Original Article

Rapid detection of IMP, NDM, VIM, KPC and OXA-48-like carbapenemases from Enterobacteriales and Gram negative non-fermenter bacteria by real time PCR and melt curve analysis

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Abstract

Carbapenemase producing microorganisms are increasingly isolated and often associated with treatment failures and outbreaks. The need for reliable and timely detection and/or confirmation of carbapenemase production is paramount, therefore a real-time PCR assay targeting IMP, NDM, VIM, KPC and OXA-48-like carbapenemases was designed and validated.

All available allele variants of the above carbepenmases were downloaded from the Beta-Lactamase DataBase (http://bldb.eu/), aligned with Clustal Omega and primers designed using Primer-BLAST. Real-time PCR monoplexes were optimized for the QuantStudio 6-Flex (Applied Biosystems) using the PowerUp SYBR Green Master Mix (Life Technologies) and validated using a panel of 204 characterised strains carrying a wide range of beta-lactamases, sometimes in combination. Melt-curve analysis was used to confirm positive results.

The *in silico* approach allowed primers to be designed in conserved regions of the KPC and NDM alignments, while three primer sets for IMP and two for VIM were necessary to ensure amplification of the different variants. One primer set was designed for OXA-48-like, however it is unlikely to detect all variants. Expected results were obtained for all 204 tested strains, with 100% sensitivity and specificity. Melt-curve analysis showed consistent Tm results for KPC, NDM and OXA-48-like; differences were instead noted for IMP and VIM as likely consequence of higher variability in the PCR target regions. Inhibition was not observed.

The assay is rapid, easy to perform and implement. It enables unequivocal detection of IMP, NDM, VIM, KPC and OXA-48-like carbapenemases even when more than one type is present simultaneously.

Introduction

Carbapenems are amongst the last line of defence to treat serious bacterial infections. However, carbapenemases (i.e. enzymes able to inactivate carbapenems) are increasingly identified in both Enterobacteriales and non-fermenter Gram-negative isolates. Carbapenemase producing organisms (CPO) are frequently associated with treatment failure and outbreaks, with the latter worsened by the fact that carbapenemases are often present on mobile genetic elements and can therefore easily spread between different bacteria [1].

In Wales, IMP, NDM, VIM, KPC and OXA-48-like (known as the "big five") are the carbapenemases predominantly identified and consistent with those found in the rest of the UK: in particular, IMP, NDM, KPC and OXA-48-like in Enterobacteriales [2] and VIM in *Pseudomonas* species [3]. Phenotypic assays employing inhibitors for the different enzymes are available for the detection of some carbapenemases. For example IMP, NDM and VIM (Class B, metallo-ß-lactamase) enzymes are inhibited by chelating agents like ethylenediaminetetraacetic acid (EDTA) and dipicolinic acid (DPA), while KPC (class A ß-lactamase) is inhibited by boronic acid; an inhibitor for OXA-48-like (class D) β-lactamases has not yet been identified, although temocillin resistance can be used as an indicator of its presence [4]. These phenotypic tests are both sensitive and specific predominantly for Enterobacteriales, while their performance is poor for *Pseudomonas* species [5, 6, 7]. Furthermore, these tests require 24hrs incubation to completion and can sometimes be difficult to interpret.

Carbapenemase screening in Enterobacteriales is performed in Wales according to EUCAST criteria, i.e. meropenem inhibition zone < 28mm and/or meropenem MIC > 0.125 mg/mL [4] with isolates flagged as potential CPOs sent to the Specialist Antimicrobial Chemotherapy Unit (SACU), Cardiff (UK) for further investigation. For *Acinetobacter* and *Pseudomonas* species all carbapenem and carbapenem plus piperacillin/tazobactam resistant isolates are referred accordingly. In SACU, phenotypic confirmation of carbapenem resistance is performed and carbapenemase production investigated using Neo-Sensitabs™ combination disks (ROSCO, Denmark) and MBL IP/IPI (*Pseudomonas* only) and MBL MP/MPI (Enterobacterales only) double ended E-Tests (bioMerieux, France), followed by in-house block-based PCR to confirm positive results.

