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Optimization and Utilization of Loop-Mediated Isothermal Amplification (LAMP) Assay to detect the blaOXA-23 gene in carbapenem-resistant *Acinetobacter baumannii, Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*

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ABSTRACT

Background: *Carbapenem-resistant Klebsiella pneumoniae (CRKP), carbapenem-resistant Pseudomonas aeruginosa (CRPA), and carbapenem-resistant Acinetobacter baumannii (CRAB) have become endemic in Southern Europe and elsewhere and has been recognized as a global health phenomenon because some of these strains have acquired broad spectrum antibiotic resistance. This study optimized a loop-mediated isothermal amplification (LAMP) assay in the detection of the blaOXA-23 gene, which are critical components of carbapenem resistance.*

Methodology: *Fourteen (14) strains CRPA, CRAB, and CRKP, were used in this study. Then the strains positive for target genes using conventional PCR were then further investigated using LAMP assay. The optimal primer ratio and reaction temperature were determined by using five strains of CRKP, three strains of CRPA and one strain of CRAB that were pre-determined positive for blaOXA-23 gene using conventional PCR.*

Results: *In this study, the detection of the blaOXA-23 gene for CRKP, CRPA, and CRAB is optimal at 61°C. Since only two primer preparations were compared in this study, it is safe to say that the 1:4 primer ratio gives better visual results than the 1:1 primer ratio. Manipulation of the temperatures and primer ratios lead us to the conclusion that changes in these factors did increase reaction speed and improve sensitivity, thus presenting optimization. With all things considered, the study presents that the antimicrobial resistance gene marker, blaOXA-23, was best detected using the LAMP assay when a temperature of 61°C and primer ratio of 1:4 was utilized.*

Conclusion: *The results demonstrate the usefulness of LAMP techniques for the diagnosis of CRKP, CRPA, and CRAB that are positive for blaOXA-23. The LAMP assay is a sensitive, rapid, and practical method in the detection of blaOXA-23.*

Keywords
*bla*OXA-23, *bla*IMP-1, Loop-mediated isothermal amplification, Polymerase chain reaction, Carbapenem-resistant.

Introduction
Multi-drug resistance has been recognized as a global phenomenon by the World Health Organization. Antimicrobial drugs are now losing effectivity against microorganisms, and the sad truth is that there is little to no investment in the development of new antimicrobials and new diagnostic methods to detect multi-drug resistant (MDR) bacteria rapidly. In the treatment of MDR gram-negative bacteria, it is considered that carbapenem is highly important [1]. The most common mechanism on how gram-negative bacteria develop resistance to carbapenem is by producing carbapenemase hydrolyzing class-D β-lactamases (CHDLS) [2]. The gram-negative bacteria *Pseudomonas aeruginosa* (*P. aeruginosa), Acinetobacter baumannii (A. baumannii),* and *Klebsiella pneumoniae*, *(K. pneumoniae),* pathogens that are commonly responsible for nosocomial infections, are frequently observed to be resistant to carbapenem which raises the problem in therapeutic options for patients [1]. The bla_{OXA-23} gene is most commonly responsible for the resistance to imipenem, especially *A. baumannii* [2]. According to Wang [3], nosocomial outbreaks in different parts of the world, including Brazil, Columbia, the UK, Korea, Tahiti in France, and China, are due to the OXA-23 producers. The first member of the IMP family found in Europe was in an *A. baumannii* isolate from Italy [4]. It was the beginning that the IMP family has been found throughout the world, with the most recent spread to the United States and Australia [5,6].

The gold standard in detecting multi-drug-resistant bacteria is the conventional Polymerase Chain Reaction (PCR). This method, however, requires non-portable laboratory instruments and entails more costly and time-consuming thermocycling protocols. The alternative Loop-Mediated Isothermal Amplification (LAMP) technique, on the other hand, offers a more uncomplicated but more rapid gene detection protocols. This study optimized *bla*OXA-23 LAMP reaction using the strains of CRKP, CRPA, and CRAB.

