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Rapid Detection of NDM, VIM, KPC and IMP Carbapenemases by Real-Time PCR

Ewa Kosykowska*, Tomasz Dzieciątkowski and Grażyna Młynarczyk

Chart and Department of Medical Microbiology, Medical University of Warsaw, Warsaw, Poland

***Corresponding author:** Ewa Kosykowska, Chart and Department of Medical Microbiology, Medical University of Warsaw, Warsaw, Poland, Tel: +48226282739; E-mail: ewa.kosykowska@wum.edu.pl

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Abstract

Carbapenems are the most potent beta-lactams, characterized by broad spectrum of activity against Gram-negative and Gram-positive bacteria. Unfortunately, the dynamic dispersal of carbapenem resistance among non-fermentative bacteria and *Enterobacteriaceae* is an over-increasing problem and might lead to dangerous limitation of treatment options. Among three different mechanisms of resistance the enzyme production is of special importance. In this case, only one small gene is enough to express carbapenem resistance. The carbapenemase genes are often a part of integrons, which carry diverse arrays of resistance gene cassettes and just one transfer event is enough to disseminate multidrug resistance. Moreover, carbapenemase genes are often located within Mobile Genetic Elements. For these reasons carbapenemases are the most epidemiologically importance. Early detection and identification of carbapenemase producers among clinical isolates can avoid nosocomial infections. We have developed a multiplex Real-Time PCR assay based on the TaqMan technology for rapid detection and identifications of the most common carbapenemases in Europe NDM, VIM, KPC and IMP. There were tested 31 isolates Enterobacteriaceae (n=15) and non-fermentative Gram-negative bacillary (n=16), which acquire NDM, VIM, KPC, IMP, GIM or OXAs carbapenemases. The whole elaborated experiment, including DNA isolation and PCR cycling, lasts up to 2 h.

Keywords: Carbapenemases detection; Real-Time PCR; NDM; VIM; KPC; IMP

Introduction

Emerging and dissemination of carbapenem-hydrolysing beta-lactamases among *Enterobacteriaceae* and non-fermentative Gram-negative bacillary such as *Pseudomonas* spp. or *Acinetobacter baumannii* over past decade is serious issue in the hospital environment. It is worthy to mention that carbapenemase producing organisms (CPOs) have primarily been recognized in hospitals but currently they are more and more common cause as well community infections [1]. Currently known mechanisms for carbapenem resistance among Gram-negative bacteria, involve decreased permeability by porins modifications, efflux pumps, increased natural cephalosporinase activity and the most frequent carbapenemase producing [2-4]. The carbapenemases hydrolyse almost all beta-lactams, are resistant to all therapeutically useful inhibitors and are co-produced with other beta-lactamases [5]. Moreover, carbapenemase genes are often a part of integrons, which carry diverse arrays of resistance gene cassettes. The most worrisome is the situation when such integron is located within some Mobile Genetic Elements (MGEs, transposons or transmissible plasmids). In this case just one transfer event is enough to disseminate multidrug resistance [1].

The most common carbapenemases in the world are NDM, VIM and IMP belonged to molecular class B and KPC, serine enzyme pertained to molecular class A [5].

Prevention of the dissemination of carbapenem resistant bacteria is of the utmost importance. Accurately identifying in the clinical laboratory is the first step in protection [1]. The purpose of the study has been to create the rapid method to detect such organisms. We have developed a multiplex real-time PCR (RT-PCR) assay, using TaqMan chemistry to detect just those species which possess some of the most frequent carbapenemases' types VIM, IMP, NDM and/or KPC.

