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# Fast and automated detection of common carbapenemase genes using multiplex real-time PCR on the BD MAX™ system

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ABSTRACT

Fast detection of carbapenemases in Gram-negative bacilli is necessary for accurate antibiotic treatment, prevention of further spreading and surveillance purposes. We analyzed the current occurrence of gene variants and designed two multiplex PCRs with hydrolysis probes.

The assay was developed for the BD MAX™ system that combines DNA extraction and PCR in a fully automated procedure providing results within 3 h and was evaluated for detection of carbapenemases from bacterial isolates and directly from rectal swabs.

The assay has a theoretic coverage of 97.1% for carbapenemases detected during the last years by the German National Reference Laboratory (NRL). A collection of 151 isolates from the NRL was used and all carbapenemase-positive bacteria (58/58) were identified correctly. The direct-PCR on rectal swabs revealed additional carbapenemase genes in 7 samples that were not identified by the culture-based method used as reference method.

The assay allows detection of carbapenemases from clinical isolates and might also help in rapid detection directly from rectal samples.

## 1. Introduction

Resistance to beta-lactam antibiotics in Gram-negative bacilli is an increasing problem worldwide. In case of resistance to carbapenems, the remaining treatment options for severe infections are limited (Kaase, 2012). Carbapenem resistance occurs mostly via carbapenemases, acquired enzymes that are able to hydrolyze different beta-lactam antibiotics. A combination of porin loss or overexpression of efflux pumps with expression of extended-spectrum beta lactamases (ESBLs) or AmpCs, depending on the bacteria, is another way of obtaining carbapenem-resistance (El Amin et al., 2005; Doumith et al., 2009). Carbapenemase genes are usually located on plasmids that allow facilitated spreading (Queenan and Bush, 2007). The first carbapenemase (NmcA) was discovered in an *Enterobacteriaceae* in 1993 (Naas and Nordmann, 1994). Over the last years, a large variety of carbapenemases have been described but the most important remain *Klebsiella pneumoniae* carbapenemase (KPC), New-Delhi-metallo-beta-lactamase (NDM), Verona Integron-encoded metallo-beta-lactamase (VIM) and the oxacillinases (OXA-48) (Albiger, Glasner et al., 2015). These four enzymes, "the big 4", are also the most prevalent carbapenemases found in *Enterobacteriaceae* over the last years in Germany (KPC: 14.7%–26.9%, NDM: 18.0%–20.5% VIM: 18.2%–24.4% and OXA-48-like: 31.4%–47.4%) (Koch-Institut, 2015; Koch-Institut, 2016; Pfennigwerth, 2017; Pfennigwerth, 2018). In the non-fermenters *Pseudomonas aeruginosa* and *Acinetobacter baumannii*, respectively VIM (79.2%–88.2%), IMP (6.5%–10.3%) and OXA-23 (66.7%–80.7%), OXA-72 (11.0%–12.7%) and OXA-58 (1.3%–7.0%), were detected more frequently (Koch-Institut, 2015; Koch-Institut, 2016; Pfennigwerth, 2017; Pfennigwerth, 2018).

Carbapenemases are classified based on their amino acid sequence into different Ambler classes. KPC, a serine beta-lactamase, belongs to Ambler class A; NDM, VIM and IMP, also known as metallo-beta-lactamases belong to class B. The oxacillinases OXA-48, OXA-72, OXA-23 and OXA-58 classify in class D (Queenan and Bush, 2007). Numerous variants exist within each class due to mutations of the genes. Newly occurring variants are a problem for molecular-based detection methods, thus regular verification of primer coverage is necessary.

For infection control it is important to know the mechanism of carbapenem resistance, thus detection methods that cover different

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carbapenemases and their variants are needed. Besides phenotypic detection methods of carbapenem resistance, a number of different genotypic assays based on monoplex or multiplex quantitative real-time PCR and using hydrolysis probes or melt-curve analysis have been developed. Eazyplex® SuperBug CRE (Amplex Biosystems GmbH, Gießen, Germany) or Check-Direct CPE assay (Check-Points, Wageningen, The Netherlands), for example, are designed to detect the "big 4" carbapenemases. There are some assays that include detection of IMP variants, such as the Cepheid Xpert Carba-R and different ESBLs and AmpCs (Dallenne et al., 2010; Naas et al., 2011; Cuzon et al., 2012). Yet, most of the commercially available assays are limited to the "big 4" carbapenemases commonly found in *Enterobacteriaceae* and do not include all gene variants.

Detection of the numerous variants of carbapenemases by PCR in a routine workflow can be highly facilitated by fully automated solutions, which can be modulated to answer changes in the local epidemiology. The BD MAX™ system is a fully integrated molecular solution that, as a peculiarity, can be used in an open-system mode, which allows user-developed protocols. The Check-Direct CPE assay has been used on a BD MAX™ for detection of carbapenemases (Antonelli et al., 2016) and additional real-time PCRs covering $bla_{IMP}$, $bla_{VIM}$, $bla_{OXA\text{-}23}$ and *mcr* colistin resistance have been published (Teo et al., 2016; Mentasti et al., 2019). Here, we performed an in-depth analysis of recently occurring carbapenemase genes to define new primers for the detection of carbapenemases in *Enterobacteriaceae* and in the non-fermenters *P. aeruginosa* and *A. baumannii*.

## 2. Materials and methods

### 2.1. Assay design

A sequence collection of beta lactamase genes from the Center for Genomic Epidemiology (https://cge.cbs.dtu.dk/services/data.php, December 2017) was used to design primers and hydrolysis probes. It was complemented with carbapenemase gene variants from the NCBI database (December 2017, Table S1). The assay was designed to detect eight common carbapenemase groups: $bla_{NDM}$, $bla_{KPC}$, $bla_{VIM}$, $bla_{IMP}$, $bla_{OXA\text{-}23\text{-}like}$, $bla_{OXA\text{-}40/24\text{-}like}$, $bla_{OXA\text{-}58\text{-}like}$ and $bla_{OXA\text{-}48\text{-}like}$.

