	9	7	2	
Other Gram-positive cocci ¶	**3**	**1**	**1**	**1**
Total	**278**	**226 (81)**	**31**	**21**

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

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

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

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

|| *S. capitis* (3), *S. schleiferi* (2), *S. auricularis* (1), *S. lugdunensis* (1), *S. warneri* (1), *S. xylosus* (1).

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

191 Table 2.

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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)**

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

† CA: number of categorical agreement (i.e. susceptible, intermediate, resistant). In bracket [ ]: no. of errors before discrepancy analysis. VME: no. of very major error (falsely susceptible), ME: no. of major error (falsely resistant), md: no. of minor discrepancies (all other errors).

‡ 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.

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

|| TMP-SXT: Trimethoprim-sulfamethoxazole