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Original article

Evaluation of loop mediated isothermal amplification (LAMP) assay for rapid detection of methicillin resistant *Staphylococcus aureus* in Tanta University Hospitals in Egypt

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ABSTRACT

Background: Methicillin-resistant *Staphylococcus aureus* (MRSA) has a long history of being a common source of healthcare-associated infections (HAIs). Early patient treatment and effective infection control strategies depend on quick MRSA diagnosis. **Aim of study:** This study aimed to assess the sensitivity and specificity of loop mediated isothermal amplification (LAMP) assay for rapid detection of MRSA. **Methods:** A total of 200 samples from patients with HAIs have been included in this study. Each sample underwent bacteriological examination. Isolation and identification of *Staphylococcus aureus* (*S. aureus*) were done by traditional cultural methods. Methicillin susceptibility was assessed phenotypically and genotypically. The phenotypic methods included cefoxitin disc diffusion and MIC detection by oxacillin E.test, while the genotypic methods included both Real-time PCR and LAMP technique for femB and mecA genes detection. **Results:** Out of 200 tested samples, 55 were *S. aureus* by conventional phenotypic methods. Both cefoxitin disc diffusion and oxacillin E.test were able to identify methicillin resistance in (78.2%) of *S. aureus* isolates. femB gene was found in all *S. aureus* isolates 55 (100%) while mecA gene was found in 44(80%) of the isolates by both Real-time PCR and LAMP. Compared to the gold standard PCR, LAMP had sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and accuracy of (100%) each. **Conclusion:** Combined detection of mecA and femB genes is reliable for diagnosis of MRSA. LAMP is a rapid, simple, sensitive, specific and relatively less costly assay for MRSA identification. LAMP can be considered a good substitute to PCR for MRSA detection.

Introduction

Methicillin resistant *Staphylococcus aureus* (MRSA) is regarded as a serious medical issue as it is a major bacterial pathogen linked to healthcare-acquired *Staphylococcus aureus* (S. aureus) infections worldwide. Furthermore, it is multidrug resistant (MDR) organism since it is resistant to other antimicrobial drugs in addition to β-lactam antibiotics [1]. For successful treatment and better infection control policy implementation, MRSA must be accurately and quickly detected [2]. It is possible to examine Methicillin resistance in *Staphylococci* using a variety of phenotypic

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techniques. However, these traditional techniques take time [3].

Molecular biology-based approaches including polymerase chain reaction (PCR) are designed to detect MRSA quickly and accurately. However, PCR-based approaches are challenging and impractical as point-of-care testing (POCT) procedures because they require specialized experimental equipment and qualified personnel, which might not be easily accessible in many resource-poor areas (4). Loop mediated isothermal amplification (LAMP), a nucleic acid amplification method innovated by **Notomi et al.** is frequently employed as a substitute to PCR-based techniques in the identification of pathogens [5, 6].

LAMP uses four or six primers that can recognize six or eight different sequences within the target DNA [5, 7]. Due to its special ability of strand displacement, the LAMP polymerase enzyme can be considered as the technique's "brain box" [8, 9]. As the reaction takes place in an isothermal condition, there is no need for expensive thermocyclers to control the temperature as with PCR; only water bath or heating block is needed [10]. By observing colorimetric changes with the naked eye, LAMP reactions allow for simple result analysis [11]. With great sensitivity and specificity, this approach can amplify a small number of DNA copies up to a million times within an hour. The WHO has recommended LAMP to fulfil all the criteria for an ideal nucleic acid amplification test for diagnostics [12].

The mecA gene mediates Methicillin resistance in Staphylococci (13) and can be found in both Coagulase-negative *Staphylococci* (CoNS) and *S. aureus*. As a result, detection of mecA alone is not enough for detection of MRSA. Testing for *S. aureus* should be done concurrently with mecA gene detection [14]. The femB gene is involved in cell wall pentaglycine side chain and interpeptide bridge formation in *S. aureus* [13]. It has been reported not to occur in CoNS and is known to participate in adjusting the level of Methicillin resistance in *S. aureus* [15].