Materials and Methods

Bacterial strains

Thirty-one reference bacterial strains were obtained from the collection of the Chair and Department of Medical Microbiology, Medical University of Warsaw and used for detection in culture in order to validate the novel detection assay of *bla*VIM, *bla*IMP, *bla*NDM, *bla*KPC genes. All of the strains were clinical isolates tested by conventional PCR for several beta-lactamases genes (*bla*VIM, *bla*IMP, *bla*GIM, *bla*KPC, *bla*NDM; in case of *Acinetobacter* spp. also: *bla*OXA-23, *bla*OXA-24, *bla*OXA-51, *bla*OXA-58) [6-9]. The only one exception was NDM-1-positive *E. coli* DH5α created by synthesis of the *bla*NDM-1 gene (Epoch Life Science, USA) and transformation of the plasmid encoding this synthesized gene (pNDM_EK) into *E. coli* DH5α. Two strains (*P. aeruginosa* WUM_5 and *P. aeruginosa* WUM_6) came from the National Medicines Institute. All bacterial strains are described in details in Table 1. MICs were determined using Etest (bioMerieux).

DNA extraction

DNA was isolated from bacterial colonies using the boiling lysis method. Strains were streaked onto Mueller Hinton (MH) agar and grown overnight at 37C. A loopful of bacterial growth was suspended in 100 µl of sterile distilled water, incubated at room temperature for 5

min, and then boiled for 10 min. After centrifugation at 13200 rpm for 10 min, the pellet was discarded and the supernatant containing DNA was used directly for PCR or stored at -20°C [6].

Analytical specificity

Analytical specificity for each assay was verified using the following comprehensive panel of different beta-lactamases producers. We used five different species of *Enterobacteriaceae*: *Escherichia coli*, *Enterobacter cloacae*, *Klebsiella oxytoca*, *Klebsiella pneumoniae*, *Serratia marcescens* and four species of non-fermentative Gram-negative bacillary: *Acinetobacter baumannii*, *Acinetobacter junii*, *Pseudomonas aeruginosa* and *Pseudomonas putida*. All of them were beta-lactamases producers (described in details in Table 1).

Design of primers and TaqMan probes

All primers and probes were designed with using LightCycler Probe Design Software 2.0 (Roche Diagnostics). Reference sequence for each of five genes was assembled from GeneBank (accession numbers: JF966383.1; JX104759.1; GU831553.1; AB182996.1; JQ060896.1). Sequence specificity was confirmed *in sillico* by nucleotide-nucleotide search in the National Centre for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/BLAST/). The detailed sequences of used primers and probes are described in Table 2.

Isolate no.	Bacterium	Genotype [a]	MIC [µg/ml]				Multiplex RT-PCR results	
			DOR [b]	ETP [b]	IMP [b]	MER [b]	530 nm	610 nm
1	*E. coli* WUM_1	*bla*NDM-1	ND	ND	ND	ND	+	-
2	*K. pneumoniae* WUM_1	*bla*NDM	3	ND	16	4	+	-
3	*E. coli* WUM_2	*bla*KPC	0,75	0,5	4	0,5	-	+
4	*E. coli* WUM_2	*bla*KPC	24	>32	>32	>32	-	+
5	*K. oxytoca* WUM_1	blaVIM, blaKPC	2	8	12	2	-	+
6	*K. oxytoca* WUM_2	blaKPC	1	1,5	0,5	1	-	+
7	*K. pneumoniae* WUM_2	blaKPC	>32	>32	16	>32	-	+
8	*K. pneumoniae* WUM_3	blaKPC	>32	>32	>32	>32	-	+
9	*K. pneumoniae* WUM_4	blaKPC	>32	>32	>32	>32	-	+
10	*K. pneumoniae* WUM_5	blaKPC	>32	>32	>32	>32	-	+
11	*K. pneumoniae* WUM_6	blaKPC	>32	>32	>32	>32	-	+
12	*K. pneumoniae* WUM_7	blaKPC	>32	>32	>32	>32	-	+
13	*K. pneumoniae* WUM_8	blaKPC	>32	>32	>32	>32	-	+
14	*E. cloacae* WUM_1	blaVIM	0,75	4	1,5	1,5	-	+
15	*P. aeruginosa* WUM_1	blaVIM-2	16	ND	12	6	-	+
16	*P. aeruginosa* WUM_2	blaVIM	>32	ND	>32	>32	-	+
17	*P. aeruginosa* WUM_3	blaVIM	32	ND	>32	>32	-	+
18	*P. aeruginosa* WUM_4	*bla*VIM	32	ND	>32	32	-	+
19	*P. putida* WUM_1	*bla*VIM	16	ND	>32	>32	-	+
20	*S. marcescens* WUM_1	*bla*VIM	>32	>32	>32	>32	-	+
21	*P. aeruginosa* WUM_5	*bla*IMP-1	>32	ND	>32	>32	+	-
22	*P. aeruginosa* WUM_6	*bla*GIM-1	>32	ND	>32	>32	-	-
23	*A. baumannii* WUM_1	*bla*OXA-24, *bla*OXA-51	>32	ND	>32	>32	-	-
24	*A. baumannii* WUM_2	*bla*OXA-24, *bla*OXA-51	>32	ND	>32	>32	-	-
25	*A. baumannii* WUM_3	*bla*OXA-24, *bla*OXA-51	>32	ND	>32	>32	-	-
26	*A. baumannii* WUM_4	*bla*OXA-51, *bla*OXA-58	32	ND	32	32	-	-

