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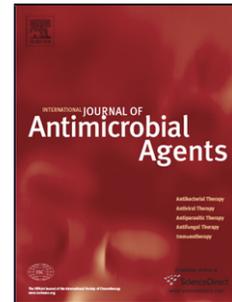
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# A set of multiplex PCRs for genotypic detection of extended-spectrum $\beta$ -lactamases, carbapenemases, plasmid-mediated AmpC $\beta$ -lactamases and OXA $\beta$ -lactamases

Guido M. Voets <sup>a,\*</sup>, A.C. Fluit <sup>a</sup>, Jelle Scharringa <sup>a</sup>, James Cohen Stuart <sup>a</sup>, Maurine A. Leverstein-van Hall <sup>a,b</sup>

<sup>a</sup> *Department of Medical Microbiology, University Medical Centre Utrecht, Utrecht, The Netherlands*

<sup>b</sup> *National Institute for Public Health and the Environment, Bilthoven, The Netherlands*

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\* Corresponding author. Present address: Department of Medical Microbiology,  
University Medical Center Utrecht, Heidelberglaan 100, Room G04.612, 3584 CX  
Utrecht, The Netherlands. Tel.: +31 88 755 6525; fax: +31 30 254 1770.

*E-mail address:* g.voets@umcutrecht.nl (G.M. Voets).

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## ABSTRACT

Worldwide, resistance of Gram-negative micro-organisms to third-generation cephalosporins and carbapenems owing to  $\beta$ -lactamases is an increasing problem. Although the CTX-M, TEM and SHV extended-spectrum  $\beta$ -lactamases (ESBLs) are most widely disseminated, other  $\beta$ -lactamase families have also recently emerged, such as plasmid-mediated AmpC  $\beta$ -lactamases and carbapenemases. Here we describe a new set of multiplex polymerase chain reactions (PCRs) with one amplification protocol enabling detection of 25 prevalent  $\beta$ -lactamase families, including ESBLs, carbapenemases, plasmid-mediated AmpC  $\beta$ -lactamases and OXA  $\beta$ -lactamases.

## 1. Introduction

The increasing prevalence of Gram-negative bacteria producing  $\beta$ -lactamases, which hydrolyse third-generation cephalosporins or carbapenems, is a worldwide problem. The most prevalent  $\beta$ -lactamases are extended-spectrum  $\beta$ -lactamases (ESBLs) of the CTX-M, TEM and SHV families [1]. However, the prevalence of Gram-negative bacteria that are resistant to third-generation cephalosporins owing to increased expression of chromosomal  $\beta$ -lactamases, such as SME and IMI-1, or the acquisition of plasmid-mediated  $\beta$ -lactamases, such as IMI-2, NDM-1, KPC, IMP, OXA, PER and VEB, as well as AmpC  $\beta$ -lactamases is increasing [2–6].

Several multiplex polymerase chain reactions (PCRs) for the detection of  $\beta$ -lactamase families have been published [7–9]. In particular, the set of multiplex PCRs by Dallenne et al. [7] detects a broad range of epidemiologically important  $\beta$ -lactamase families. However, this set does not detect the SME, IMI, NDM, NMC-A, GIM, SIM and SPM  $\beta$ -lactamase families as well as several OXA groups within the OXA family.

The aim of this study was to develop a set of multiplex PCRs for the detection of the majority of genes encoding clinically important  $\beta$ -lactamases causing third-generation cephalosporin or carbapenem resistance using a single amplification protocol.

To achieve this aim, seven multiplex PCRs were developed for the detection of plasmid-mediated AmpC  $\beta$ -lactamases (ACC, ACT, DHA, CMY, FOX, LAT, MIR and

MOX), metallo-carbapenemases (GIM, NDM, SIM and SPM), serine carbapenemases (IMI, SME and NMC-A) and OXA  $\beta$ -lactamases (OXA groups 23, 24, 48, 1, 2, 51, 4 and 58) using Super *Taq* polymerase (HT Biotechnology Ltd., Cambridge, UK) (Table 1). For detection of genes encoding CTX-M, TEM, SHV, GES, VEB, PER, KPC, VIM and IMP  $\beta$ -lactamase families, four multiplex PCRs of Dallenne et al. (multiplex I, II, IV and VI) [7] were evaluated using the same amplification protocol as used for our PCRs, including Super *Taq* polymerase instead of the AmpliTaq polymerase (Sigma-Aldrich, St-Quentin-Fallavier, France) originally used by the authors.

