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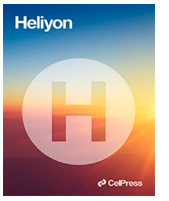

Research article

Loop-mediated isothermal amplification assay detects multiple alleles of *bla*$_{\text{OXA-51-like}}$ genes in *Acinetobacter baumannii* and other Gram-negative bacteria despite primer-template mismatches

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ABSTRACT

The known intrinsic and polymorphic *bla*$_{\text{OXA-51-like}}$ genes of *Acinetobacter baumannii* were recently reported in other non-*A. baumannii* Gram-negative pathogens. Accurate detection of this potentially transferrable carbapenemase gene in the clinical setting is critical. This study developed a loop-mediated isothermal amplification (LAMP) assay targetting multiple alleles of *bla*$_{\text{OXA-51-like}}$ genes. Specifically, an alignment-based primer design, *in silico* primer screening, and *in vitro* assay confirmation were conducted. Both *in silico* and *in vitro* results revealed the tolerance of the LAMP assay to up to five primer-template mismatches outside the 3′-end primer regions. Within 90 min, the LAMP assay also detected the gene targets in other Gram-negative bacteria with known and novel *bla*$_{\text{OXA-51-like}}$ genes. Finally, it showed a superior limit of detection (as low as 10^1 CFU/mL) compared with polymerase chain reaction, and high specificity against non-targets. This study developed a highly adaptable LAMP assay to monitor *bla*$_{\text{OXA-51-like}}$ genes in the clinical setting and provided important insights into LAMP primer design and screening.

1. Introduction

The World Health Organization identified carbapenem-resistant Gram-negative bacteria as critical priority pathogens that pose the greatest threat to humans [1]. Infections caused by these bacteria have limited treatment options, resulting in high hospitalization and mortality rates [2]. The mechanisms of resistance among these pathogens are highly diverse, with the production of carbapenemases as the most prevalent in carbapenem-resistant *Acinetobacter baumannii* (CRAb). CRAb is now a growing concern in the global clinical setting, with up to 90 % prevalence in certain parts of Europe, Asia, and Africa [3]. The global increase in CRAb incidence may be due to the rapid spread of carbapenemase genes across Gram-negative species.

The *bla*$_{\text{OXA-51-like}}$ is a subfamily of chromosomal class D β-lactamase genes in *A. baumannii* that confers significant hydrolyzing

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activities against carbapenems [4]. As an intrinsic gene, it has been used as a target for confirming species identity and investigating CRAb in hospital outbreaks [5,6]. However, in the past years, there have been reports of $bla_{\text{OXA-51-like}}$ in non-*A. baumannii* Gram-negative bacterial species [7–9]. The spread of these genes is due to their association with insertion sequences, which makes them transferrable to other pathogenic Gram-negative species through transposition [10]. Therefore, $bla_{\text{OXA-51-like}}$ may also be used to indicate the spread of antibiotic resistance genes in the hospital and environment.

Within the class D β-lactamase genes, the $bla_{\text{OXA-51-like}}$ subfamily is the biggest, with >370 alleles (http://bldb.eu/accessed on August 1, 2023). The diversity of sequences under this subfamily is due to single-nucleotide polymorphisms (SNPs) dispersed throughout the gene. Hence, when designing molecular probes for nucleic acid amplification tests (NAATs), the high variations among the alleles should be considered. The loop-mediated isothermal amplification (LAMP) assay, a type of NAAT, is dependent on at least six different primers that form loop structures facilitating the subsequent amplification of the target DNA region [11,12]. It is performed using *Bst* polymerase, whose activity depends on the high fidelity of the 3′-ends of the primers serving as the initial point of DNA elongation [13]. Hence, any mismatch on the 3′-end of the primers was believed to result in low amplification efficiency.

The dynamics of the molecular interactions between the LAMP primers and templates are still understudied, with mismatches considered as a culprit for suboptimal assay [14,15]. Unfortunately, the extent of the effect of the mismatches was not thoroughly investigated, especially those outside the 3′-end primer region. In this study, we developed a LAMP assay that detects multiple variants of the $bla_{\text{OXA-51-like}}$ alleles from *A. baumannii* and other Gram-negative species by fine-tuning the parameters of primer design and screening. We also explored the effects of different outer and inner primer mismatches in the performance of the LAMP assay using both *in silico* and *in vitro* experimental models.

2. Materials and methods

2.1. Collection of reference DNA samples

The protocol for this study was acknowledged by The Medical City–Institutional Review Board (GCS-2020-050). All isolates were acquired from the microbial biobank of The Medical City, Philippines. Only microbiological data were collected from the isolates. Genomic DNA samples were isolated using the boil lysis method [16] from clinical *A. baumannii* strains with sequence-confirmed $bla_{\text{OXA-51-like}}$ variants and standard strains of the American Type Culture Collection (ATCC) (*A. baumannii* ATCC BAA-1605 and ATCC 19606). Additional DNA from ATCC and the National Collection of Type Cultures (NCTC) standard multidrug-resistant Gram-negative strains were also obtained. Finally, DNA from three *Klebsiella pneumoniae* and five *Pseudomonas aeruginosa* clinical isolates with previous polymerase chain reaction (PCR)-confirmed $bla_{\text{OXA-51-like}}$ genes were also collected (Supplementary File 1). All DNA samples were checked for quality and quantity using Spectrostar Nano UV–Vis Spectrophotometer (BMG Labtech, Germany).