The number of isolates referred to SACU for carbapenemase confirmation doubled from approximately 250 to 500 per year between 2015 and 2018, with an accompanying 100% rise in the number of CPO

confirmed per year (from 19 to 38). Nevertheless, the incidence in Wales (population approximately 3,125,000 inhabitants estimated in June 2017 [8]) remains low when compared to data reported from other Countries [9]. Referral numbers are predicted to rise further as hospitals undertake more rigorous screening programmes to detect CPO in admission patients as per recommendations [10]. The increase in requests for carbapenemase confirmation plus the importance of rapidly detecting CPO for effective infection control management, demanded a development of the above described confirmatory approach adopted by SACU. These methods are time consuming, labour intensive and involve the use of the toxic and carcinogenic compound, ethidium bromide, to visualise PCR results by gel electrophoresis (although safer intercalating agents are available nowadays). The need for a more rapid and reliable approach has been identified with the aim to reduce the time to issue confirmatory reports and better assist clinicians and infection control teams. Real-time PCR is a well-established methodology for detecting genetic markers in bacteria. It can provide results much faster than block-based PCR and does not normally require the use of high risk reagents. Therefore, a real-time PCR assay based on SYBR Green chemistry has been designed to detect the “big five” carbapenemases and thoroughly validated using a large panel of previously characterised Gram-negative isolates.

Materials and Methods

Assay design

IMP, NDM, VIM, KPC and OXA-48-like allele sequences were downloaded (as of December 2018) from the Beta-Lactamase DataBase [11] and aligned using Clustal Omega [12]. Alleles with less than 90% sequence identity to the rest of the alignment were excluded and separate primers were designed where a strain carrying them was available to the authors for *in vitro* testing. Ten different primer sets per target were designed using Primer-BLAST [13] and then compared to the relevant alignment to identify the pair that at least *in silico* was able to amplify the highest number of alleles. Where necessary, degenerate bases (a maximum of 2 per primer) were added to cover non-conserved positions. Primer sequences and amplicon sizes are detailed in Table 1.

Bacterial strains

A total of 204 previously characterised Gram-negative isolates from 19 different *species* isolated world-wide were selected from the Authors', Public Health England, NCTC and ATCC collections (Supplementary data). One hundred and thirteen carried one of the "big five" carbapenemases while 22 carried two different markers simultaneously. The remaining 69 were negative for "big five" carbapenemases however some carried genes for other β-lactamases (such as AmpC, CTX-M, GES, IMI, L1 & L2, OXA-10, OXA-23, OXA-51, OXA-58, TEM, SHV, SME, SPM and VEB). The 135 "big five" CPOs carried a total of 40 IMPs (including IMP-1, IMP-4, , IMP-6, IMP-7, IMP-8, IMP-10, IMP-11, IMP-13, IMP-14, IMP-29 and IMP-62), 27 VIMs (including VIM-1, VIM-2, VIM-4, VIM-7, VIM-10, VIM-19 and VIM-29), 31 NDMs (NDM-1, NDM-3, NDM-4, NDM-5, NDM-7), 26 KPCs (including KPC-2, KPC-3, KPC-4 and KPC-23) and 33 OXA-48-like markers (including OXA-48, OXA-181, OXA-204, OXA-232, OXA-244 and OXA-245).

After overnight aerobic growth on blood agar at 35±1°C, bacterial growth approximately equivalent to a third of a loopful (10μL loop) was re-suspended in 250 μL of nuclease-free water and heat killed at 100°C for 10 min. Supernatant was separated by centrifugation at 12,000 *g* for 2 min and diluted 1:20 in nuclease-free water prior to testing. An extraction control (i.e. 250 μL of nuclease-free water) was always included to discount cross contamination. A panel of seven strains was used as positive control in every experiment, namely *Escherichia coli* NCTC 13476 (IMPa), *Serratia marcescens* SACU 1212 (IMPb), *Pseudomonas aeruginosa* SACU 1205 (IMPc), *Klebsiella pneumoniae* NCTC 13440 (VIM), *Klebsiella pneumoniae* NCTC 13443 (NDM), *Klebsiella pneumoniae* NCTC 13438 (KPC) and *Klebsiella pneumoniae* NCTC 13442 (OXA-48).

