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Standardization of inhouse developed real time polymerase chain reaction (PCR) for universal carbapenemase resistant genes amongst *Enterobacterales* isolated from septicemic patients in intensive care settings

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Abstract

Objectives: To develop inhouse real time polymerase chain reaction (PCR) for carbapenemase anti-microbial resistant genes comprising of *imipenem (IMP)*, *Klebsiella pneumoniae carbapenemase (KPC)*, *oxacillinase-48(OXA-48)*, *New Delhi metallo-beta-lactamase (NDM)* and *verona integron-encoded metallo-beta-lactamase (VIM)*, keeping multiplex PCR via biofire filmarray (BFA) as the gold standard, and to calculate its diagnostic accuracy.

Method: The cross-sectional, interdisciplinary, diagnostic accuracy (DA) study was conducted from May 9 2024, to June 30 2025, at the Microbiology Department of the Armed Forces Institute of Pathology, National University of Medical Sciences, Rawalpindi, Pakistan. The sample comprised septicemic inpatients fulfilling Sepsis 3 guidelines. Blood cultures found positive by best available control technology, showing

28 Gram-negative rods on direct staining, were selected for cultures proceedings along with
29 BFA and real time PCR processing. Data was analysed using SPSS 27.

30 **Results:** Of the 116 patients, 45(38.8%) were aged ≤ 12 years with a mean of
31 5 ± 3.1 years, and 71(61.2%) were aged ≥ 12 years with a mean of 48 ± 26.3 years. Males
32 were 77(66.4%) and females were 39(33.6%). Of the 116 samples, 102(87.9%) blood
33 culture were found positive. The insilico-designed primers for *IMP*, *KPC*, *OXA-48* and
34 *NDM* were successfully optimised and validated. No isolate was found positive for *VIM*,
35 so its primers cannot be optimised. The inhouse real time PCR demonstrated a DA of
36 98.9%, sensitivity of 100% and specificity of 98.4% on comparison with blood culture
37 identification panel of BFA.

38 **Conclusion:** The optimisation and validation of primers via real time PCR for *IMP*,
39 *KPC*, *OXA-48* and *NDM* were successfully completed, showing a high diagnostic
40 accuracy.

41 **Key Words:** Septicaemia, Biofire filmarray, Real time PCR, *IMP*, *KPC*, *NDM*, *OXA-48*.
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43

44 Introduction

45 In 2018, a report by the World Health Organisation (WHO) labelled antimicrobial
46 resistance (AMR), including multidrug resistant (MDR), extensively drug resistant
47 (XDR) and pan-drug resistant (PDR) streams, as one of the biggest global health
48 challenges of the 21st century.^{1,2} It was also predicted that till the availability of new
49 anti-microbials, the existing scenario might worsen and may lead to 10 million deaths
50 annually by 2050, necessitating the formulation of national action plans linking with
51 global action plans to reduce high morbidity and mortality rates, especially in low
52 middle income countries (LMICs).^{1,3}

53 The amalgamation of carbapenem-resistant *Enterobacteriales* (CRE) in septicemic
54 patients admitted in intensive care units (ICUs) has become the proverbial tip of the
55 iceberg.⁴⁻⁶

56 Depending on amino acid homology, Ambler classification is used for beta (β)-
57 lactamase categorisation as A, B, C and D. The CRE is mostly noticeable for classes A,
58 B and D, while the *klebsiella pneumoniae* carbapenemase (KPC) group is clinically
59 present in class A carbapenemase. Class B carbapenemases included imipenem (IMP),
60 New Delhi metallo- β -lactamase (NDM) and verona integron-encoded metallo- β -
61 lactamase (VIM). Class D has oxacillinase (OXA) carbapenemases rendering their
62 capability to hydrolyse oxacillin.²

63 The labelled gold standard bacterial culture process is based on an accurate, but
64 time-consuming test.⁷ The highly sensitive and specific real-time polymerase chain
65 reaction (PCR) assay brought about a revolution by decreasing turnaround time,
66 contributing to improved patient management.⁶ Also, the United State Food and Drug
67 Administration (FDA) has allowed the usage of blood culture identification (BCID)
68 panel of biofire filmarray (BFA), which simultaneously and rapidly detects genomic
69 characterisation of bacterial and AMR genes.⁸
70 There are very few virtuous microbiological laboratory facilities in Pakistan, and LMIC,
71 and no DA studies from Pakistan regarding inhouse real time PCR for carbapenemase
72 genes could be found in literature. The current study was planned to fill the gap in
73 literature by developing an inhouse real time PCR for carbapenemase-resistant genes,
74 including IMP, KPC, NDM, OXA-48 and VIM, keeping multiplex PCR via BCID panel
75 of BFA as the gold standard, and to calculate its diagnostic accuracy.

