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Rapid Detection of Carbapenemase Production Directly from Blood Culture by Colorimetric Methods: Evaluation in a Routine Microbiology Laboratory

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ABSTRACT The aim of this study was to evaluate the two rapid colorimetric methods (CNPt-Direct and Blue-Carba) for the detection of carbapenemase production directly from blood culture in a routine microbiology laboratory. The methods were initially evaluated on spiked blood cultures with 61 carbapenemase-positive isolates. Afterwards, they were used in blood cultures (314 samples were evaluated) obtained from patients in a routine microbiology laboratory during a period of 6 months. The colorimetric methods were compared to the conventional culture of blood. The results of the spiked blood cultures indicated that both colorimetric methods presented positive results for the vast majority (95%) of the isolates harboring KPC, NDM, and IMP genes. However, the assay failed to detect many GES- and OXA-48-like-positive isolates (65% positive results). In the second part of the study, a total of 314 blood cultures from patients were evaluated, and 33 yielded *Enterobacteriaceae* isolates resistant to meropenem (30 isolates were positive for carbapenemases according to PCR). The colorimetric tests correctly detected 24 out of the 30 carbapenemase-positive isolates directly from the blood vial (80% positive results). Overall positive percent agreement and negative percent agreement were 80% and 100%, respectively. The colorimetric assays are simple and cost-effective methods that can be implemented in a routine microbiology laboratory, diminishing the time necessary to detect carbapenemase-producing isolates from 24 to 48 h to 3 to 5 h. Moreover, according to our results, the positive colorimetric test results do not need to be confirmed and can be immediately provided to the attending physician.

KEYWORDS blood culture, carbapenemase, colorimetric methods, rapid test

Carbapenemase-producing Gram-negative rods are microorganisms causing hospital infections, including bloodstream infections (BSI), which are associated with high morbidity and mortality worldwide (1). The best outcomes for BSI are achieved with early start of adequate therapy (2); nevertheless, precise diagnosis of bacteremia usually takes at least 48 h, and for this reason, broad-spectrum antibiotics are, in most cases, administered empirically. Moreover, the empirical treatment for patients with symptoms of systemic infection is usually a combination of antibiotics, which can be neurotoxic and nephrotoxic (3, 4). Therefore, it is paramount to shorten the time to provision of appropriate therapy for patients with BSI (5). Fast identification of the bacteria, as well as of their resistance profile in the routine microbiology laboratory, plays a crucial role in the treatment of patients.

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The Carba NP test is a colorimetric rapid test based on *in vitro* hydrolysis of imipenem using phenol red as a pH indicator that is used to detect carbapenemase production from bacterial isolates (6). The CNPt-Direct test is a simplified version of Carba NP that alternatively uses Triton X-100 as an enzymatic extractor (7). Another colorimetric test, Blue-Carba, has the same principle as the Carba NP and CNPt-Direct tests but uses bromothymol blue as a pH indicator and does not need the enzymatic extraction step (8). These colorimetric tests have already been extensively evaluated for the detection of carbapenemase production from pure bacterial colonies (9, 10) and eventually from spiked blood culture bottles (11). Other techniques for the detection of carbapenemase production directly from blood culture have been published (12); however, they are usually based on molecular detection. In fact, only a few studies also assessed the use of colorimetric assays directly from blood cultures in a routine laboratory; however, these studies used different protocols, which are either more expensive or longer than the one we have used in our study (13, 14).

The aim of this study was to evaluate two simple and rapid protocols, CNPt-Direct and Blue-Carba (colorimetric assays), for the detection of carbapenemase production directly from blood culture bottles, i.e., without isolation of bacteria in pure culture. The colorimetric methods were initially evaluated on spiked blood cultures with carbapenemase-positive isolates. Afterwards, the methods were used in blood cultures obtained from patients in a routine microbiology laboratory during a period of 6 months.