The alignment of the carbapenemase sequences was done with MUSCLE in MEGA 7.0.26. For alignment the following carbapenemase variants were selected: $bla_{NDM\text{-}1}$ to $bla_{NDM\text{-}21}$, $bla_{KPC\text{-}1}$ to $bla_{KPC\text{-}32}$, $bla_{VIM\text{-}1}$ to $bla_{VIM\text{-}45}$, $bla_{IMP\text{-}1}$ to $bla_{IMP\text{-}69}$ and $bla_{OXA\text{-}1}$ to $bla_{OXA\text{-}566}$. The aim was to cover as many gene variants as possible with one set of primers and probe. To analyze variants clustering together, phylogenetic trees were created with the UPGMA method in MEGA 7.0.26. Either all sequences or a selection of sequences were used to create a consensus sequence. The design of the primers and probes was done with the IDT primer tool (Integrated DNA Technologies, Inc.). To define two multiplex PCRs, possible interactions between all oligonucleotides were analyzed with the multiple primer analyzer (Thermos Fisher Scientific). As a positive control primers and probe for the detection of 16S rDNA were included to estimate the impact of PCR inhibition on negative results (Nadkarni et al., 2002). The first multiplex PCR was designed to detect $bla_{NDM}$, $bla_{KPC}$, $bla_{VIM}$, $bla_{IMP}$ and 16S rDNA, the second multiplex PCR is for detection of $bla_{OXA\text{-}23\text{-}like}$, $bla_{OXA\text{-}40/-24\text{-}like}$, $bla_{OXA\text{-}58\text{-}like}$ and $bla_{OXA\text{-}48\text{-}like}$. Sequences, amplicon size and melting temperature are detailed in the table S2.

### 2.2. Bacterial isolates

To test and validate the assay, two collections of bacterial isolates from the German National Reference Laboratory (NRL) were used. Isolates of the first collection ($n = 151$) were consecutively collected during a half-month period (Jan, 21st to Feb, 6th 2013), consisting of carbapenemase-positive bacteria ($bla_{NDM}$: $n = 5$, $bla_{KPC}$: $n = 8$, $bla_{VIM}$: $n = 23$, $bla_{OXA\text{-}23}$: $n = 12$, $bla_{OXA\text{-}48}$: $n = 9$, $bla_{NDM}$ and $bla_{OXA\text{-}48}$: n = 1), isolates harboring resistance genes, not included in our assay ($bla_{GES}$: n = 5, $bla_{CMY}$: n = 5, $bla_{SHV}$: n = 1, $bla_{DHA}$: n = 1) and beta-lactamase-negative bacteria ($n = 81$). The second set was a random collection with isolates harboring less common carbapenemase variants, especially different $bla_{IMP}$ and $bla_{OXA}$ variants, and bacteria harboring two different carbapenemases simultaneously ($n = 32$). Bacterial species were determined by MALDI TOF (Bruker Daltonics, Germany). The isolates were well characterized by several phenotypic tests, like susceptibility testing, the modified Hodge Test and the combined disk test with EDTA and boronic acid, additionally PCR and sequencing was performed at the NRL (Hofko et al., 2014).

In addition, bacterial isolates and the corresponding rectal swabs with a known carbapenemase were collected from the University Hospital Heidelberg during a procedure of routine patient screening at hospital admission. The isolates were regrown on BD™ Columbia Agar with 5% Sheep Blood (Becton Dickinson GmbH, Heidelberg, Germany) at 37 °C.

### 2.3. DNA extraction

The chelex-based DNA extraction was performed as described by Martin-Platero et al. in 2010 (Martin-Platero et al., 2010). For purifying the DNA ethanol precipitation was carried out, i.e., adding 0.3 M ammonium acetate and 80% (v/v) of ethanol absolute. Samples were inverted and incubated at −80 °C for one hour. After incubation, the samples were centrifuged (20 min, 13,300 rpm, 4 °C). The supernatant was discarded and two washing steps followed by adding 300 µl of 70% ethanol, mixing and centrifugation (20 min, 13,300 rpm, 4 °C). After drying the pellet, it was resuspended with 56 °C nucleic acid-free water. DNA quality was determined with the NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific); quantification was done with the Quant-iT™ PicoGreen™ dsDNA Assay Kit (Thermo Fisher Scientific).

DNA extracted with the chelex-based method was used to analyze the PCR performance, since a known amount of DNA is needed for determining the limit of detection (LOD). The validation of carbapenemase detection in clinical samples was performed on the BD MAX™ system, by using the BD MAX™ ExK™ DNA-2 Kit for DNA extraction and PCR in an automated closed system.

### 2.4. Multiplex PCR in PCR-only mode

Quantitative real-time PCRs were performed on the BD MAX™ system. For detection of all eight carbapenemases two master mixes (MM1 and MM2) with a final volume of 12.5 µl were prepared. Each master mix consisted of 2.5 µl primaQUANT 5× qPCR-Probe-MasterMix (Steinbrenner GmbH, Wiesenbach, Germany), a forward (F) and reverse (R) primer, the hydrolysis probe (P), PCR-grade water and 4 µl DNA. The following final concentrations of primers and probes were used for MM1: IMP-F/R, 16S-F/R: 0.3 µM; KPC-F/R, NDM-F/R, VIM-F/R: 0.4 µM; IMP-P (CY®5: 650 nm–670 nm): 0.2 µM, 16S-P (Cy5.5: 684 nm–708 nm): 0.25 µM, KPC-P (JOE: 529 nm–555 nm), NDM-P (FAM: 495 nm–520 nm), VIM-P (Texas Red®: 597 nm–616 nm): 0.15 µM; and for MM2: primers: 0.4 µM, OXA-23-P (FAM: 495 nm–520 nm), OXA-40/24-P (JOE: 529 nm–555 nm): 0.25 µM; OXA-58-P (Texas Red®: 597 nm–616 nm), OXA-48-P (CY®5: 650 nm–670 nm): 0.15 µM. Then, 10 µl of MM1 and MM2 were transferred to the BD MAX™ PCR cartridge. PCR was run in the PCR-only mode with the following program: 98 °C, 3 min; 98 °C, 5 s; 57 °C, 47.8 s for 3 cycles; 98 °C, 5 s; 61 °C, 43 s for 37 cycles.