76

77 **Materials and Methods**

78 The cross-sectional, interdisciplinary, DA study was conducted from May 9, 2024, to
79 June 30, 2025, at the Microbiology Department of the Armed Forces Institute of
80 Pathology (AFIP), Rawalpindi, Pakistan, after approval from the institutional ethics
81 review board. Though mainly hosted by the AFIP, the study was done in collaboration
82 with Armed Forces Post Graduate Medical Institute (AFPGMI), National University of
83 Medical Sciences (NUMS), Health Research Institute (HRI), National Institute of Health
84 (NIH), National Institute of Genomics and Advanced Biotechnology (NIGAB),

85 National Agriculture Research Centre (NARC), Islamabad, Pakistan. The neonatal ICU
86 (NICU), paediatric ICU (PICU), medical ICU (MICU), surgical ICU (SICU), acute
87 medical unit (AMU), and male and female oncology wards of Pak Emirates Military
88 Hospital (PEMH) and Combined Military Hospital (CMH), Rawalpindi.

89 The sample was raised using non-probability convenience sampling technique.⁹ The
90 sample size was calculated using sensitivity and specificity formula from the WHO
91 calculator,¹⁰ keeping BFA sensitivity 99%, specificity 100%, expected prevalence
92 0.15(15%), confidence level 95% (1.96) and desired precision 0.05.^{11,12}

93 The inclusion criteria was divided into three categories; patient selection based upon
94 Sepsis 3 guidelines, blood sample selection criteria for BFA proceedings, and the criteria
95 for real time PCR. The admitted patients in ICUs fulfilling Sepsis 3 criteria (fever
96 $\geq 101^{\circ}\text{F}$, tachypnea ≥ 22 breaths per min, tachycardia ≥ 100 beats per minute, and systolic
97 blood pressure [SBP] ≤ 100 mmHg), whether or not responding to antibiotics after 03
98 days in hospital, were included.¹³ For laboratory diagnostic protocols, presence of blood
99 complete picture (CP) with neutrophilic leucocytosis and deranged inflammatory markers
100 were the preliminary requirements. Among the inflammatory markers, data related to C-
101 reactive protein (CRP), activated partial thromboplastin time (APTT), prothrombin time
102 (PT), international normalised ratio (INR), fibrinogen degradation products (FDPs) and
103 d-Dimers was necessary. This step helped reduce bias regarding patient selection. Further,
104 blood sample selection was limited for BFA proceedings. Only best available control
105 technology (BacT) alert signalled positive culture bottled showing gram-negative rods
106 (GNRs) on gram staining. These were selected and included for BFA and ultimately
107 real time PCR.

108 The exclusion criteria were the positively signalled blood culture bottles by BacT alert
109 yielding more than two bacterial growths, gram-positive cocci or presence of candida
110 species. Patients or their attendants no willing to participate were also excluded.

111 Data was collected after taking informed consent in all cases. A proforma was used to
112 gather required details for correlation with laboratory results, including length of hospital
113 stay (LOS), past clinical history, history of ICU stay, presenting complaints, clinical and

laboratory tests details in line with Sepsis 3 criteria, antibiotics used in ICU, and available laboratory tests results carried out in hospitals. Another proforma was used to gather result of blood culture and sensitivity by disc diffusion method, gram staining of positively signalled bottles by BacT alert (BioMerieux S.A., Marcy-l'Étoile, France), results of BCID panel of BFA, insilico designed primers used for optimisation and validation by real time PCR, and the final results.

