Improved blood culture workflow for faster identification of KPC-producing *Enterobacterales*

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Abstract

Carba-NP original report for blood cultures described the need of subculture and mechanical lysis before testing, reaching the turnaround time of approximately 4 hours for sample preparation. We tested 100 consecutive blood cultures positive for Gram-negative bacilli on the Gram stain from a large clinical laboratory. Bacterial pellets were prepared by centrifugation and submitted to Carba-NP and Blue-Carba tests and used further to prepare smears for Vitek MS. Results obtained with colonies grown on sheep blood agar using the same methodologies were used as the gold standard. Carbapenemase genes were confirmed by PCR and DNA sequencing. Vitek MS identified correctly 86% of the samples. Of note, 7% of the samples were incorrectly reported by the instrument as containing a single isolate. KPC-2 was the predominant carbapenemase detected. There was 100% concordance for both negative and positive results for Carba-NP. In contrast, for Blue-Carba the concordance for positive results was 92.8%, and 41% of strains negative for carbapenemases presented a yellowish color on control well turning the test non-interpretable. The turnaround time for sample preparation for preparing the pellet was 13 min, and no subculture or mechanical lysis is needed when detecting KPC production in *Enterobacterales*.

Keywords KPC · Carbapenemases · Carba-NP · Blue-Carba · Blood culture

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The central idea of this work—using bacterial pellets from clinical positive blood cultures for Carba-NP and Blue Carba—was presented at the 28th Brazilian Congress of Microbiology held in 2015 at Florianópolis, Brazil and was awarded the bioMérieux prize.

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Introduction

Carbapenemase production in *Enterobacterales* is a major concern worldwide. Among them, *Klebsiella pneumoniae* carbapenemases (KPCs) are the most prevalent worldwide [1]. The expression of these enzymes leads to resistance to most clinically available β -lactam antibiotics, drastically reducing the therapeutic options and treatment effectiveness against infections caused by these microorganisms. Infections caused by KPC-producing *Enterobacterales* have been associated with higher mortality, particularly in blood-stream infections and the time under inadequate therapy is one of the variables influencing mortality [2]. The faster carbapenemase-producing *Enterobacterales* (CPE) are detected, the earlier adequate empirical therapy can be initiated, potentially increasing patient's survival [3].

Two rapid methods—Carba-NP and Blue-Carba—have been proposed for the rapid detection of CPE and are based

on acid production during imipenem hydrolysis [4, 5]. Carba-NP has been demonstrated to detect CPE directly from spiked blood cultures but requires subculture of 300 µL of the positive blood culture broth in a 20-ml brain infusion broth containing imipenem and ZnSO₄ before centrifugation and mechanical lysis of the bacterial pellet, resulting in a turnaround time of 3 h and 50 min for sample preparation. Additionally, the method has not been tested in the blood culture routine in a clinical microbiology laboratory or coupled with rapid species identification [6]. The Blue-Carba method was originally described for use with bacterial colonies grown on solid media [5]. To date, there are no reports on the use of Blue-Carba method to test bacterial pellets obtained from positive blood cultures.

This work aimed to evaluate if two rapid colorimetric carbapenemase detection methods would be adequate when using bacterial pellets, without subculture or mechanical lysis, prepared from positive clinical blood cultures.

Materials and methods

Blood culture vials

Blood samples were collected in Bactec Plus aerobic and anaerobic blood bottles in a clinical microbiology laboratory routine and incubated in Bactec FX (Becton-Dickinson) instruments until they were detected as positive. Vials were selected for analysis based on Gram-stain result indicating the presence of Gram-negative bacilli. Vials were processed for bacterial pellet preparation in a maximum of 12 h after they were detected as positive by the instrument. A total of 100 consecutive samples, one per patient, were included in the study.

Preparation of the bacterial pellet

A volume of 12 mL of blood culture was transferred to a 15-mL disposable conic tube. Bacteria isolation was performed accordingly to Hoyos-Mallecot et al. 2014 [7] with modifications. The sample was centrifuged at 600×g for 10 min, for decanting eukaryotic cells. An aliquot of 2 mL of the supernatant was transferred to each of five sterile microtubes, totalizing 10 mL of the supernatant. The microtubes were centrifuged at 20,000×g for 1 min, and the supernatant was discarded. A 1 mL of sterile reagent grade water was added to each tube and vortexed, following a 1-min centrifugation at 20,000×g. This step was repeated once, and bacterial pellets were used for the Carba-NP [4] and Blue-Carba [5] tests.