Methodology
Collection of Bacterial Isolates
The fourteen (14) non-duplicate bacterial strains used in this study that includes five CRAB, five CRKP, and four CRPA, which were collected from two selected tertiary hospitals in Metro Manila, Philippines (Table 2). The information for the sources of clinical isolates was limited to age, gender, room assignments, and the source of specimens as to anatomic sites. The sampling sites have previously identified these up to species level and determination of antimicrobial resistance profile using the VITEK ® 2 system. The panel of antibiotics was based on the Clinical and Laboratory Standards Institute (CLSI) 2018 guidelines. Once identified as CRKP, CRPA, and CRAB, these strains were collected and included in this study. The collection of clinical isolates has been approved by the Ethics and Research Board of the sampling sites. For the storage and transport, the bacterial strains were inoculated in nutrient agar for transport. The protocol from the Guidance on Regulations for the Transport of Infectious Substances (2013-2014) published by WHO in 2012 was followed [7]. The bacterial strains were then sub-cultured and purified using MacConkey agar plates and incubated for at least 24 hours at 37ºC [8]. Pure isolates were cultured in 8 ml Tryptic Soy Broth media for 18-24 hours at 35-37ºC, and DNA extraction was conducted.

Molecular Assay
DNA Extraction
All fourteen (14) bacterial strains were tested using conventional PCR for the detection of bla_{OXA-23} and bla_{IMP-1}. The DNA extraction was conducted using the Macherey-Nagel NucleoSpin® Microbial DNA Kit following the manufacturer's protocol [9]. The nucleic acid purity and concentration of all DNA extract were spectrophotometry analyze using Thermo Scientific µDrop™ Plate.

Detection of *bla*OXA-23 and *bla*IMP-1 using conventional PCR
Prior to LAMP techniques, the presence of *bla*OXA-23 and *bla*IMP-1 in 14 strains was detected using conventional PCR. The DNA extracts were then amplified using BIO-RAD T100™ Thermal Cycler. The extracted DNA was amplified using the KAPA Taq ReadyMix PCR kit [10]. The following primers were used to amplify the target genes: forward primer 5'- TGAGCAAGTTATCTGTATTC-3' and reverse primer 5'- TTAGTTGCTTGGTTTTGATG-3' for *bla*IMP-1 (740 bp) [11]. The forward primer for *bla*OXA-23-like is 5'-GATCGGATTGGAGAACCAGA-3' and the reverse primer is 5'- ATTTCTGACCGCATTTCCAT-3' (501 bp) (see Table 1) [12]. The amplification of the DNA extracts was based on the cycling conditions for amplifying DNA from the protocol of KAPA Taq ReadyMix but with optimization of the annealing temperature based on the melting temperature of the forward and reverse primer [10]. Gel electrophoresis was done using the Cleaver SCIENTIFIC™ Electrophoresis machine. One kb DNA ladder, 1% agarose gel, 1x TAE buffer, Gel Red™ Dye, and 6x VIVANTIS™ loading dye was used.

Detection of *bla*OXA-23 using LAMP Assay
None of the strains tested positive for *bla*IMP-1 using PCR, whereas, nine strains tested positive for *bla*OXA-23 using PCR were then further tested for the presence of *bla*OXA-23 using LAMP technique. The optimization of *bla*OXA-23 using the LAMP technique was done using Five (5) strains of carbapenem-resistant *K. pneumoniae* (CRKP), three (3) strains of carbapenem-resistant *P. aeruginosa* (CRPA), and one (1) strain of carbapenem-resistant *A. baumannii* (CRAB). The LAMP assay methodology was based on a study done by Nicolasora et al. (2014) with minor modifications [13]. In the blaOXA-23 detection, the LAMP primers by Kim et al. (2015) was used (Table 2) [14]. Instead of using electrophoresis, the LAMP assay was viewed with fluorescence. Gel Red™ Dye was used [13].