Internal process control

An assay detecting a 76 bp fragment of the *green fluorescent protein, gfp* from *Aequorea victoria* [14] was included to discount PCR inhibition. A custom-made plasmid (Eurofins, Germany) containing the entire *gfp* sequence (Genbank Accession: M62653) was diluted to a working concentration of 1 pmol/μL before being added to the GFP reaction mix.

Real Time PCR

Monoplex PCR assays were optimized for the QuantStudio 6-Flex (Applied Biosystems) using the PowerUp SYBR Green MasterMix (Life Technologies) and Microamp Fast Optical 96-Well Reaction Plate 0.1 mL (Thermo-Fisher Scientific). Briefly, reactions were performed in a final volume of 10 μL containing 5 μL of MasterMix, 2.5 μL of template, 2 μL of PCR grade water and 0.5 μL of relevant primer mix. The final concentration of each primer is listed in Table 2. The GFP reaction mix also contained 0.5 μL per reaction of pGFP [1 pmol/μL], with 1.5 μL of PCR grade water to make 10 μL final volume. After an initial uracil-DNA-glycosylase step at 50°C for 2 min, the Dual-Lock™ DNA polymerase was activated at 95°C for 2 min followed by 35 cycles of denaturation at 95 °C for 1 sec and annealing/extension at 60°C for 20 sec. Amplification results were analysed with the threshold set at 0.5 ΔRn for all targets and the baseline set between 5 and 15 cycles only for the GFP target. Melting curve analysis was performed as follows: 95°C for 15 sec (ramp rate = 1.6°C/sec), 60°C for 1 min (ramp rate = 1.6°C/sec) and 95°C for 15 sec (ramp rate = 0.15°C/sec) with fluorescence fluctuation analysed during the latter.

Results

In silico analysis

The *in silico* approach allowed primers to be designed within 100% conserved regions of the KPC and NDM alignments. For the remaining carbapenemases, a single primer set able to amplify all alleles, was not identified. Three primer sets (tested separately) were necessary to detect IMP variants and two sets (tested in the same tube) for VIM variants. One primer set was designed for the OXA-48-like group, however despite detecting the most common variants it is unlikely to detect OXA-54, OXA-436 and OXA-535 due the suboptimal sequence identity (i.e. < 90%) to the rest of the group. . Clustal alignments of PCR fragments with primers binding sites highlighted are available in the Supplementary data.

Thirty-seven KPC variants were listed on the Beta-Lactamase DataBase, however two sets (i.e. KPC-1 & KPC-2 and KPC-9 & KPC-23) were identical while KPC-20 was “assigned” but no sequence was available. Specific primers were designed in a conserved region of the remaining 34 KPC alleles. Primer

175 binding sites were 100% conserved and no single nucleotide polymorphisms (SNPs) were identified in
176 the region between the primers.
177
178 A specific primer set was designed in a conserved region of the alignment of all available 24 NDM
179 variants, with no exclusion. Primer binding sites were 100% conserved and only one SNP (found in
180 NDM-10) was identified in the region between the primers.
181
182 Among the 79 described IMP variants, six (i.e. IMP-36, IMP-39, IMP-46, IMP-50, IMP-57 and IMP-65)
183 appeared as “assigned” but no sequence was available. Moreover, IMP-8 and IMP-47 share identical
184 sequence, consequently, the actual number of analysed variants was 72. Clustal analysis showed
185 considerable sequence variability, therefore it was not possible to identify a single primer set able to
186 detect all alleles. The Clustal generated tree was arbitrarily split in three sections (Figure 1) and a set
187 of primers designed for each section (i.e. IMPa, IMPb and IMPc). Degenerate bases had to be inserted
188 in all six primer sequences to ensure amplification of different variants.
189
190 Among the 60 described VIM variants, VIM-21, VIM-22 and VIM-55 were “assigned” but no sequence
191 was available. VIM-7 showed low sequence identity with the rest of the alleles (e.g. 81% identity with
192 VIM-1), consequently it was excluded from the alignment. Specific primers were designed in a
193 conserved region of the remaining 56 VIM alleles and one degenerate base was introduced in the
194 forward primer. As previously mentioned, a separate set of primers was also designed specifically for
195 VIM-7 as this variant was available to the authors for *in vitro* testing.
196
197 Only OXA-48-like variants with a definitive assignment by NCBI (30 in total) were downloaded from the
198 Beta-Lactamase DataBase (i.e. OXA-48, OXA-54, OXA-162, OXA-163, OXA-181, OXA-199, OXA-204,
199 OXA-232, OXA-244, OXA-245, OXA-247, OXA-252, OXA-370, OXA-405, OXA-416, OXA-436, OXA-
200 438, OXA-439, OXA-484, OXA-505, OXA-514, OXA-515, OXA-517, OXA-519, OXA-535, OXA-538,
201 OXA-546, OXA-547, OXA-566 and OXA-657). After Clustal analysis, OXA-54, OXA-436 and OXA-535
202 were excluded from the alignment due to low sequence identity (84%, 84% and 85% respectively with
203 OXA-48). In this instance specific primers were not designed as they were not available to the authors
204 for *in vitro* testing. Primers for the remaining 27 OXA-48-like variants were designed in a conserved

