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Development and evaluation of a multiplex real-time PCR for the detection of IMP, VIM, and OXA-23 carbapenemase gene families on the BD MAX open system

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ABSTRACT

A multiplex real-time polymerase chain reaction (PCR) assay was developed for the detection of clinically prevalent IMP, VIM, and OXA-23 gene families. The assay was designed to work on the BD MAX open platform which is a fully automated system for all PCR processes including sample extraction to PCR resulting. A total of 107 well-characterized carbapenem resistant *Enterobacteriaceae* were evaluated and the results were 100% concordant with the reference test isolates. This assay will serve to complement PCR screens that detect the major carbapenemase families of NDM, KPC, and OXA-48-like.

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Resistance to carbapenem antimicrobials represents one of the most pressing threats to public health. Of utmost concern is carbapenemase-producing *Enterobacteriaceae* (CPE) which is typically multidrug resistant making CPE infections challenging to treat due to limited therapeutic options and consequently associated with higher mortality rates (Lutgring and Limbago, 2016).

Nosocomial outbreaks involving bacteria carrying each of the 5 most prevalent carbapenemase families (KPC, OXA-48-like, NDM, VIM, and IMP) have been described globally (Nordmann and Poirel, 2014), with the distribution of carbapenemase types differing geographically. Local molecular surveillance of carbapenemase genotypes over the last 4 years, indicate that NDM, KPC, and OXA-48-like predominate although IMP-like enzymes are also not uncommon (Koh et al., 2013; Teo et al., 2013). In 2014, *Escherichia coli* bearing OXA-23 was first detected (La et al., 2014) and has since become established in our healthcare setting (data provided by National Public Health Laboratory [NPHL], Singapore).

The key to limiting the spread of CPE is timely infection prevention and control measures facilitated by rapid diagnostic methods for CPE (Lutgring and Limbago, 2016; Nordmann and Poirel, 2014). The ability to perform molecular carbapenemase detection directly from rectal swabs presents a promising alternative to culture, offering the potential for same-day turnaround time and, consequently, faster implementation of infection control measures. Commercial assays marketed for direct CPE detection includes the Xpert Carba-R Assay, performed on the GeneXpert Systems (Cepheid; Sunnyvale, CA) which is designed for carbapenemase gene families KPC, NDM, VIM, IMP-1, and OXA-48-like. The BD MAX CRE Assay for use on the BD MAX System (BD Diagnostics; Sparks, MD) detects KPC, NDM and OXA-48-like. A third-party reagent mix, Check-Direct CPE (Check-Points BV; Wageningen, The Netherlands) for use on the BD MAX platform enables detection of KPC, NDM, VIM, and OXA-48-like. A noncommercial assay based on melting-curve analysis for the identification of carbapenemases has been demonstrated on the BD MAX platform (Hofko et al., 2014). Both the GeneXpert and the BD MAX System are fully integrated and automated platforms for multiplex real-time polymerase chain reaction (PCR) testing. Currently, only the Xpert Carba-R assay is approved as an in vitro diagnostics test.

The BD MAX system is unique in having open system assay flexibility, allowing in-house developed assays to be run on the system. Taking advantage of the BD MAX open system, we developed a multiplex CPE PCR assay detecting IMP-like, VIM-like, and OXA-23-like. This assay can complement the BD MAX CRE Assay and will offer coverage of the most prevalent carbapenemase types.

One hundred and seven isolates CPE were used for evaluation, of which 8 were noncarbapenemase producing (Table 2). These isolates were obtained from the NPHL reference laboratory, where submission of carbapenem-resistant microorganisms from public hospitals was mandatory for surveillance purposes. Characterization of β-lactamase genes was performed by PCR assays targeting serine carbapenemases (bla_{KPC}, bla_{GES}, $bla_{\text{IMI-1}}$ and $bla_{\text{NMC-A}}$), metallo-β-lactamases (bla_{NDM}, bla_{VIM} and bla_{IMP}), and OXA-type ($bla_{\text{OXA-48-like}}$ and $bla_{\text{OXA-23-like}}$) carbapenemases (Balm et al., 2013; Teo et al., 2012). Full gene