MATERIALS AND METHODS

Multiplex PCR for carbapenemase genes. The presence of carbapenemase genes was investigated by a high-resolution melting (HRM) real-time multiplex PCR described previously by Monteiro et al. (15). Briefly, the DNA from each isolate was extracted, and amplifications were performed in 25 ml of the master mix containing 12.5 ml of 2× high-resolution melt (HRM) PCR master mix (Qiagen, Germany). The multiplex real-time PCR was performed using specific primer sets (KPC-F TCGCTAAACTCGAACAGG, KPC-R TTACTGCCCGTTGACGCCCAATCC, NDM-F TTGGCCTTGCTGTCCTTG, NDM-R ACACCAGTGACAATATCACCG, GES-F CTATTACTGGCAGGGATCG, GES-R CCTCTCAATGGTGTGGGT, OXA-48-F TGTTTTTGGTGGCATCGAT, OXA-48-R GTAAMRATGCTTGGTTCGC, IMP-F GAGTGGCTTAATTCTCRATC, IMP-R AACTAYCCAATAYRTAAC, VIM-F GTTTGGTCGCATATCGCAAC, and VIM-R AATGCGCAGCACCAGGATAG). The multiplex real-time PCR conditions were as follows: 95°C for 5 min; 35 cycles of 95°C for 20 s, 55°C for 45 s and 72°C for 30 s; and a melt curve step (from 65°C, gradually increasing by 0.1°C/s to 95°C, with fluorescence data acquisition every 1 s).

Isolates for the spiked blood culture (protocol evaluation). From a previous surveillance study (16), 61 carbapenem-resistant *Enterobacteriaceae* (CRE) isolates were selected for the first part of the study (protocol evaluation). These isolates carried at least one carbapenemase gene (19 bla_{KPC}, 6 bla_{GES}, 17 bla_{NDM}, 4 bla_{IMP}, 11 $bla_{OXA\text{-}48\text{-}like}$, and 4 $bla_{OXA\text{-}48\text{-}like}$ + bla_{NDM}) and included 20 *Klebsiella pneumoniae*; 1 *Klebsiella oxytoca*; 21 *Enterobacter cloacae* complex; 1 *Klebsiella aerogenes*; 2 *Enterobacter* sp.; 6 *Escherichia coli*; 4 *Morganella morganii*; 4 *Kluyvera intermedia*; 1 *Providencia rettgeri*, and 1 *Citrobacter freundii* isolate.

Spiked blood culture assay. The first part of the study used blood culture bottles (bioMérieux) with addition of 10 ml of sterile human blood spiked with a bacterial inoculum of 10^3 CFU/ml (final concentration). The inoculum was prepared by diluting 0.5 McFarland suspension (10^8 CFU/ml) in sterile saline with the CRE isolate derived from an overnight culture. Spiked blood culture bottles (BacT/Alert FA Plus; bioMérieux) were incubated in an automated system (BacT/Alert) until the flask was flagged as positive.

The positive-flagged blood culture was evaluated according to a modified assay proposed by Dortet et al. (11). Briefly, an aliquot of 300 μl of the blood culture was inoculated in 20 ml of Luria broth (LB) supplemented with 0.12 μg/ml of imipenem (final concentration) and 70 μg/ml of $ZnSO_4$ (final concentration). The LB was incubated at 37°C for 2 h, with agitation. The bacterial pellet was recovered by centrifugation at 4,000 × *g* for 15 min. The bacterial pellet was resuspended in 1 ml of sterile water and distributed in four wells (100 μl each), in a microtiter plate.

Colorimetric assays. The CNPt-Direct and Blue-Carba solutions were prepared according to Pasteran et al. (7) and Pires et al. (8), respectively, with minor modifications as follows: the concentration of all components was duplicated (final solution, 2× concentrated). An aliquot of 100 μl of the 2× concentrated Carba NP-Direct solution, without antibiotic, was added to one of the four wells containing 100 μl of bacterial inoculum, resulting in a 1× concentrated solution. The same was done for CNPt-Direct solution supplemented with antibiotic, as well as for the Blue-Carba solution supplemented and not supplemented with antibiotic. The microtiter plate was incubated at 37°C for a maximum of 2 h. The CNPt-Direct and Blue-Carba were considered positive when the color in the well containing the antibiotic changed from red to yellow/orange and from green/blue to yellow, respectively.