Table 1. Primers used in the study

Assay	Target Gene	Primer Name	Sequence (5' to 3')	Sources
PCR	*bla*OXA-23	F	5'-GATCGGATTGGAGAACCAGA-3'	[12]
		R	5'- ATTTCTGACCGCATTTCCAT-3'	
	*bla*IMP-1	F	5'- TGAGCAAGTTATCTGTATTC-3'	[11]
		R	5'- TTAGTTGCTTGGTTTTGATG-3'	
LAMP	*bla*OXA-23	FIP	CGCGGCTTAGAGCATTACCATATAG – F1C GAAAAAAACACCTCAGGTGTGCTGG F2	[14]
		BIP	TGTTGAATGCCCTGATCGGATTGG – B1C GACCTTTTCTCGCCCTTCCATTTAAAT B2	
		F3	AACCCCGAGTCAGATTGTTCAAGG	
		B3	GCTTCATGGCTTCTCCTAGTGTC	

Legend: *bla*OXA-23=oxacillinase, *bla*IMP-1= imipenemase, pcr = polymerase chain reaction, LAMP= loop-mediated isothermal amplification

Optimization of *bla*OXA-23 LAMP reaction

The optimal primer ratio and reaction temperature were determined by using five strains of CRKP, three strains of CRPA and one strain of CRAB that were pre-determined positive for *bla*OXA-23 gene using conventional PCR.

The temperature for which Bst polymerase is known to amplify between 57°C and 65°C [15]. In determining which temperature allows for optimum amplification, the LAMP reaction was observed at 57°C, 61°C, and 63°C. The primer ratio is known to affect the LAMP reaction; therefore, the ideal primer ratios were determined. The effects of primer ratios (1:1 and 1:4) in the LAMP reaction were noted to select the ideal and optimal ratio. PCR grade water was used as a negative control.

Results and Discussion

Polymerase Chain Reaction

In the study performed, 9 (64.29%) out of 14 bacterial strains were tested positive for bla_{OXA-23} (501bp) (Figures 1 and 2). Whereas no bacterial strains tested positive for *bla*IMP-1 using PCR, as seen in figure 3. These results indicate that the acquisition of *bla*IMP-1 may not play a major role in carbapenem resistance in the Philippines.