2.2. Analysis of the $bla_{OXA\text{-}51\text{-}like}$ genes in non-A. baumannii samples

All non-*A. baumannii* DNA samples underwent $bla_{\text{OXA-51-like}}$ PCR with *A. baumannii* ATCC 19606 and *Staphylococcus aureus* ATCC 29213 as positive and negative controls, respectively. PCR assays were conducted in a 25 μL reaction with 12.5 μL of a 2 × GoTaq Green PCR master mix (Promega, Wisconsin, USA), 10 μM of primers [17], at least 25 ng/μL template DNA, and nuclease-free water. The reaction was performed in a GeneExplorer thermal cycler (Bioer, Hangzhou, China) following a published protocol [17]. All PCR assays were conducted in two independent trials. Amplicons were visualized in 2 % agarose gel using MyGel Instaview® gel electrophoresis (Accuris, Kyonggi, Korea). PCR was confirmed by observing amplicons with a size of 825 bp. Gel profiles were presented as raw images and compiled using Microsoft PowerPoint (Microsoft, Washington, USA). The $bla_{\text{OXA-51-like}}$ amplicons generated from the non-*A. baumannii* samples were bidirectionally sequenced using Sanger sequencing (Macrogen, Seoul, Korea) to determine the allelic profiles. Amplicons from *A. baumannii* ATCC BAA-1605 and ATCC 19606 were sequenced as controls. The complete DNA and amino acid sequences were compared with all known $bla_{\text{OXA-51-like}}$ variants using the β-lactamase database-embedded Basic Local Alignment Search Tool (BLDB BLAST; http://www.bldb.eu:4567/, accessed on August 1, 2023) [18]. The recent consensus for β-lactamase nomenclature was followed in naming alleles [19]. All original sequences were deposited in GenBank (https://www.ncbi.nlm.nih.gov/genbank/, accessed on September 5, 2023) with accession numbers OR449063–OR449072.

2.3. LAMP primer design for $bla_{OXA\text{-}51\text{-}like}$ genes

The primer design workflow suggested by Shirshikov et al. was followed with slight modifications [20]. Briefly, the sequences of 34 unique $bla_{\text{OXA-51-like}}$ alleles from our available isolates, together with the native $bla_{\text{OXA-51}}$ gene, were gathered from the GenBank database (Supplementary File 2). The chosen alleles correspond to those that have counterpart samples for the *in vitro* experiments, ensuring accurate confirmation of our *in silico* hypothesis on primer-template mismatch tolerance later on. The sequences were aligned in Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/, accessed on January 14, 2023) using default parameters. The multiple sequence alignment file was then uploaded to MorphoCatcher (http://morphocatcher.ru/, accessed on January 20, 2023) to detect and mark sites with high variations. The generated file was uploaded to PrimerExplorer v.5 (http://primerexplorer.jp/lampv5e/index.html, accessed on January 20, 2023) using AT-rich sequence parameters. The resulting primer sets were checked for the absence of primer secondary structures using the default parameters of Oligo Calculator v.3.27 (http://biotools.nubic.northwestern.edu/OligoCalc.html, accessed on January 25, 2023). The top primer set (TMC6: F3, B3, Forward Inner Primer (FIP) = F2 + F1c, and Backward Inner Primer (BIP) = B2 + B1c) was chosen based on the set parameters. The final primer sequences are TMC6–F3

(5′-GCCAAACTCAACAAAGCTATGG-3′), TMC6–B3 (5′-CGGAATAGCGGAAGCTTTCA-3′), TMC6–FIP (5′-GCTCAAGGCCGATCA AAGCATTAAG–TCGTGCTTCGACCGAGTA-3′), and TMC6–BIP (5′-ACAGAAGTATTTAAGTGGGACGGGC–CCTAGGGTCATGT CCTTTTCC-3′).

2.4. In silico analysis of the LAMP primers

To determine the mismatches of the designed LAMP primer set on the target genes, the primers were aligned to the available $bla_{\text{OXA-51-like}}$ alleles from our reference sequences using Clustal W in Bioedit v.7.2.5. The alignment was visualized as conservation plots. Meanwhile, to determine the initial performance of the chosen primer set, an *in silico* simulation was performed using the electric LAMP program (eLAMP) with modified parameters [14]. The TMC6 primer set and comparable primers from the literature (Primer Sets 1 and 2) [21,22] were compiled into one comma-separated values file. Meanwhile, sequences from 373 known $bla_{\text{OXA-51-like}}$ alleles were compiled into one FASTA file and used as the template for the *in silico* simulation. The simulation was performed using combinations of different primer mismatch parameters (Supplementary File 3). Amplification results were scored from 0 to 3 (0 for no amplification and 1–3 for amplification with one to three matching 3′-end sequences). Scores were visualized as a heatmap using Microsoft Excel (Microsoft, Washington, USA). The *in silico* performance of the TMC6 primer set was compared with Primer Sets 1 and 2 using analysis of variance with Tukey's test in R v.4.3.1.

2.5. LAMP assay development

The LAMP assay was performed in a 25 μL reaction with 12.5 μL of a 2 × DNA Polymerase Buffer B (LGC, Biosearch Technologies, London, UK), 1 μL 100 mM of $MgSO_4$ (New England Biolabs, Massachusetts, USA), 10 mM of a deoxynucleotide triphosphate mix (Vivantis, Selangor, Malaysia), 5 μM of F3 and B3 primers, 12 μM of FIP and BIP, 20–25 ng of template DNA, 8 units of *Bst* DNA Polymerase Exonuclease Minus (LGC, Biosearch Technologies, London, UK), and nuclease-free water. All reference DNA from the isolates with sequence-confirmed $bla_{\text{OXA-51-like}}$ alleles underwent the LAMP assay. DNA from *S. aureus* ATCC 29213 was used as the negative control. All assays were performed in at least two independent trials using a GeneExplorer thermal cycler (Bioer, Hangzhou, China) set at 64 °C for at least 60 min and terminated at 80 °C for 2 min. Amplification products were visualized by adding a 1000 × SYBR Green I dye (Invitrogen, California, USA) to the completed reactions and by 2 % gel electrophoresis, as described earlier. Colorimetric and gel profiles were presented as raw images and compiled using Microsoft PowerPoint (Microsoft, Washington, USA).