So, the purpose of this study was to develop two LAMP assays to identify MRSA targeting mecA and femB genes and assess sensitivity and specificity of LAMP assay for rapid MRSA detection.

Materials and methods

Study design

This descriptive cross-sectional study was executed on 200 patients who were admitted during the period of research from July 2020 to July 2022 to Tanta University Hospitals. All patients included in the study had signs and symptoms of any type of healthcare- associated infections (HAIs) (Infections occur on or after the 3rd calendar day of admission where day of admission is calendar day 1) [16]. All participants in this research provided written, informed consent.

Ethical consideration

The protocol of this study was approved by the research ethics committee of Tanta University's faculty of medicine (approval code 33812/5/20).

Isolation and identification of *S. aureus*

As soon as possible, the different samples were transported to the Microbiology Department Lab for additional processing. First, a different code was applied to each sample, then cultured on blood, nutrient and mannitol salt agars (Oxoid, UK). The plates were incubated at 37°C for 24 hours. By using colony morphology, Gram staining and biochemical reactions (catalase and coagulase test), colonies were identified.

Identification of MRSA isolates

Phenotypic detection of MRSA

Cefoxitin disc diffusion test with disc content of 30 µg and MIC for oxacillin by E-test strips (Liofilchem, Italy) were used to test all *S. aureus* isolates for the MRSA phenotype. The results interpretation was carried out according to CLSI 2022 [17].

Genotypic detection of MRSA

Genotypic detection of MRSA was carried out by mecA and femB genes detection in all *S. aureus* isolates. For mecA and femB genes the workflow was processed separately. MRSA ATCC 33591 was used as a positive control for mecA and femB genes. *Enterococcus faecalis* ATCC 29212 and *Escherichia coli (E. coli)* ATCC 25922 were used as negative controls for both genes. DNA extraction (Boiling method) [18].

In 5ml of brain heart infusion broth, a single or two isolated colonies were grown for 18 to 24 hours. 1ml of this suspension was centrifuged at 13,000 rpm for 5 min. The pellet was suspended in 100 µL of TE buffer (Invitrogen, Thermo Fisher Scientific) after the supernatant was discarded. The suspension was

boiled for 10 min at 100 °C, immediately put right on ice. For 5 min, the suspension was centrifuged at 13,000 rpm at room temperature. The supernatant was used as a DNA template for PCR and LAMP assays. The nanodrop spectrophotometer (Thermo Scientific, NY, U.K.) was used to measure the concentration and purity of the crudely extracted DNA at wavelengths of A260 and A260/A280, respectively.

I. Uniplex SYBR green Real-time PCR for mecA and femB genes detection

PCR reaction mix was prepared in accordance with manufacturing instructions of QuantiFast® SYBR® Green PCR (Qiagen). A total of 20 µl of PCR reaction mixtures was made; each reaction contained 1.3 µl of DNA tempate, 0.7 µl of each primer (Applied Biosystems-USA), the used primers were summarized in **table (1)** (19) and 10 µl of QuantiFast® SYBR® Green PCR Master Mix, then the volume was brought to 20 µl by adding 7.3 µl of nuclease free water. The Rotor-Gene Q PCR thermocycler (Qiagen) was used and programmed as follows: initial denaturation at 95°C for 5 minutes, then 40 cycles of denaturation at 95°C for 15 seconds, annealing at 55°C for 30 seconds and extension at 72°C for 30 seconds.

The results were analyzed by using Q-Rex Software, and the curves were interpreted according to the threshold. Cycle threshold (CT) for each sample was calculated. Melting curve was obtained using a ramping rate of 0.5°C / 30 s for 55–95°C. The melting temperature (Tm) of each sample was compared to the Tm of the positive control (MRSA ATCC 33591). A Tm within the range of 74.40 ± 0.6°C was regarded as positive and amplicon-specific for mecA gene. A Tm within the range of 78 ± 0.6°C was regarded as positive and amplicon-specific for femB gene.

