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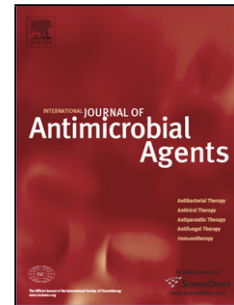
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Real-time TaqMan PCR for rapid detection of genes encoding five types of non-metallo- (class A and D) carbapenemases in *Enterobacteriaceae*

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ABSTRACT

A real-time TaqMan multiplex polymerase chain reaction (PCR) assay was developed to detect genes encoding five types of serine carbapenemases (GES, IMI/NMC, KPC, OXA-48 and SME). The assay was validated using control strains known to produce each of these types of enzyme and was then further assessed by 'blindly' testing 59 previously characterised clinical isolates, including 19 with serine (KPC or OXA-48) carbapenemases, 22 with metallo- (IMP, VIM or NDM) carbapenemases, and 18 with carbapenem resistance contingent upon extended-spectrum β -lactamase (ESBL) or AmpC production combined with porin loss. The assay detected and correctly assigned the serine carbapenemases in all five positive control strains and in 19 clinical isolates. No false-positive results were seen for isolates with metallo-enzymes or for those that lacked a carbapenemase. The five serine carbapenemase genotypes could also be distinguished by melt-curve analysis or the molecular size of the amplicons.

1. Introduction

Carbapenems are frequently the only therapeutic option available for treatment of severe hospital or community-acquired infections caused by multiresistant Enterobacteriaceae with AmpC β -lactamases or extended-spectrum β -lactamases (ESBL) [1]. The emergence and spread of carbapenem-hydrolysing β -lactamases ('carbapenemases') amongst Enterobacteriaceae over the past decade therefore represents a serious public health threat. Furthermore, the genes encoding these enzymes are often carried on mobile genetic elements, e.g. plasmids, that also confer resistance to many other antibiotic classes [2]. Carbapenemase-producing Enterobacteriaceae have been responsible for hospital outbreaks that presented major therapeutic and infection control challenges [3] and have been associated with increased mortality, length of stay and costs [4].

This clinical and public health importance means that there is an urgent need to monitor the dissemination both of the enzymes and of resistant producer clones [5]. This is complicated by the fact that the carbapenemases involved are diverse, including serine (class A and D) and metallo- (class B) enzymes. Multiplex polymerase chain reactions (PCRs) have been designed for metallo-carbapenemases [6] and for intrinsic and acquired OXA carbapenemases from Enterobacteriaceae [7,8], but there are no previous multiplexed assays aimed at detection of the class A and D enzymes increasingly encountered in Enterobacteriaceae.

We therefore sought to develop a multiplex PCR assay to detect and differentiate all alleles encoding known molecular class A and D serine carbapenemases in carbapenem-resistant Enterobacteriaceae. The assay was developed both in real-time and conventional formats in order to make it widely accessible to clinical diagnostic laboratories.

2. Materials and methods

2.1. Design of primers and TaqMan probes

Reference gene sequences for each of five enzyme families were assembled from GenBank (<http://www.ncbi.nlm.nih.gov/GenBank>) as follows: for GES, accession nos. GU169702, GQ337064, GQ139471, FN554543, FJ854362, FJ820124, EU598463, EF219164, EF219163, EF202187, EF190326, DQ660416, DQ333893, DQ236171, DQ236170, AY953375, AY494718, AF329699, AF156486 and AB113580, representing alleles 1, 3, 4, 5, 7, 10, 11, 12 and 13; for IMI/NMC, Z21956, U50278, GU015024, DQ173429, AY780889 and AJ536087, representing alleles 1, 2 and 3; for KPC, EF062508, DQ899729, GQ229417, GQ140348, FJ624872, FJ473382, FJ234412, EU729727, EU555534, EU447304, EU400222, EU176013, EU176011, AY700571, AY522950, AY210886, AF481906, AF395881 and AF297554, representing alleles 1, 3, 4, 5, 6, 7, 8, 9 and 10; for OXA-48, AY236073 is the only representative sequence and was therefore aligned against its closest relatives (identified through a BLAST search) to ensure the design of the primers and probe were *bla*_{OXA-48}-specific, i.e. AY500137 (OXA-54), CP000469 (*Shewanella* sp. ANA-3), CP000444 (*Shewanella* sp. MR-7), CP000681 (*Shewanella putrefaciens*) and AY343493 (OXA-55); and for SME, AY584237, U60295, AF275256 and Z28968,

representing alleles 1–3. Sequences were aligned using MegAlign (ClustalV method) and Lasergene (version 8.0) (<http://www.DNAstar.com>), conserved sites were identified and consensus primer pairs were designed (Table 1) and supplied by Metabion (Martinsried, Germany).