Bacterial identification

An aliquot of the bacterial pellet was tooth-picked and used to prepare two smears on the Vitek-MS slide. One microliter of

the formic acid solution at 70% (Sigma-Aldrich) was added before the smear was allowed to dry. One microliter of the matrix (α -Cyano-4-Hydroxycinnamic Acid—10 mg/L) was then added. After smears were completely dry, the slide was introduced into the Vitek MS system and identification was carried out using the Myla® software. In order to evaluate the concordance between the two methods of smear preparation, identification to the species level was also carried out after colonies were grown on sheep blood agar, using the same instrument and procedure.

Carbapenemase detection using Carba-NP

The method was performed according to Dortet et al. [8], with modifications. The bacterial pellet was suspended with 100 µL of lysis buffer produced in-house (2% IGEPAL CA-630 in Tris-HCl 20 mM pH 7.55 ± 0.05) instead of BPER® (Thermo) and incubated at ambient temperature during 15 min. For the preparation of imipenem solution, 10 mg of the imipenem/cilastatin commercial product (ABL, Brazil) was used. For each sample tested, 50 µL aliquot of solution A, containing phenol red and zinc sulfate [8], without imipenem, and the same volume of solution A containing imipenem were placed in individual wells of a 96-well microdilution plate. A total of 50 µL of the lysed bacterial suspension were added to each of the two wells. The *K. pneumoniae* ATCC BAA1705 strain was used as a positive control in each testing lot. The plate was covered with an adhesive plastic film and incubated at 37 °C without shaking. Readings were performed after 15 min, 30 min, 1 h, and 2 h of incubation. Interpretation of color changes was performed as previously recommended [8] and detailed in Fig. 1. The Carba-NP test was also performed with colonies grown on sheep blood agar in order to evaluate the concordance with results obtained with bacterial pellets.

Fig. 1 Interpretative criteria used in this work for color changes. NC, negative control without imipenem; IMP, solution with imipenem

Carbapenemase detection using Blue-Carba

The Blue-Carba test was performed as previously described by Pires et al. 2013 [5], with the following modifications: the bacterial pellet was suspended in 50 μ L of sterile water, 0.04% bromothymol blue (BTB) solution containing 10-mM zinc sulphate (pH 6.80 ± 0.05) and 10 mg of commercial imipenem/cilastatin (ABL, Brazil) were used. For each sample tested, 75 μ L of BTB solution and 75 μ L of BTB solution containing imipenem (10 mg/mL) were added to a 96-well microdilution plate. For each of the two wells, 25 μ L of the bacterial suspension were added. The plate was covered with an adhesive plastic film and incubated at 37 °C. The *K. pneumoniae* ATCC BAA1705 strain was used as a positive control in each testing lot. Readings were performed after 15 min, 30 min, 1 h, and 2 h of incubation. Interpretation of color changes was performed as previously recommended [5] and detailed in Fig. 1. The Blue-Carba test was also performed with colonies grown on sheep blood agar to evaluate the concordance with results obtained with bacterial pellets.

Genotypic identification of carbapenemases

Isolates grown on sheep blood agar, showing a positive Carba-NP or Blue-Carba test were tested for the presence of *bla*_{OXA-48-like} [9] and *bla*_{KPC} [10] as previously described and for the presence of *bla*_{NDM} genes using the following primers and probe: NDM F: 5' –CTATCTCG ACATGCCGGGTTT – 3'; NDM R: 5' – CCGCCATC CCTGACGAT – 3'; NDM S: 5' VIC – TCGCTTCC AACGGTTT – MGB 3'. The full *bla*_{KPC} gene sequences were determined using primers 5'CTAGCTCCACCTTC AAACAAGGAA and 3'TGGGTGGGCCAATAGATGAT for amplification. Sequencing was carried out using the Big Dye Terminator kit version 3.1 in a 3130xl Genetic Analyzer (Applied Biosystems) with the same primers used for PCR plus primers 5'GCCGCCAATTTGTT GCTGAA and 3'GCAGAGCCCCAGTGTCAGTTT.

Estimation of hands-on and turnaround time for carbapenemase detection and species identification

The estimated time for the procedure to be completed was computed during the manipulation of the samples using a timer. The timer was stopped while in centrifugation and incubation times for calculation of the hands-on time. This time was included in the calculation of the total turnaround time.

Results

Clean bacterial pellets were obtained from all blood culture samples tested

A total of 100 consecutive, one per patient, aerobic, and anaerobic blood bottles were included in this study. Blood cells and the supernatant containing bacterial cells were successfully separated with differential centrifugation. All samples generated clear bacterial pellet with no visible amount of red blood cells after the two washing step with water.

