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Primer Evaluation for PCR and its Application for Detection of Carbapenemases in *Enterobacteriaceae*

Patrik Mlynarcik,[1,*] Magdalena Roderova,[1] and Milan Kolar[1]

[1]Department of Microbiology, Faculty of Medicine and Dentistry, Palacky University Olomouc, Olomouc, Czech Republic

*Corresponding author: Patrik Mlynarcik, Department of Microbiology, Faculty of Medicine and Dentistry, Palacky University Olomouc, 775 15, Olomouc, Czech Republic. Tel: +420-585632406, E-mail: patrik.mlynarcik@upol.cz

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Abstract

Background: During the last decade, the prevalence of carbapenem-resistant *Enterobacteriaceae* in human patients has increased. Carbapenemase-producing bacteria are usually multidrug resistant. Therefore, early recognition of carbapenemase producers is critical to prevent their spread.

Objectives: The objective of this study was to develop the primers for single and/or multiplex PCR amplification assays for simultaneous identification of class A, class B, and class D carbapenem hydrolyzing β-lactamases in *Enterobacteriaceae* and then to evaluate their efficiency.

Materials and Methods: The reference sequences of all genes encoding carbapenemases were downloaded from GenBank. Primers were designed to amplify the following 11 genes: bla_{KPC}, bla_{OXA}, bla_{VIM}, bla_{NDM}, bla_{IMP}, bla_{SME}, bla_{IMI}, bla_{GES}, bla_{GIM}, bla_{DIM} and bla_{CMY}. PCR conditions were tested to amplify fragments of different sizes. Two multiplex PCR sets were created for the detection of clinically important carbapenemases. The third set of primers was included for detection of all known carbapenemases in *Enterobacteriaceae*. They were evaluated using six reference strains and nine clinical isolates.

Results: Using optimized conditions, all carbapenemase-positive controls yielded predicted amplicon sizes and confirmed the specificity of the primers in single and multiplex PCR.

Conclusions: We have reported here a reliable method, composed of single and multiplex PCR assays, for screening all clinically known carbapenemases. Primers tested in silico and in vitro may distinguish carbapenem-resistant *Enterobacteriaceae* and could assist in combating the spread of carbapenem resistance in *Enterobacteriaceae*.

Keywords: Enterobacteriaceae, Drug Resistance, Multiplex Polymerase Chain Reaction, Carbapenems

1. Background

Carbapenems are considered to be one of the few drugs that are useful for the treatment of infections caused by multiresistant Gram-negative bacteria. The emergence of carbapenem-resistant *Enterobacteriaceae* is a serious public health concern due to the large spectrum of resistant genes and the lack of therapeutic options (1, 2). Therefore, there is an ongoing effort in the development of earlier and more sensitive detection of carbapenemase producers. With regard to carbapenem resistance, it must be taken into account that carbapenemase-producing bacteria may sometimes exhibit only a slight increase of minimal inhibitory concentration (MIC) values for carbapenems, which reflects the importance of molecular approaches to phenotypic tests (3).

Enterobacteriaceae, such as *Escherichia coli*, *Klebsiella pneumoniae* and *Enterobacter* spp., commonly cause nosocomial pneumonias and infections in the bloodstream, urinary tract and intra-abdominal region (4, 5). Carbapenem resistance mechanisms in *Enterobacteriaceae* include the following: (i) enzymatic inactivation by β-lactamases; (ii) modification of outer-membrane proteins (porins) and penicillin-binding proteins; and (iii) efflux pumps (6). Carbapenem-hydrolyzing β-lactamases belonging to molecular class A (e.g., KPC, GES, IMI, SME), class B (e.g. IMP, VIM, NDM, GIM) and class D (e.g. OXA-23 and OXA-48), are the main source of antibiotic resistance in *Enterobacteriaceae*. Genes encoding these types of carbapenemases are extensively reported among *E. coli* and *K. pneumoniae* isolates from many European countries (1).

The carbapenem-hydrolyzing class D β-lactamase OXA-48 has been identified in a *K. pneumoniae* isolate from Europe (7, 8), OXA-23 in *Proteus mirabilis* from France (9), OXA-162 in *K. pneumoniae* isolates from Hungary (10), OXA-181 in *K. pneumoniae* from Romania (11) and *Citrobacter freundii* from France (12), OXA-232 in *K. pneumoniae* from France (13) and OXA-244 and 245, both in *K. pneumoniae* from Spain (14). Additionally, OXA-247 was first described in *K. pneumoniae* in Argentina (15). Recently, $bla_{OXA-51-like}$, bla_{OXA-58} and bla_{DIM-1} carbapenemase genes have been found in a large variety of enterobacterial species (16).