### 2.5. Limit of detection

To determine the LOD, one representative isolate harboring a carbapenemase gene of each group was selected (*A. baumannii*: $bla_{NDM\text{-}1/-6}$, *K. oxytoca*: $bla_{KPC\text{-}3}$, *E. cloacae*: $bla_{VIM\text{-}1}$, *P. aeruginosa*: $bla_{IMP\text{-}8/-24}$, *A. baumannii*: $bla_{OXA\text{-}23}$, *A. baumannii*: $bla_{OXA\text{-}72}$, *A. baumannii*: $bla_{OXA\text{-}58}$, *S. marcescens*: $bla_{OXA\text{-}48}$). Genomic copy numbers were calculated based

on the measured DNA concentration and the average genome size of each bacterial species. Copy numbers were used instead of CFU and DNA concentrations, to make interpretation independent on the efficiency of the DNA extraction and the bacterial species. Yet, there can still be variations due to different copy numbers of the carbapenemase-encoding plasmids. As the calculation was done for one copy of the carbapenemase gene, the LOD should be seen as an approximation. The DNA samples were adjusted to $10^5$ copies/μl and 10-fold serial dilutions to one copy/μl were prepared. Based on the slope of the standard curve, PCR efficiency was calculated by using the equation: $E = \left(-1+10^{\frac{-1}{slope}}\right)*100$. The multiplex PCRs were performed on the BD MAX™ system in the PCR-only mode described above.

### 2.6. Combined extraction and PCR on the BD MAX™ system

The sample buffer tube was inoculated with one bacterial colony. The DNA was extracted with the BD MAX™ ExK™ DNA-2 Kit (4 snap configuration). To dilute the elution buffer, 50 μl of PCR-grade water were added to the extraction stripes. For extraction, the BD MAX™ ExK™ DNA-2 protocol (type 3: liquid MM with primers and probes) was used. The PCR was performed with the program described above.

### 2.7. Carbapenemase detection directly from rectal swabs

In total, 66 rectal swabs (ESwab™, Copan) with multidrug-resistant Gram-negative bacteria previously detected by selective culture screening were collected from the University Hospital Heidelberg in the years 2017–2019. Swabs were selected for direct PCR if bacterial growth on a chromID® ESBL plate, inoculated with 10 μl rectal swab medium, could be observed (bioMérieux Deutschland GmbH). ChromID® ESBL plates were used to distinguish between the German 3MRGN and 4MRGN classification. The colony counts on the chromID® ESBL plates were used to estimate the bacterial load (CFU/ml) per swab. One or more representative isolates were selected for analyzing the resistance to meropenem and imipenem using VITEK®2 system (bioMérieux Deutschland GmbH, Nürtingen, Germany). The bacterial species was determined by MALDI TOF (Bruker Daltonics, Germany). To identify the carbapenemase type, isolates were tested by multiplex SYBR green real-time PCR (Hofko et al., 2014), used as a reference method. Direct PCR was performed by adding 100 μl of the rectal swab medium to the sample buffer tube of the ExK DNA-2 Kit. Details on the PCR procedure are given above.

## 3. Results

### 3.1. Primer design

Nine sets of primers and probes were designed to detect eight common carbapenemases and most of their gene variants (Table S2). The aim was to cover at least 95% of the carbapenemases detected by the NRL during the last years in Germany, which presented to be a manageable threshold for our PCR design. The design of primers and probes was based on the phylogeny of the different carbapenemase variants. All currently known *bla*$_{KPC}$ and *bla*$_{NDM}$ variants were detectable. Due to high sequence variations, the *bla*$_{VIM}$-set covered all variants except *bla*$_{VIM-7}$ (Fig. S1). *Bla*$_{IMP}$ and *bla*$_{OXA}$ type carbapenemase genes showed a high degree of sequence diversity, which is why not all variants are detectable (Table 1, Fig. S2).

### 3.2. Assay performance

The assay performance was determined on the BD MAX™ system, using the PCR-only mode. One representative target of each carbapenemase was selected to define the LOD (*bla*$_{NDM-1/-6}$, *bla*$_{KPC-3}$, *bla*$_{VIM-1}$, *bla*$_{IMP-8/-24}$, *bla*$_{OXA-23}$, *bla*$_{OXA-72}$, *bla*$_{OXA-58}$, *bla*$_{OXA-48}$). PCR was done with serial dilutions in four replicates. For *bla*$_{NDM}$, *bla*$_{VIM}$, *bla*$_{OXA-23}$, *bla*$_{OXA-72}$, *bla*$_{OXA-58}$ and *bla*$_{OXA-48}$ the detection of 3.2 copies/PCR was possible. The LOD for *bla*$_{KPC}$ and *bla*$_{IMP}$ was 32 copies/PCR. The PCR efficiency for *bla*$_{NDM}$, *bla*$_{OXA-58}$, *bla*$_{OXA-72}$ and *bla*$_{OXA-48}$ was in the desired range from 90% to 110%. *Bla*$_{KPC}$ (88%) and *bla*$_{OXA-23}$ (89%) were almost in this range. Only *bla*$_{VIM-1}$ and *bla*$_{IMP-8/24}$ showed poorer efficiencies of 80% and 77% respectively (Fig. 1).