II. LAMP assay for mecA and femB genes detection

Primer's design

The Primer Explorer V5 program (http://primerexplorer.jp/lampv5e/index.html) (Eiken Chemical, Japan) was used in order to design LAMP primers for MRSA detection using the mecA and femB nucleotide sequences that were previously published (GenBank accession no. BA000017). Two outer primers, F3 and B3, as well as two inner primers, FIP and BIP (BioLabs, New England), were included **(Table 2).**

Amplification and detection of products

The LAMP assay was optimized using MRSA control strain ATCC 33591 by incubation at various temperatures ranging from 58 to 65 °C.

WarmStart Colorimetric LAMP 2X Master Mix Typical LAMP Protocol (M1800, New England, BioLabs) was used to set up the reactions. A 25 µl reaction mixture including 12.5µl WarmStart Colorimetric LAMP 2X Master Mix, 2.5µl LAMP Primer Mix (0.2 µM each of F3 and B3 and 1.6 µM each of FIP and BIP), 1 µl target DNA and 9 µl nuclease free water was used to conduct the LAMP reaction. Tubes were incubated in water bath at 63°C for 60 minutes. The tubes were taken out of the water bath and checked visually for color alterations. Positive reactions changed to yellow, whereas negative reactions stayed pink (pH indicator).

Statistical analysis

SPSS software statistical computer program for Windows, version 21 (IBM Corp., Armonk, New York, USA) was used to statistically analyze the acquired data. Numbers and percentages were used to portray qualitative data. To evaluate consistency between variables, kappa agreement was performed. Values ≤ 0 represent no agreement, 0.01-0.20 represent none to slight, 0.21- 0.40 represent fair, 0.41- 0.60 represent moderate, 0.61-0.80 represent substantial, and 0.81-1.00 represent nearly perfect agreement. For sensitivity, specificity, PPV, NPV, and accuracy, ROC curves were created. According to AUC (Area under curve), an approximate classification of a diagnostic test's accuracy is as follows: [0.90-1 = excellent, 0.80-0.90 = good, 0.70-0.80 = fair, 0.60-0.70 = poor, 0.50-0.60 = fail).

Table 1. List of primers used in Real-time PCR for mecA and femB genes

Primer name	Primer sequence (5´-3´)
***mec*A F**	AAATATTATTAGCTGATTCAGGTTAC
***mec*A R**	CGTTAATATTGCCATTATTTTCTAAT
***fem*B F**	CCGTATTGGTTATATCATCTATA
***fem*B R**	GGGTGTTTTACCTTCAAG

Table 2. List of primers used in LAMP for mecA and femB genes

Primer name	Primer sequence (5´-3´)
***mec*A gene**	
FIP	TCCCTTTTTACCAATAACTGCATCATATGTTGGTCCCATTAACTCT
BIP	AAGCTCCAACATGAAGATGGCCGATTGTATTGCTATTATCGTCAA
F3	GCGACTTCACATCTATTAGGT
B3	GCCATCTTTTTTCTTTTTCTCT
***fem*B gene**	
FIP	TACCTTCAAGGTTTAATACGCCCATCATCATGGCTTTACAACTGAG
BIP	ACACCCGAAACATTGAAAAAGACACTTTAACACCATAGTTTATCGCTT
F3	TGTTTAAATCACATGGTTACGAG
B3	TCACGTTCAAGGAATCTGA

Results

Isolation of *S. aureus* from clinical specimens

Out of 200 samples, 194 isolates were recovered. *S. aureus* represented 55 (28.4%) of total isolates.

Phenotypic identification of MRSA

Out of 55 *S. aureus* isolates, 43 (78.2%) were MRSA by using both cefoxitin disc diffusion and oxacillin E.test. Oxacillin E.test revealed that 30 (69.8%) MRSA isolates exhibited high levels of resistance (>32 µg /ml).