Further, the presence of carbapenemases and extended-spectrum β-lactamases (ESBLs) were also described: (i) bla_{TEM-1}, bla_{SHV-11}, $bla_{CTX-M-15}$ and bla_{OXA-9} genes were present in the *K. pneumoniae* isolates harboring bla_{OXA-48}; (ii) bla_{TEM-1} and bla_{OXA-1} genes were found in *E. coli* harboring $bla_{OXA-162}$ and bla_{OXA-48}; (iii) bla_{SHV-5} in *C. freundii* harboring $bla_{OXA-162}$; and (iv) bla_{TEM-1} and $bla_{CTX-M-15}$ in *Enterobacter cloacae* carrying bla_{OXA-48} (17).

Moreover, *E. coli* and *K. pneumoniae* clinical isolates producing CTX-M-2 and CTX-M-92 and a *K. pneumoniae* isolate with CTX-M-3 were detected, and each had the OXA-2 type beta-lactamase (18). In addition, KPC-2 and TEM-1 enzymes were identified in *K. pneumoniae* isolates, and VIM-1 and TEM-1 in *P. mirabilis*, both of which were positive for bla_{OXA-10} (19). In this context, a recent study has demonstrated that OXA-2 and OXA-10 are in fact carbapenem-hydrolyzing class D beta-lactamases (CHDLs), also called oxacillinases (OXA) (20). Interestingly, development of carbapenem resistance in CTX-M-1-producing *Enterobacteriaceae* has been reported (21, 22). Furthermore, it has been reported that CMY-2 β-lactamase plays a role in carbapenem resistance ("trapping" of meropenem) (23).

The rates of hydrolysis by OXA-type carbapenemases are weak. However, genes encoding OXA overcome this deficiency by possessing efficient promoters, leading to their overexpression and to increased carbapenem resistance (24). Thus, rapid and useful detection methods are important for the implementation of appropriate infection control measures to prevent the further spread of ESBLs and carbapenemases.

2. Objectives

The aim of the present study was to develop the primers for single and/or multiplex PCR amplification assays for identification of all known genes that confer carbapenem resistance in *Enterobacteriaceae* and evaluate the amplification efficiency of the primers.

3. Materials and Methods

3.1. Bacterial Isolates

Fifteen carbapenemase-producing strains were used as positive controls for optimizing the multiplex PCR assay; six reference strains and nine clinical isolates. The reference strains used were: (i) IMP-type (NCTC 13476), producing *E. coli*; (ii) KPC-3 positive *K. pneumoniae* (NCTC 13438); (iii) NDM-1 positive *K. pneumoniae* (NCTC 13443); (iv) VIM-10 positive *Pseudomonas aeruginosa* (NCTC 13437); (v) OXA-23 positive *Acinetobacter baumannii* (NCTC 13301); and (vi) OXA-48 positive *K. pneumoniae* (NCTC 13442). The clinical isolates were: (i–v) *K. pneumoniae* that produce VIM (V602B), KPC-2 (V117), KPC-3 (V514) and KPC (V601, V646); (vi–viii) *P. aeruginosa*-producing IMP (V7424) and VIM (V109, V7393); and (ix) NDM-1 positive *A. baumannii* (V509). All the clinical strains were provided by Ing. J. Hrabák, Ph.D. from the Department of Microbiology, Faculty of Medicine and University Hospital in Plzen, Charles University in Prague, Plzen, Czech Republic.

3.2. Design of Group-Specific Primers for Single and Multiplex PCR Assays

Two multiplex PCRs were designed in this study: a bla_{KPC}/$bla_{OXA-48-like}$/bla_{VIM} multiplex PCR and a bla_{NDM-1}/bla_{IMP} variants/$bla_{OXA-23-like}$ mutliplex PCR. The sequences of genes that encode carbapenemases (KPC, VIM, IMP, SME, IMI, GES, NDM, OXA) so far described (http://www.lahey.org/studies/; last accessed February 2015) were downloaded from the GenBank databases and were aligned using Geneious Pro 4.8.5 (Biomatters Ltd, Newark, NJ, USA) to identify highly homologous regions suitable for designing primers. Two sets of primers were tested against reference standard strains, as well as clinical isolates, in a single PCR reaction and then in a multiplex format. These reference strains included NCTC 13476, NCTC 13438, NCTC 13443, NCTC 13437, NCTC 13301 and NCTC 13442. Representative V602B, V117, V514, V601, V646, V7424, V109, V7393 and V509 were used as clinical isolates. Further, eleven pairs of primers were designed but not tested. The primer sequences, concentrations and calculated lengths of the corresponding amplicons are listed in Table 1.