**Table 1**
In silico and experimentally detectable carbapenemase variants by multiplex real-time PCR.

| Carbapenemase gene | In silico detectable variants | Experimentally tested variants |
|---|---|---|
| *bla*$_{NDM}$ | *bla*$_{NDM-1}$-*bla*$_{NDM-21}$ | *bla*$_{NDM-1}$, *bla*$_{NDM-5}$, *bla*$_{NDM-6}$ |
| *bla*$_{KPC}$ | *bla*$_{KPC-1}$-*bla*$_{KPC-32}$ | *bla*$_{KPC-2}$, *bla*$_{KPC-3}$ |
| *bla*$_{VIM}$ | *bla*$_{VIM-1}$-*bla*$_{VIM-45}$, except *bla*$_{VIM-7}$ | *bla*$_{VIM-1}$, *bla*$_{VIM-2}$, *bla*$_{VIM-26}$, *bla*$_{VIM-28}$ |
| *bla*$_{IMP}$ | *bla*$_{IMP-1-3}$, *bla*$_{IMP-5-10}$, *bla*$_{IMP-14-16}$, *bla*$_{IMP-18-20}$, *bla*$_{IMP-22-25}$, *bla*$_{IMP-28-30}$, *bla*$_{IMP-32}$, *bla*$_{IMP-40-43}$, *bla*$_{IMP-45}$, *bla*$_{IMP-48}$, *bla*$_{IMP-49}$, *bla*$_{IMP-52}$, *bla*$_{IMP-53}$, *bla*$_{IMP-55}$, *bla*$_{IMP-56}$, *bla*$_{IMP-58}$, *bla*$_{IMP-60}$, *bla*$_{IMP-62}$, *bla*$_{IMP-66}$,*bla*$_{IMP-69-73}$, *bla*$_{IMP-75-80}$ | *bla*$_{IMP-1}$, *bla*$_{IMP-2}$, *bla*$_{IMP-7}$, *bla*$_{IMP-8}$, *bla*$_{IMP-13}$, *bla*$_{IMP-14}$, *bla*$_{IMP-15}$, *bla*$_{IMP-16}$, *bla*$_{IMP-19}$, *bla*$_{IMP-24}$, *bla*$_{IMP-28}$ |
| *bla*$_{OXA-23-like}$ | *bla*$_{OXA-23}$, *bla*$_{OXA-27}$, *bla*$_{OXA-49}$, *bla*$_{OXA-73}$, *bla*$_{OXA-102}$, *bla*$_{OXA-103}$, *bla*$_{OXA-105}$, *bla*$_{OXA-133}$, *bla*$_{OXA-134}$, *bla*$_{OXA-146}$, *bla*$_{OXA-165-171}$, *bla*$_{OXA-225}$, *bla*$_{OXA-239}$, *bla*$_{OXA-366}$, *bla*$_{OXA-398}$, *bla*$_{OXA-422}$, *bla*$_{OXA-423}$, *bla*$_{OXA-435}$, *bla*$_{OXA-440}$, *bla*$_{OXA-469}$, *bla*$_{OXA-481-483}$, *bla*$_{OXA-565}$, *bla*$_{OXA-657}$, *bla*$_{OXA-806-818}$ | *bla*$_{OXA-23}$ |
| *bla*$_{OXA-40/24-like}$ | *bla*$_{OXA-40/24}$, *bla*$_{OXA-25}$, *bla*$_{OXA-26}$, *bla*$_{OXA-72}$, *bla*$_{OXA-139}$, *bla*$_{OXA-160}$, *bla*$_{OXA-207}$, *bla*$_{OXA-437}$, *bla*$_{OXA-653}$ | *bla*$_{OXA-72}$ |
| *bla*$_{OXA-58-like}$ | *bla*$_{OXA-58}$, *bla*$_{OXA-96}$, *bla*$_{OXA-97}$, *bla*$_{OXA-164}$, *bla*$_{OXA-397}$, *bla*$_{OXA-420}$, *bla*$_{OXA-467}$, *bla*$_{OXA-512}$ | *bla*$_{OXA-58}$ |
| *bla*$_{OXA-48-like}$ | *bla*$_{OXA-48}$, *bla*$_{OXA-162}$, *bla*$_{OXA-163}$, *bla*$_{OXA-181}$, *bla*$_{OXA-199}$, *bla*$_{OXA-204}$, *bla*$_{OXA-232}$, *bla*$_{OXA-244}$, *bla*$_{OXA-245}$, *bla*$_{OXA-247}$, *bla*$_{OXA-370}$, *bla*$_{OXA-405}$, *bla*$_{OXA-416}$, *bla*$_{OXA-438}$, *bla*$_{OXA-439}$, *bla*$_{OXA-484}$, *bla*$_{OXA-505}$, *bla*$_{OXA-514}$, *bla*$_{OXA-517}$, *bla*$_{OXA-519}$, *bla*$_{OXA-538}$, *bla*$_{OXA-547}$, *bla*$_{OXA-566}$, *bla*$_{OXA-567}$, *bla*$_{OXA-788}$, *bla*$_{OXA-793}$ | *bla*$_{OXA-48}$, *bla*$_{OXA-162}$, *bla*$_{OXA-181}$, *bla*$_{OXA-204}$, *bla*$_{OXA-232}$, *bla*$_{OXA-244}$ |

### 3.3. Validation of the automated assay

The assay was validated as a fully automated PCR with the first collection of isolates from the NRL ($n = 151$ isolates). The collection contained 58 carbapenemase positive strains, 12 isolates with ESBL or AmpC resistance genes and 81 beta-lactamase-negative isolates. All 58 carbapenemases were identified correctly. The oligonucleotides were specific for the particular carbapenemase and their tested variants, as neither amplification of ESBLs (*bla*$_{GES}$, *bla*$_{SHV}$) and AmpC beta-lactamases (*bla*$_{DHA}$, *bla*$_{CMY}$) nor of beta-lactamase-negative bacteria could be observed, leading to a sensitivity and specificity of 100% (Table 2).