For blood culture and sensitivity, recommended protocols for sample collection, BacT alert processing and Clinical and Laboratory Standards Institute (CLSI) guidelines 2024 were followed.¹⁴

The positively signalled blood culture bottles showing the presence of GNRs on gram staining were selected to proceed for BCID panel of BFA.¹⁵⁻¹⁷ The test has the ability to target multiple bacterial genes; gram-positive, gram-negative, yeasts and AMR genes including *IMP*, *KPC*, *NDM*, *VIM*, *OXA-48*, mobilized colistin resistant gene 1 (*mcr-1*), methicillin resistant gene A (*mecA*), methicillin resistant gene C (*mecC*), methicillin resistant gene A and C (*mecA/C*), methicillin resistant extremity junction gene (*MREJ*), vancomycin A and B resistant genes (*van A/B*) and Cefotaximase-Munich resistant gene (*CTX-M*).¹⁸

The pure growth from blood culture bottles detected positive by BCID panel of BFA for bacterial and carbapenemase genes *NDM*, *OXA-48*, *KPC*, *IMP* and *VIM* were ultimately optimised and validated by real time PCR using Bio-perfectus nucleic acid extraction rapid kit (magnetic bead method).¹⁹ The manufacturer guidelines were followed for DNA extraction and real time PCR proceedings.^{6,19}

This was followed by validation regarding sequences of primer selection for real time PCR. Insilico blast sequencing was done using NCBI.²⁰ All the selected genes had amplicon size of ≤ 200 bp. The positive blood samples via gold standard BFA were used as reference and standard. The details of insilico designed primers for universal carbapenemase genes was noted in detail as mentioned below:²⁰

For insilico designed primers of KPC: F: GAACAGGACTTTGGCGGCT/ R: GCAT T

TTTGCCGTAACGGAT were used. The amplicon length was 191 and annealing temperature of 58°C was selected.^{21,22} For insilico designed primers of IMP: F: ACTGTGCGCCATGGAAATGT/R: AGGTTGGGGTGTAGTCACAA were used. The amplicon length was 169 and annealing temperature of 58°C was selected.^{21,22} For VIM: F: AACGCAGTCGTTTGATGGCG/ R:CGCGGTCGTCATGAAAGTG were used. The amplicon length was 185 and annealing temperature of 60°C was selected.^{21,22} For NDM: F: AGCTCGCACCGAATGTCTG/ R: TTGATCTCCTGCTTGATCC were used. The amplicon length was 172 and annealing temperature of 56°C was selected.^{20,21} For OXA-48, F: AACATAAATCACAGGGCGT/ R:GTCTGTCCATCCCACTTAA were selected. The amplicon length was 187 and annealing temperature of 54°C was selected.^{22,23}

Data was analysed using SPSS 27. For qualitative analysis, frequencies and percentages were used to identify statistical inference. For DA, sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and likelihood ratios were used for inference. The calculations for positive likelihood ratio (PLR) of real time PCR was calculated using formula 'PLR = Sensitivity / 1- Specificity'. The negative likelihood ratio (NLR) was calculated using the formula 'NLR = 1- Specificity / Sensitivity'.²⁴

Results

Of the 116 patients, 45(38.8%) were aged ≤ 12 years with a mean of 5 ± 3.1 years, and 71(61.2%) were aged ≥ 12 years with a mean of 48 ± 26.3 years. Of the 116 samples, 102(87.9%) blood culture were found positive.

Of the 116 samples, 102(87.9%) blood culture was found positive. Gram staining showed the presence of GNRs in 81(69.82%) specimens. One type of bacterial growth was yielded in 76(65.5%) specimens, while two types of bacterial growth was seen in 20(17.2%) specimens, and 6(5.1%) specimens either remained negative or revealed three types of bacteria, and were excluded.

In terms of phenotypic expression, one type of bacteria was noted in 76(65.5%) cases, with the highest frequency being for *Burkholderia cepacia* (BC) 15(19.7%) and *Serratia*

171 *marcescens* (SM) 15(19.7%). This was followed by *Acinetobacter baumannii* (AB) in
172 14(18.4%), *Klebsiella pneumoniae* (KP) 13(17.1%) and *Escherichia coli* (*E.coli*)
173 9(11.8%) cases.

174 Of the 81(69.8%) specimens yielding GNRs, 66(81.4%) yielded positive results via
175 BFA proceedings. Among them, one type of bacteria was isolated from 52(78.78%)
176 specimens, while two types of bacteria were seen in 13(19.69%) specimens. Three types
177 of bacterial isolates were seen in 1(1.51%) specimen and was excluded.