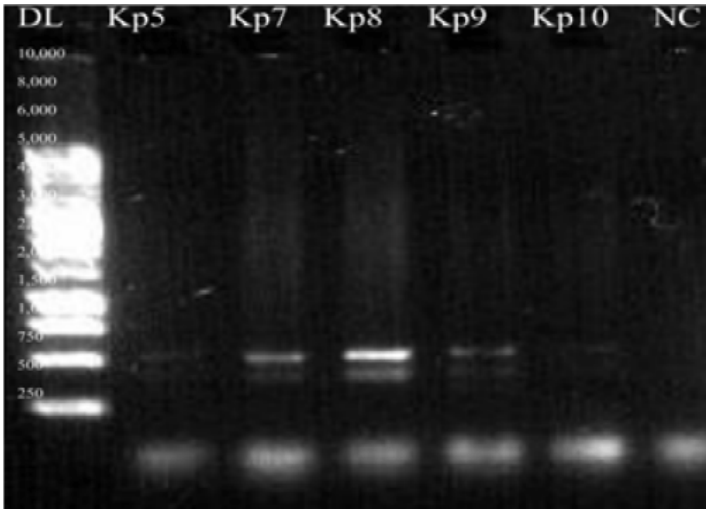


Figure 1

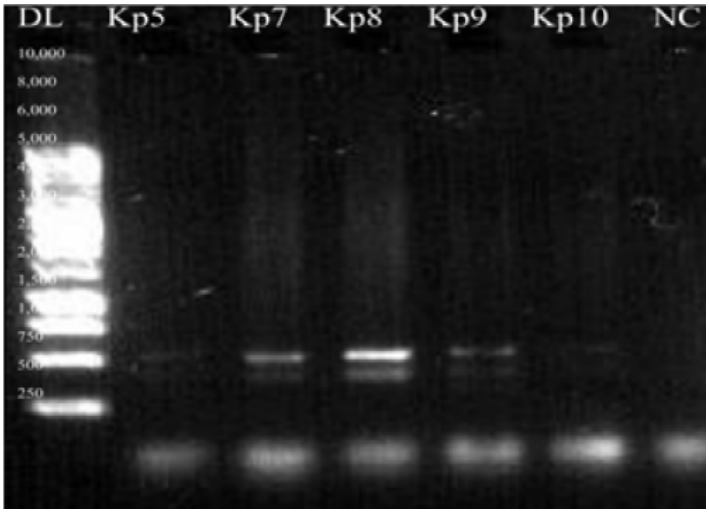


Figure 2

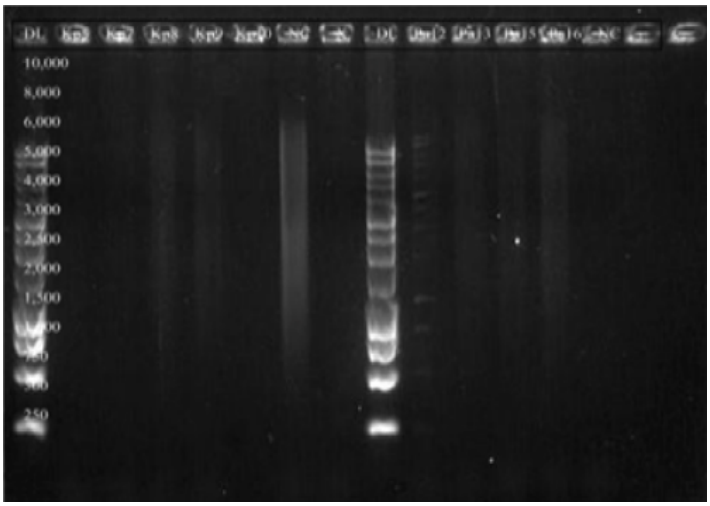


Figure 3

Loop-Mediated Isothermal Amplification Techniques

The detection of the bla_{OXA-23} gene in CRKP was optimized at 61°C, being equally sensitive, whether using a 1:1 or 1:4 primer ratio. However, if temperatures of 57°C and 63°C were to be used, acquiring more visible results is made possible by optimizing the primer ratio to 1:4. On the other hand, CRPA was optimized at a temperature of 61°C and 63°C, being equally sensitive, whether with the use of a 1:1 or 1:4 primer ratio. However, if a temperature of 57°C is used, a primer ratio of 1:4 would still acquire more visible results. Lastly, CRAB was optimized at a temperature of 61°C and 63°C, being equally sensitive, whether with the use of a 1:1 or 1:4 primer ratio. However, using a temperature of 57°C, a primer ratio of 1:4 still acquired more visible results (Figures 4,5, and 6).

This study shows that the detection of the *bla*OXA-23 gene for CRKP, CRPA, and CRAB is optimal at 61ºC. Since only two primer preparations were compared in this study, it is safe to say that the 1:4 primer ratio gives better visual results than the 1:1 primer

Table 2: Bacterial Strains from the Sampling Sites and the Results of conventional PCR

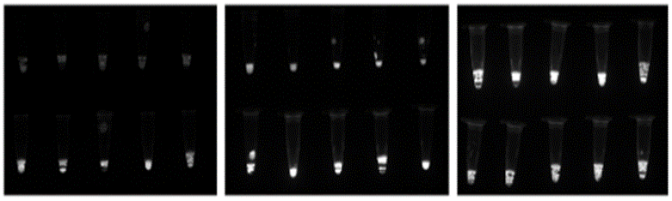
Sampling Sites	Date of Collection	Bacterial Strains	Sample Code	*bla*OXA-23	*bla*IMP-1
Hospital 1	June 2018	*Carbapenem-resistant Acinetobacter baumannii* (CRAB)	Ab8	+	-
Hospital 1	April 2018		Ab9	-	-
Hospital 2	September 2018		Ab11	-	-
Hospital 2	September 2018		Ab12	-	-
Hospital 2	October 2018		Ab13	-	-
Hospital 2	April 2018	*Carbapenem-resistant Klebsiella pneumoniae* (CRKP)	Kp5	+	-
Hospital 2	September 2018		Kp7	+	-
Hospital 2	September 2018		Kp8	+	-
Hospital 1	November 2018		Kp9	+	-
Hospital 1	November 2018		Kp10	+	-
Hospital 2	December 2018	*Carbapenem-resistant Pseudomonas aeruginosa* (CRPA)	Pa12	+	-
Hospital 2	December 2018		Pa13	-	-
Hospital 2	January 2019		Pa15	+	-
Hospital 1	January 2019		Pa16	+	-