2.6. Analytical performance testing of the LAMP assay

The limit of detection (LOD) of the LAMP assay was assessed using different dilutions of *A. baumannii* TMC36 ($bla_{\text{OXA-1071}}$ with no primer–template mismatch) and *A. baumannii* ATCC 1605 ($bla_{\text{OXA-69}}$ with multiple primer–template mismatches) to achieve cell concentrations of 10^0–10^6 colony forming units (CFU)/mL. Serial dilutions were performed from 0.5 McFarland standardized cultures in nuclease-free water, and colony counts were confirmed through plating. DNA samples from each dilution were extracted as described previously and used as templates for both LAMP and PCR assays. The LOD was defined as the lowest tested sample concentration that yielded a positive assay result. Meanwhile, to determine the specificity of the LAMP assay, at least 20–25 ng of DNA from the 15 $bla_{\text{OXA-51-like}}$-negative standard multidrug-resistant Gram-negative strains was used as the templates. LAMP assays were performed as with the previous experiments.

3. Results

3.1. PCR and sequencing confirmed $bla_{\text{OXA-51-like}}$ genes in other Gram-negative clinical isolates

The $bla_{\text{OXA-51-like}}$ PCR analysis revealed the presence of faint amplicon bands in the three *K. pneumoniae* and four *P. aeruginosa* clinical isolates (Supplementary Files 4-5). Sequence analyses of the resulting amplicons confirmed the genes to be known ($bla_{\text{OXA-66}}$, $bla_{\text{OXA-769}}$, and $bla_{\text{OXA-1070}}$) and novel ($bla_{\text{OXA-1217}}$, $bla_{\text{OXA-1218}}$, $bla_{\text{OXA-1219}}$, and $bla_{\text{OXA-1220}}$) alleles (GenBank: OR449065–OR449072). Conversely, all other reference multidrug-resistant, non-*A. baumannii* Gram-negative strains tested negative for the gene (Supplementary Files 4-5). Our controls *A. baumannii* ATCC BAA-1605 and *A. baumannii* ATCC 19606 had the confirmed $bla_{\text{OXA-69}}$ and $bla_{\text{OXA-98}}$ alleles, respectively (GenBank: OR449063–OR449064).

3.2. Primer design workflow generated primers with ideal characteristics

The initial multiple sequence alignment of the 34 reference alleles showed SNPs dispersed throughout the $bla_{\text{OXA-51-like}}$ genes (Supplementary File 6). The MorphoCatcher program determined sites with low average mutational index, chose the best template (Supplementary File 7), and marked the template sequence with informative sites for the primer design. The output from the MorphoCatcher was used in PrimerExplorer v.5, which generated a total of 13 primer designs within the target gene positions (160–700 bp). The top five primers with higher dimerization energies were chosen and screened using the set parameters. The TMC6 primer set was finally selected on the basis of its ideal characteristics (Supplementary File 8).