The Vitek MS system reports using bacterial pellets were correct in 86% of the samples tested

Considering the identification from colonies obtained on solid media as the gold standard, the largest fraction of the samples (78%) contained *Enterobacterales*, while anaerobic and microaerophilic bacteria and non-fermenters were detected, respectively in 4% and 20% of the samples (Table 1). The Vitek-MS® system reports using smears prepared with bacterial pellets were correct in 86% of all samples tested. Nine samples (9%) with two or more species were detected in this study. Of these, seven (7%) were incorrectly reported as containing a single species, while the remaining samples containing two or more species were correctly reported as no reliable identification.

The Carba-NP test is superior to the BLUE-Carba for detection of CPE directly from blood culture vials

In this study, 11 (11.6%) of all *Enterobacterales*, two *Aeromonas* sp. strains and one *E. meningoseptica* strain were positive for Carba-NP when bacterial pellets were tested. There was a 100% concordance for both negative and positive Carba-NP results when results obtained with bacterial pellets were compared with those obtained with colonies grown on sheep blood agar. PCR and sequencing reactions evidenced that all *Enterobacterales* harbored the *bla*_{KPC-2}, except one *E. coli* strain that contained a new *bla*_{KPC-2-like} gene. No *bla*_{OXA-48-like} or *bla*_{NDM} genes were detected among carbapenemase-producing strains.

Concerning the Blue-Carba test, all KPC-producing *Enterobacterales* and the *E. meningoseptica* were detected as positive using bacterial pellets but one carbapenemase-producing *Aeromonas* strain was not detected by the Blue-Carba using the bacterial pellet; consequently, the concordance for positive results was 92.8%. When negative results obtained from gold standard method were compared to those from bacterial pellets, in 41% of the samples, the BLUE-Carba showed a yellowish color after adding the bacterial suspension to the control well, turning the test uninterpretable (Fig. 2). This phenomenon was observed

Table 1 Results obtained with smears prepared from the bacterial pellet and colonies on sheep blood agar using the Vitek MS (bioMérieux)

MALDI-ToF-MS identification from bacterial pellet	MALDI-ToF-MS from colonies grown on sheep blood agar	Number of samples	CarbaNP positive	Enzyme
Correctly indicating a single species		84		
<i>Escherichia coli</i>	<i>E. coli</i>	31	1	KPC-new
<i>Klebsiella pneumoniae</i>	<i>K. pneumoniae</i>	17	7	KPC-2
<i>Pseudomonas aeruginosa</i>	<i>P. aeruginosa</i>	8		
<i>Enterobacter cloacae</i> complex	<i>E. cloacae</i> complex	5		
<i>Serratia marcescens</i>	<i>S. marcescens</i>	3		
<i>Bacteroides</i> sp.	<i>Bacteroides</i> sp.	3		
<i>Burkholderia cepacia</i> complex	<i>B. cepacia</i> complex	2		
<i>K. oxytoca</i>	<i>K. oxytoca</i>	2		
<i>Ralstonia mannitolilytica</i>	<i>R. mannitolilytica</i>	2		
<i>Aeromonas</i> sp.	<i>Aeromonas</i> sp.	1	1	Other*
<i>Acinetobacter baumannii</i> complex	<i>A. baumannii</i> complex	1		
<i>Campylobacter jejuni</i>	<i>C. jejuni</i>	1		
<i>Elizabethkingia meningoseptica</i>	<i>E. meningoseptica</i>	1	1	Other*
<i>Klebsiella aerogenes</i>	<i>K. aerogenes</i>	1		
<i>Proteus mirabilis</i>	<i>Proteus mirabilis</i>	1		
<i>Pseudomonas stutzeri</i>	<i>P. stutzeri</i>	1		
<i>Ralstonia pickettii</i>	<i>R. pickettii</i>	1		
<i>Raoultella ornithinolytica</i>	<i>R. ornithinolytica</i>	1		
<i>Salmonella</i> sp.	<i>Salmonella</i> sp.	1		
<i>Stenotrophomonas maltophilia</i>	<i>S. maltophilia</i>	1		
Incorrectly indicating a single species but with two or more species on solid media		7		
<i>Citrobacter freundii</i>	<i>Streptococcus agalactiae</i> + <i>C. freundii</i>	1		
<i>E. cloacae</i> complex	<i>K. pneumoniae</i> + <i>E. cloacae</i> complex	1	1	KPC-2
<i>E. coli</i>	<i>Proteus mirabilis</i> + <i>E. coli</i>	1		
<i>E. coli</i>	<i>E. coli</i> + <i>Aeromonas</i> sp.* + <i>K. oxytoca</i>	1	1	Other*
<i>K. pneumoniae</i>	<i>K. pneumoniae</i> + <i>E. cloacae</i> complex	1		
<i>K. pneumoniae</i>	<i>K. pneumoniae</i> + <i>P. aeruginosa</i>	2	1	KPC-2
No reliable identification due to the presence of two or more species on solid media		2		
	<i>A. haemolyticus</i> + <i>Leclercia adecarboxylata</i>	1		
	<i>E. cloacae</i> complex + <i>E. coli</i>	1		
No reliable identification but a single species on solid media		7		
	<i>E. cloacae</i> complex	2		
	<i>E. coli</i>	2		
	<i>K. pneumoniae</i>	1	1	KPC-2
	<i>Morganella morganii</i>	1		
	<i>R. ornithinolytica</i>	1		
Total number of <i>Enterobacterales</i> detected		95		
Total number of samples tested		100		