Legend: *bla*OXA-23=oxacillinase, *bla*IMP-1= imipenemase, pcr = polymerase chain reaction, LAMP= loop-mediated isothermal amplification, *Ab= Acinetobacter baumannii, Kp= Klebsiella pneumoniae, Pa= Pseudomonas aeruginosa, CRAB = Carbapenem-resistant Acinetobacter baumannii,* CRKP = *Carbapenem-resistant Klebsiella pneumoniae, CRPA = Carbapenem-resistant Pseudomonas aeruginosa*

Table 3: Bacterial Strains from the Sampling Sites and tested positive for *bla*OXA-23 using PCR and its results using LAMP Techniques

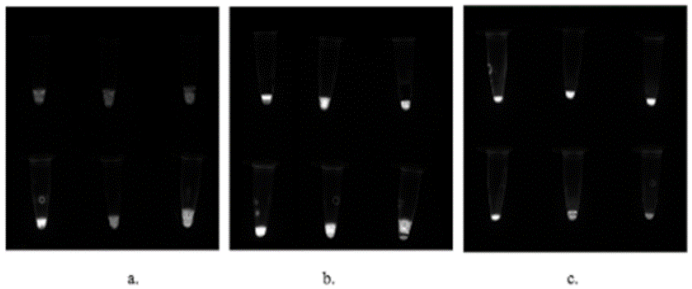
Sampling Sites	Date of Collection	Bacterial Strains	Sample Code	*PCR bla*OXA-23	*LAMP blaOXA-23*
Hospital 1	June 2018	*Carbapenem-resistant Acinetobacter baumannii* (CRAB)	Ab8	+	+
Hospital 2	April 2018	*Carbapenem-resistant Klebsiella pneumoniae* (CRKP)	Kp5	+	+
Hospital 2	September 2018		Kp7	+	+
Hospital 2	September 2018		Kp8	+	+
Hospital 1	November 2018		Kp9	+	+
Hospital 1	November 2018		Kp10	+	+
Hospital 2	December 2018	*Carbapenem-resistant Pseudomonas aeruginosa* (CRPA)	Pa12	+	+
Hospital 2	January 2019		Pa15	+	+
Hospital 1	January 2019		Pa16	+	+

Legend: *bla*OXA-23=oxacillinase, *bla*IMP-1= imipenemase, pcr = polymerase chain reaction, LAMP= loop-mediated isothermal amplification, *Ab= Acinetobacter baumannii, Kp= Klebsiella pneumoniae, Pa= Pseudomonas aeruginosa, CRAB = Carbapenem-resistant Acinetobacter baumannii,* CRKP = *Carbapenem-resistant Klebsiella pneumoniae, CRPA = Carbapenem-resistant Pseudomonas aeruginosa*



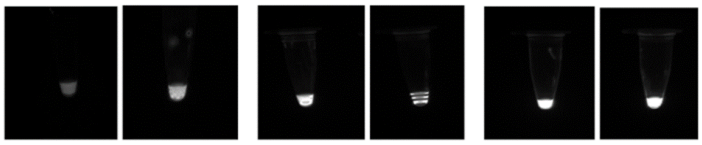
a. b. c.

Figure 4. *Klebsiella pneumoniae* isolates under 365 nm view box
a. Top (L-R): K5G, K7G, K8G, K9G, K10G; Bottom (L-R): K5H, K7H, K8H, K9H, K10H;
b.Top (L-R): K5E, K7E, K8E, K9E, K10E; Bottom (L-R): K5F, K7F, K8F, K9F, K10F;
c. Top (L-R): K5C, K7C, K8C, K9C, K10C; Bottom (L-R): K5D, K7D, K8D, K9D, K10D



a. b. c.

Figure 5. *Pseudomonas aeruginosa* isolates under 365 nm view box
a. Top (L-R): P12G, P15G, P16G; Bottom (L-R): P12H, P15H, P16H;
b. Top (L-R): P12E, P15E, P16E; Bottom (L-R):P12F, P15F, P16F;
c. Top (L-R): P12C, P15C, P16C; Bottom (L-R): P12D, P15D, P16D



a. b. c.