Detection of mecA and femB genes in *S. aureus* isolates by Real-time PCR

Out of 55 *S. aureus* isolates, 44 (80%) were identified as MRSA (had femB and mecA genes). femB gene was revealed in all *S. aureus* isolates 55 (100%) while mecA gene was revealed in 44(80%) of the isolates **(Figure 1, 2).**

Detection of mecA and femB genes in *S. aureus* isolates by LAMP assay

Out of 55 *S. aureus* isolates, 44 (80%) were identified as MRSA (femB and mecA positive). femB gene was detected in all *S. aureus* isolates 55 (100%) while mecA gene was detected in 44(80%) of the isolates **(Figure 1, 3).** There was perfect (100%) agreement between the result of mecA/femB Real-time PCR and mecA/femB LAMP assays **(Table 3).**

Comparison between various laboratory methods for detection of MRSA

Out of 55 *S. aureus* isolates, 43 (78.2%) were identified as MRSA by phenotypic methods (cefoxitin disc diffusion and oxacillin E.test), while 44 (80%) were identified as MRSA by genotypic methods (LAMP and Real-time PCR). This indicated that 1 (1.8%) *S. aureus* isolate carried mecA gene but was cefoxitin and oxacillin sensitive **(Table 4).**

LAMP was rapid compared to other methods. It can significantly detect MRSA with an excellent area under ROC curve (AUC=1.000) with sensitivity, specificity, PPV, NPV and accuracy of (100%) each. The cost of LAMP is relatively cheaper compared to the gold standard PCR. Phenotypic methods (cefoxitin disc diffusion and oxacillin E.test) were time consuming compared to LAMP and the gold standard PCR, having ROC curve with AUC= 0.989, sensitivity (97.7%), specificity (100%), PPV (100%), NPV (91.7%) and accuracy (98.2%) **(Table 5)** and **(Figure 4).**

Table 3. mecA/femB LAMP assay diagnostic performance in comparison to the mecA/femB Real-time PCR assay.

Assay and target gene	Results				Kappa agreement
	Positive		Negative		
	N	%	N	%	
Real-time PCR *mec*A *fem*B	44 55	80 100	11 0	20 100	1.000 (100%)
LAMP *mec*A *fem*B	44 55	80 100	11 0	20 100	

*Kappa agreement between *mec*A/*fem*B LAMP assay and *mec*A/*fem*B Real-time PCR assay is perfect

Table 4. Comparison between various laboratory methods for detection of MRSA.

Detection method		MRSA	
		N	%
Phenotypic methods	Cefoxitin disc diffusion	43	78.2
	Oxacillin E.test	43	78.2
Genotypic methods	LAMP	44	80
	Real-time PCR	44	80

Table 5. Comparison of the different parameters between MRSA identification methods.

Method	Speed**	Cost	Sensitivity	Specificity	PPV	NPV	Accuracy
Cefoxitin disc diffusion	24 h	+	97.7%	100%	100%	91.7%	98.2%
Oxacillin E.test	24 h	++	97.7%	100%	100%	91.7%	98.2%
LAMP	1.5 h	+++	100%	100%	100%	100%	100%
Real-time PCR*	3 h	+++++	100%	100%	100%	100%	100%

* Real-time PCR is the gold standard test
** Duration is calculated after the result of culture
PPV: positive predictive value
NPP: negative predictive value

Figure 1. Detection pattern of mecA and femB genes in *S. aureus* isolates by Real-time PCR and LAMP