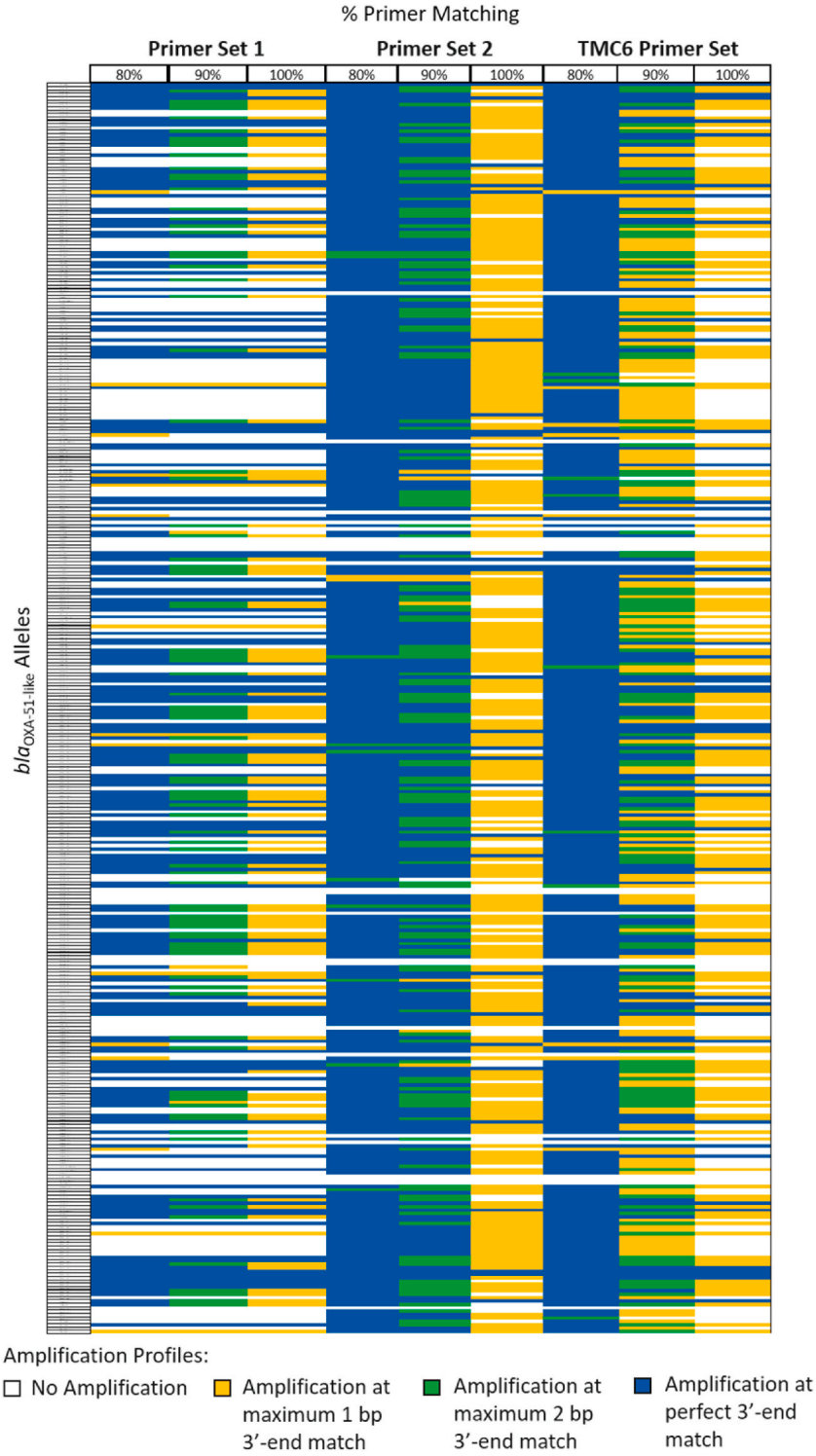

Fig. 1. The heatmap generated from the *in silico* simulation using the eLAMP program indicated the high sensitivity of the TMC6 primer set similar to that of published Primer Set 2 [21] and superior performance of the TMC6 primer set compared with that of published Primer Set 1 [22] at 80 % primer–template matching and complete (three out of three) 3′-end primer fidelity conditions in detecting 373 different known alleles of $bla_{\text{OXA-51-like}}$ genes. Percentage primer matching refers to the primer–template matching rate outside the 3′-end primer regions. The heatmap colors represent the amplification profiles of the primers at set parameters. Unmarked cells refer to the absence of amplification in the eLAMP reaction.

3.3. *In silico* LAMP showed high amplification success rates

The *in silico* LAMP analysis showed that the TMC6 primer set had high mismatch tolerance in detecting various *bla*$_{\text{OXA-51-like}}$ alleles (Fig. 1). The primer set amplified 334 of the 373 alleles with as low as 80 % overall primer matching and exact 3′-end fidelity (Supplementary File 9). The TMC6 primer set was found to have comparable *in silico* performance with that of Primer Set 2 [21] and superior sensitivity compared with that of Primer Set 1 [22] (Fig. 1). These results were confirmed by the statistically insignificant differences (p-values >0.05) between TMC6 primers and Primer Set 2 in all set conditions and a significant difference (p-value <0.001) between Primer Set 1 and the TMC6 primer set at 80 % primer–template matching (Supplementary File 10).

3.4. *LAMP assay confirmed the mismatch tolerance of the designed primers*

The reference DNA samples for the *in vitro* experiments were grouped based on the characteristics of their *bla*$_{\text{OXA-51-like}}$ alleles (Fig. 2). Primer–template mismatches can be found in all primer positions (outer, middle, and inner) and directions (forward and backward), with a higher occurrence of mismatches in the backward primers. None of the outer and middle primers have a 3′-end mismatch with the template, and none of the inner primers have a 5′-end mismatch. The LAMP assay showed positive amplification reactions for all the tested *bla*$_{\text{OXA-51-like}}$ alleles regardless of their primer–template mismatch groupings (Fig. 3; Supplementary File 11). The efficiency of the LAMP reaction is not dependent on the number of mismatches as most of the representatives have close band intensity profiles regardless of the grouping and at almost similar starting DNA template concentrations (20–25 ng/μL).

3.5. *LAMP assay detected bla*$_{\textit{OXA-51-like}}$ *alleles in other Gram-negative bacteria*

Using the grouping in Fig. 2, the *bla*$_{\text{OXA-51-like}}$ alleles of the clinical *K. pneumoniae* and *P. aeruginosa* isolates were identified to be