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sequencing was routinely performed for all OXA-48-like, IMP-like, VIM-like, and OXA-23-like amplicons. Carbapenem nonsusceptibility due to porin loss associated with extended-spectrum β-lactamases and AmpC overproduction was not investigated. The primer and probes were designed using reference carbapenemase sequences obtained from the National Center for Biotechnology Information sequence database (Table 1). Based on phylogeny, IMP variants can be divided into at least 7 clusters (Pournaras et al., 2013). Local molecular epidemiology of IMP enzymes indicates that majority of IMP isolated were IMP-1 followed by IMP-4 and IMP-8 (Table 1). The IMP-1-like primers and probe were designed to detect members of the IMP-1 cluster, whereas IMP-8-like primers and probe detect variants of the IMP-2 cluster (Table 1). The variants picked up by VIM-like and OXA-23-like primers and probe are also described in Table 1. Prior to real-time PCR optimization on the BD MAX platform, the primer pairs were first tested singly and then as a multiplex PCR against selected test isolates from Table 2, using conventional agarose gel-based PCR to ensure specificity.

For the open-system assay, the extraction kit BD MAX™ EXK™ DNA-2 KIT and PCR master mix BD MAX™ DNA MMK sample processing control (SPC) was used. BD MAX™ DNA MMK is a lyophilized PCR reagent mix containing an SPC. The multiplex PCR reaction mix using in-house primers and probes (Table 1) was prepared as recommended in the BD MAX™ DNA MMK (SPC) package insert. Briefly, a sample extraction and PCR run was setup as follows: a total of 200 μl of a 0.5 McFarland standard bacterial suspension $\approx 10^8$ CFU/mL, was pipetted into the sample buffer tube (SBT) of the BD MAX ExK DNA-2 kit. The SBT was replaced with a septum cap, vortexed briefly, and placed into the system rack. For each specimen, a unitized reagent strip was slotted into the system rack and assembled by snapping into assigned tube positions: a PCR Master Mix Tube, a DNA extraction tube, and an empty conical PCR tube. Into the conical PCR tube, a 2-fold concentrated primer-probe mixture containing 0.3 μM of each primer and 0.1 μM of each probe (Table 1) totalling a volume of 12.5 μL, was pipetted into the conical PCR tube. After extraction, the BD MAX system combines the nucleic acid eluate with primer-probe mixture and 12 μL of the mixture is then loaded into the PCR cartridge for amplification. The following PCR cycling parameters was used: 98 °C for 10 min, followed by 42 cycles of 98 °C for 20 seconds and 58 °C for 50 seconds. The PCR gain and threshold for fluorescence detection were set at 50 and 100, respectively. During multiplex PCR evaluation, all IMP-like, VIM-like, and OXA-23–positive isolates yielded cycle threshold (Ct) values ranging from 10 to 20.8. The cutoff Ct value for classification of IMP-like, VIM-like, and OXA-23-like positive isolates was <35. Similarly, for the SPC to be valid, the Ct cutoff was <35. The PCR result logic was set such that a result would be called positive only when Ct values for the individual FAM, Tex 615, Cy5 channels, together with the SPC were both <35.

Assay linearity and limit of detection were determined by performing serial 10-fold dilutions of a bacterial cultures of *Klebsiella pneumoniae* National Collection of Type Cultures (NCTC) 13,439 VIM-1, *E. coli* OXA-23, *Enterobacter cloacae* IMP-1, and *E. coli* IMP-8 from 10 to 10^7 CFU/mL. Ct values obtained from each bacterial dilution were graphed on the y axis versus the log of the dilution on the x axis. The amplification efficiency (E) was determined from the slope of the standard curve according to this equation: $E = 10^{-1/\text{slope}}$. The detection limit was 10^2 CFU/ml for the 4 carbapenemase genes. A high degree of correlation was observed between the bacterial load and the Ct values, *K. pneumoniae* NCTC 13439 VIM-1 ($R^2 = 0.998$), *E. coli* OXA-23 ($R^2 = 0.996$), *E. cloacae* IMP-1 ($R^2 = 0.996$), and *E. coli* IMP-8 ($R^2 = 0.991$) with amplification efficiencies of 100%, 96.3%, 105.2%, and 97.1%, respectively.