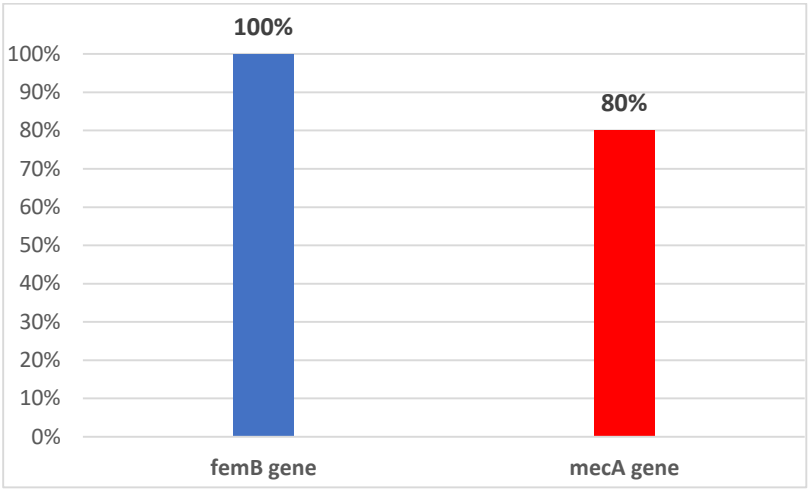
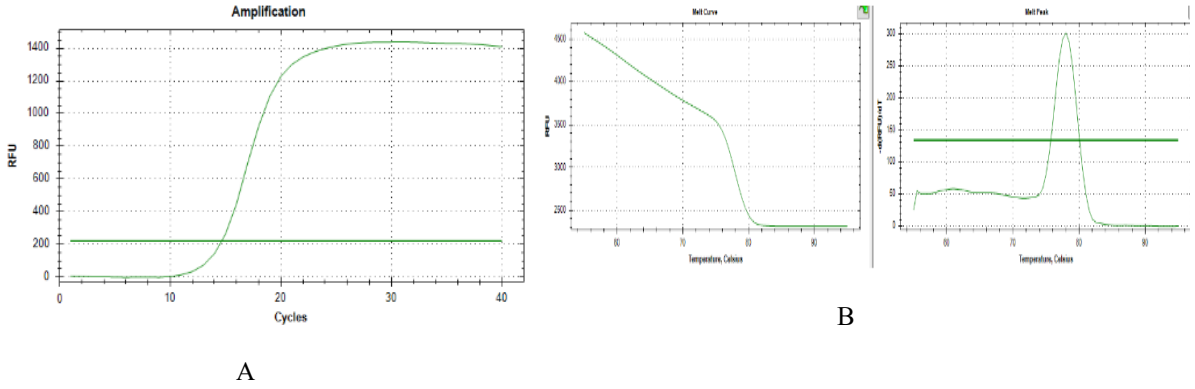
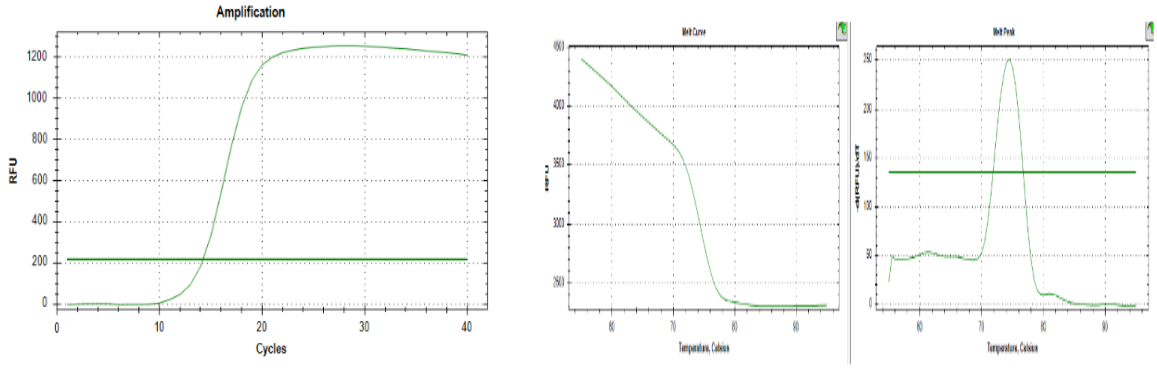


Figure 2. A: Real-time PCR curve showing positive amplification of femB gene
B: Melting curve and Melting Peak analysis for femB gene with single well-defined peak at 78 °C
C: Real-time PCR curve showing positive amplification of mecA gene
D: Melting curve and Melting Peak analysis for mecA gene with single well-defined peak at 74.4 °C