3.3. DNA Extraction and Multiplex PCRs

DNA preparation was performed by suspending a colony of each bacterial strain in 100 μL of distilled water, boiling at 98°C for 10 minutes and centrifuging the cell extract for five minutes at 13,000 rpm. A multiplex PCR assay was designed to detect and differentiate one family of class A carbapenemase (KPC), three families of class B carbapenemases (IMP, NDM and VIM) and two families of class D carbapenemases (OXA) in two reactions. Both multiplex PCRs were performed with six pairs of specific primers (Table 1), which were used to amplify fragments (different in size) of 340 bp (KPC), 597 bp (OXA-48), 247 bp (VIM), 439 bp (NDM), 183 bp (IMP) and 736 bp (OXA-23). The PCR reaction mixture contained: 0.5 μL DNA (50 ng) in 24.5 μL complete reaction buffer with $MgCl_2$ (containing 100 mmol/L Tris-HCl [pH 8.8], 500 mmol/L KCl, 1% Triton X-100, 15 mmol/L $MgCl_2$) (Top-Bio, Czech Republic; 4 μL), dNTP (10 mM, 0.5 μL), 15 pmol of each primer (0.5 μL) and Taq DNA polymerase (Top-Bio, Czech Republic; 0.2 μL).

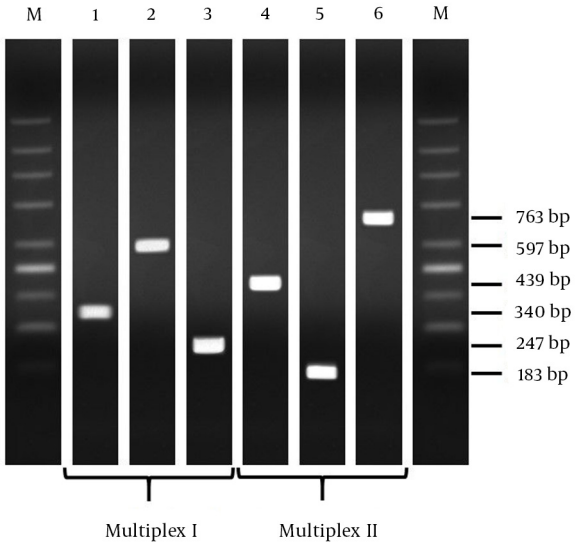
The PCR conditions were as follows: initial denaturation at 95°C for five minutes, 35 cycles at 95°C for one minute, at different annealing temperatures for one minute and 72°C for one minute, followed by a single, final elongation step at 72°C for five minutes. The annealing temperature was optimal at 56°C for amplification of the bla_{KPC}, bla_{OXA} and bla_{VIM} genes and optimal at 52°C for amplification of the bla_{NDM}, bla_{IMP} and bla_{OXA} genes (Table 1). Amplicons were visualized after running at 100 V for 90 minutes on a 1.5% agarose gel containing ethidium bromide (Figure 1). A 200–1500 bp DNA ladder (Top-Bio, Czech Republic) was used as a size marker.

Table 1. Sequences of Primers Used for Single and Multiplex PCR for Detection of Genes Encoding Carbapenemases in *Enterobacteriaceae*