## 2. Material and methods

Primers were designed using the Lasergene 8 program (DNASTAR, Madison, WI). Optimisation of the amplification conditions of each PCR, both the newly presented PCRs in this paper as well as the complementary PCRs of Dallenne et al. [7], resulted in the following PCR protocol. DNA was isolated using a NucleoSpin<sup>®</sup> Tissue Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. DNA concentrations of the samples were equalised to ca. 50 ng/ $\mu$ L and 1  $\mu$ L was added to each multiplex PCR in a 25  $\mu$ L reaction mixture containing 1 $\times$  PCR buffer, 50  $\mu$ M of each deoxynucleotide triphosphate, 1 U Super *Taq* and a variable concentration of primers and MgCl<sub>2</sub> (Table 1). Amplification was carried out as follows: initial denaturation for 1 min at 94 °C, 30 cycles of 30 s at 94 °C, 40 s at 60 °C and 1 min at 72 °C, and a final elongation step for 1 min at 72 °C. Amplification products were visualised after running at 100 V for 1 h on a 1% agarose gel containing GelRed<sup>™</sup> (Biotium Inc., Hayward, CA). A 1-kb DNA ladder

(Invitrogen, Breda, The Netherlands) was used as a size marker. DNA sequencing was performed by BaseClear (Leiden, The Netherlands). In silico PCR simulations were performed using nucleotide BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and MegAlign (DNASTAR Lasergene 8).

### 3. Results and discussion

Amplification products of the expected sizes were obtained with all control strains, confirming the group specificity of the primers (Fig. 1). In addition, the expected amplification products were also obtained with the adapted complementary PCRs described by Dallenne et al. [7] (data not shown). To determine the specificity of the primers for allotypes for which no positive control strain was available, in silico PCR simulation was performed. All allotypes to which the primers anneal, allowing for one mismatch except for the 3'-nucleotide, and allotypes with more mismatches but in the same target gene group were identified (Table 1). In silico PCR simulation was also performed to check for any unwanted cross-reaction with members of the Enterobacteriaceae, *Acinetobacter baumannii* and *Pseudomonas aeruginosa*. The 3'-end of the SPM-GBM-R primer anneals with 21 of 27 bp to the ACC deaminase gene of a *Pseudomonas* spp. (GenBank accession no. EU520401) and the 3'-end of O58-GD3M-R anneals with 17 of 22 bp to the biotin sulfoxide reductase gene of *Escherichia coli* (GenBank accession no. CP001846) and also with 17 of 22 bp to the molybdopterin guanine dinucleotide-containing S/N-oxide reductase gene of *E. coli* (GenBank accession no. CP001637). Cross-reaction of the SPM-GBM-R primer was not a problem with the *P. aeruginosa* positive control. Cross-reactions of O58-GD3M-R may result in poorer resolution of the MGD3 multiplex PCR when used in combination with *E. coli*.

It should be noted that the MGC2 multiplex PCR yielded a 160-bp non-specific amplification product (Fig. 1B, lanes 1 and 2). DNA sequencing showed that this product was identical to part of a CMY-2 group gene in the control strain (lanes 1 and 2). In six isolates that were expected to remain negative, another 160-bp non-specific amplification product was noted. DNA sequencing showed that this product was part of an ACT-like AmpC (GenBank accession no. EF078894.1) (data not shown). The CMY-2 group gene amplification resulted from annealing of the ACT-GCM2-R primer and CY2-GC2M-F primer to genes of the CMY-2 group despite several mismatches. The ACT-like AmpC gene amplification resulted from annealing of the ACT-GCM2 primers to the ACT-like AmpC gene. However, these amplification products were not considered to be a problem for interpretation of the multiplex PCR because they are smaller than the expected specific products. Furthermore, the amplification products were derived from a CMY-2 group or ACT-like gene, which are both targets of the MGC2 multiplex PCR. In conclusion, we describe a set of multiplex PCRs that, when used in combination with the multiplexes I, II, IV and VI of Dallenne et al. [7], can detect a wide range of  $\beta$ -lactamases using the same amplification conditions. This enables detection of the majority of clinically important  $\beta$ -lactamases causing third-generation cephalosporin or carbapenem resistance.

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**Competing interests**

None declared.