For further validation the second set of collected isolates from the

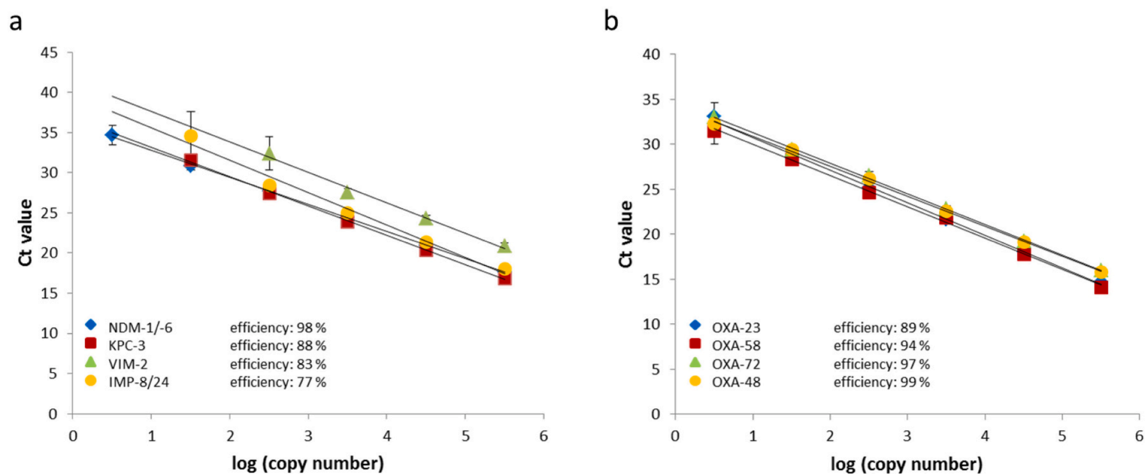


**Fig. 1.** Determination of the limit of detection and PCR efficiencies. a: standard curve for MM1; the detection limit for $bla_{NDM}$ and $bla_{VIM}$ is 3.2 copies, for $bla_{KPC}$ and $bla_{IMP}$ 32 copies. PCR efficiency in MM1 is 98% for $bla_{NDM}$, 88% for $bla_{KPC}$, 80% for $bla_{VIM}$ and 77% for $bla_{IMP}$. b: standard curve for MM2; the detection limit for the different $bla_{OXA}$ variants is 3.2 copies. PCR efficiencies are ranging from 89% to 99% in MM2.

**Table 2**
Consecutive collection of carbapenemase-positive and -negative isolates, used for the validation of the assay. $Bla_{GES}$, $bla_{CMY}$, $bla_{DHA}$ and $bla_{SHV}$ were considered as "negative".

| Beta-lactamase NRL result | Bacterial species | Number of isolates | BD MAX™ result | Concordant | Discordant |
|---|---|---|---|---|---|
| $bla_{VIM-1}$ | *K.oxytoca E.cloacae K.pneumoniae E.coli* | 13 | $bla_{VIM}$ | 13 | 0 |
| $bla_{VIM-2}$ | *P.aeruginosa* | 10 | $bla_{VIM}$ | 10 | 0 |
| $bla_{KPC-2}$ | *K.pneumoniae* | 4 | $bla_{KPC}$ | 4 | 0 |
| $bla_{KPC-3}$ | *K.pneumoniae* | 4 | $bla_{KPC}$ | 4 | 0 |
| $bla_{NDM-1/-6}$ | *E.coli, K. pneumoniae, A.baumannii* | 5 | $bla_{NDM}$ | 5 | 0 |
| $bla_{NDM-1/-6}$, $bla_{OXA-23}$ | *A.baumannii* | 1 | $bla_{NDM}$, $bla_{OXA-23-like}$ | 1 | 0 |
| $bla_{OXA-23}$ | *A.baumannii* | 12 | $bla_{OXA-23-like}$ | 12 | 0 |
| $bla_{OXA-48}$ | *K.pneumoniae E.coli* | 9 | $bla_{OXA-48-like}$ | 9 | 0 |
| $bla_{GES-1}$ | *P.aeruginosa* | 5 | negative | 5 | 0 |
| $bla_{CMY-2}$ | *E.coli P.mirabilis* | 3 | negative | 3 | 0 |
| $bla_{CMY-2/22}$ | *P.mirabilis* | 2 | negative | 2 | 0 |
| $bla_{SHV-12}$ | *E.cloacae* | 1 | negative | 1 | 0 |
| $bla_{DHA}$ | *E.coli* | 1 | negative | 1 | 0 |
| Negative | *P.aeruginosa, E.cloacae, K. pneumoniae, E.aerogenes, E.coli, A. baumannii, P. mirabilis, M. morganii, A. pittii, A.junii, X.maltophila, C.freundii* | 81 | negative | 80 | 1[a] |

[a] The detection of 16S positive control failed due to inhibition. There was no detectable signal for a carbapenemase gene.

NRL was tested, containing less common carbapenemase variants and combinations. Different $bla_{OXA}$ ($bla_{OXA-58}$, $bla_{OXA-72}$, $bla_{OXA-204}$, $bla_{OXA-162}$, $bla_{OXA-181}$, $bla_{OXA-232}$, $bla_{OXA-244}$) and $bla_{IMP}$ ($bla_{IMP-1}$, $bla_{IMP-13}$, $bla_{IMP-16}$, $bla_{IMP-7}$, $bla_{IMP-14}$, $bla_{IMP-15}$, $bla_{IMP-4/28}$, $bla_{IMP-2/19}$) types were identified correctly as well as $bla_{NDM-5}$ and $bla_{VIM-28}$ positive bacteria. Additionally, isolates that harbor two different carbapenemases in combination could be detected by the multiplex PCR ($bla_{KPC-2}$ + $bla_{VIM-26}$, $bla_{NDM-1/6}$ + $bla_{OXA-48}$, $bla_{OXA-23}$ + $bla_{OXA-58}$, $bla_{NDM-5}$ + $bla_{OXA-181}$, Table S3).