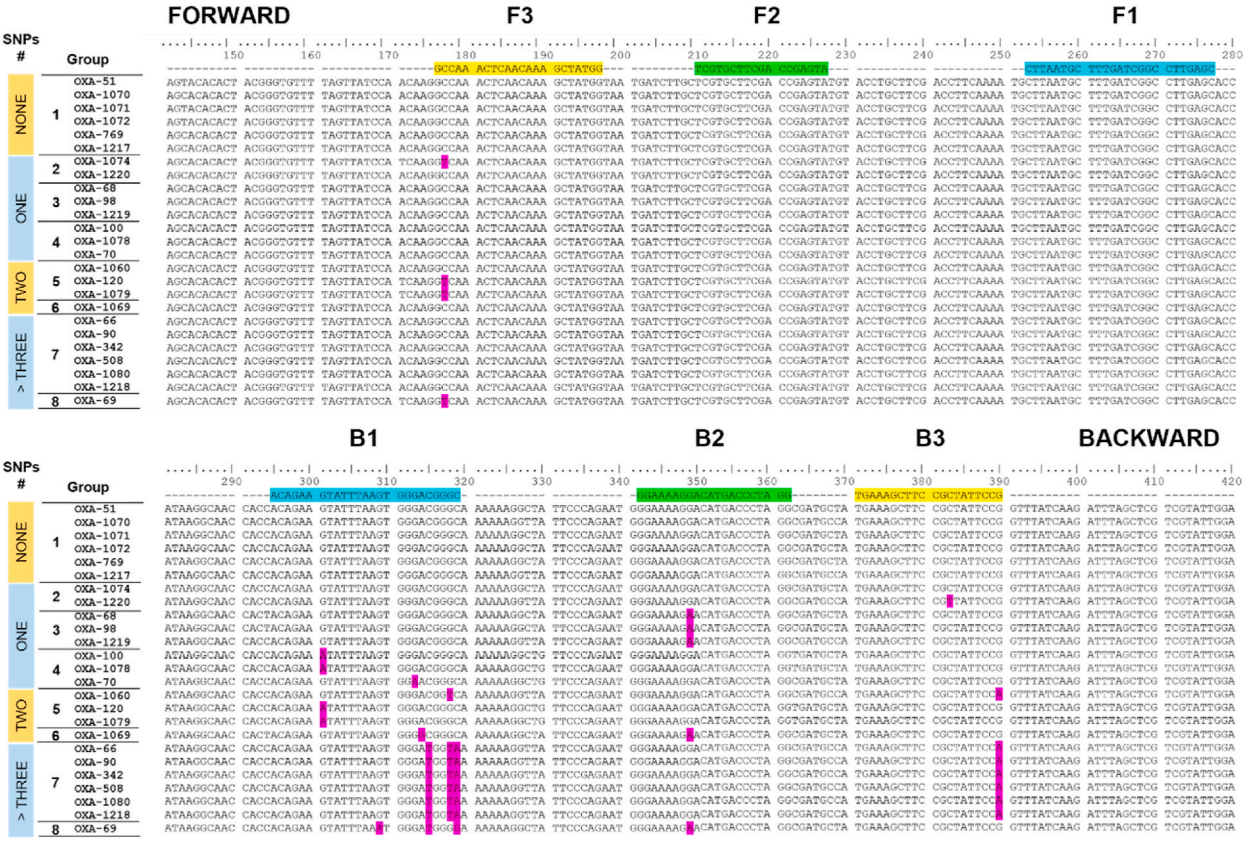

Fig. 2. The primer binding position map of the TMC6 primer set showed single-nucleotide polymorphisms (SNPs) or false priming sites (purple) in most of the target genes tested. Outer (yellow), middle (green), and inner (blue) binding sites are shown for both forward (F1–F3) and backward (B1–B3) primers. The number of SNP mismatches between the primer and template ranges from zero to five. Groups 1, 2, 3, and 4 refer to targets with no false priming sites (no mismatch), those with one outer primer mismatch, those with one middle primer mismatch, and those with one inner primer mismatch, respectively. Groups 5 and 6 refer to targets with one outer and one inner primer mismatches and those with one middle and one inner primer mismatches, respectively. Group 7 are targets with one outer and three inner primer mismatches (for a total of four). Finally, Group 8 is a target with one outer, one middle, and three inner primer mismatches (for a total of five).

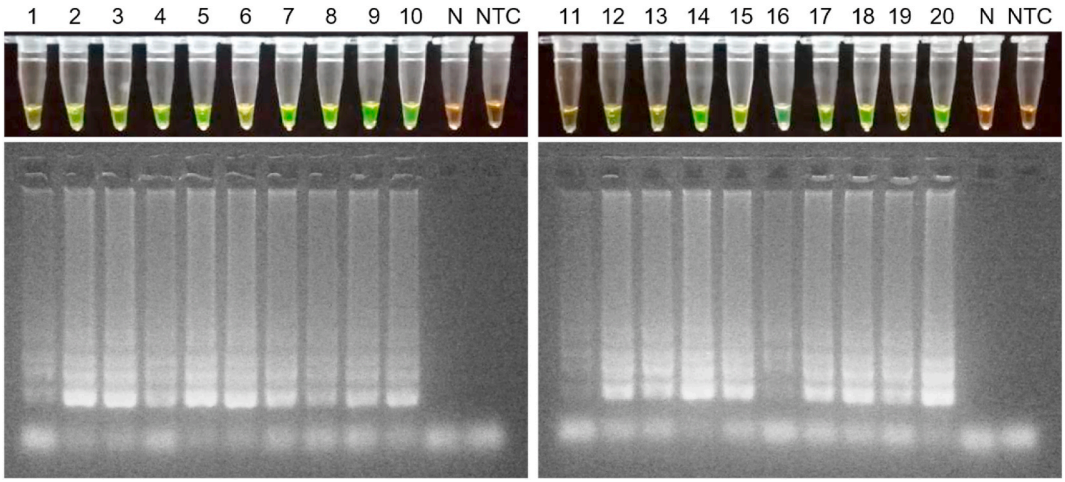

Fig. 3. The LAMP assay showed positive detection (as a green fluorescence and ladder-like gel profile) of the $bla_{\text{OXA-51-like}}$ genes in all reference *A. baumannii* samples regardless of the target allele. Groups 1 (lanes 1–3: $bla_{\text{OXA-1070}}$, $bla_{\text{OXA-1071}}$, and $bla_{\text{OXA-1072}}$), 2 (lane 4: $bla_{\text{OXA-1074}}$), 3 (lanes 5–7: $bla_{\text{OXA-68}}$ and two $bla_{\text{OXA-98}}$), 4 (lanes 8–10: $bla_{\text{OXA-70}}$, $bla_{\text{OXA-100}}$, and $bla_{\text{OXA-1078}}$), 5 (lanes 11–13: $bla_{\text{OXA-120}}$, $bla_{\text{OXA-1060}}$, and $bla_{\text{OXA-1079}}$), 6 (lane 14: $bla_{\text{OXA-1069}}$), 7 (lanes 15–19: $bla_{\text{OXA-66}}$, $bla_{\text{OXA-90}}$, $bla_{\text{OXA-342}}$, $bla_{\text{OXA-508}}$, and $bla_{\text{OXA-1080}}$), and 8 (lane 20: $bla_{\text{OXA-69}}$) alleles with different levels of primer–template mismatch showed almost similar levels of amplification in the LAMP assay. N, negative control (*S. aureus* ATCC 29213); NTC, no-template control (nuclease-free water). Raw images are provided as Supplementary File 11.

under Groups 1 (bla_{OXA769}, $bla_{\text{OXA-1070}}$, and $bla_{\text{OXA-1217}}$), 2 ($bla_{\text{OXA-1220}}$), 3 ($bla_{\text{OXA-1219}}$), and 7 ($bla_{\text{OXA-66}}$ and $bla_{\text{OXA-1218}}$). These alleles were successfully detected in the LAMP assay as well. However, the LAMP products from the *K. pneumoniae* and *P. aeruginosa* DNA templates were significantly detected only after at least 90 min of reaction time (compared with only 60 min in the initial assays, Fig. 4A and B; Supplementary File 12). The intensity and sizes of the LAMP products are not dependent on the number of primer–template mismatches.