A total of 107 isolates were validated (Table 2) for the IMP-like, VIM-like, and OXA-23-like multiplex assay on the BD MAX open system. There were 63 positives ($n = 38$ IMP-like, $n = 2$ VIM-like, $n = 2$ VIM-4 and IMP-4, and $n = 21$ OXA-23) and 44 negatives. It was of interest to note that the PCR assay easily detected isolates cocarrying VIM-4 and IMP-4. In summary, the multiplex PCR results were 100% concordant with the reference PCR screening and sequencing identifications. During the evaluation, 11 isolates were initially unresolved due to failure in the amplification of the SPC. Upon repeat testing, all 11 isolates gave a proper result. We have not obtained an explanation from the manufacturer as to why there was a technical failure of the SPC although we do note that SPC failure appears randomly, without isolate bias.

An aspect of the study that was not included was assay evaluation using either spiked CPE fecal samples or direct screenings from clinical rectal swabs. In our current laboratory setting, conventional gel-based PCRs are used for the detection of carbapenemase-producers from cultures originating from carbapenem-resistance selective media. The

Table 1
Primer and probes used in BD MAX open system for real-time multiplex PCR detection of IMP-like, VIM-like, and OXA-23-like.

bla target	Primer/Probe	Sequence (5′–3′)	Amplicon (bp)	Variants detected	Reference GenBank sequence
IMP-1-like	IMP-1 F	TTTTGTTTTGCAGCATTGCTAC	113	IMP-1, IMP-4, IMP-3*, IMP-6*, IMP-10*, IMP-25*, IMP-30*, IMP-34*, IMP-40*, IMP-42*, IMP-52*, IMP-55*	IMP-1; KC675185
	IMP-1 R	GCCCCACCCGTTAACTTCTT			
	IMP-1 probe	6-FAM-CTTTGCCAGATTTAAAAATTG-Black hole quencher 1			
IMP-8-like	IMP-8 F	TCTCAATCTATTCCCACGTATGC	104	IMP-2*, IMP-8, IMP-16* IMP-19*, IMP-20*, IMP-22*, IMP-24*	IMP-8; HE605039
	IMP-8 R	AGCCAATAACTAACTCCGCTAAA			
	IMP-8 probe	6-FAM-AAAGACGGTAAGGTGCAAGCTAAA-Black hole quencher 1			
VIM-1-like	VIM-1 F	CGCACTTTCATGACGACCGCGT	118	VIM-1, VIM-2*, VIM-4, VIM-5*, VIM-8*,VIM-9*, VIM-10*, VIM-12*,VIM-14*, VIM-15*, VIM-16*, VIM-17*, VIM-19*, VIM-20*,VIM-23*, VIM-24*, VIM-25*,VIM-26*, VIM-27*, VIM-28*, VIM-29*, VIM-30*, VIM-31*, VIM-32*, VIM-33*, VIM-34*, VIM-35* VIM-36*, VIM-37*,VIM-38* VIM-39*, VIM-40*, VIM-41* VIM-42*VIM-43*,VIM-44* VIM-45*,VIM-46*,VIM-49*	VIM-1; DQ489717
	VIM-1 R	GTGGGAATCTCGTTCCCCTC			
	VIM-probe	TEX 615-GTGGCAACGTACGCATCACCGT-Black hole quencher 2			
OXA-23-like	OXA-23 F	CCTCAGGTGTGCTGGTTATT	96	OXA-23, OXA-49*, OXA-73*, OXA-133*, OXA-134*, OXA-146*, OXA-165*, OXA-167*, OXA-168*, OXA-169*, OXA-170*, OXA-171*, OXA-225*, OXA-239*, OXA-469*	OXA-23; JF731028
	OXA-23 R	ATGTAGAGGCTGGCACATATTC			
	OXA-23 probe	Cy5-TATGGTAATGCTCTAAGCCGCGCA-Black hole quencher 2			

*In silico predicted primer binding was evaluated using nucleotide Basic Local Alignment Search Tool (BLAST) at http://blast.ncbi.nlm.nih.gov/Blast.cgi and Resistance Determinants DataBase (RED-DB) at http://www.fibim.unisi.it/REDDB/

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