The sequences of the five TaqMan probes, the 5' reporter and 3' quencher dyes are detailed in Table 1.

2.2. Positive control strains

Isolates producing KPC (*Klebsiella pneumoniae*), OXA-48 (*K. pneumoniae*), SME (*Serratia marcescens*), IMI/NMC (*Pantoea* sp.) and GES (*Escherichia coli*) enzymes were retrieved from the collection of carbapenemase-producers held at the Health Protection Agency's Antibiotic Resistance Monitoring Reference Laboratory (ARMRL) (London, UK) for use as positive PCR controls.

2.3. Clinical isolates used for assay validation

The assay was further assessed using a panel of 59 clinical isolates with defined mechanisms of carbapenem resistance, including 19 with serine (KPC or OXA-48) carbapenemases, 22 with metallo- (IMP, VIM or NDM) carbapenemases, and 18 with carbapenem resistance contingent upon ESBL or AmpC production in combination with porin loss (Table 2). The isolates had been referred to ARMRL from many different laboratories, mostly in the UK, and belonged to multiple pulsed-field gel electrophoresis (PFGE)-defined strains. Inclusion of multiple representatives of some strains was in some instances unavoidable, e.g. as many isolates with KPC enzymes

belonged to the internationally-disseminated *K. pneumoniae* sequence type ST258 clone.

This panel was tested 'blind' in Cambridge, i.e. without knowledge of the expected results, with the results subsequently decoded in ARMRL.

2.4. DNA extraction and PCR assays

A 10 μL loopful of pure organism grown on Colombia blood agar (Oxoid, Basingstoke, UK) was emulsified in 300 μL of TE [10 mM Tris, 1 mM ethylene diamine tetra-acetic acid (EDTA), pH 8.0], and DNA was extracted using a modification of the method described by Narayanan et al. [9]. A final DNA concentration of 1 ng/ μL was used in the PCR assay. Once optimal reaction conditions had been determined empirically using the Rotor-Gene Q (5-plex HRM) apparatus (<http://www.qiagen.com/>; QIAGEN, Crawley, UK), the TaqMan PCR assay was tested initially on an individual basis with each of the five control extracts at dilutions of 10^{-1} , 10^{-2} and 10^{-3} . This was then repeated as a pentaplex.

The PCR assay was run using the 59 'blind' clinical carbapenem-resistant isolates, the five positive controls, and water as a negative control. Each reaction tube contained 5 μL of DNA extract solution, 12.5 μL of Platinum[®] Quantitative PCR SuperMix-UDG (Invitrogen, Paisley, UK), 0.5 μL of each 20 pmol/ μL forward and reverse primers, 0.1 μL of 10 pmol/ μL IMI/NMC TaqMan probe, 0.2 μL of each of the other four 10 pmol/ μL TaqMan probes and 1.5 μL of RNase-free water. Cycling conditions comprised: two holding periods, each 2 min, one at 50 °C and the other at

95 °C followed by 30 cycles of PCR, each cycle consisting of 10 s at 95 °C and 60 s annealing at 60 °C, acquiring fluorescence on all five available channels (Crimson/Quasar 705, Green/6FAM, Orange/ROX, Red/CY5 and Yellow/VIC). Real-time data were analysed with Rotor-Gene Q software (version 1.7).