**Ethical approval**

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**Fig. 1.** Multiplex polymerase chain reactions (PCRs). All multiplex PCR products were separated on a 1% agarose gel. (A) Multiplex PCR for detection of the carbapenemase families SME, IMI and NMC-A. Lane M, molecular size marker (in bp); lane 1, Nord-03 + Nord-04; lane 2, Nord-03; lane 3, Nord-04; and lane 4, negative control. (B) Lanes 1–5, multiplex PCR for CIT, EBC and FOX groups of plasmid-mediated AmpC  $\beta$ -lactamases; lanes 6–10 multiplex PCR for OXA-1, -2 and -51 groups. Lane M, molecular size marker (in bp); lane 1, CP040 + CP038 + CP093; lane 2, CP040; lane 3, CP038; lane 4, CP093; lane 5, negative control; lane 6, CP116 + CP082 + NCTC-13420; lane 7, CP116; lane 8, CP082; lane 9, NCTC-13420; and lane 10, negative control. (C) Multiplex PCR for detection of the metallo-carbapenemase families SIM, GIM, SPM and NDM. Lane M, molecular size marker (in bp); lane 1, PSA096 + PSA097 + PSA098 + NCTC-13443; lane 2, PA097; lane 3, PSA098; lane 4, NCTC-13443; lane 5, PSA096; and lane 6, negative control. (D) Lanes 1–5, multiplex PCR for OXA-23, -24 and -48 groups; lanes 6–9, multiplex PCR for OXA-10 and OXA-58 groups. Lane M, molecular size marker (in bp); lane 1, CP081 + NCTC-13303 + NCTC-13442; lane 2, CP081; lane 3, NCTC-13303; lane 4, NCTC-13442; lane 5, negative control; lane 6, CP089 + NCTC-13305; lane 7, CP089; lane 8, NCTC-13305; and lane 9, negative control. (E) Multiplex PCR for CMY-1, MOX, DHA and ACC groups of plasmid-mediated AmpC  $\beta$ -lactamases. Lane M, molecular size marker (in bp); lane 1, CP043 + CP039 + CP044; lane 2, CP043; lane 3, CP039; lane 4, CP044; and lane 5, negative control.

**Table 1**

Group-specific primers used in the multiplex PCRs

Multiplex	$\beta$ -Lactamase(s) targeted <sup>a</sup>	Primer name	Sequence (5'→3')	Positive control	Amplicon size (bp)	Primer concentration (pmol/ $\mu$ L)	MgCl <sub>2</sub> concentration (mM)
MGA	SME-1 to SME-3	SME- GAM-F	GAG GAA GAC TTT GAT GGG AGG AT	Nord-03 [10]	334	0.8	1.5
		SME- GAM-R	TCC CCT CAG GAC CGC CAA G			0.7	
	IMI-1 to IMI-3, NMC-A	IMI-GS- F	GGT GTC TAC GCT TTA GAC ACT GGC TC	Nord-04 [11]	536	0.5	
		IMI-GS- R	GCA CGA ATA CGC GCT GCA CCG G			0.6	
MGB	GIM-1	GIM- GBM-F	CGT TGC CAG CTT TAG CTC AGG	PSA098 (this study)	279	0.3	2.5

	GIM-	GCA ACT TGA			0.3
	GBM-R	TAC CAG CAG TGC G			
SIM-1	SIM-	TTG CGG AAG	PSA097	613	0.3
	GBM-F	AAG CCC AGC CAG	[12]		
	SIM-	GCG TCT CCG			0.3
	GBM-R	ATT TCA CTG TGG C			
NDM-1	NDM-	CCC GGC CAC	NCTC-	129	0.7
	GBM-F	ACC AGT GAC A	13443		
	NDM-	GTA GTG CTC			0.6
	GBM-R	AGT GTC GGC AT			
	<sup>b</sup>				
SPM-1	SPM-	GGG TGG CTA	PSA096 [9]	447	1.2
	GBM-F	AGA CTA TGA AGC C			
	SPM-	GCC GCC GAG			1.4
	GBM-R	CTG AAT CGG			

MGC1	CMY-1, CMY-8 to CMY-11, CMY-19; MOX-1 to MOX-4 and [MOX-5 to MOX-7]	CY1-	GCT GCT CAA	CP043,	522	0.5	1.0
		GC1M-F	GGA GCA CAG GAT CCC G	CP113 [13]			
DHA-1 to DHA-3, DHA-7		CY1-	GGC ACA TTG			0.5	
		GC1M-R	ACA TAG GTG TGG TGC ATG				
		DHA-	CTT TCA CAG	CP039 [13]	403	0.5	
		GC1M-F	GTG TGC TGG GTG CG				
ACC-1 to ACC-4		DHA-	CCG TAC GCA			0.6	
		GC1M-R	TAC TGG CTT TGC GC				
		ACC-	TCC AGC CGC	CP044 [13]	365	1.1	
		GC1M-F	TGA TGC AGA AGA AT				
		ACC-	CCA YGC TTT			1.0	
		GC1M-R	TAG ATA AGC CAT CAG CTG				