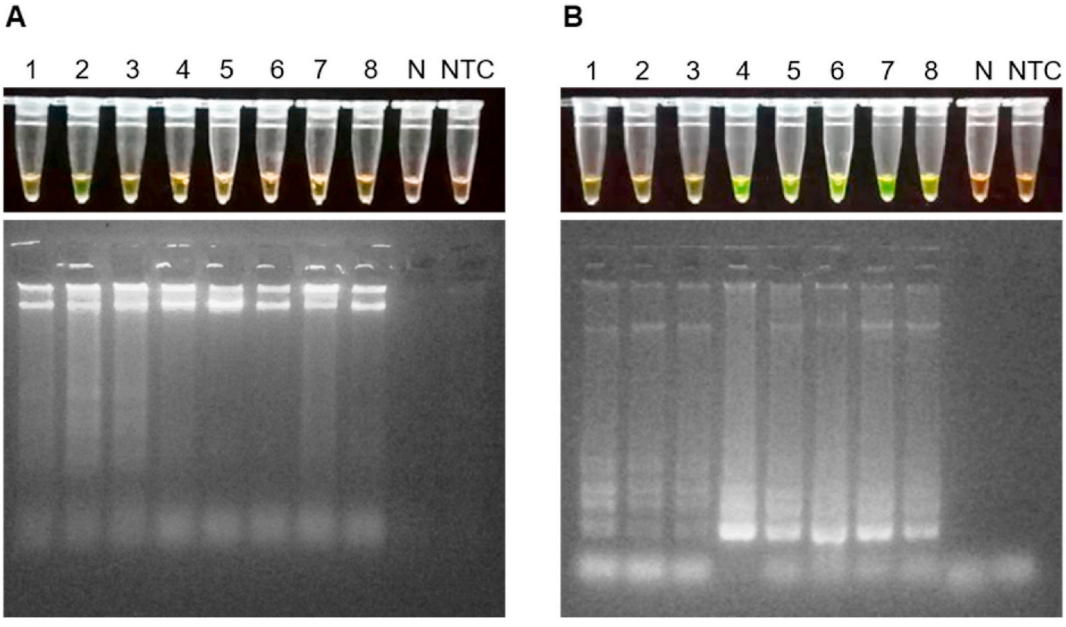

Fig. 4. The LAMP assay showed detectable positive results (as a green fluorescence and ladder-like gel profile) for $bla_{\text{OXA-51-like}}$ genes in non-*A. baumannii* clinical samples after a 90-min reaction time regardless of the target allele. (A) The standard 60-min LAMP assays showed minimal to no positivity for all samples in colorimetric and gel electrophoresis profiles. (B) The positivity was enhanced when the assays were extended to 90 min. Lanes 1–3: *K. pneumoniae* TMCK01, TMCK03, and TMCK04 with $bla_{\text{OXA-1217}}$, $bla_{\text{OXA-769}}$, and $bla_{\text{OXA-66}}$, respectively. Lanes 4–8: *P. aeruginosa* TMCP02, TMCP03, TMCP04, TMCP12, and TMCP14 with $bla_{\text{OXA-1070}}$, $bla_{\text{OXA-1218}}$, $bla_{\text{OXA-1219}}$, $bla_{\text{OXA-1220}}$, and $bla_{\text{OXA-66}}$, respectively. N, negative control (*S. aureus* ATCC 29213); NTC, no-template control (nuclease-free water). Raw images are provided as Supplementary File 12.

3.6. The developed LAMP assay has a superior LOD than PCR and high specificity

The LAMP assay showed robust detection of the $bla_{\text{OXA-51-like}}$ genes in *A. baumannii* in as low as 10^1 CFU/mL (Fig. 5A; Supplementary File 13). This observation is true whether there is no primer–template mismatch ($bla_{\text{OXA-1071}}$) or in templates with up to five mismatches ($bla_{\text{OXA-69}}$; Supplementary Files 14-15). Conversely, PCR was only able to detect the targets at 10^6 CFU/mL cell concentration. Regarding specificity, the LAMP assay did not produce any spurious LAMP products when tested against the $bla_{\text{OXA-51-like}}$-negative standard Gram-negative strains (Fig. 5B).