Upon completion of the run, a cycle threshold (Ct) was calculated by determining the signal strength at which the fluorescence exceeded a threshold limit. This was manually set for each assay, but reproducibly resulted in a normalised fluorescence ranging between 0.01 and 0.02 normalised fluorescence units for all five channels. Any sample demonstrating a fluorescence signal above this value was regarded as positive. In the case of real-time amplification, with a melt-curve analysis performed at the end of the run, the individual TaqMan probes were replaced with 1 µL of LCGreen[®] Plus (Idaho Technology Inc., Salt Lake City, UT) and the temperature of the annealing/extension step was decreased to 55 °C for 60 s. Fluorescence acquisition was restricted to the green channel and a melt cycle was included at the end of the amplification consisting of a hold at 55 °C for 90 s, followed by slow heating at a transition rate of 0.5 °C per step (held for 5 s) to 95 °C with continuous acquisition.

2.5. Capillary gel electrophoresis

PCR amplicons were analysed on a QIAxcel instrument (QIAGEN) using the QIAxcel DNA high-resolution kit alongside QX DNA size marker 50–800 bp and the QX alignment marker 15 bp/1 kb according to the manufacturer's protocols.

2.6. Sequence analysis

Amplicons from TaqMan assays for the five control strains were subjected to DNA sequencing (BigDye[®] Terminator Kit; Applied Biosystems, Warrington, UK) to confirm their identities.

3. Results

The TaqMan PCR assay was validated using control strains known to produce each of the five carbapenemase types and then by 'blindly' testing 59 previously characterised clinical isolates, including 19 with serine (KPC or OXA-48) carbapenemases, 22 with metallo- (IMP, VIM or NDM) carbapenemases, and 18 with carbapenem resistance contingent upon ESBL or AmpC production in combination with porin loss. These represent the main carbapenemase types circulating in the UK: GES, IMI and SME are all extremely rare.

The assay detected and correctly assigned the serine carbapenemases in all 5 positive control strains and in all 19 clinical isolates with a relevant enzyme (8 with KPC and 11 with OXA-48) (Table 2). Clearly-defined amplification curves were observed for all positive samples in their predicted fluorescence channels (Fig. 1a). No false-positive results were observed for isolates with metallo-enzymes ($n = 22$) or with ESBLs/AmpC combined with porin loss ($n = 18$).

It was possible to replace the TaqMan probes with the intercalating dye LCGreen[®] Plus and still distinguish the five carbapenemase genes on the basis of their melt curves (Fig. 1b). Furthermore, capillary electrophoresis (Fig. 1c) of 24 amplicons

(derived from the 5 positive controls and 19 positive clinical isolates) confirmed that their molecular sizes (i) allowed the five carbapenemase genes to be distinguished and (ii) corresponded to those predicted from sequence analysis.

BLAST search analysis of the amplicon sequences of the five positive controls gave 100% matches for their assigned genotypes.

4. Discussion

Carbapenem-resistant Enterobacteriaceae are an increasingly serious public health concern, and management may be compromised by problems arising with phenotypic methods of detection. Not all carbapenemase-producers show high carbapenem minimum inhibitory concentrations (MICs) and there is overlap in the MIC distributions for isolates in which carbapenem resistance depends on ESBL/AmpC plus porin loss [3]. For carbapenemase-producers, there is a clear infection control issue, with multiple outbreaks reported and with the risk of further horizontal transfer of the carbapenemase plasmids among strains. By contrast, where carbapenem resistance is contingent on porin loss in an ESBL- or AmpC-producer, it cannot be transferred horizontally; moreover, isolates with this combination of mechanisms are rarely associated with outbreaks, suggesting reduced fitness in many cases.

We describe a multiplex PCR assay for the identification of five genotypes of serine carbapenemases with three ways of analysing the results. To our knowledge, this is the first report of a multiplex real-time PCR assay for the identification of serine

carbapenemases, although a set of multiplex PCR assays in which genes for GES, OXA-48-like and KPC genes are sought has been described [10].

In the case of GES, the assay described here will amplify all known GES alleles and as a consequence will detect those that do not contain the glycine to serine substitution at amino position 170 conferring carbapenemase activity [11]; these will therefore lack measurable or significant activity against carbapenems. The PCR target encompasses the informative sequence, so if a GES-positive isolate is identified, the PCR product can be retrieved and sequenced to determine whether glycine or serine is at that position and thus assess carbapenemase activity. Whilst clinical isolates producing KPC and OXA-48 enzymes were available, we had only single control isolates with GES, SME and IMI/NMC enzymes, these being very rare. However, the assay identified the correct gene in each of these three positive controls (Fig. 1).