205 region with one ambiguity introduced within the forward primer while the binding site of the reverse
206 primer was 100% conserved.
207
208 ***In vitro* analysis**
209
210 The eight monoplex real-time PCR assays (i.e. IMPa, IMPb, IMPc, NDM, VIM, KPC, OXA-48 and GFP)
211 were performed in 96-well plates with PCR mixes added in rows; DNA extracts were tested in column
212 1 to 10, while column 11 and 12 were used to test extraction and positive controls respectively. The
213 time necessary to complete PCR amplification and melt curve analysis was approx. 40min. Testing of
214 undiluted DNA extracts resulted in strong PCR inhibition (as revealed by absence of- or delayed GFP
215 amplification), consequently 1:20 dilutions were prepared prior to testing. Formation of primer dimers
216 was observed with the NDM assay, therefore oligonucleotide sequences were reviewed *in silico* and
217 concentrations decreased *in vitro* to improve results (Table 2).
218
219 DNA extracts of *Escherichia coli* NCTC 13476 (IMP), *Klebsiella pneumoniae* NCTC 13440 (VIM-1),
220 *Klebsiella pneumoniae* NCTC 13443 (NDM-1), *Klebsiella pneumoniae* NCTC 13438 (KPC-3) and
221 *Klebsiella pneumoniae* NCTC 13442 (OXA-48) were mixed to generate all possible target combinations
222 (for simplicity only IMPa among the three IMP primer sets was included in this analysis). In all cases,
223 the assay successfully detected all the carbapenemases present in the reaction mix, regardless of if
224 only one or all five were present simultaneously (Supplementary data).
225
226 Melt-curve analysis was used to confirm positive results by comparing dissociation curves to that of the
227 relevant positive control. Minor Tm variations were observed for NDM, VIM, KPC, OXA-48-like and GFP
228 (0.15 < St Dev < 0.30), while greater differences were noted with the three IMP monoplexes (0.49 < St
229 Dev < 0.62).
230
231 The expected result was initially obtained for 203 of the 204 previously characterised isolates. *Klebsiella*
232 *pneumoniae* SACU 27698 was meant to be negative for the “big five” carbapenemases, instead it
233 produced unequivocal amplification with IMPa (Ct = 17.49) and IMPb (Ct = 30.49) primer sets and
234 melting curves consistent with those of *E. coli* NCTC 13476 and *S. marcescens* SACU 1212 (i.e. IMPa

and IMPb positive controls). Further analysis with in-house block-based PCR confirmed presence of IMP metallo-β-lactamase, while Whole Genome Sequencing (WGS) by MiSeq (Illumina) characterised it as IMP-4 (data not shown). Indeed, the newly designed assay provided the expected result for all 204 tested isolates (100% sensitivity and specificity) including those carrying two "big five" carbapenemases simultaneously. Cross-reaction with the other resistance markers present in the above mentioned isolates was not observed.