*Other—carbapenemase other than KPC, NDM, or OXA48-like

with *E. coli* and *K. pneumoniae*. There was no difference regarding the detection time for both methods. From the group of samples containing carbapenemase-producing strains ($n = 14$), the largest amount (8) was identified within 15 min of incubation while one was detected in 30 min, two in 30 min, and three in 2 h.

Turnaround and hands-on times

The hands-on time for a single sample, from the moment the blood culture sample was transferred to the 15-ml tube, to the moment the carbapenemase tests and the Vitek MS slide were ready was 13 min. The total turnaround for processing a single

Fig. 2 Carba-NP and BLUE-Carba tests. Control wells are located on columns 1 and 3, while wells containing imipenem are located on columns 2 and 4. Columns 1 and 2 correspond to the Carba-NP test, while columns 3 and 4 correspond to BLUE-Carba. Strain 27 is a KPC-2-producing *K. pneumoniae*. Strains 26, 28, 29, and 30 are negative for carbapenemase production. Note control wells with acidic reaction for samples 28, 29, and 30 (column 3). The four wells at the bottom of the figure correspond to the KPC-producing control strain *K. pneumoniae* ATCC BAA1705

sample, and including the final reading after 2 h of incubation, was 2 h and 31 min.

Discussion

In this work, we demonstrated the feasibility of performing both species identification by MALDI-ToF MS and carbapenemase detection using a colorimetric method from bacterial cells pellets obtained from positive blood cultures showing Gram-negative bacilli on the microscopy.

Many articles in the literature have described different methods for obtaining bacterial pellets from positive blood culture vials to be used for identification by MALDI-ToF [11, 12]. In this work, we were able to get a correct identification in 86% of all samples tested. Our findings are lower than a recent publication that reported a 95% success rate in obtaining a correct identification for samples containing

Gram-negative bacilli [13], but in this study, we had 7% error rate due to the presence of polymicrobial bacteremia that influences the performance of the equipment. Compared to the method pointed out by these authors as the best one, our method is simpler and does not require the use of ammonium chloride solution for lysis of red blood cells, which would turn the bacterial pellet inappropriate for use in preparing suspensions for susceptibility tests or carbapenemase detection.

The Carba-NP test has been tested with spiked blood cultures [8], but no reports have emerged demonstrating its performance in the clinical microbiology laboratory setting on a routine basis, nor has a comparison between Carba-NP and BLUE-Carba for this use been reported. In this work, we compared the performance of Carba-NP and BLUE-Carba in the detection of CPE directly from positive blood culture vials. We found a 100% concordance for both positive and negative results for the Carba-NP. Our findings are in accordance with the original work of Dortet et al. 2014 [8] which reported a 100% sensitivity and specificity for KPC producers. These authors reported a maximum time of 30 min for detection of KPC producers, while in this work, most of the CPE were detected in 15 min and some of them were detected only after 2 h of incubation. One possibility for this divergence is the fact that they used spiked blood cultures and we used clinical samples that differ concerning the presence of antimicrobials, phagocytes, antibodies, and the number of bacterial cells present in each vial. The Blue-Carba detected all KPC-producing strains, but the yellowish color on 41% of the control wells would be a limitation for the detection of weak carbapenemases as OXA-48-like.

Our work has a limitation concerning that no NDM or OXA-48-like enzymes were not detected; consequently, the adequacy of both methods to detect these enzymes using bacterial pellets remains to be defined.

In summary, we demonstrated that a simple concentration method, with a 13 min turnaround time, can be used to prepare bacterial pellets to carry out the Carba-NP and Blue-Carba tests for KPC detection and prepare smears for analysis with the Vitek MS, without the need of sub-culture or mechanical lysis.

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