The assay could be applied to any isolate with resistance or reduced susceptibility to any carbapenem, but could be reserved for carbapenem-resistant Enterobacteriaceae showing no synergy between carbapenems and either EDTA or dipicolinic acid, i.e. when a metallo-carbapenemase is not suspected [12]. The amplicons could be distinguished and carbapenemase identified by (i) real-time probe-based PCR, (ii) amplicon melt-curve analysis or (iii) amplicon molecular size determination on gel electrophoresis. The assay would be suitable for laboratories without sophisticated real-time instruments, expediting detection of carbapenem-resistant Enterobacteriaceae and offering rapid guidance both for antibiotic treatment

and infection control purposes. Positive isolates should none the less be referred to national reference laboratories for national epidemiological purposes.

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Competing interests

None declared.

Ethical approval

Not required.

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Fig. 1. (a) Amplification curves for the real-time pentaplex polymerase chain reaction (PCR), showing the five controls and six of the clinical specimens. Orange channel, OXA-48 control (purple) and positive clinical isolates (blue); red channel, KPC control (orange) and positive clinical isolates (blue); green channel, IMI control (green); crimson channel, GES control; yellow channel, SME control (purple). (b) Melt-curve analysis of amplicons using LCGreen[®] Plus showing the five positive controls and three each of the clinical isolates containing OXA-48 (two of the curves merged) and KPC. (c) Capillary electrophoresis of amplicons. Lanes 1–3, clinical isolates with OXA-48; lanes 4–6, clinical specimens with KPC; lane 7, GES control; lane 8, IMI control; lane 9, KPC control; lane 10, OXA-48 control; lane 11, SME control; M, size markers.

Table 1

Carbapenemase pentaplex primers and probes

Gene	Primer/probe	Sequence (5'→3' direction)	Nucleotide positions	Reference sequence	Amplicon size (bp)
KPC	kpc F	GCAGCGGCAGCAGTTTGTGATT	602–624	AY700571	184
	kpc R	GTAGACGGCCAACACAATAGGTGC	785–762		
	probe	CY5-CAGTCGGAGACAAAACCGGAACCTGC-BHQ3	685–710		
OXA-48	oxa48 F	TTCGGCCACGGAGCAAATCAG	2679–2699	AY236073	240
	oxa48 R	GATGTGGGCATATCCATATTCATCGCA	2918–2892		
	probe	ROX-CTGGCTGCGCTCCGATACGTGTAACCTTATTG-BHQ2	2751–2721		
SME	sme F	TGTAGGTGACAARACTGGGAGCTGTG	829–854	Z28968	165
	sme R	GCAATACGTGATGCTTCCGCAATAG	993–969		
	probe	VIC-CGGCATAATCATTGCGCA-NFQMGB	884–868		
IMI	imi F	GAGGGTATGACTAAATTCATGCGGTCTGA	442–469	DQ173429	116
	imi R	GCAGGTGTAGATGTGTCACGYTCATCG	557–531		
	probe	6FAM-CGTTGGGAGTTAGATC-NFQMGB	496–511		

GES	ges F	CGGTTTCTAGCATCGGGACACAT	313–335	AY494718	263
	ges R	CCGCCATAGAGGACTTTAGCMACAG	575–551		
	probe	Quasar 705- CGACCTCAGAGATACTAACTACGCCTATTGC- BHQ2	510–539		