27	*A. baumannii* WUM_5	*bla*OXA-51, *bla*OXA-58	12	ND	32	32	-	-
28	*A. baumannii* WUM_6	*bla*OXA-51	4	ND	6	8	-	-
29	*A. baumannii* WUM_7	*bla*OXA-51, *bla*OXA-58	24	ND	>32	32	-	-
30	*A. baumannii* WUM_8	*bla*OXA-51	32	ND	32	32	-	-
31	*A. junii* WUM_1	*bla*OXA-58, *bla*VIM	2	ND	16	4	-	+

Table 1: Results of multiplex RT-PCR and MICs of bacterial isolates used in this study. [a] Genotype was determined by conventional PCR in our labalatory; [b]DOR – doripenem; ETP – ertapenem; IMP - imipenem; MER – meropenem; ND - MICs not determined because of natural resistance for ertapenem *Pseudomonas* spp. and *Acinetobacter* spp. and in case of nonclinical isolate *bla*NDM-1 positive strain; + positive result; (-) negative result.

Real-Time PCR

Investigations were performed using amplification mixture TaqMan Master Kit® (Roche Diagnostics). Besides chemicals supplied by kit producer, reaction mixture contained 1 µl of template DNA, 1,5 µM of each probe, 1,75; 2 or 2,25 µM of adequate primers (as described in Table 2) in a final volume of 10 µl. The best results of amplification were obtained with: activation of thermostable hot-start DNA polymerase for 10 min at 95°C, followed by 40 cycles comprising: denaturation (10 s at 95°C), primers annealing (10 s at 60°C) and strand elongation (15 s at 72°C). After the end of cycling, material was cooled down to 40°C for 60 s. Fluorescence levels were measured at wavelengths: 530 nm (FAM dye) and 610 nm (LC-Red 610 dye). The limit of detection (LOD) of each assay was determined by analysis of serial decimal dilutions of used DNA in range 101-106 copies. Each dilution was prepared and analyzed in six independent replications. Probity analysis was used to calculate the LOD concentration [10].

All described experiments were performed in two independent repetitions, using LightCycler 2.0 instrument (Roche Diagnostics).

Interpretation of results

A sample was considered positive by RT-PCR if it crossed the threshold before a crossing point (*Cp*) of 35 cycle and negative if the *Cp* was greater than 35. Positive result at wavelength 530 nm (FAM dye) stated the presence of *bla*IMP or *bla*NDM, whereas positive result at wavelength 610 nm (LC-Red 610 dye) meant the presence of *bla*VIM or *bla*KPC.