A B



C D

Figure 3. Loop mediated isothermal amplification of femB and mecA genes: (A and C) before amplification, (B and D) after amplification showing color change to yellow indicating positive gene detection (tube 1: positive control, tube 6: negative control)

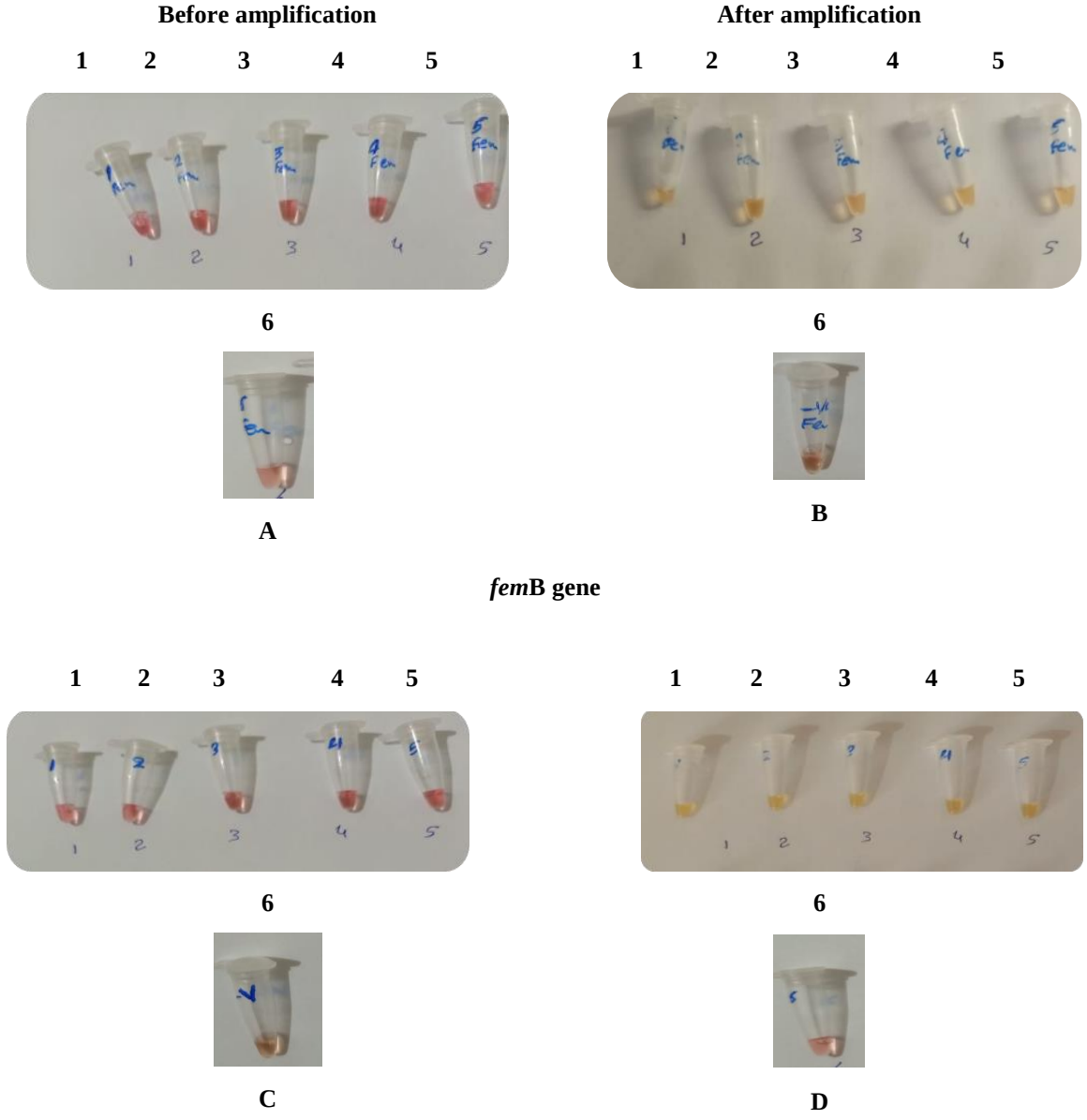
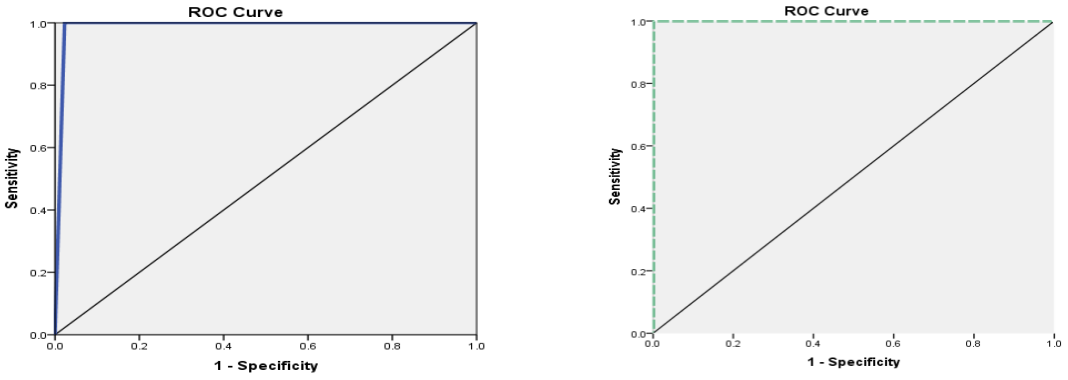