Table 2

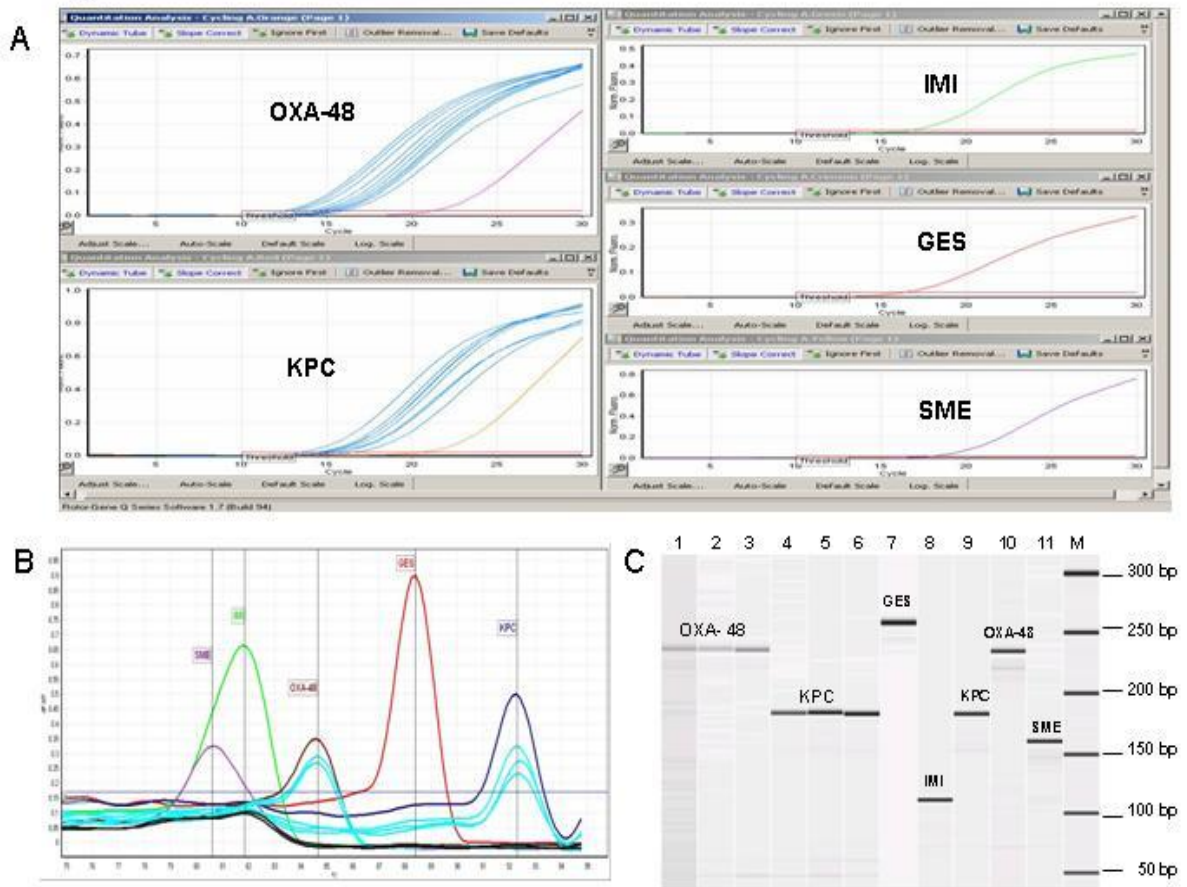
Carbapenem-resistant clinical isolates screened by polymerase chain reaction (PCR)

Carbapenem resistance mechanism	Species	Positive multiplex result
OXA-48 (<i>n</i> = 11)	10 <i>Klebsiella</i> spp.; 1 <i>Escherichia coli</i>	11 × OXA-48
KPC (<i>n</i> = 8)	7 <i>Klebsiella</i> spp.; 1 <i>Enterobacter</i> spp.	8 × KPC
IMI/NMC (<i>n</i> = 1)	Control isolate	1 × IMI/NMC
SME (<i>n</i> = 1)	Control isolate	1 × SME
GES (<i>n</i> = 1)	Control isolate	1 × GES
IMP (<i>n</i> = 12)	7 <i>Klebsiella</i> spp.; 3 <i>Enterobacter</i> spp.; 1 <i>E. coli</i> ; 1 <i>Pantoea</i> spp.	0
VIM (<i>n</i> = 3)	3 <i>Klebsiella</i> spp.	0
NDM (<i>n</i> = 7)	3 <i>E. coli</i> ; 2 <i>Citrobacter</i> spp.; 1 <i>Klebsiella</i> spp.; 1 <i>Enterobacter</i> spp.	0
ESBL/AmpC + porin loss (<i>n</i> = 18)	9 <i>Klebsiella</i> spp.; 6 <i>Enterobacter</i> spp.; 3 <i>E. coli</i>	0

ESBL, extended-spectrum β-lactamase.

[THIS FIGURE SHOULD BE REPRODUCED IN COLOUR]

Figure 1



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