### 3.4. Carbapenemase detection directly from rectal swabs

Apart from using the automated PCR for bacterial isolates, direct detection from rectal swabs, e.g. during screening procedures, might be envisioned. For this purpose, 66 rectal swabs from Heidelberg hospital patients were examined. Using the multiplex SYBR green real-time PCR (Hofko et al., 2014) the carbapenemase type of each isolate, analyzed during routine procedure, was determined. Concordant results were achieved in 77.3% (51/66 swabs), discordant results in 12.1% (8/66 swabs) (Table 3). Using the direct PCR, six samples were false negative (3× $bla_{VIM-1}$, 2× $bla_{KPC}$, 1× $bla_{IMP-1}$), and for one sample ($bla_{NDM}$) the direct PCR failed due to inhibition, since no signal for 16S was detectable. In one case different carbapenemase types were detected (Table S4). The colony counts on the chromID® ESBL plates of these six false-negative samples were all in the range of 1–5 colonies, which resembles a bacterial load of 100 CFU/ml-500 CFU/ml in the rectal swab. In 7 cases, additional carbapenemase genes were detected in the direct PCR samples that were not identified during routine diagnostics (10.6%, Table 3, Table S4).

## 4. Discussion

The aim of this study was to design a PCR-based assay for detecting eight common carbapenemases and their variants. This resulted in the design of two multiplex PCRs that could be run in a fully-automated manner to facilitate detection of carbapenemases from suspicious bacterial isolates or directly from rectal swabs.

Multiple PCRs for the detection of carbapenemases have been published and are commercialized. However, a drawback of existing commercial assays is the fixed composition of included targets, whereas a drawback of in-house assays is their greater demand for molecular expertise. So far, few carbapenemase assays have been reported for the BD MAX™ (Hofko et al., 2014; Antonelli et al., 2016; Teo et al., 2016; Teo et al., 2018). With the recent improvement allowing the use of two master mixes for one extraction the number of possible targets increased. The available assays cover almost all known variants of the "big 4" carbapenemases, but especially for $bla_{IMP}$ and $bla_{OXA}$ some variants occurring in Germany in recent years (Koch-Institut, 2015; Koch-

**Table 3**
Carbapenemase detection directly from rectal swabs compared to differentiated isolates. In 77.3% (51/66 swabs) the carbapenemase genes were found in rectal swabs and in the differentiated isolate. Discordant results were obtained in 12.1% (8/66 swabs), since the carbapenemase genes could not be identified in the direct PCR-sample but were detected in isolates from selective agar or a different carbapenemase gene was detected. By direct PCR additional carbapenemase genes were detected from rectal swabs (7/66, 10.6%), which were not found in routine diagnostics.

| Carbapenemases detected in cultured isolate from rectal swab by SYBR green PCR | Number of rectal swabs | Results of direct PCR from rectal swabs | | | |
|---|---|---|---|---|---|
| | | Concordant | Detection of new carbapenemase by direct PCR | Detection of different carbapenemase by direct PCR | Discordant |
| $bla_{OXA\text{-}48\text{-}like}$ | 30 | 29 | 1 | 0 | 0 |
| $bla_{OXA\text{-}23\text{-}like}$ | 4 | 3 | 0 | 1 | 0 |
| $bla_{KPC}$ | 9 | 5 | 2 | 0 | 2 |
| $bla_{NDM}$ | 11 | 7 | 3 | 0 | 1 |
| $bla_{NDM}$, $bla_{OXA\text{-}48\text{-}like}$ | 2 | 1 | 1 | 0 | 0 |
| $bla_{VIM\text{-}1}$ | 6 | 3 | 0 | 0 | 3 |
| $bla_{VIM\text{-}2}$ | 3 | 3 | 0 | 0 | 0 |
| $bla_{IMP\text{-}1}$ | 1 | 0 | 0 | 0 | 1 |

Institut, 2016; Pfennigwerth, 2017; Pfennigwerth, 2018) cannot be detected. With the multiplex PCR developed by Teo et al. in 2016 and 2018 (Teo et al., 2016, Teo et al., 2018) $bla_{IMP\text{-}7}$, $bla_{IMP\text{-}13}$, $bla_{IMP\text{-}14}$, $bla_{IMP\text{-}15}$ and $bla_{IMP\text{-}28}$ cannot be identified (Teo et al., 2016, Teo et al., 2018). The BD MAX Check-Points CPO assay is failing to detect $bla_{IMP\text{-}14}$, $bla$IMP-15, $bla_{IMP\text{-}16}$, $bla_{IMP\text{-}18}$, $bla_{IMP\text{-}22}$, $bla_{IMP\text{-}23}$ and $bla_{IMP\text{-}28}$ (Antonelli et al., 2016). The Cepheid Xpert Carba-R assay is designed for detecting $bla_{NDM}$, $bla_{KPC}$, $bla_{VIM}$, $bla_{IMP\text{-}1}$ and $bla_{OXA\text{-}48\text{-}like}$, but $bla_{OXA\text{-}23\text{-}like}$, $bla_{OXA\text{-}58\text{-}like}$ and $bla_{OXA\text{-}40/24\text{-}like}$ are not included in the panel. Additionally, $bla_{IMP\text{-}4}$, $bla_{IMP\text{-}6}$, $bla_{IMP\text{-}10}$ and $bla_{IMP\text{-}11}$ are detectable with this assay but $bla_{IMP\text{-}7}$, $bla_{IMP\text{-}13}$ and $bla_{IMP\text{-}14}$ cannot be identified (Traczewski et al., 2018). These $bla_{IMP}$-variants as well as $bla_{OXA\text{-}40/24\text{-}like}$ and $bla_{OXA\text{-}58\text{-}like}$ previously not included but frequently found in *A. baumannii* (Hofko et al., 2014; Antonelli et al., 2016; Teo et al., 2016; Teo et al., 2018) can be detected with our new designed assay (Table 1). However the Cepheid Xpert Carba-R assay, the BD MAX Check-Points CPO assay and the assay designed by Teo et al. in 2016 (Teo et al., 2016) are able to detect $bla_{IMP\text{-}4}$, which is not included in our assay (Antonelli et al., 2016; Traczewski et al., 2018).