PCR Name	Targeted Gene	Primer Name	Sequence (5' to 3' Direction) [a]	Length (Bases)	Amplicon Size, bp	TM in °C	Primer CONCENTRATION, pmol/μL	Reference
Multiplex I KPC, OXA and VIM	bla_{KPC} type	Forward	TGTTGCTGAAGGAGTTGGGC	20	340	56	15 pmol	This study
		Reverse	ACGACGGCATAGTCATTTGC	20				
	$bla_{OXA-48-like}$ including OXA-199 and OXA-370	Forward	AACGGGCGAACCAAGCATTTT	21	585 or 597		15 pmol	This study
		Reverse	TGAGCACTTCTTTTGTGATGGCT	23				
	bla_{VIM} type	Forward	CGCGGAGATTGARAAGCAAA	20	247		15 pmol	This study
		Reverse	CGCAGCACCRGGATAGAARA	20				
Multiplex II NDM, IMP and OXA	bla_{NDM-1}	Forward	TAAAATACCTTGAGCGGGC	19	439	52	15 pmol	This study
		Reverse	AAATGGAAACTGGCGACC	18				
	bla_{IMP} variants except, IMP-3, IMP-16, IMP-27, IMP-31, IMP-34 and IMP-35	Forward	GAGTGGCTTAATTCTCRATC	20	183		15 pmol	Monteiro et al., 2012 (25)
		Reverse	CCAAACYACTASGTTATCT	19				Ellington et al., 2007 (26)
	$bla_{OXA-23-like}$	Forward	GTGGTTGCTTCTCTTTTTCT	20	736		15 pmol	This study
		Reverse	ATTTCTGACCGCATTTCCAT	20				
PCR/Multiplex[b]	$bla_{SME(1-5)}$	Forward	TATGGAACGATTTCTTGGCG	20	300	56		This study
		Reverse	CTCCCAGTTTTGTCACCTAC	20		58		
	bla_{IMI} type	Forward	CTACGCTTTAGACACTGGC	19	481	57		This study
		Reverse	AGGTTTCCTTTTCACGCTCA	20		56		
	bla_{IMP-27}	Forward	AAAGGCACTGTTTCCTCACA	20	169	56		This study
		Reverse	TCGCCAGCCAATAACTAACC	20		58		
	$bla_{NDM(2-10,12)}$	Forward	ATGACCAGACCGCCCAGAT	19	380	60		This study
		Reverse	GAGATTGCCGAGCGACTTG	19		60		
	$bla_{IMP(3,34)}$	Forward	GTGGTTCTTGTAAATGCTGAGG	22	532	60		This study
		Reverse	CCGCCTGCTCTAATGTAAGT	20		58		
	$bla_{GES(1-22,24)}$	Forward	GAAACCAAACGGGAGACGC	19	207	60		This study
		Reverse	CTTGACCGACAGAGGCAACT	20		60		
	bla_{GIM-1}	Forward	TCGACACACCTTGGTCTGAA	20	477	58		Ellington et al., 2007 (26)
		Reverse	AACTTCCAACTTTGCCATGC	20		56		
	$bla_{OXA-2-like}$	Forward	ATTTCAAGCCAAAGGCACGA	20	568	56		This study
		Reverse	GCCACTCAACCCATCCTACC	20		63		
	$bla_{OXA-10-like}$	Forward	ACGGAAAGCCAAGAGCCAT	19	354 or 357	57		This study
		Reverse	CCCACACCAGAAAAACCAGT	20		58		
	$bla_{OXA-51-like}$	Forward	TGTACCTGCTTCGACCTTCA	20	435	58		This study
		Reverse	TCCCCAACCACTTTTTGCGT	20		58		
	$bla_{OXA-58-like}$	Forward	GCCATTCCCCAGCCACTTTTA	21	470	61		This study
		Reverse	CACGCATTTAGACCGAGCAA	20		58		
	bla_{DIM-1}	Forward	CGGCTGTTTTGTGCGTAG	18	215	56		This study
		Reverse	GCGTTCGGCTGGATTGATT	19		57		
	bla_{CMY-2}	Forward	GCCGTTGCCGTTATCTAC	18	511	56		This study
		Reverse	AATCTTTTTGTTCGTTCTGCG	21		55		

[a] For degenerate primers: R = A or G; S = G or C; Y = C or T.
[b] Designed primers but not tested.

Figure 1. Detection of Genes Encoding Carbapenemases in Carbapenem-resistant Control Strains and Clinical Isolates by Multiplex PCR



Notes, lane 1, control bla_{KPC} gene; lane 2, control bla_{OXA-48} gene; lane 3, control bla_{VIM} gene; lane 4, control bla_{NDM-1} gene; lane 5, control bla_{IMP} gene; lane 6, control bla_{OXA-23} gene. M, Molecular mass markers (200 - 1500 bp DNA ladder).

