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Note

A multiplex real-time PCR for the direct, fast, economic and simultaneous detection of the carbapenemase genes *bla*KPC, *bla*NDM, *bla*VIM and *bla*OXA-48

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ABSTRACT

A novel multiplex real-time PCR was designed to detect the clinically most important carbapenemase genes, *bla*KPC, *bla*VIM, *bla*NDM and *bla*OXA-48. The multiplex assay was verified testing genomic DNA of 24 carbapenemase-producing strains. It was validated using a blinded panel of 82 carbapenemase-producing and 50 non-producing isolates by direct colony PCR.

In humans, *Enterobacteriaceae* can cause a wide variety of infections. They are commonly treated with fluoroquinolones or beta-lactam antibiotics (Mikasa et al., 2016). Beta-lactams comprise four major groups, penicillins, cephalosporins, monobactams and carbapenems (Nordmann et al., 2012a, 2012b). Nearly 50% of all antibiotics currently prescribed belong to the beta-lactams (Van Boeckel et al., 2014). Especially third and fourth generation (3G/4G) cephalosporins are important for the treatment of enterobacterial infections. Their overuse in humans and livestock (Halloran, 2012; Landers et al., 2012; o'Neill, 2015) lead to the emergence of *Enterobacteriaceae* that are resistant due to production of extended spectrum beta-lactamases (ESBL). Carbapenems are commonly and increasingly used as last line antibiotics for the therapy of infections with such ESBL producers, or as initial treatment before the causative organism is identified and its susceptibility pattern is determined. The use of carbapenems consequently leads to a selective pressure favoring different resistance mechanisms. These include AmpC overexpression accompanied by a porin loss (Doumith et al., 2009; Martinez-Martinez et al., 1999; Nordmann et al., 2012a, 2012b) or the production of carbapenemases which are able to hydrolyze most, or even all beta-lactam antibiotics. The corresponding carbapenemase genes are usually plasmid-located and they are associated with various mobile genetic elements like transposons, integrons and insertion sequences (Diene and Rolain, 2014).

Recent reports have shown a dramatic increase of the prevalence of carbapenemase-producing organisms (CPOs) as well as a rapid worldwide spread (Canton et al., 2012; Livermore, 2012; Livermore et al., 2011). The carbapenemase genes *bla*KPC, *bla*NDM, *bla*OXA-48 and/or *bla*VIM are currently most prevalent.

The treatment options for infections with CPOs are limited, especially given that carbapenemase genes usually are co-localized on mobile genetic elements together with additional beta-lactamase genes and genes conferring resistance to fluoroquinolones and/or aminoglycosides. Currently, only few antibiotics (*i.e.*, colistin, tigecycline or fosfomycin) remain to treat infections with such multiresistant CPOs (Livermore et al., 2011).

Therefore, a rapid molecular identification of carbapenemase genes in Gram-negative bacteria is crucial for infection control, prevention, surveillance and for a quick confirmation of results from other procedures such as susceptibility tests. Furthermore, it may have a significant impact on the selection of an appropriate initial treatment and could therefore be of great benefit for intensive care patients.

Conventional methods for detection of CPOs in patient samples include the use of selective culture media and standard susceptibility testing followed by confirmatory tests such as the Modified Hodge test (Carvalhaes et al., 2010; Hrabak et al., 2014) or the Nordmann-Poirel (NP) test (Tijet et al., 2013). These methods are often time consuming.

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Thus several PCR-based assays have been developed that are able to detect carbapenemase genes directly in patient samples [*e.g.*, Cepheids Xpert® Carba-R (Cortegiani et al., 2016, Tato et al., 2016), Check-Points Check-Direct CPE® test (Huang et al., 2015, Lau et al., 2015)] or to identify carbapenemase genes in bacterial culture material [Check-Points Check-MDR Carba (Boran et al., 2015); Alere Technologies CarbDetect AS-2 (Braun et al., 2014)].

In addition, other methods were developed recently that could lead to the development of new diagnostic tests to target CPOs. These include molecular beacons, metabolite profiling of the carbapenemases directly by liquid chromatography mass spectrometry (LC-MS/MS) and modern quantum dot technologies based on cadmium ion electronic detection systems (Ipe et al., 2013).

We aimed to develop a specific and sensitive multiplex real-time PCR to detect the four clinically most important carbapenemase genes in CPOs using direct and fast colony PCR without any sample preparation prior to amplification.

Serial dilutions of genomic DNA preparations of 24 carbapenemase-producing reference strains were used for verification and assessment of assay performance (calculated as limit of detection, LoD). For validation, a blinded panel of carbapenemase-producing (n = 82) and non-producing (n = 50) isolates were used with direct colony PCR. All strains and isolates were initially genotyped by DNA-microarray (CarbDetect AS-2, Alere Technologies GmbH, Jena, Germany) and characterized by susceptibility tests with the VITEK-2 system (card AST-248, bioMerieux, Nürtingen, Germany). All isolates were cultured on tryptone yeast agar overnight at 37 °C. Genomic DNA was extracted using the Qiagen DNeasy Blood & Tissue Kit (Qiagen GmbH, Hilden, Germany) according to manufacturer's instructions. The DNA concentration was determined spectrophotometrically (Nanodrop ND-1000, Thermo Fisher Scientific, Dreieich, Germany). Dilution series were prepared, calculated and adjusted to cover the range of 10^5 to 10^1 genomic equivalents (GE).