As the multiplex PCR was designed for the BD MAX™ system, which is beneficial for large laboratory use, other tests for the identification of carbapenemase-positive bacteria might be more suitable for locations with less carbapenemase prevalence and smaller diagnostic microbiological laboratories, like the carbapenem inactivation method (van der Zwaluw et al., 2015), CARBA-5 (Hopkins et al., 2018) or RESIST-4 (Cointe et al., 2018).

Based on the data of the German NRL in the years 2014–2017 (Koch-Institut, 2015; Koch-Institut, 2016; Pfennigwerth, 2017; Pfennigwerth, 2018), 98.2% of the carbapenemases in *Enterobacteriaceae*, 94.0% in *P. aeruginosa* and 96.1% in *A. baumannii* are theoretically detectable with this assay, which leads to an overall theoretical sensitivity of 97.1%. Compared to the detection of carbapenemases in *Enterobacteriaceae* and *A. baumannii* the theoretical coverage of carbapenemases in *P. aeruginosa* was slightly lower (94.0%). Especially $bla_{IMI}$ and $bla_{GIM}$ carbapenemases occurred more frequently in *P. aeruginosa* during the last years (Koch-Institut, 2015, Koch-Institut, 2016, Pfennigwerth, 2017, Pfennigwerth, 2018), which are not included in our assay.

The assay was validated with a consecutive collection of 151 well characterized isolates from the NRL leading to a sensitivity and specificity of 100% by testing from bacterial colony, which is comparable to other assays (Hofko et al., 2014; Teo et al., 2016; Lund et al., 2018; Teo et al., 2018; Mentasti et al., 2019).

Our assay showed a good LOD in the PCR-only mode, since 1 copy/μl for $bla_{NDM}$, $bla_{VIM}$ and $bla_{OXA}$ and 10 copies/μl for $bla_{KPC}$ and $bla_{IMP}$ were detectable, which equals 3.2 copies and 32 copies per PCR, respectively (Fig. 1). The detection limit of other assays was 73 CFU/ml to 900,000 CFU/ml (Antonelli et al., 2016), 100 CFU/ml (Teo et al., 2016; Teo et al., 2018) and 100 CFU/ml to 1,000 CFU/ml, respectively (Lund et al., 2018). It is difficult to compare our LOD with those of other assays, as we used copy numbers of bacterial genomes instead of CFU for quantification. However, we can estimate that 1 copy/μl equals approximately 1 CFU/μl (1000 CFU/ml). Thus, dependent on the target our LOD seems to be in line with the assays described by Antonelli et al. in 2016 with a LOD ranging from 73 CFU/ml to 90,000 CFU/ml (Antonelli et al., 2016), and Lund et al. in 2018 with a LOD between 100 CFU/ml and 1,000 CFU/ml (Lund et al., 2018), but might be less sensitive than the assays reported by Teo et al., 2016 (Teo et al., 2016) and Teo et al., 2018 (Teo et al., 2018) with LODs of 100 CFU/ml.

The results encouraged us to use the assay for direct testing from rectal swabs. Such a procedure reduces the time to results, since an overnight culture is not necessary. Carbapenemase genes were found in 59/66 rectal swabs (89.4%) by direct-PCR as compared to detection by culture screen on chromID® ESBL plates (Table 3, Table S4). Other assays designed for testing directly from rectal swabs showed higher sensitivities ranging from 93.1% to 100% (Antonelli et al., 2016) and 97.5%, respectively (Saliba et al., 2019). When testing directly from rectal swabs, six samples gave false-negative results, in one case the PCR failed due to inhibition. Other published assays also reported that some carbapenem resistant isolates can be overseen (limited sensitivity in direct testing) (Antonelli et al., 2016; Lund et al., 2018; Saliba et al., 2019). One possible reason could be the quantity of bacteria in the primary sample. This can be tested by counting the colonies on chromID® ESBL plates. Typically, only one to five colonies grew on one plate (resembling 100–500 CFU/ml), indicating that the amount of bacteria represents a detection limit for direct PCR application. However, in one case a $bla_{KPC}$ isolate with four colonies could be detected with a Ct value of 26.4. Besides the number of bacteria, the number of plasmids, which usually encode the resistance gene, could play a role in the detection limit. $Bla_{KPC}$ was described on low-copy number plasmids like IncF plasmids as well as on high-copy number Col-like plasmids (Chavda et al., 2015; Stoesser et al., 2017). In this case, perhaps the number of $bla_{KPC}$ carrying plasmids could compensate the low bacterial load in the rectal swab. Compared to other assays with efficiencies between 95% and 105.2% (Teo et al., 2016; Lund et al., 2018), the amplification of $bla_{VIM}$ and $bla_{IMP}$ showed poorer efficiencies (80%, 77%) in our assay, which could be another reason for the false-negative results (3× $bla_{VIM\text{-}1}$, 1× $bla_{IMP\text{-}1}$) obtained from rectal swabs, using the direct PCR. The efficiencies for $bla_{NDM}$, $bla_{KPC}$ and $bla_{OXA}$, however, are in line with available assays.