Overall, amplification results produced mean Ct values of ca. 16 and Standard Deviation (SD) values between 0.88 and 1.45 (Table 3). Among the 40 isolates carrying IMP markers, 36 gave positive results with two of the three IMP primer sets used (i.e. a strong result in the expected Ct range and a weak result with Ct > 25 respectively). IMP alleles from part A of the Clustal generated tree gave strong IMPa results and weak IMPb results, alleles in part B gave strong IMPb results and weak IMPc results, while alleles in part C of the tree gave IMPc strong results and weaker IMPb results (Supplementary data). Four IMPs of which three IMP-13, were only amplified by one set of primers.

Discussion

We describe here the design and validation of an array of monoplex real-time PCRs optimised for the QuantStudio 6-flex platform (ThermoFischer Scientific) to rapidly detect and differentiate the "big five" carbapenemases, (i.e. IMP, NDM, VIM, KPC and OXA-48) from Enterobacteriales and non-fermenter Gram-negative isolates. A rational approach that took into account all the relevant allele sequences available from the Beta-Lactamase Database (as of December 2018) was applied *in silico* to design specific primers and to then predict whether they would also detect those allele variants that were not available to the authors for *in vitro* testing. A wide collection of previously characterised Gram-negative isolates was then tested *in vitro* to fully validate the newly designed monoplexes. An assay targeting the *gfp* [14] was simultaneously tested as an internal control to discount inhibition.

As resistance markers (especially IMP in this case) evolve rapidly causing emergence of new allele variants, SYBR Green chemistry combined with melting curve analysis in monoplex PCR reactions was

chosen over multiplexing with primers and probes. Newly described SNPs in primers and/or probe binding sites would potentially result in assays having to be redesigned and then revalidated. This is time consuming and costly. The absence of probes in this assay reduces the number of binding sites from three to two and consequently the likelihood that a newly described SNP falls into them. If indeed a new SNP is identified in either of the two primer binding sites and it is decided that they need to be redesigned, the use of separate monoplexes allows to limit the revalidation to just the affected monoplex instead of having to perform a major revalidation like when a multiplex approach is used. Furthermore, the use of separate monoplexes provides greater flexibility to combine testing of different panels of targets if the need arises: one or more of the monoplexes described here could be quickly replaced with others validated separately to detect different resistance markers (e.g. GIM, SIM, SPM, IMI, etc) without the need to perform any further validation on the entire assay panel.

The *in silico* analysis showed that this assay should be able to detect all the IMP, NDM, VIM and KPC variants described as of December 2018. Among the OXA-48-like group, OXA-54 and OXA-535, described in the chromosome of *Shewanella species* [15, 16] and OXA-436 identified on a plasmid in *Enterobacter species*, *Citrobacter species* and *K. pneumoniae* in Denmark [17] are unlikely to be detected.

The *in vitro* experiments proved the assay to be 100% sensitive and specific on a large panel of previously characterised isolates available to the authors. As stated by Lund *et al.* (2018), assays are often validated using a limited selection of target variants that are available locally; furthermore, testing high numbers of unsequenced isolates might simply result in redundant analysis of a small number of variants which then provides little indication about the actual sensitivity and specificity of a particular assay. These issues were addressed in this study by applying a robust *in silico* approach to confidently predict amplification of those variants that were not available for *in vitro* testing and by then including (where possible) isolates where the “big five” allele variant had previously been characterised (i.e. by Sanger sequencing and/or by WGS).

Interpretation of melting profiles can be problematic when dissociation curves do not overlap with that of the positive control. This was often observed with isolates carrying IMP genes as a likely