CNPt-Direct and Blue-Carba in a routine microbiology laboratory. The second part of the study consisted of applying the colorimetric assays in a routine microbiology laboratory. The routine micro-

TABLE 1 Results of the colorimetric assays from spiked blood culture

β-Lactamase class and identification (no. of isolates)	Bacterial species identification (no. of isolates)[a]	Blood culture (no. of isolates positive/total no. of isolates [%])	
		CNPt-Direct	Blue-Carba
Class A carbapenemases			
KPC (19)	*Klebsiella pneumoniae* (13P), *Enterobacter cloacae* complex (1P), *Enterobacter aerogenes* (1P), *Escherichia coli* (1P + 1N), *Morganella morganii* (1P + 1N)	17/19 (89)	17/19 (89)
GES (6)	*Klebsiella pneumoniae* (1P), *Providencia rettgeri* (1P), *Kluyvera intermedia* (2P + 2N)	4/6 (66)	4/6 (66)
Class B carbapenemases			
NDM (17)	*Klebsiella oxytoca* (1P), *Klebsiella pneumoniae* (2P), *Enterobacter cloacae complex* (7P), *Enterobacter* sp. (2P), *Escherichia coli* (2P), *Morganella morganii* (2P), *Citrobacter freundii* (1P)	17/17 (100)	17/17 (100)
IMP (4)	*Klebsiella pneumoniae* (3P), *Enterobacter cloacae* complex (1P)	4/4 (100)	4/4 (100)
Class D carbapenemase			
OXA-48-like (11)	*Klebsiella pneumoniae* (1P), *Enterobacter cloacae* complex (4P + 4N), *Escherichia coli* (2P)	7/11 (64)	7/11 (64)
Carbapenemase coproducer			
OXA-48-like + NDM (4)	*Enterobacter cloacae* complex (4P)	4/4 (100)	4/4 (100)

[a]No. of isolates from a species that presented positive or negative results in the test is given. P, positive; N, negative.

biology laboratory is located in an 840-bed University Hospital (Hospital de Clínicas de Porto Alegre), located in Porto Alegre, southern Brazil. The routine microbiology laboratory receives around 18 blood samples per day to be cultured. The blood culture was processed according to conventional culture of blood in an automated system (BacT/Alert), followed by isolation of the bacteria in solid media (pure culture) for identification and susceptibility testing. Identification of the bacteria was made in the Vitek system (bioMérieux), and the susceptibility test was performed using the disc diffusion method, according to CLSI guidelines (17). If the isolate was resistant to meropenem, a set of carbapenemase genes (bla_{KPC}, bla_{NDM}, bla_{IMP}, bla_{VIM}, bla_{GES}, and $bla_{OXA-48-like}$) was investigated by a multiplex high-resolution melting (HRM) real-time PCR, as described above. The colorimetric assays were included in the daily routine of the laboratory as follows: every working day (Monday to Friday), at 12:00 PM, aliquots from all blood culture bottles with Gram-negative rods that flagged positive in the last 24 h were submitted to the CNPt-Direct and Blue-Carba tests as above. If the assay was positive, the attending physician was immediately informed. In addition, aliquots from every blood culture submitted to this study were cultured in Mueller-Hinton (MH) agar, and, after overnight incubation, the pure colonies were submitted to CNPt-Direct and Blue-Carba tests.