Target	Primer/probe name	Sequence (5' → 3')	Amplicon size (bp)	Concentration [µM]	Reference sequence[b]
	VIMf	TCTCCACGCACTTTCATGAC	124	2	JF966383.1
	VIMr2	GTGGGAATCTCGTTCCCCTC		2	
*bla*VIM	s_qVIM	**LC.red 610** -GGCAACGTACGCATCACCGTC- **BHQ-2** [a]		1,5	
	KPCf	ATATCTGACAACAGGCATGAC	95	1,75	JX104759.1
	KPCr	CCAACTCCTTCAGCAACAAA		1,75	
*bla*KPC	s_qKPC	**LC.red 610** -AATACAGTGATAACGCCGCCG- **BHQ-2**		1,5	
	IMP1f	GGCTTAATTCTCGATCTATCCC	114	1,75	GU831553.1
	IMP1r	CTAGCCAATAGTTAACTCCGC		1,75	
*bla*IMP-1 like	s_qIMP1	**FAM** -GACGGTAAGGTTCAAGCCACAAAT- **BHQ-1**		1,5	
	IMP2f	TAGAGTGGCTTAATTCTCAATCTATTCC	120	2	AB182996.1
	IMP2r	CTAGCCAATAACTAACTCCGC		2	
*bla*IMP-2 like	s_qIMP2	**FAM** -AACTTCTTAAAAAAGACGGTAAGGTGCA- **BHQ-1**		1,5	
	NDM1f2	GATCCTCAACTGGATCAAGC	128	2,25	JQ060896.1
	NDM1r2	CATTGGCATAAGTCGCAATC		2,25	
*bla*NDM	s_qNDM	**FAM** -GACTCACGCGCATCAGGACAA- **BHQ-1**		1,5	

Table 2: Primers and probes used in this study. [a] Probes' 5' reporter and 3' quencher dyes are bolded. [b] Gene Bank accession no.

Results

Designed primers and probe of the *bla*KPC amplicon *in silico* show total identity to all 12 alleles (*bla*KPC-2 to *bla*KPC-13). Likewise, NDM1f, NDM1r primers and s_qNDM probe are identical to all known *bla*NDM (*bla* NDM-1-7) alleles. Amplicon VIM covers most of the 33 alleles but not all. However, the most common VIM alleles are detectable. VIMf, VIMr primers and s_qVIM probe are identical to regions of blaVIM-1, 2, 4, 5, 8, 9, 10, 12, 14-17, 19-33 alleles. *bla*VIM-3, 6, 7, 11, 13 alleles have from one to five single nucleotide polymorphisms (SNPs) within these regions which are covered by those three oligonucleotides. *bla*VIM-18 allele has 12 nt deletion in the region which covers VIMr primer. *bla*IMP-like genes are much more diverse than the others and we decided to design two amplicons. Such solution allowed to detect the majority of the most frequent *bla*IMP alleles. IMP-1f, IMP-1r primers and s_qIMP1 probe are identical to *bla*IMP-1, 3, 6, 10, 25 and 30 alleles. IMP-2f, IMP-2r primers and s_qIMP2 probe are identical to *bla*IMP-2, 8, 19, 20 and 24 alleles.

Analytical specificity

The specificity of the primers and probes for the detection of all investigated genes were evaluated by the BLAST search program, available at www.ncbi.nlm.nih.gov. No matches to the primers and probe sequences other than those which were investigated were found.

Additionally, to verify analytical specificity, the multiplex RT-PCR assay was checked in 31 DNA templates from nine different Gram-negative species, beta-lactamases producers (described in details in Table 2) and the results are in accordance with expected. No cross-reactivity or nonspecific amplification was observed for any of the assays tested with these tested organisms.