178 The carbapenem resistance via phenotypic expression was seen in 21(18.1%) blood
179 samples of septicemic patients. Of them, 15(12.9%) patients were aged ≤ 12 years and
180 6(5.17%) patients were aged >12 years. The maximum resistance was observed for KP
181 7(6.03%), followed by SM 6(5.17%). Next in sequence was AB 5(4.3%), *E.coli*
182 2(1.72%) and *Pseudomonas aeruginosa* (PA) 1(0.86%). Based upon the results of BFA
183 for carbapenemase genes, overall 16(13.7%) specimens having *NDM* gene in KP
184 7(8.6%) and SM 6(5.17%) were subjected to real time PCR test. Besides, 11(9.48%)
185 specimens having *IMP* gene in KP 9(7.75%), followed by AB 5(4.31%) and *E.coli*
186 2(1.72%) were subjected to real time PCR test. Also, 7(6.03%) specimens having KP
187 with *KPC* gene and 8(6.89%) specimens having SM with *OXA-48* gene were subjected
188 to real time PCR test.

189 The DA of real time PCR against gold standard BCID panel of BFA was calculated from
190 total selected 94 (N) specimens. The DA was calculated by using a table of 2 x 2. True
191 positives were 31 (n), false positives was 01(n), false negative was zero(n) and true
192 negatives were 62 (n). The DA of real time PCR against gold standard BCID panel of
193 BFA revealed 98.9% results. The sensitivity of real time PCR for carbapenem resistant
194 gene detection was 100%, specificity was 98.4%, PPV was 96.872% and NPV was
195 100%. PLR of real time PCR showed a result of 50, while the NLR showed a result of
196 0.2. It was interpreted that the in view of PLR and NLR values, a strong positive
197 diagnostic evidence was established for real time PCR to confirm AMR genes compared
198 to BFA via BCID panel.

199 Real time PCR optimisation and validation details for universal carbapenemase genes
200 was noted in detail (Table - I), and so were the melting temperatures and amplification
201 details (Figures 1-2).

202

203 Discussion

204 As per global data for 2024, bloodstream infections (BSI) amongst the highest causes
205 of morbidity and mortality in ICU settings.²⁵ Therefore, International Surviving Sepsis
206 Campaign (ISSC) suggested abrupt commencement of escalating therapy.
207 Simultaneously adoption of cautious measures was advised to reduce AMR at the
208 beginning of such management.²³ In 2020, the WHO published a report confirming 49
209 million people with sepsis globally and 11 million deaths annually, paving for declaring
210 sepsis as a global health priority.²⁶ A Chinese study in 2022 concluded sepsis-induced
211 mortality rate of 25% in hospitals and 40% in ICUs.²⁷

212 The current results revealed 82(70.6%) positive cases of septicemia. These were patients
213 found to be positive via both BFA and phenotypic identification methods. An earlier
214 study reported 16.09% positivity rate for blood cultures.²⁸ The disparity can be due to a
215 strict inclusion criteria in the current study which followed the Sepsis 3 guidelines.¹³
216 Besides, neutrophilic leukocytosis along with deranged inflammatory markers CRP,
217 FDPs, PT, APTT, INR and d-Dimers were mandatory elements of the inclusion criteria.
218 The current outcomes revealed the highest prevalence for BC 15(12.9%) and SM
219 15(12.9%). Next in sequence were AB 14(12.06 %), KP 13(11.2%) and *E.coli* 9(7.75%)
220 cases. A study in India showed the highest incidence of coagulase-negative staphylococci
221 (21.6%) in septicemic patients. This was followed by KP (19.3%) and *Acinetobacter*
222 species(spp.) (17.8%).²³

223 Another study reported the highest incidence of gram-negative aerobes 496(45%),
224 followed by *Acinetobacter spp.* 164(34%), KP 135(28%), *E.coli* 123(26%) and
225 salmonella typhi 29(6.1%). Gram-positive aerobes, like *Staphylococcus aureus*
226 (*S.aureus*) were seen in 40% cases and enterococcus spp. in 4.2%. A study in 2025
227 revealed the highest frequency for KP, SM and AB.²⁹

228 The current study showed that 15.5% cases were true positive (TP) for carbapenemase
229 resistance genes, 6.9% were false positive (FP), 1.7% were false negative (FN) and 56.9%
230 were true negative (TN). The BCID panel of BFA showed 20.6% patients with variable
231 carbapenemase resistant genes. These findings are similar to Centres for Disease Control
232 and Prevention (CDC) data published in 2025 which showed that the annual incidence of
233 CRE has risen to 18% compared to data from 2019-23.³⁰ Another study in 2025 reported
234 the highest incidence of CRE in KP 86.9% cases.³¹