RESULTS

Spiked blood culture. A total of 61 CRE isolates were included in the evaluation of the colorimetric assays of the spiked blood culture, and all of them presented the same results by the two methods (CNPt-Direct and Blue-Carba) for carbapenemase detection. Therefore, both colorimetric assays allowed the detection of carbapenemase production in 87% (53/61) of carbapenemase-positive isolates. It was possible to detect 89% (17/19) of KPC producers and 67% (4/6) of GES producers (class A carbapenemases). Among the metallo-β-lactamases, 100% of NDM (17/17) and 100% (4/4) of IMP isolates presented positive results. The OXA-48-like carbapenemases (class D) presented 64% (7/11) positivity. All carbapenemase coproducer (NDM + OXA-48-like) isolates were positive in the colorimetric assays (Table 1).

Colorimetric assays in a routine microbiology laboratory. The colorimetric assays were applied in a routine microbiology laboratory for 6 months, and a total of 314 blood samples were tested. According to conventional blood culture, 33 isolates (only *Enterobacteriaceae* were evaluated) were resistant to meropenem and were identified as *K. pneumoniae* (30 isolates), *E. coli* (2), and *Serratia marcescens* (1) (Table 2). Five isolates with intermediate meropenem results were considered susceptible in all statistical analysis in this study. It was possible to identify a carbapenemase gene in 30 of the meropenem-resistant isolates, as follows: the KPC gene was identified in 27 isolates, and the GES and NDM genes were identified in 1 isolate each. One isolate was a coproducer of NDM and KPC.

TABLE 2 Results of the colorimetric assays applied in a routine microbiology laboratory

β-Lactamase class and identification(s) (no. of isolates)[a]	Bacterial species identification	Resistance to meropenem[b]	Colorimetric assay results			
			Pure culture		Blood culture	
			CNPt-Direct	Blue-Carba	CNPt-Direct	Blue-Carba
Class A carbapenemases						
KPC (27)	*Klebsiella pneumoniae*	+	+	+	+	+
	Klebsiella pneumoniae	+	+	+	−	−
	Klebsiella pneumoniae	+	+	+	+	+
	Klebsiella pneumoniae	+	+	+	+	+
	Klebsiella pneumoniae	+	+	+	+	+
	Klebsiella pneumoniae	+	+	+	+	+
	Klebsiella pneumoniae	+	+	+	+	+
	Klebsiella pneumoniae	+	+	+	+	+
	Klebsiella pneumoniae	+	+	+	−	−
	Klebsiella pneumoniae	+	+	+	+	+
	Klebsiella pneumoniae	+	+	+	+	+
	Klebsiella pneumoniae	+	+	+	+	+
	Klebsiella pneumoniae	+	+	+	+	+
	Klebsiella pneumoniae	+	+	+	+	+
	Klebsiella pneumoniae	+	+	+	+	+
	Klebsiella pneumoniae	+	+	+	+	+
	Klebsiella pneumoniae	+	+	+	+	+
	Klebsiella pneumoniae	+	+	+	−	−
	Klebsiella pneumoniae	+	+	+	+	+
	Klebsiella pneumoniae	+	+	+	+	+
	Klebsiella pneumoniae	+	+	+	−	−
	Klebsiella pneumoniae	+	+	+	+	+
	Klebsiella pneumoniae	+	+	+	+	+
	Klebsiella pneumoniae	+	+	+	+	+
	Klebsiella pneumoniae	+	+	+	+	+
	Klebsiella pneumoniae	+	+	+	+	+
GES (1)	*Serratia marcescens*	+	−	−	−	−
Class B carbapenemase						
NDM (1)	*Klebsiella pneumoniae*	+	+	+	+	+
Carbapenemase coproducers						
KPC + NDM (1)	*Escherichia coli*	+	+	+	−	−
Negative for carbapenemases tested (3)	*Klebsiella pneumoniae*	+	−	−	−	−
	Klebsiella pneumoniae	+	−	−	−	−
	Escherichia coli	+	−	−	−	−
All negative (281)		−				

[a]Carbapenemases genes were investigated by real-time polymerase chain reaction-high resolution melting (RT-PCR-HRM) for the following genes: bla_{NDM-1}, bla_{KPC}, $bla_{OXA-48-like}$, bla_{GES}, and bla_{IMP}.
[b]Resistance to meropenem was evaluated in the antibiogram of the conventional protocol.