After positive result, it is recommended to conduct the next RT-PCR with single pair of primers (monoplex RT-PCR) to find out which exactly carbapenemase tested isolate contains. For example, if tested strain revealed signal on 610 nm, it is worthy to conduct two independent reactions with KPC primers and probes and with VIM oligonucteotides. Differentiation of carbapenemase type is also possible by cheaper and time-consuming methods such as phenotypic tests. The results of LOD in developed RT-PCR assays, determined in DNA copies per reaction tube are shown in Table 3 (supplementary information).

Detected gene in a given RT-PCR assay	LOD (DNA copies/reaction)	95% confidence interval (DNA copies/reaction)
bla KPC	107	81-129
bla IMP-1	56	44-67
bla NDM-1	194	175-231
bla VIM-2	98	82-113

Table 3: Limits of detection (LOD) of the RT-PCR assays.

Discussion

Early identification of carbapenemase producing organisms (CPOs) among clinical isolates should be obligatory to prevent their prevalence. Infections caused by CPOs are the most worrisome because of the high mortality rate. Additionally, carbapenemases genes might disperse rapidly among intestinal microbiota and others bacteria especially easily in hospital environments under antibiotic selective pressure [11].

In the routine laboratory, detection of carbapenem resistant isolates is mainly based on phenotypic methods such as double-disk test (DDT) with EDTA inhibitor to detect metalo-beta-lactamases (MBLs) producers and modified Hodge test (MHT) with phenyl boronic acid as an inhibitor to detect KPC-positive bacteria or modifications of above-mentioned tests [12,13]. Temocillin susceptibility is ascertaining to identify OXA-48 producers. These phenotypic methods characterised by different specificity and sensitivity often need technical changes and are time-consuming. Currently, there are commercially available more convenient phenotypic method, Etest to detect MBL producers and the other one for KPC identification [14,15]. Recently, many different methods are evaluated to detect CPOs but without differentiation into enzyme type. The first step is similarly in all methods described below. It is based on the *in vitro* hydrolysis of carbapenem (imipenem, meropenem, ertapenem, less doripenem) by bacterial colonies or their extract. The detection of decreased carbapenem concentration is performed by several different manners. Matrix assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) assays reveals others view in mass spectra of the beta-lactam molecule, its salts, and/or its degradation products [16,17]. Hydrolysis of beta-lactam may be conduct with using a bacterial culture or the extract. CarbaNP is a biochemical test for detection of CPOs directly from bacterial culture, which is detected by changes in pH values revealed by the indicator bromothymol blue [18]. Carbapenem inactivation method (CIM) is a cost-effective and highly robust method. After incubation disc containing meropenem with tested strain the disc is plated on MH agar plate inoculated with a susceptible *E. coli* indicator strain. If the tested strain produced a carbapenemase, the meropenem was inactivated allowing uninhibited growth of the *E. coli* indicator strain [19]. Protocol for spectrophotometric detection is more time-consuming and labor-intensive than methods above. After 18 h incubation, double centrifugations and sonication the supernatant is used to test for its hydrolysis activity by UV spectrophotometry using imipenem as a substrate at a wavelength of 297 nm [20].

The new, described method in confrontation to other available in literature such as multiplex RT-PCR with melting curves described by Monteiro or Hofko is a little more expensive but the results are more unequivocal [21,22]. Similarly, Nijhuis provided a multiplex RT-PCR to detect carbapenemase genes by using molecular beacon probes, which are more difficult with interpretations than TaqMan probes [23].

According to our knowledge, this is the first report of a multiplex RT-PCR assay with TaqMan probes to detect such set of enzymes, dedicated for *Enterobacteriaceae* and non-fermentative bacilli. The experiment is easy to conduct and lasts only about 1 h. Unfortunately, expensive equipment and reagents are required therefore it is solution only for specialist laboratories.

In conclusion, we have described a multiplex RT-PCR assay for identification and partial classification of four selected carbapenemases: VIM, IMP, NDM and KPC. The whole experiment, including DNA isolation and PCR cycling, lasts up to 2 h.

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