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Rapid detection of carbapenemase genes by multiplex real-time PCR

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Objectives: To develop a single multiplex real-time PCR assay to detect six different genetic types of carbapenemases already identified in Enterobacteriaceae (KPC, GES, NDM, IMP, VIM and OXA-48).

Methods: A total of 58 bacterial isolates were tested. Thirty were previously characterized as resistant to carbapenems and documented by PCR and sequencing analysis to carry the following genes: bla_{KPC} type, bla_{GES} type, bla_{IMP} type, bla_{VIM} type, bla_{OXA-48} and bla_{NDM-1}. These positive strains included 21 Enterobacteriaceae, 1 *Acinetobacter baumannii* and 8 *Pseudomonas aeruginosa* isolates. The remaining 28 isolates previously tested susceptible to carbapenems and were negative for these genes. Bacterial DNA was extracted using the easyMag extractor (bioMérieux, France). The real-time PCR was performed using the Rotor-Gene 6000 instrument (Corbett Life Science, Australia) and specific primers for each carbapenemase target were designed using the DNAStar software (Madison, WI, USA).

Results: Each one of the six carbapenemase genes tested presented a different melting curve after PCR amplification. The melting temperature (T_m) analysis of the amplicons identified was as follows: bla_{IMP} type (T_m 80.1°C), bla_{OXA-48} (T_m 81.6°C), bla_{NDM-1} (T_m 84°C), bla_{GES} type (T_m 88.6°C), bla_{VIM} type (T_m 90.3°C) and bla_{KPC} type (T_m 91.6°C). No amplification was detected among the negative samples. The results showed 100% concordance with the genotypes previously identified.

Conclusions: The new assay was able to detect the presence of six different carbapenemase gene types in a single 3 h PCR.

Keywords: resistance, β-lactamases, Enterobacteriaceae

Introduction

The emergence and spread of carbapenem-hydrolysing β-lactamases amongst Enterobacteriaceae over the past decade represents a serious issue in the hospital environment. This fact is worrying, especially because these enzymes also hydrolyse almost all antimicrobial β-lactams and often are also resistant to commercially available β-lactamase inhibitors. The genes coding for these enzymes are frequently located in mobile genetic elements, facilitating the dissemination of resistance among different bacteria.[1,2] Carbapenemase-producing Gram-negative bacteria have been associated with increasing mortality and with serious hospital outbreaks that present major therapeutic and infection control challenges.[3] For all these reasons, the inter- and intra-hospital spread of these enzymes has become a major clinical concern and rapidly identifying the organisms carrying these genes might be the best way we have to reduce or prevent this problem in healthcare centres.

New breakpoints established by the CLSI to detect carbapenem-resistant Enterobacteriaceae contributed positively to better screening of strains expressing these important mechanisms of resistance.[4] Increased carbapenem MICs in Enterobacteriaceae can be a result of two different mechanisms of resistance: (i) hyperproduction of class C β-lactamases or extended-spectrum β-lactamases (ESBLs) in combination with porin alteration; and/or (ii) carbapenemase production by serine carbapenemase and/or metallo-β-lactamases.[3] Thus, the new breakpoints established by the CLSI can be an excellent screening test, but they do not identify the resistance mechanism. Therefore, tests based on molecular techniques are considered the standard tests for the identification of carbapenemase genes.[5]

Molecular tests have been described to identify carbapenemase-producing Gram-negative bacteria. Some assays use multiplex real-time PCR to identify serine carbapenemase genes or metallo-β-lactamase genes.[6,7] Assays that target more than one class of carbapenemase (A, B and D) have been developed, but all of them use conventional PCR.[8,9] None of them represented a single sensitive and specific assay that is designed to rapidly detect all of the main carbapenemases.

Table 1. Primers used in this study

Target	Primer name	Sequence (5′–3′)	Amplicon size (bp)	Primer concentration (μM)[a]	T_m[b]	Reference
bla_{KPC} type	KPC-F	TCGCTAAACTCGAACAGG	785	0.2	91.6	10
	KPC-R	TTACTGCCCGTTGACGCCCAATCC				
bla_{NDM-1}	NDM-F	TTGGCCTTGCTGTCCTTG	82	0.2	84	this study
	NDM-R	ACACCAGTGACAATATCACCG				
bla_{GES} type	GES-F	CTATTACTGGCAGGGATCG	594	0.2	88.6	this study
	GES-R	CCTCTCAATGGTGTGGGT				
bla_{OXA-48}	OXA-48-F	TGTTTTTGGTGGCATCGAT	177	0.2	81.6	this study
	OXA-48-R	GTAAMRATGCTTGGTTCGC				
bla_{IMP} type	IMP-F	GAGTGGCTTAATTCTCRATC	120	1.2	80.1	6
	IMP-R	AACTAYCCAATAYRTAAC				
bla_{VIM} type	VIM-F	GTTTGGTCGCATATCGCAAC	382	0.2	90.3	6
	VIM-R	AATGCGCAGCACCAGGATAG				

[a]Final concentration in the multiplex real-time PCR.
[b]Melting point calculated by the Rotor-Gene 6000 using Type-it HRM.