235 The current results revealed 72°C as the annealing and melting temperature (T_m) for
236 *KPC*, *NDM* and *OXA-48* genes. For *IMP* it was 58°C. This is comparable with data
237 showing T_m 90.01°C for *KPC*, 89.45°C for *NDM*, 80.51°C for *OXA-48* and 77.02°C for
238 *IMP* genes.³²

239 The calculations for DA of real time PCR against gold standard BCID panel of BFA
240 revealed 98.9% results, sensitivity 100%, specificity 98.4%, PPV 96.872% and NPV
241 100%. The PLR and NLR showed values of 50 and 0.2, respectively. From these values,
242 it was inferred that a strong positive diagnostic evidence was established for real time
243 PCR to confirm carbapenemase resistance genes *KPC*, *NDM*, *OXA-48*, *IMP* and *VIM*
244 compared to BCID panel of BFA. Despite thorough literature search, the data was found
245 scarce for comparison of DA of BFA to detect carbapenemase resistance with phenotypic
246 expression. Published literature had highlighted the rapid turnaround time for real time
247 PCR.³³ Only one study carried out in 2022 showed 94.9% sensitivity of BCID panel with
248 95% confidence interval (CI) to identify carbapenemase resistant genes.³⁴

249 The current study has limitations as the whole genome sequencing was not carried out.
250 Comparative analysis was not done between multiplex PCR via BFA and real time PCR
251 by direct evaluation of blood sample in brain heart infusion (BHI) bottle along with their
252 corresponding pure growths.

253 Despite the limitations, however, the current findings suggest that successfully validated
254 and optimised primers for *NDM*, *OXA-48*, *IMP* and *KPC* carbapenemase genes can be
255 used in clinical settings for early detection of resistant genes. Also, real time PCR for

the detection of carbapenemase genes can serve as a cost-effective alternative method in LMICs.

Conclusion

The primers' optimisation and validation via real time PCR for *IMP*, *KPC*, *OXA-48* and *NDM* were successfully completed. Real time PCR for the detection of carbapenemase genes can serve as a cost-effective, accurate, rapid and alternative method in LMICs to detect AMR genes.

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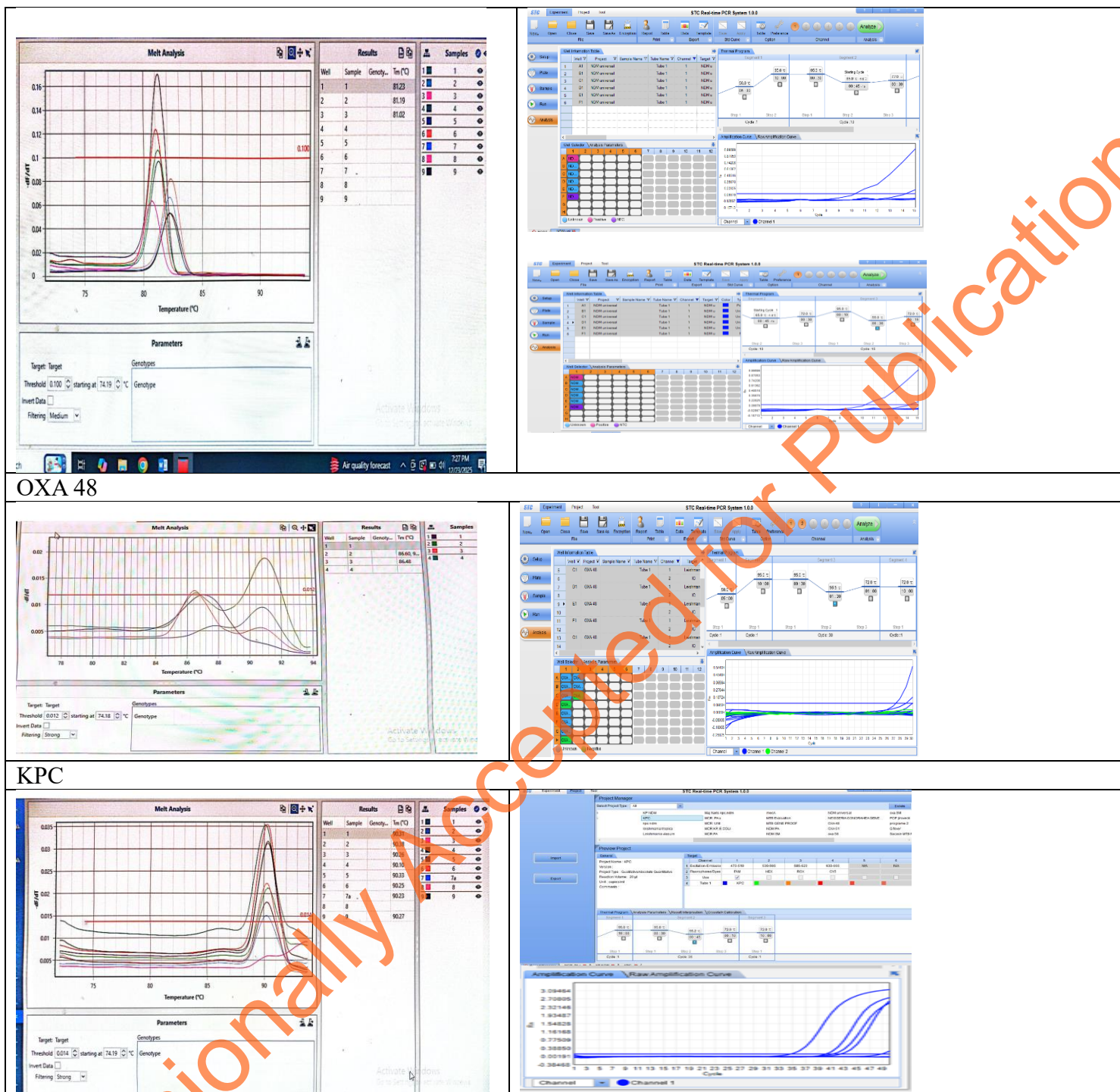
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397 **Table I: Optimised and validated universal carbapenemase genes by real-time**
398 **polymerase chain reaction (PCR).**