For colony PCR, all used isolates were cultured directly from cryocultures (Microbank™, Pro—Lab Diagnostics, Canada) which were stored at − 80 °C and plated on tryptone yeast agar for 16 h at 37 °C. Subsequently, a single colony per isolate was picked, re-suspended in 10 µl of water and heated at 95 °C for 10 min. Afterwards the suspension (10 µl) was used directly for PCR.

Primer and TaqMan probes were designed with Alere primer design software packages determining highly conserved sequence regions for each target to cover all known and published alleles so far (August 2016; for primer mapping to all used design input sequences see Table S1). Detailed information about lengths, sequences and physical parameters of primers and TaqMan probes, and the lengths of resulting amplicons is given in Table 1. TaqMan probes were designed using different fluorophores [*i.e.*; 6FAM (green), HEX (yellow), ROX (orange) and Cy5 (red)] for the respective targets covalently attached to the 5′-end.

The real time PCRs were performed using a Corbett Rotor-Gene 6000 Cycler (Qiagen, Hilden, Germany) and the RNA UltraSense™ One-Step Quantitative RT-PCR System Kit (ThermoFischer, Dreieich, Germany). Real-time PCR assays were carried out in 25 µl reaction volumes containing final concentrations of 200 nM of each primer and 320 nM of each TaqMan probe. Finally, 2 µl of a DNA preparation, as described above, were added. The PCR program for the amplification consisted of the following steps: 4 min of initial denaturation at 95 °C followed by 40 cycles of denaturation at 95 °C for 30 s, initial annealing at 50 °C for 30 s and extension at 72 °C for 60 s. The data were collected during the annealing phase and they were analyzed using the Rotor-Gene 6000 software 2.3.1 (Qiagen GmbH, Hilden, Germany). For colony PCR, 10 µl of cell suspension were added to 15 µl of PCR reaction mix. Final concentrations of primer and probes were identical as described above.

The sensitivity (true positive rate) of the assay was determined using a panel of 70 well-characterized carbapenem-resistant strains that, by array hybridization (CarbDetect AS-2), were shown to harbor one or several of the four target genes of the multiplex PCR described herein. These included blaKPC, n = 17; blaNDM, n = 16; blaVIM, n = 16; blaOXA-48-like, n = 17; blaKPC + blaOXA-48-like, n = 2; blaNDM + blaOXA-48-like, n = 1 and blaVIM + blaOXA-48-like, n = 1. (Table S2).

The specificity (true negative rate) of the assay was evaluated with a panel of 62 well-characterized strains that either harbored no carbapenemase genes at all (n = 50), or other carbapenemase genes (blaGIM n = 2; blaOXA-23 n = 2; blaOXA-40-like n = 2; blaIMP n = 2; blaOXA-55 n = 2; blaOXA-51-like n = 2) than those covered by the multiplex PCR described herein (Table S3).

The limit of detection for all target genes was 10^1 GE in both, single and multiplex reactions. Specific signals for all target genes were detected in all reference strains, including strains containing more than one carbapenemase gene (Fig. 1).

Although the RNA UltraSense™ One-Step Quantitative RT-PCR System Kit (ThermoFischer, Dreieich, Germany) is able to amplify both DNA and RNA, the tests for the limit of detection were done using RNA free DNA to ensure that the results weren't influenced by RNA expression levels, which depend on multiple factors like growth phase, media and selective pressure by antibiotics.

In direct colony PCRs, theoretically both, target DNA and RNA molecules might be amplified. For practical purposes this would be rather helpful as the presence of RNA would increase the absolute number of target molecules possibly resulting in a higher overall sensitivity.

For colony PCR, tryptone yeast agar was used for cultivation (16 h, 37 °C) and no PCR inhibition was observed. However, other growth media (including broth from blood culture bottles) should be evaluated separately in order to rule out possible interfering or inhibiting factors.

The validation showed that *bla*KPC, *bla*NDM, *bla*VIM and/or *bla*OXA-48 genes can reliably be detected in clinical isolates *via* colony PCR. That also included isolates containing multiple target genes and/

Table 1
List of oligonucleotides. Primers and probes used for multiplex real-time PCR detecting the carbapenemase genes *bla*KPC, *bla*NDM, *bla*VIM and *bla*OXA-48.

Name	Sequence (5′–3′)	Tm (°C)	Amplicon (bp)	Accession no.
KPC-FW	CTGTATCGCCGTCTAGTTCTG	55.4		EU447304
KPC-RV	AGTTTAGCGAATGGTTCCG	53.0	101	
KPC-TMP	6FAM-TGTCTTGTCTCTCATGGCCGCTGG-BHQ1	64.0		
NDM-FW	GCATTAGCCGCTGCATT	53.4		
NDM-RV	GATCGCCAAACCGTTGG	53.7	100	FN396876
NDM-TMP	ROX-ACGATTGGCCAGCAAATGGAAACTGG-BHQ2	63.7		
OXA48-FW	TTCCCAATAGCTTGATCGC	52.7		
OXA48-RV	CCATCCCACTTAAAGACTTGG	53.5	70	AY236073
OXA48-TMP	HEX-TCGATTTGGGCGTGGTTAAGGATGAAC-BHQ1	63.9		
VIM-FW	TGGCAACGTACGCATCACC	58.4		
VIM-RV	CGCAGCACCGGGATAGAA	56.9	143	Y18050.2
VIM-TMP	Cy5-TCTCTAGAAGGACTCTCATCGAGCGGG-BHQ3	63.6		