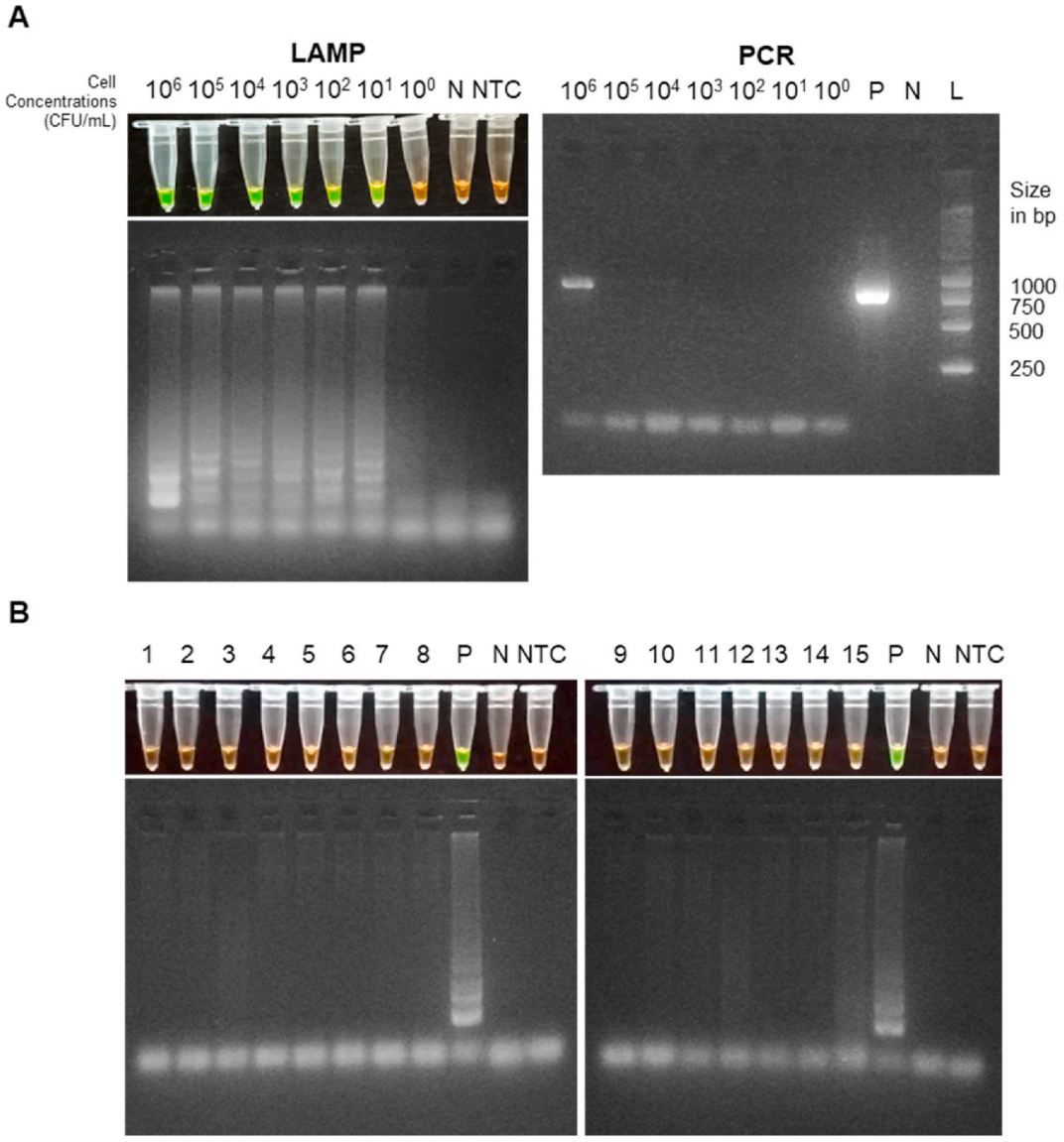

Fig. 5. The LAMP assay detected (as a green fluorescence and ladder-like gel profile) the different allele targets in as low as 10^1 CFU/mL cell concentration (vs. 10^6 CFU/mL limit of detection for PCR) and no spurious amplification in templates without the $bla_{\text{OXA-51-like}}$ targets. (A) The LAMP assay showed a lower limit of detection compared with PCR regardless of no (*A. baumannii* TMC36 with $bla_{\text{OXA-1071}}$) or up to five (*A. baumannii* ATCC BAA-1605 with $bla_{\text{OXA-69}}$; Supplementary File 14) mismatches in the target template. The cell concentrations used are 10^6 to 10^0 CFU/mL as confirmed from plate counts. (B) Specificity testing showed that the LAMP assay did not produce false-positive results when tested against other multidrug-resistant Gram-negative pathogens with no $bla_{\text{OXA-51-like}}$ genes based on PCR (lanes 1–8: *K. pneumoniae* ATCC 700603, ATCC 13883, ATCC BAA-1705, BAA-2146, NCTC 13438, NCTC 13439, NCTC 13440, NCTC 13442; lane 9: *En. cloacae* ATCC BAA-1143; lanes 10–14: *Es. coli* ATCC 35218, ATCC BAA-2469, NCTC 13353, NCTC 13476, NCTC 13846; lane 15: *P. aeruginosa* ATCC 27853). For the LAMP gel profiles: N, negative control (*S. aureus* ATCC 29213); NTC, no-template control (nuclease-free water); P, positive control (*A. baumannii* ATCC 19606). For the PCR gel profiles: L, 1-kb DNA ladder; P, positive control (*A. baumannii* ATCC 19606); N, negative control (*S. aureus* ATCC 29213). The expected $bla_{\text{OXA-51-like}}$ amplicon size is 825bp. Raw images are provided as Supplementary Files 13 and 15.

4. Discussion

NAATs play an important role in detecting and monitoring antibiotic resistance in healthcare and community settings. LAMP is considered among the promising NAATs due to its high adaptability and accuracy [23]. However, it can be challenging to design primers that will ensure high sensitivity and specificity, especially for highly polymorphic targets like most antibiotic resistance genes. The conventional LAMP primer design promised versatility in terms of targeting common sites in different sequences [12]. However, limitations exist in using large datasets as target references and in setting limits on the number and positions of allowable mismatches. As multiple variants of the *bla*$_{\text{OXA-51-like}}$ genes exist, we developed a LAMP assay that could detect different alleles of the genes even in the presence of up to five mismatches in the primer binding regions. The developed assay was also found to detect the presence of the gene when acquired by other Gram-negative bacteria.

The presence of *bla*$_{\text{OXA-51-like}}$ genes from non-*A. baumannii* species was reported in *Acinetobacter nosocomialis* [24], Enterobacterales [8,25], and *P. aeruginosa* [9] in the last 15 years. Our study showed the presence of the same genes in clinical *K. pneumoniae* and *P. aeruginosa* isolates. More importantly, we showed that four of the detected genes correspond to new alleles (*bla*$_{\text{OXA-1217}}$ to *bla*$_{\text{OXA-1220}}$). Our results provide preliminary evidence on the potential cross-species transfer of the intrinsic *bla*$_{\text{OXA-51-like}}$ carbapenemase genes among non-*A. baumannii* Gram-negative clinical isolates in a Philippine hospital setting. The presence of these genes in other species as a transferrable element may further spread the carbapenem resistance phenotype, eventually rendering β-lactam antibiotics ineffective in several Gram-negative pathogens [26]. Although only four of the non-*A. baumannii* isolates reported are carbapenem-resistant, the presence of the carbapenemase genes gives the pathogens the potential to become resistant later. Herein, we propose *bla*$_{\text{OXA-51-like}}$ genes to be important markers in monitoring the spread of carbapenem resistance in Gram-negative pathogens.

LAMP assay development starts by understanding the peculiarities of primer design. Although targeting conserved gene regions is straightforward in PrimerExplorer v.5, using multiple representative sequences for highly polymorphic targets can be challenging. In the original design manual, uploading multiple alignment sequences is possible, but only limited to up to 2000 bp of input sequence. The workflow used in our study addressed this limitation using Clustal Omega, which allows for unlimited sequence length input [20]. Additionally, the MorphoCatcher program allowed for the creation of an annotated input file that can be interpreted by PrimerExplorer v.5 [20]. This combination provided an efficient method of representing multiple variations of polymorphic genes as input sequences for primer design.

Technologists only rely on empirical testing of multiple primer sets to decide which primers will work [27]. The eLAMP program solved this limitation by allowing the simulation of different primer–template conditions and predicting LAMP amplification with allowance for mismatches [14]. The program could be a crude way to test the initial sensitivity of the primers against variants of polymorphic targets. Our results indicate that the assay could tolerate up to 20 % mismatch (up to 4-bp mismatch per primer, assuming an average primer length of 20 bp) *in silico*. We hypothesize that these *in silico* conditions could provide good estimates of the allowable number of mismatches for LAMP primer design, as also supported by another study [14]. Given its potential predictive power, the eLAMP results can be used to screen for the best primer to synthesize, eventually saving both time and resources for technologists developing LAMP assays.