295 consequence of high sequence variability of the IMP PCR products. Nevertheless, when an IMP allele
296 was present, more than one primer set was often able to detect it (usually two primers sets, one
297 producing a strong result with Ct between 14 and 18, and another producing Ct > 18) allowing confident
298 identification of true positives. It is noteworthy that variations in Tm results were observed also for those
299 PCR products that were not meant to contain any SNP (e.g. KPC and GFP) therefore it seems that the
300 actual Tm value cannot be reproducibly obtained by the PCR platform between different PCR runs. A
301 Tm standard deviation of 0.20 was calculated for the GFP amplified fragment (which is identical in every
302 PCR run) and this value could be considered as an indication of the Tm uncertainty of measurement
303 intrinsic to this procedure. Different Tm values were also noted for VIM alleles, however in this case, it
304 was due to VIM-7 being quite different (only 81% nucleotide identity with VIM-1), with a Tm of approx.
305 84°C compared to Tm of approx. 81°C for the remaining VIM alleles. This was consistently observed
306 and was actually helpful to differentiate between VIM-7 and the other VIM alleles.
307
308 In at least one instance, the assay provided more sensitive and reliable results than standard phenotypic
309 analysis: *K. pneumoniae* SACU 27698 previously characterised as negative for the “big five”
310 carbapenemases, was instead found to be IMP positive and later characterised by WGS to carry IMP-
311 4. This result triggered an investigation that identified the above isolate as part of a cluster involving five
312 patients (data not shown) which clearly proves the added value of the here described real time PCR
313 assay. Retrospective analysis on the isolate showed that combination disks did not to show metallo-β-
314 lactamase activity (i.e. only 2 mm difference between the meropenem and the meropenem/DPA disks)
315 while the MP/MPI double-ended strip provided a weakly positive result (MIC ratio = 12). With this in
316 mind, it was decided to modify the SACU approach for investigation of carbapenemases. The real time
317 PCR assay described here became the first line method to investigate presence of the “big five”
318 carbapenemases. Positive PCR results are reported immediately without performing further analysis,
319 however as negative PCR results cannot completely exclude presence of a carbapenemase, further
320 investigations need to be performed. As already mentioned, new “big five” allele variants might not be
321 detected by this assay or a carbapenemase not included in this assay (e.g. GIM, SIM, SPM, etc) could
322 be present. Finally, novel carbapenemases, not known as yet, would equally not be detected.
323 Therefore, all PCR negative isolates are tested with combination disks and double ended strips to
324 phenotypically exclude both carbapenemase and Extended Spectrum ß-lactamase (ESBL) activity

before negative results are reported. In those cases where carbapenemase activity is observed despite a negative PCR results, alternative methods (such as sequence analysis) would be applied to investigate the cause of the resistant phenotype.

In certain cases, “big five” variants detected by this assay might actually confer either weak or undetectable level of carbapenemase resistance. Indeed, KPC-14 and KPC-28 have been demonstrated to have reduced carbapenemase activity [19] while OXA-163, OXA-247 and OXA-405 have been shown to have no carbapenemase activity [20, 21] due to amino acid deletions in the active site. Isolates carrying these variants are likely to generate discrepant PCR and phenotypic results thus further testing (e.g. sequence analysis of the “big 5” variant) should be performed. In particular, KPC-28 has also been characterised to have an amino acid substitution (i.e. H274Y) causing a 50-fold increase in ceftazidime hydrolysis and consequently high MIC to ceftazidime/avibactam, an effective antimicrobial option for KPC producing bacteria [19].

The assay described herein allows testing of 10 isolates at a time (plus extraction control and positive control) using 96-well plates. Given the short PCR running time (ca. 40 min), DNA could be extracted simultaneously from a greater number of isolates to allow a quick second PCR experiment to be prepared while the first is running; up to 20 isolates could be then tested in less than 90 min. Alternatively, for high through put 384-well plates may be used, allowing up to 46 strains (plus controls) to be tested simultaneously. The assay was deemed easy to perform by members of staff with little or no experience with real-time PCR and melt-curve analysis. Importantly, the real time PCR assay allowed unequivocal result interpretation even when more than one “big five” carbapenemase resistance marker was present. Finally, the new algorithm improved the reference service by reducing the time necessary to report positive results and at the same time reducing the risk of reporting false-negative results.

In summary the assay was easily implemented, allowed faster and more reliable positive results than previously obtained by SACU to be available for clinicians and infection control specialists. The potential wider benefits of the assay include reducing costs to the hospital from keeping potential positive patients

in side rooms, inappropriate therapy of positive patients and increased screening costs because of delays in positive results.