The purpose of this study was to develop a single multiplex real-time PCR assay to identify the most common types of serine-β-lactamase (KPC, GES and OXA-48) and metallo-β-lactamase (IMP, VIM and NDM), already described in Enterobacteriaceae isolates, using high-resolution melting curves.

Materials and methods

Bacterial isolates

A total of 58 Gram-negative isolates with decreased susceptibility to carbapenems were tested in this study. Thirty of them, previously characterized by PCR and sequencing analysis, harbour the following carbapenemase genes: bla_{KPC} type ($n=15$; all of them isolated at Tampa General Hospital, Tampa, FL, USA), bla_{GES} type ($n=3$; samples kindly provided by JMI Laboratories, North Liberty, IA, USA and LEMC-ALERTA Laboratories, São Paulo, Brazil), bla_{IMP} type and bla_{VIM} type ($n=5$ and $n=3$; samples kindly provided by LEMC-ALERTA Laboratories), bla_{OXA-48} ($n=3$; samples kindly provided by JMI Laboratories and LEMC-ALERTA Laboratories) and bla_{NDM-1} ($n=1$; ATCC BAA-2146). These positive strains included 21 Enterobacteriaceae (bla_{KPC} type, bla_{IMP} type, bla_{OXA-48}, bla_{NDM-1} and bla_{GES} type), 1 *Acinetobacter baumannii* (bla_{IMP} type) and 8 *Pseudomonas aeruginosa* isolates (bla_{IMP} type, bla_{VIM} type and bla_{GES} type). The other 28 Enterobacteriaceae isolates were negative for the six carbapenemases tested.

DNA extraction

Bacterial DNA was extracted using the NucliSens easyMAG platform with NucliSens magnetic extraction reagents (bioMérieux, France), according to the manufacturer's recommendations. The extracted DNA was recovered in 60 μL of the elution buffer. The DNA extraction takes approximately 1 h.

Design of primers

The details of the reference genes used in this assay were obtained from the following homepage: http://www.lahey.org/studies/. These genes were: class A carbapenemases encoding GES and KPC type, class D oxacillinases encoding OXA-48 and class B metalloenzymes encoding NDM, IMP and VIM.[1,2,5] The sequences of these genes were downloaded from the GenBank web site (http://www.ncbi.nlm.nih.gov/genbank/). Based on the comprehensive analyses and alignments of each carbapenemase type, primers were specifically designed to amplify all alleles of each carbapenemase gene family described above. The melting temperature (T_m) of the amplification product of each carbapenemase gene family was determined by the Lasergene software package (DNASTAR, Madison, WI). VIM, IMP and KPC pairs of primers were adapted from previously published sequences.[6,10] To confirm the specificity of the real-time PCR assays, the primers were evaluated in a single PCR format to ensure that they correctly amplified their respective loci and that the amplicons showed the expected T_m. Subsequently, the multiplex format was optimized by assaying different primer pair concentrations. The size of each of the PCR products was verified by electrophoresis in a 2% agarose gel. All primers were synthesized by IDT (Coralville, IA, USA). Primer sequences and references are listed in Table 1.

Multiplex real-time PCR

Amplifications were performed in 25 μL of the Master Mix reaction containing 12.5 μL of 2× high-resolution melt (HRM) PCR Master Mix (HotStartTaq *Plus* DNA polymerase, Type-it HRM PCR buffer, EvaGreen dye, Q-solution, dNTP mix of ultrapure quality and RNase-free water) (Qiagen®, Germany), a sufficient quantity of sterile water, primers and 1 μL of the DNA template. The pairs of primers were optimized to a final concentration of 0.2 μM, except for IMP-F and IMP-R, which were optimized to a final concentration of 1.2 μM. The PCR run was performed using the Rotor-Gene 6000 instrument (Corbett Life Science, Valencia, CA, USA). All the PCR runs were performed using the six positive controls and RNase-free water as a negative control. The 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). The Rotor-Gene instrument automatically calculated the negative derivative of fluorescence measured at 533 nm and generated melting peaks by plotting with regard to temperature (−dF/dT).

Data analysis

To analyse the results obtained in this new multiplex real-time PCR assay, each one of the six positive controls was tested in quadruplicate in the same run and in three different runs. The T_m mean, SD and coefficient

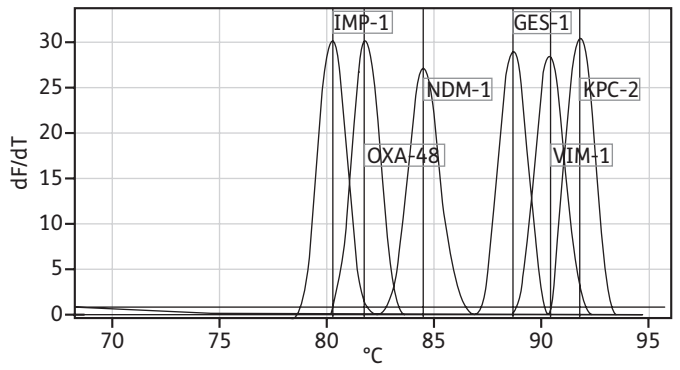

Figure 1. Results from the real-time multiplex PCR melting curves of the amplicons generated by primers targeting the six carbapenemases types. The gene targets, from left to right, are as follows: bla_{IMP} type (T_m 80.1°C), bla_{OXA-48} (T_m 81.6°C), bla_{NDM-1} (T_m 84°C), bla_{GES} type (T_m 88.4°C), bla_{VIM} type (T_m 90.3°C) and bla_{KPC} type (T_m 91.6°C).