Figure 6. *Acinetobacter baumannii* isolates under 365 nm view box
(a.) A8G, A8H; (b.) A8C, A8D; (c.) A8E, A8F

ratio. Figures 4-6 show the difference in fluorescence brightness between 1:1 and 1:4 primers for the three bacterial strains where the 1:4 primer ratio is giving off better fluorescence. Manipulation of the temperatures and primer ratios lead us to the conclusion that changes in these factors did increase reaction speed and improve sensitivity, thus presenting optimization. With all things considered, the study presents that the antimicrobial resistance gene marker, $bla_{\text{OXA-23}}$, was best detected using the LAMP assay when a temperature of 61°C and primer ratio of 1:4 was utilized. The *bla*OXA-23 gene was detected using the LAMP technique in all nine (9) strains tested positive using the conventional PCR (100 % sensitivity). Figures 4-6 show fluorescence in all five (5) strains of CRKP, all three (3) strains CRPA, and one (1) strain of CRAB.

A study conducted by Ghosh et al. [16] in targeting the Foc in *Fusarium oxysporum* f. sp. *ciceri*s found that LAMP was optimal at 63°C for 60 min. The results also revealed that the detection limit was at 10 fg of the target material. Frisch and Niessen [17] developed and optimized the LAMP method to detect idh (isoepoxydon dehydrogenase) gene in patulin-producing *Penicillium* spp. According to the study, positive results were already obtained after 30 minutes of incubation at 63.9 – 65.6ºC. It was then recommended that further experiments be carried out in 65 ºC. The detection limit was determined to be 2.5 pg per reaction. [18] Caipang et al. [19] were able to optimally detect white spot syndrome virus (WSSV) at 65 ºC incubation for 60 minutes and Vibrio spp. at 59 – 67 ºC for 60 minutes in *Litopenaeus vannamei* in selected sites in the Philippines. All these considered, it can be concluded that optimal temperature in using LAMP for detection of the target gene varies from organism to organism.

The results in this study supported other recent studies that LAMP assay is as sensitive as the conventional PCR in the detection of blaOXA-23.

The LAMP assay has exhibited higher or at least similar sensitivity to PCR in the diagnosis of different causative agents, including viruses, bacteria, parasites, and fungi [20]. Also, several studies have reported that LAMP has higher sensitivity than PCR in detecting certain genes: cagA gene in *Helicobacter pylori* [21], cnm gene in *Streptococcus mutans* [22], and 529 bp repeat element in *Toxoplasma gondii* [23]. According to Kashir and Yaquinudin [24], LAMP is said to have ~ 100 fold more sensitive than conventional RT-PCR in detecting various forms of coronavirus RNA in patient samples. A study done by Ghosh et al. [16] showed that by optimizing LAMP assay at 63°C, gene detection of EF-1alpha of *Fusarium oxysporum f. sp. ciceris* was optimal. The higher sensitivity of LAMP over PCR can be explained by the LAMP's ability to detect the target gene at significantly lower concentrations. For example, the detection limit of Foc in *Fusarium oxysporum* using LAMP was found to be 10 fg while 100 pg using PCR. Another study conducted by Abbasi et al. [18] detecting *Leishmania donovani* DNA at a very low concentration of less than 100 fg is approximately 10^4 more sensitive than conventional PCR.

Our study and the study by Kim et al. (2016) [14], presented LAMP as a useful molecular diagnosis assay for CRPA and CRAB carrying *bla*OXA-23. In addition, our study also revealed its usefulness in the detection of *bla*OXA-23 gene in CRKP. Due to the LAMP method's simplicity, it is considered an attractive diagnostic technique, especially in underdeveloped countries with poor facilities [15,23]. It offers a practical alternative for the detection of viral and bacterial antigens [19].

Conclusion

The results demonstrate the usefulness of LAMP techniques for the diagnosis of CRKP, CRPA, and CRAB carrying *bla*OXA-23 gene. The LAMP assay is a sensitive, rapid, and practical method in the detection of blaOXA-23.

Acknowledgement

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