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Compliance with Ethical Standards

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456 **Table 1.** Summary of primer sets used in this study (FP = Forward Primer; RP = Reverse Primer;
457 ambiguities are underlined; * VIM and VIM-7 tested in the same tube).
458

Primer set	Target	Name	Sequence (5′→3′)	Amplicon (bp)	Reference
1	IMP(a)	IMPa_FP	TGACGCAAATDTAGAAGCTTGGC	101	This study
		IMPa_RP	CRTCTCCARCTTCACTGTGAC		
2	IMP(b)	IMPb_FP	GAYGCCTATCTRATTGACACTCC	79	This study
		IMPb_RP	TATARCCGCGCTCMACAAACCA		
3	IMP(c)	IMPc_FP	KGAYGCAAATTTAGAAGCTTGGCCA	142	This study
		IMPc_RP	CCCTTTAACAGCCTGYTCCCA		
4	NDM	NDM_FP	CAACTTTGGCCCGCTCAAGG	100	This study
		NDM_RP	GCAGCCACCAAAAGCGATGT		
5*	VIM	VIM_FP	CGAAAAACACAGCGGCMCTTCT	73	This study
		VIM_RP	GTGGAGACTGCACGCGTTAC		
	VIM-7	VIM-7_FP	GGTGGCTGTGCAGTTCATGAG	122	This study
		VIM-7_RP	ACGACCTCTGCTTCCGGATAC		
6	KPC	KPC_FP	CCACTGGGCGCGCACCTATT	51	This study
		KPC_RP	TGTTAGGCGCCCGGGTGTAG		
7	OXA-48-like	OXA-48_FP	CTGGTGGGTYGGTTGGGTTGA	89	This study
		OXA-48_RP	GCAGCCCTAAACCATCCGATGT		
8	GFP	GFP_FP	CCTGTCCTTTTACCAGACAACCA	76	[14]
		GFP_RP	GGTCTCTCTTTTCGTTGGGATCT		

459

460 **Table 2.** Final concentration of primers used in this study (* VIM and VIM-7 tested in the same tube).

Primer set	Target	Primer Name	Final Conc. [nM]
1	IMPa	IMP(a)_FP	500
		IMP(a)_RP	500
2	IMPb	IMP(b)_FP	500
		IMP(b)_RP	500
3	IMPc	IMP(c)_FP	500
		IMP(c)_RP	500
4	NDM	NDM_FP	300
		NDM_RP	300
5*	VIM	VIM_FP	400
		VIM_RP	400
	VIM-7	VIM7_FP	100
		VIM7_RP	100
6	KPC	KPC_FP	500
		KPC_RP	500
7	OXA-48-like	OXA-48_FP	400
		OXA-48_RP	400
8	GFP	GFP_FP	300
		GFP_RP	300