However, our assay identified different or new carbapenemase genes that were not detected by the routine procedure (8/66 swabs). These carbapenemase-positive bacteria perhaps were not viable/cultivable anymore due to antibiotic treatment or unculturability of the carrier, since mixed populations can be present in the rectal swab. Another possibility is that the resistance gene is present but the expression level is low or the gene is not expressed at all, which leads to phenotypical sensitivity. For culture-negative rectal swabs, isolates might have grown on selective agar with a further enrichment culture. Identification of

additional carbapenemases is in line with reported results from other validation of molecular assays such as Check-Direct CPE real-time PCR (Antonelli et al., 2016) and GeneXpert® (Saliba et al., 2019). The data indicate that neither molecular nor culture screening detect all present carbapenem resistant bacteria.

The following limitations could be observed: To determine the relative LOD genomic copy numbers of the bacteria were used. Thus, depending on the copy numbers of the carbapenemase encoding plasmid the LOD may vary between different isolates (Chavda et al., 2015; Stoesser et al., 2017; Shen et al., 2020). Whether the carbapenemase detection from direct PCR-samples is more sensitive than culture-based methods cannot be determined from our test setting, since only carbapenemase-positive rectal swabs were used for analyzing the assay performance, and is thus subject to further investigation. For identification of carbapenemase-positive bacteria chromID® ESBL plates were used as a selective media. The detection limit of this agar is dependent on the bacterial species, the present carbapenemase and also the minimum inhibitory concentration to the antibiotics (Nordmann et al., 2012; Vrioni et al., 2012; Göttig et al., 2020). Especially OXA-48-producing bacteria that do not co-express an ESBL are inhibited in growth (Göttig et al., 2020), which might also be an explanation for detecting more carbapenemases by direct PCR. Another drawback of our assay is the limited number of isolates and clinical samples with different carbapenemase variants that could be experimentally tested. Particularly $bla_{\text{VIM-4}}$ and $bla_{\text{VIM-11}}$ were detected more frequently by the NRL during the last years (Koch-Institut, 2015; Koch-Institut, 2016; Pfennigwerth, 2017; Pfennigwerth, 2018; Pfennigwerth, 2019; Pfennigwerth, 2020). Other $bla_{\text{VIM}}$, $bla_{\text{NDM}}$, $bla_{\text{KPC}}$, $bla_{\text{IMP}}$ and $bla_{\text{OXA}}$ variants, not present in our collection, occurred in less than 1% of the analyzed carbapenemase positive bacteria by the NRL from 2014 to 2019 (Koch-Institut, 2015, Koch-Institut, 2016, Pfennigwerth, 2017, Pfennigwerth, 2018, Pfennigwerth, 2019, Pfennigwerth, 2020). Nonetheless if new carbapenemase variants appear during routine diagnostics, they can be validated by PCR. Additionally, rare carbapenemases like $bla_{\text{GIM}}$ or $bla_{\text{GES}}$ cannot be detected, which could lead to false negative results. This limitation could be resolved, however, by adding more probes to the assay. Furthermore, our assay does not identify the bacterial species, which would be helpful for antibiotic treatment.

## 5. Conclusion

The developed assay for the BD MAX™ system provides a time effective method to detect carbapenemases that frequently occur in Germany from bacterial colonies in less than 3 h. The automatization of the assay allows an easy performance of DNA extraction and real-time PCR without requiring in-depth experience. The assay is rapid and requires little hands-on time. Due to the usage of the BD MAX™ in an open mode, the user-developed protocol could be adapted in case new variants appear or epidemiology changes. With two multiplex PCRs eight important carbapenemases and most of their known variants occurring in *Enterobacteriaceae* and the non-fermenters *P. aeruginosa* and *A. baumannii* could successfully be detected. The assay showed high sensitivity and specificity, and might also be adapted to initial screening of patients possibly colonized with carbapenemase harboring bacteria. The shorter time to results can avoid isolation of patients and will help to prevent spread of antimicrobial resistances and possible outbreaks by allowing timely initiation of infection control measures.

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## Authors' contributions

Conception and design of the study: A.H.D., K.H.; acquisition, analysis and interpretation of data: K.P., S.B., A.H.D.; drafting the article or revising it critically for important intellectual content: K.P., S.B., M. B., K.H., A.H.D.

## Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mimet.2021.106224.

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## Glossary

*CFU:* colony forming unit
*Ct:* cycle threshold
*Cy5:* cyanine5
*Cy5.5:* cyanine5.5
*ESBL:* extended-spectrum β-lactamase
*FAM:* 6-carboxyfluorescein
*GES:* Guiana extended-spectrum β-lactamase
*GIM:* German imipenemase
*IMP:* imipenem-hydrolyzing β-lactamase
*JOE:* 6-Carboxy-4′,5'-Dichloro-2′,7'-Dimethoxyfluorescein, Succinimidyl Ester
*KPC:* *Klebsiella pneumoniae* carbapenemase
*LOD:* limit of detection
*MALDI-TOF:* Matrix-assisted laser desorption/ionization-time of flight
*MUSCLE:* Multiple Sequence Comparison by Log-Expectation
*NCBI:* National Center for Biotechnology Information
*NDM:* New Delhi metallo-β-lactamase
*NRL:* National Reference Laboratory
*OXA-23:* oxacillinase-23
*OXA-40/24:* oxacillinase-40/24
*OXA-48:* oxacillinase-48
*OXA-58:* oxacillinase-58
*PCR:* polymerase chain reaction
*UPGMA:* Unweighted Pair Group Method with Arithmetic mean
*VIM:* Verona integron-encoded metallo-β-lactamase