of variation (CV%) were calculated to access the inter- and intra-run reproducibility of the assay.

Results and discussion

This study was performed using high-resolution melting curve analysis. The same T_m from each gene was detected when the positive control strains (bla_{KPC-2}, bla_{GES-1}, bla_{OXA-48}, bla_{IMP-1}, bla_{VIM-1} and bla_{NDM-1}) were tested by simplex real-time PCR and by multiplex real-time PCR assay. The T_m analysis of the amplicons identified was as follows: bla_{IMP} type (T_m 80.1°C), bla_{OXA-48} (T_m 81.6°C), bla_{NDM-1} (T_m 84°C), bla_{GES} type (T_m 88.6°C), bla_{VIM} type (T_m 90°C) and bla_{KPC} type (T_m 91.6°C) (Figure 1).

Among the 58 strains tested, a concordance of 100% was observed when results of multiplex real-time PCR assay were compared with genotypes previously identified by Sanger sequencing. PCR products were also visualized and determined to be of the appropriate size by agarose gel electrophoresis. Results showed sizes compatible with fragments of 82, 177, 188, 382, 594 and 785 bp for bla_{NDM-1}, bla_{OXA-48}, $bla_{IMP-type}$, $bla_{VIM-type}$, $bla_{GES-type}$ and bla_{KPC-2}, respectively. No amplification was observed when the 28 non-carbapenemase-producing enterobacterial isolates were tested.

T_m values of genes bla_{KPC-2}, bla_{NDM-1} and bla_{OXA-48} were very similar among runs. However, the T_m values of bla_{GES}, bla_{VIM} and bla_{IMP} presented minor variations among variants of the same gene, as follow: bla_{GES-1} (T_m 88.65°C), bla_{GES-5} (T_m 88.4°C) and bla_{GES-16} (T_m 88.4°C), bla_{VIM-1} (T_m 90.35°C) and bla_{VIM-2} (T_m 90°C), and bla_{IMP-1} (T_m 80.1°C), bla_{IMP-13} (T_m 79.0°C), bla_{IMP-16} (T_m 79.6°C) and bla_{IMP-18} (T_m 78.4°C). These differences in the T_m for allelic variants likely were linked to the GC content of the amplicons. Even though our study demonstrated these differences among T_m of the same gene type, the precision of interpretation of the results and concordance with the previous publication by Mendes *et al*.[6] were not affected.

The mean T_m, SD and CV% of the multiplex real-time PCR assay were calculated from results of quadruplicates amplified in three different runs. The T_m, SD and CV% values for each positive control of carbapenemase in the three different runs (inter-run) were as follows: KPC-2 (91.73, 0.05 and 0.05%), GES-1 (88.65, 0.12 and 0.14%), IMP-1 (80.34, 0.10 and 0.13%), VIM-1 (90.38, 0.03 and 0.03%), NDM-1 (84.48, 0.05 and 0.06%) and OXA-48 (81.70, 0.08 and 0.09%). The T_m, SD and CV% values for quadruplicates of each positive control of carbapenemase (intra-run) were: KPC-2 (91.63, 0.12 and 0.13%), GES-1 (88.57, 0.20 and 0.23%), IMP-1 (80.15, 0.14 and 0.17%), VIM-1 (90.30, 0.17 and 0.19%), NDM-1 (84.33, 0.24 and 0.28%) and OXA-48 (81.54, 0.18 and 0.22%). These results show excellent reproducibility of this new assay.

To our knowledge, this is the first report of a multiplex real-time PCR assay, in a single reaction, for the identification of the most common types of serine carbapenemases and metallo-β-lactamases (KPC, GES, OXA-48, IMP, VIM and NDM-1) described in Enterobacteriaceae isolates. This assay provides for the rapid, sensitive and specific detection and identification of these important genes of resistance. Considering the demonstrated potential for rapid horizontal and vertical transmission of these genes, the accurate and timely identification of these resistance genes will be an important tool to help infection control measures and guide the appropriate choice of antimicrobial therapy. The entire assay, including DNA extraction, sample preparation, multiplex PCR run and results analysis was performed in 3 h.

Conclusions

In summary, the multiplex real-time PCR assay developed in this study is a fast (3 h) and reliable assay for rapid screening and identification of the most relevant genes identified in Enterobacteriaceae carbapenemase-positive clinical isolates.

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Transparency declarations

None to declare.

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