Sr. No	AMR Genes	Primers Validation Details for Real Time PCR.	Amplicon length (bp)	Optimised Thermal programme
1.	OXA – 48 [Universal]	F: AACATAAATCACAGGGCGT R: GTCTGTCCATCCCCTTAA	187 bp	-
		Segment 1/ Cycle 1	Step 1	55.0 °C / 05:00
		Segment 2/ Cycle 1	Step 1	95.0°C /10:00
		Segment 3/ Cycle 30	Step 1	95.0 °C /00:30
			Step 2	56.2°C / 01:30
			Step 3	72.0°C / 01:00
2.	NDM[Universal]	F: AGCTCGCACCGAATGTCTG R: TTGATCTCCTGCTTGATCC	172bp	
		Segment 1 / Cycle 1	Step 1	55.0 °C / 06:00
			Step 2	95.0°C /10:00
		Segment 2/ Cycle 10	Step 1	95.0°C /30:00
			Step 2	65.0 °C -1.0°C /00:45 -1s
			Step 3	72.0 °C /00:30
		Segment 3 / Cycle 15	Step 1	95.0°C /00:10
			Step 2	55.0 °C /00:30
			Step 3	72.0 °C /00:15
		Segment 4/ Cycle 1	Step 1	72.0°C /10:00
3.	KPC [Universal]	FP: GTATCGCCGTCTAGTTCTGC RP: GGTCGTGTTCCCTTTAGCC	191 bp	-
		Segment 1/ Cycle 1	Step 1	95.0°C / 10:00
		Segment 2/ Cycle 35	Step 1	95.0 °C / 00:30
			Step 2	55.2° C / 00:45
			Step 3	72° C / 00:10
		Segment 3 / Cycle 1	Step 1	72° C /10:00
4.	IMP [Universal]	F: ACTGTGCGCCATGGAAATGT R: AGGTTGGGGTGTAGTCACAA	169	-
		Segment 1/ Cycle 1	Step 1	50.0° C / 02:00
		Segment 2/ Cycle 1	Step 1	95.0° C / 00:10
		Segment 3/ Cycle 40	Step 1	95° C / 00:15
			Step 2	56° C / 00:45
			Step 3	72° C / 00:10

399 AMR: Antimicrobial resistance, IMP: Imipenem, KPC: Klebsiella pneumoniae carbapenemase, OXA-
400 48: Oxacillinase 48, NDM: New Delhi metallo-beta-lactamase.