Figure 4. ROC curve for: (A) phenotypic methods, (B) LAMP



Discussion

MRSA has long been known to be a frequent cause of HAIs, leading to increased mortality, longer hospital stay and higher costs to healthcare systems. Among Gram-positive bacteria, MRSA possess the greatest risk. It is highly prevalent around the world [20, 21]. Adequate and suitable treatment, surveillance and infection control of MRSA require its prompt detection and confirmation [3].

PCR has been used to detect MRSA. However, because of the limited instruments and equipment in developing countries, it is difficult to operate as POCT. However, in recent years, interest in isothermal amplification has increased. In comparison to other methods, LAMP is less expensive, takes less time and requires less complex equipment. It is a highly sensitive and specific isothermal amplification technique [22, 23].

In this study, MRSA was detected phenotypically by cefoxitin disc diffusion and oxacillin E.test and genotypically by mecA/femB real-time PCR and mecA/femB LAMP.

In the current study, *S. aureus* represented (28.4%) of all isolates. This result was consistent with the finding of Elshabrawy and his colleges [24] at Mansoura University Hospital, who stated that *S. aureus* was isolated from (27.9%) of patients. Also, **Mahmood et al.** [25] at Sohag University Hospital isolated *S. aureus* from (28.2%) nosocomial wound infections. On the other hand, high rate (63.1%) and low rate (5.3%) of nosocomial *S. aureus* were detected by **Garoy et al.** [26] and **Hormozi et al.** [27] respectively.

The difference between the present study results and the other researchers can be explained by variable samples size. Also, isolates show variable results in different geographical distribution between different countries, different places in the same country and different hospitals in the same place. The sticking to infection control measures is another factor [28].

According to both cefoxitin disc diffusion and oxacillin E. test conducted during this study, the prevalence of MRSA was (78.2%). These results matched with that obtained by Abdel-Maksoud and his co-workers [29], who reported that MRSA isolates constituted (76%) of Staphylococcal infections by disc diffusion method. Also, **Alfeky et al.** [30] revealed that (81.2%) of the isolates were MRSA. On the contrary, low percentages of MRSA (17.5%, and 31.2%) were detected by Dilnessa and Bitew [31] and **Khanal et al.** [32] respectively.

The dissimilarity between the results may be due to difference in the clinical samples collected, samples size, locality, different prevention protocols as well as the indiscriminate use of antibiotics (topical and systemic) and its accessibility [33].