Considering the blood cultures of the 30 isolates with carbapenemase genes, the colorimetric tests correctly detected carbapenemase production in 24 cases (80%), namely, 23 *K. pneumoniae* KPC producers and one *K. pneumoniae* NDM producer. Four *K. pneumoniae* KPC producers were negative by the direct colorimetric test, although after isolation and pure culture test they presented positive results; the same was observed for the *E. coli* KPC + NDM coproducer. One *Serratia marcescens* isolate that was resistant to meropenem and was a GES producer was negative by the direct colorimetric tests; nevertheless, when the pure culture was tested, it was also negative. The three meropenem-resistant isolates with negative results in the PCR for carbapenemase genes, as well as the 281 isolates susceptible to meropenem, were all negative by the colorimetric tests. As also found with the spiked blood evaluation, the CNPt-Direct and Blue-Carba tests presented the same results when used in the routine microbiology laboratory. Overall, the positive percent agreement and negative percent agreement of the colorimetric assays were 80% and 100%, respectively.

DISCUSSION

The evaluation of the colorimetric assays performed on spiked blood culture allowed the detection of carbapenemase production in 87% (53/61) of all carbapenemase-positive isolates. It was noteworthy that most of the false-negative results of the colorimetric assays were due to isolates harboring the OXA-48-like and GES carbapenemases, which are considered weak carbapenemases. If we do not consider these isolates, the positivity of the colorimetric assays increases to 95% (42/44). Other studies, performed directly from the colonies, also found lower detection rates of the colorimetric assays for isolates harboring these types of enzymes (8, 9). Dortet et al. (11) performed a similar study (spiked blood cultures) and obtained 100% specificity and 97% overall sensitivity. The increased sensitivity is probably due to a more robust enzyme extraction process used in Dortet's study, which used the Carba NP test for carbapenemase detection with the cell lysis buffer B-PER (bacterial protein extraction reagent; Thermo Scientific Pierce, Rockford, IL) as the enzyme extractor, in addition to microbead tubes and a mechanical extraction procedure.

In the second part of our study, 314 blood samples were evaluated and rendered 30 carbapenemase-producing *Enterobacteriaceae* isolates. The colorimetric assays performed directly in the blood culture detected only 24 of 30 cases (80%) of carbapenemase-positive isolates, which indicates that these assays cannot be used as a standalone screening method, since negative results are not trustable and need further tests. However, all blood samples which presented growth of Gram-negative rods either susceptible to meropenem (281 isolates) or negative for carbapenemase genes (3 isolates) were also negative in the colorimetric assays. Therefore, all of the positive results obtained in the colorimetric tests presented 100% agreement compared to the standard method, and no false-positive result was observed, which indicates that positive results do not need to be confirmed and can be provided to the attending physician immediately. This would contribute significantly to provision of early appropriate therapy for patients with BSI.

Comparing the results of colorimetric assays with those obtained by conventional methods, it is possible to say that the former required considerably less time for the diagnosis of carbapenemase production by microorganisms causing bloodstream infections (from 24 to 48 h to 3 to 5 h), after the blood bottles flagged positive. In addition, the colorimetric assays are cost-effective, since they do not require expensive equipment, special reagents, or laborious work.

The results of this study indicate that the colorimetric methods directly from the blood culture bottles can be easily implemented in a routine microbiology laboratory and would be important in regions with high prevalence of carbapenemases (in particular KPC) among *Enterobacteriaceae* isolates. However, it is necessary to perform further studies to increase the rate of detection of carbapenemase-producing isolates directly from the blood culture bottles in order to improve the positive percent agreement of the colorimetric methods.

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