401 -----

NDM



402 **Figure 1: Melting curves and amplification details of *New Delhi metallo-beta-***
 403 ***lactamase (NDM), oxacillinase-48 (OXA48) and *Klebsiella pneumoniae****
 404 ***carbapenemase (KPC).***

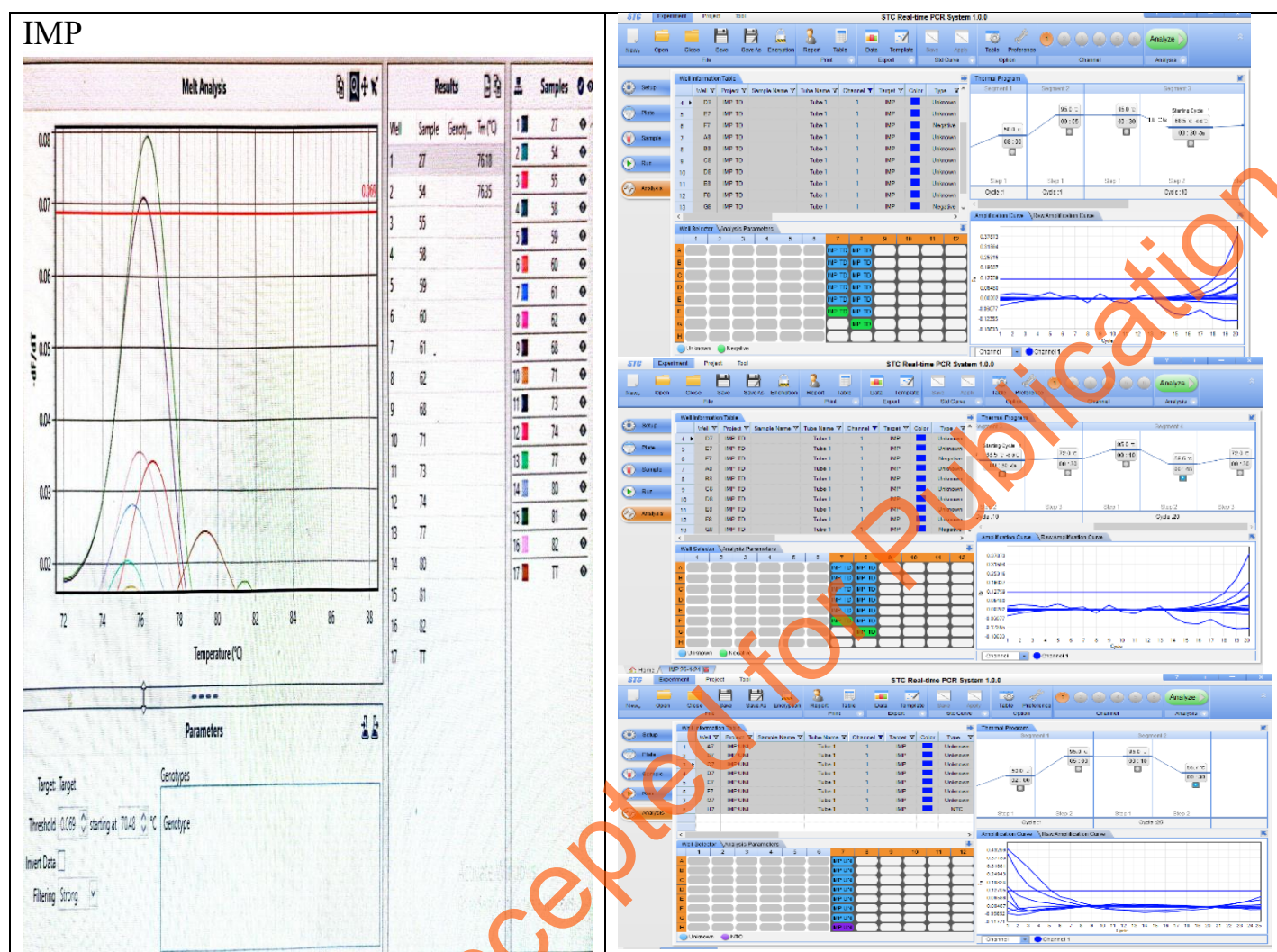


Figure 2: Melting curves and amplification details of imipenem (IMP).

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HZ: Synopsis writing, funding grant proposal write up, conducting entire research, compilation, data analysis and final approval.

IAM: Supervision, funding grant proposal write up, research conduction, compilation, data analysis and final approval.

MF: Supervision, guidance and facilitation for procurement of primers – real time PCR and protocol finalisation.

MAA: Data gathering, records and samples collection.