The eLAMP analysis revealed a high sensitivity of the TMC6 primer set to at least 89.5 % of all known *bla*$_{\text{OXA-51-like}}$ alleles. This performance is comparable with that of Primer Set 2 [21]. The difference lies in considering multiple sequence alignment data in our primer design workflow, resulting in an additional 18 *in silico* amplified targets with exact primer–template matches and 3′-end fidelity. Conversely, Primer Set 1 [22] showed a more selective *in silico* amplification compared with the TMC6 primer set. A quick evaluation of their primers showed nonideal primer characteristics such as low GC rates of F3 and BIP (35%–38 %) and the presence of potential primer secondary structures. This observation highlights the importance of evaluating individual primer characteristics before choosing the best set for the assay.

To determine if the eLAMP results complement the actual LAMP assay, DNA templates with different variants of the *bla*$_{\text{OXA-51-like}}$ alleles were used. Our results showed that the developed LAMP was able to tolerate up to a total of five primer mismatches if the 3′-ends of the individual primers were exactly matching. Previous studies hypothesized that general LAMP primer mismatches may result in nonamplification of the target [14,28]. However, this hypothesis was not supported by *in vitro* data. Our results add to the limited experimental evidence showing mismatch tolerance of 2–3 bp in middle primers and at the 5′-ends [15,29]. Our study provided evidence that mismatches at the 5′-ends of the outer primers (F3 and B3) and middle positions within BIP (B1c and B2) are tolerated under the standard assay. Therefore, technologists may add these considerations in primer design workflows, making allowance for mismatches, especially for highly polymorphic antibiotic resistance gene targets. Our *in vitro* results supported the *in silico* results, showing up to approximately 20 % primer–template mismatch tolerance (outside the 3′-end) of the LAMP assay.

Previous LAMP assays targeting *bla*$_{\text{OXA-51-like}}$ genes were used for rapid *A. baumannii* detection and investigations of hospital outbreaks [21,22]. However, none of these assays has been tested in detecting the transferrable gene in non-*A. baumannii* isolates or in novel *bla*$_{\text{OXA-51-like}}$ alleles. Our developed LAMP assay addressed these gaps by detecting the target genes in *K. pneumoniae* and *P. aeruginosa,* and four novel *bla*$_{\text{OXA-51-like}}$ alleles (*bla*$_{\text{OXA-1217}}$ to *bla*$_{\text{OXA-1220}}$). However, longer assay times are necessary for properly detecting the genes in non-*A. baumannii* samples. One possible explanation is the potentially low initial quantity of the mobile genetic elements carrying the *bla*$_{\text{OXA-51-like}}$ genes in non-*A. baumannii* isolates. Our PCR results support this hypothesis by showing only faint target amplicons for the tested *K. pneumoniae* and *P. aeruginosa*. Increasing the LAMP reaction time to 90 min provided an advantage in preventing false-negative or inconclusive results. A 90-min LAMP assay has been described in the literature for testing targets in mobile genetic elements from crude DNA samples [30].

Our developed LAMP assay showed superior LODs compared with PCR, making it more sensitive in detecting the target antibiotic resistance genes even at low concentrations [31]. Additionally, no spurious amplification was detected in DNA samples from other

multidrug-resistant Gram-negative pathogens without the target gene, suggesting high specificity [32]. The rapid nature of the reaction (relative to its PCR counterpart), coupled with high sensitivity and specificity, makes the developed LAMP assay ideal in fast-paced epidemiological and clinical investigations adaptable to low-resource clinical settings.

Overall, this study presented limitations. First, we only used selected reference sequences for the primer design. The chosen alleles correspond to those that have counterpart samples for the *in vitro* experiments, ensuring accurate confirmation of our *in silico* hypothesis on primer-template mismatch tolerance. Second, only a few non-*A. baumannii* strains with $bla_{\text{OXA-51-like}}$ genes were used for additional *in vitro* testing. Despite this low sample availability, our study provided initial evidence of the potential transferability of the $bla_{\text{OXA-51-like}}$ genes in non-*A. baumannii* Gram-negative pathogens, which could be further studied in future investigations. Finally, only limited DNA samples possessing different alleles with primer-template mismatches were used in the *in vitro* experiments. The meticulous primer design workflow, extensive complementary *in silico* analysis, and confirmatory *in vitro* experimental design helped supplement this limitation.

5. Conclusions

Our results imply the need for more efforts to monitor transferrable resistance genes. Poor resistance detection and control may ultimately result in increased resistance rates and improper patient management. The developed LAMP assay is useful in detecting multiple alleles of the $bla_{\text{OXA-51-like}}$ genes, which are emerging to be important markers for the spread of antibiotic resistance. Meanwhile, the LAMP assay development workflow described herein also provided important insights into mismatch considerations, which can be used in designing assays against other polymorphic resistance gene targets. Future studies may delve into designing closed-tube (minimizing potential cross-contamination of products), or allele-specific LAMP assays for other applications.

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Data availability statement

All data are provided within the manuscript and its supplementary files. All original annotated sequences have been deposited in the GenBank database under the accession numbers OR449063–OR449072.

Ethics declaration statement

This study was reviewed and acknowledged by The Medical City Institutional Review Board, with a research registry number: GCS-2020-050. Full review and/or approval by the ethics committee was not needed for this study because only samples stored in the hospital biobank, without any patient-related data, were used for the experiments and analyses.

CRediT authorship contribution statement

Mark B. Carascal: Writing – review & editing, Writing – original draft, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Lawrence S. Macalalad:** Methodology, Investigation, Formal analysis, Data curation. **Joy Ann Petronio-Santos:** Validation, Supervision, Resources, Formal analysis, Conceptualization. **Raul V. Destura:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Conceptualization. **Windell L. Rivera:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

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

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