4. Results

After optimizing the PCR (amplification) conditions, all positive controls (reference strains and clinical isolates) yielded amplicons of the predicted sizes and confirmed the specificity of the primers used (Figure 1). The primer paris were tested in mixed (Table 1, Figure 1) and individual reactions (data not shown). The two multiplex PCR assays were 6 validated with a panel of fifteen characterized Gram-negative bacterial strains. This collection includes 5 class A carbapenemases (5 KPC), 8 class B carbapenemases (2 IMP, 2 NDM and 4 VIM) and 2 class D carbapenemases (2 OXA). The resistance genes of the control isolates were correctly determined by each multiplex PCR (accuracy 100%; Figure 1). Non-specific amplification generating fragments of unexpected size was not observed. In addition, eleven pairs of primers have been proposed but not tested experimentally in PCR and/or multiplex reactions (Table 1).

5. Discussion

The emergence of carbapenem resistance in *Enterobacteriaceae* has become a substantial clinical problem, since carbapenemase production cannot be easily inferred from the antimicrobial resistance profiles, thus, dissemination of these enzymes among nosocomial pathogens (e.g., *Enterobacteriaceae*) is a threat to public health and must be closely monitored (phenotypic and genotypic tests). In addition, delay in detection of multidrug-resistant *Enterobacteriaceae* results in longer hospitalizations and increased healthcare costs (26).

Recently, multiplex PCR assays for the detection of bla_{IMP}, bla_{VIM}, bla_{OXA}, bla_{NDM} and bla_{KPC} have been described (25-30). Unfortunately, the primers used had lower detection ranges (many homologs are missed). For example, the multiplex PCR assay described by Kaase et al. (29) targeted VIM and IMP enzymes, among other carbapenemases, and allowed the detection of 22 variants of IMP (IMP-1 to IMP-22) and 13 variants of VIM (VIM-1 to VIM-13). However, according to the Lahey Clinic website, to this day over 41 IMP and 39 VIM derivatives have been documented. This suggests that specificity of those primers may be impaired due to sequence variations. Nijhuis et al. (30) targeted IMP, OXA-48-like types (OXA-48, 162, 163, 181, 204, 232, 244, 245, 370) and other carbapenemase genes. The assay reported in this study allowed the detection of 33.3% of IMP variants (14/42) and was not able to detect OXA-23 and OXA-247.

This study shows that primers in two multiplex PCRs are able to detect of KPC, VIM, NDM-1, OXA (OXA-23-like and OXA-48-like enzymes) and IMP variants (except IMP-3, IMP-16, IMP-27, IMP-31, IMP-34 and IMP-35) present in *Enterobacteriaceae* clinical isolates. Due to the success of the first primer sets, the additional untested sets (Table 1) are predicted to detect the remaining NDM- and IMP-type enzymes and other carbapenemases. Therefore, the combination of primer sets presented in this study will cover all known carbapenemase genes found in *Enterobacteriaceae*.

The multiplex PCR assay described in this study is a fast, low-cost, efficient and accurate test for rapid screening of *Enterobacteriaceae* isolates with respect to drug resistant genes for bla_{KPC}, bla_{VIM}, bla_{IMP}, bla_{NDM} and bla_{OXA} and cover 100% (19/19), 100% (39/39), 85.7% (36/42), 9.1% (1/11) and 8.5% (25/293) of the genes described in the Lahey database, respectively. The proposed untested primers may be also used for detection of the remaining carbapenemases in single and/or multiplex PCR reactions (Table 1). The resulting assays could collectively cover 92.9% (39/42), 100% (11/11), 100% (5/5), 100% (4/4), 95.8% (23/24) and 52.6% (154/293) of the bla_{IMP}, bla_{NDM}, bla_{SME}, bla_{IMI}, bla_{GES}, bla_{OXA} and other genes, respectively.

We included here all known carbapenemase genes that have been identified in clinical isolates of *Enterobacteriaceae* (bla_{KPC}, $bla_{OXA,}$ bla_{VIM}, bla_{NDM}, bla_{IMP}, bla_{SME}, bla_{IMI}, bla_{GES}, bla_{GIM}, bla_{DIM} and bla_{CMY}). In conclusion, primers tested in silico and in vitro may be used in single and/or multiplex PCR for screening encountered carbapenemases in *Enterobacteriaceae*, as well as for monitoring their emergence and spread (e.g., outbreaks).

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Footnotes

Authors' Contribution: Patrik Mlynarcik designed the primers; Patrik Mlynarcik and Magdalena Roderova car-

ried out the experiments; Patrik Mlynarcik, Magdalena Roderova and Milan Kolar prepared the manuscript.

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