461

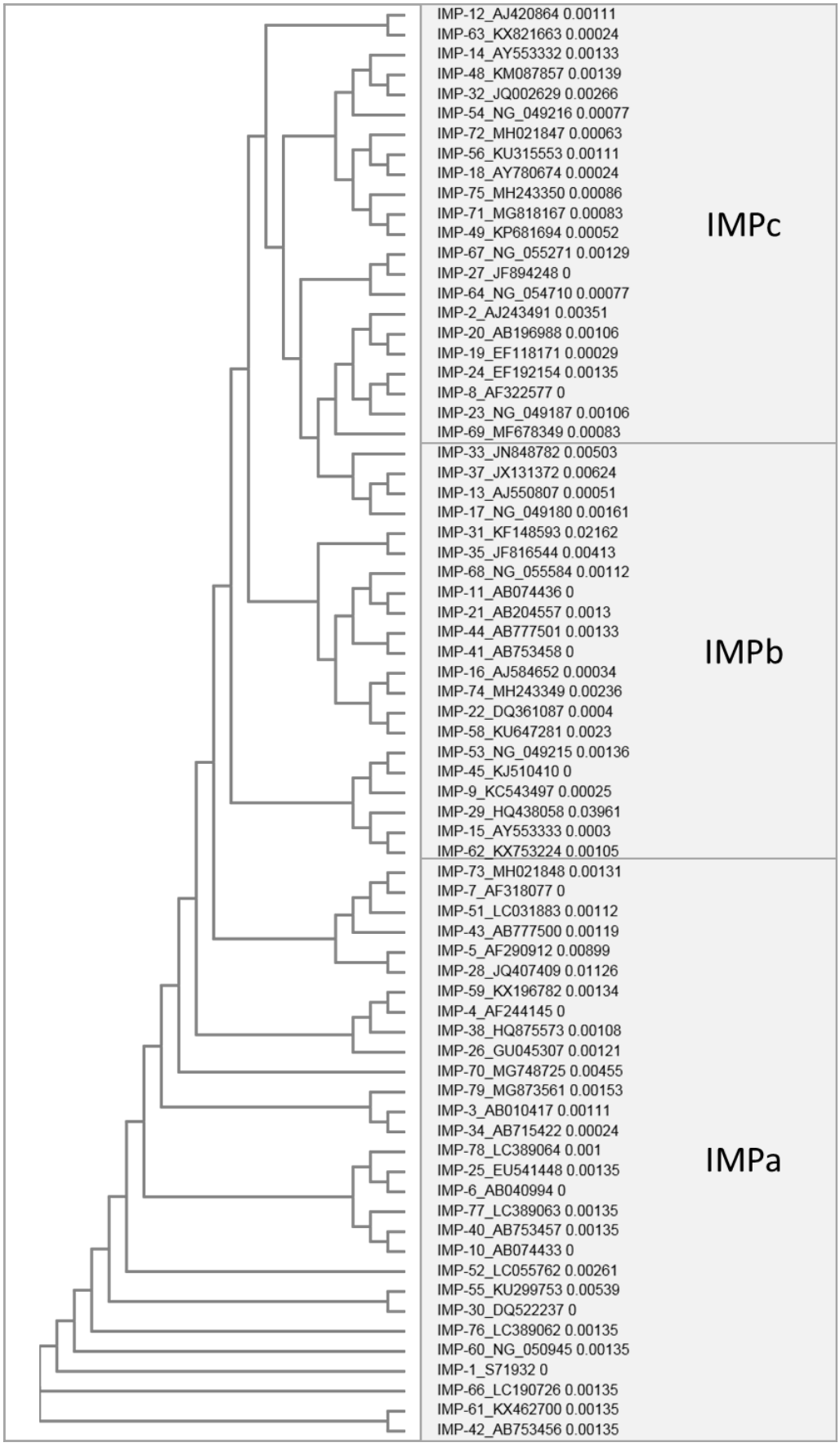
462 **Table 3.** Summary of Cycle threshold (Ct) and melting temperature (Tm) results for the primer sets
463 used in this study (* VIM and VIM-7 tested in the same tube; ** only one strain containing VIM-7 was
464 available for testing, consequently Min, Max and SD were not calculated).

465

Primer set	Target	Ct				Tm (°C)			
		Min	**Mean**	**Max**	**SD**	**Min**	**Mean**	**Max**	**SD**
1	IMPa	14.67	**16.73**	20.35	1.43	76.92	**77.74**	78.47	0.49
2	IMPb	14.43	**16.66**	18.94	1.45	75.13	**76.31**	77.21	0.77
3	IMPc	14.88	**16.06**	16.98	0.88	76.92	**77.51**	78.06	0.62
4	NDM	13.73	**16.41**	18.83	0.99	84.61	**84.86**	85.58	0.21
5*	VIM	14.11	**16.09**	18.23	0.95	80.43	**80.73**	81.07	0.15
	VIM-7**	-	**15.49**	-	-	-	**84.12**	-	-
6	KPC	13.52	**16.81**	18.50	1.13	82.90	**83.40**	83.83	0.30
7	OXA-48-like	13.63	**16.16**	18.60	1.21	78.05	**78.26**	78.78	0.18
8	GFP	21.36	**23.36**	24.28	0.44	77.51	**77.77**	78.83	0.20

466

467 **Figure 1.** Clustal Omega generated tree of the IMP alleles available from the Beta-Lactamase
468 DataBase.



469