MGC2	CMY-2-group <sup>c</sup> , LAT-1, LAT-4 <sup>d</sup>	CY2- GC2M- F	ACT GGC CAG AAC TGA CAG GCA AA	CP040, CP122, CP168 [13]	466	1.7	1.0
		CY2- GC2M- R	GTT TTC TCC TGA ACG TGG CTG GC			1.7	
	ACT-1 to ACT-3, MIR-1 to MIR-4, MIR-8 and [ACT- 5] <sup>e</sup>	ACT- GC2M- F	TCG GTA AAG CCG ATG TTG CGG	CP038 [13]	302	0.6	
		ACT- GC2M- R	CTT CCA CTG CGG CTG CCA GTT			0.6	
	FOX-1 to FOX-8	FOX- GC2M- F	CAT GGG GTA TCA GGG AGA TGC C	CP093 [13]	218	0.1	
		FOX- GC2M- R	GCC GCT GCT CGC CCA TCG			0.1	

MGD1	OXA-23, -27, -49, -73, -133, -146	O23-	CCT GAT CGG	NCTC-	516	0.6	2.0
		GDM-F	ATT GGA GAA CCA G	13421, CP081 [13]			
		O23-	GAT GCC GGC			0.7	
		GDM-	ATT TCT GAC				
		R	CG				
	OXA-24 (= -33, -40), -25, -26, -72, -139, -160 and [OXA-143, -182]	O24-	GGT CGA TAA	NCTC-	237	0.3	
GDM-F		TTT TTG GTT AGT TGG CCC	13302 , NCTC- 13303				
		O24-	CCA TTA GCT			0.3	
		GDM-	TGC TCC ACC				
		R	CAA CCA G				
	OXA-48, -54 and [OXA-181]	O48-	CCA AGC ATT	NCTC-	389	1.1	
GDM-F		TTT ACC CGC ATC KAC C	13442				
		O48-	GYT TGA CCA			1.2	
		GDM-	TAC GCT GRC				
		R	TGC G				

MGD2	OXA-1 (=30), -31, -47	O1-	CAA CGG ATT	CP116 [13]	198	0.1	2.5
		GD2M-	AAC AGA AGC				
		F	ATG GCT CG				
		O1-	GCT GTR AAT			0.1	
OXA-2, -3, -15, -21, -32, -34, -36, -46, -53, -141, -144, -161 and [OXA-118, -119]		GD2M-	CCT GCA CCA				
		R	GTT TTC CC				
		O2-	GAC CAA GAT	CP082,	256	0.8	
		GD2M-	TTG CGA TCA	CP087			
		F	GCA ATG CG	[13]			
		O2-	CYT TGA CCA			0.8	
OXA-51 group <sup>f</sup>		GD2M-	AGC GCT GAT				
		R	GTT CYA CC				
		O51-	GAC CGA GTA	NCTC-	497	1.6	
		GD2M-	TGT ACC TGC	13420			
		F	TTC GAC C				
		O51-	GAG GCT GAA			1.5	
	GD2M-	CAA CCC ATC					
	R	CAG TTA ACC					

MGD3	OXA-10 group <sup>g</sup> and [OXA-5]	O10-	CGC CAG AGA	CP089 [13]	138	1.1	2.0
		GD3M- F	AGT TGG CGA AGT AAG				
		O10-	GAA ACT CCA			1.1	
		GD3M- R	CTT GAT TAA CTG CGG				
	OXA-58, -96, -97, -164	O58-	GTG CTG AGC	NCTC-	630	0.5	
		GD3M- F	ATA GTA TGA GTC GAG C	13305			
		O58-	GGT CTA CAG			0.6	
		GD3M- R	CCA TTC CCC AGC C				

PCR, polymerase chain reaction; NCTC, National Collection of Type Cultures.

<sup>a</sup> All allotypes without brackets have no or one mismatch with the primers, except for the 3'-nucleotide, designed for this target group.

<sup>b</sup> Primer sequence from David Livermore, Health Protection Agency, UK (pers. corr.).

<sup>c</sup> CMY-2, -4 to -7, -12 to -16, -18, -21, -23, -24, -27 to -36, -38 to -41, -43 to -45, -47 to -49, -53 to -55, and -59.

<sup>d</sup> Also known as the EBC group [14].

<sup>e</sup> Also known as the CIT group [14]

<sup>f</sup> OXA-51, -64 to -71, -75 to -80, -82 to -84, -86 to -95, -98 to -100, -106 to -113, -115 to -117, -128, -130 to -132, -138, -144, -148 to -150, and -172 to -180.

<sup>g</sup> OXA-4, -7, -10, -11, -13, -14, -16, -17, -19, -28, -56, -74, -101, -129, -142, -145 and -147.