Regarding the result of the two uniplex Real-time PCR used to identify MRSA; out of 55 *S. aureus* isolates, (80%) of the isolates were identified as MRSA (had femB and mecA genes). femB gene was detected in all *S. aureus* isolates (100%) while mecA gene was detected in (80%) of the isolates. This finding was in line with that reported by **Lee et al. [**34] who revealed that (76.5%) of *S. aureus* isolates were identified as MRSA by detecting femB and mecA genes by PCR. Also, **Pournajaf et al.** [35] reported that among 127 *S. aureus* isolates, femB gene was amplified in all strains tested by PCR and 79 (62.2%) strains contained mecA gene. Moreover, **Khosravi et al.** [2] and **Abdelwahab et al.** [36] in Egypt found that mecA gene was detected in (80.8%) and (82.2%) of *S. aureus*, respectively by PCR.

However, Mohanasoundaram and Lalitha, [37] found that femB and mecA genes were found in all MRSA isolates by PCR but femB was absent in (4%) isolates that were methicillin sensitive *S. aureus* (MSSA). They justified their findings by stating that though femB gene is found only in S. aureus, its absence does not indicate that the isolate is not *S. aureus* as femB gene can be rarely mutated. However, femB negativity amongst *S. aureus* was mostly found in MSSA. Mutation in the fem operon will convert a mecA-positive *S. aureus*, Methicillin susceptible. **Awadalla et al.** [38] at Ain Shams University Hospital, Egypt found that mecA gene was detected in 50 out of 51 phenotypically diagnosed MRSA by using PCR. The presence of alternative resistance mechanisms, such as large amounts of produced beta-lactamase or change in mecA as a result of the mutations, may be the cause of methicillin resistance with negative mecA gene [39]. The mecC gene, a homolog of mecA, is also responsible for methicillin resistance in *S. aureus* [40].

In the current study, LAMP was used to identify MRSA; out of 55 *S. aureus* isolates, 44 (80%) were identified as MRSA (femB and mecA positive. There was (100%) agreement between the

result of mecA/femB Real-time PCR and mecA/femB LAMP assays. Supporting to the present study results, **Hanaki et al.** [41] found the same agreement between LAMP & PCR for detection of these genes. Moreover, the study by **Chen et al.** [19] revealed that mecA/femB/nuc LAMP assays and the mecA/femB/nuc triplex Real-time PCR were (100%) consistent with each other. In comparison with the study of **Koide et al.** [42] on sputum samples, The LAMP for mecA gene showed (93.2%) agreement with PCR. As compared to PCR, which detected mecA gene in 13 samples, LAMP only found the gene in nine samples. This finding was explained by the possibility that the sputum samples included more inhibitors of the LAMP reaction. **Aliasgharian et al.** [43] found that LAMP assay was more efficient than PCR in detecting mecA gene in 30 MRSA-positive blood cultures as PCR missed the diagnosis of one MRSA isolate that was detected by LAMP. LAMP has lower detection limit than PCR when performed directly on samples, that is how they explained this finding.

Out of 55 *S. aureus* isolates, 43 (78.2%) were identified as MRSA by phenotypic methods, while 44 (80%) were identified as MRSA by genotypic methods (LAMP and Real- time PCR). This indicated that 1 (1.8%) *S. aureus* isolate carried mecA gene but was cefoxitin and oxacillin sensitive. This result was in agreement with Salem-Bekhit [44] who revealed that femB and mecA genes were found in all of the phenotypically proven MRSA isolates by PCR and only one isolate carried mecA gene but was oxacillin sensitive.

In this study, LAMP was rapid compared to other methods. It can significantly detect MRSA with an excellent area under ROC curve (AUC=1.000) with sensitivity, specificity, PPV, NPV and accuracy of (100%) each. The cost of LAMP is relatively cheap compared to the gold standard PCR. Phenotypic methods were time consuming compared to LAMP and the gold standard PCR, having ROC curve with AUC= 0.989, sensitivity (97.7%), specificity (100%), PPV (100%), NPV (91.7%) and accuracy (98.2%). These results agreed with that of **Koide et al.** [42] who found that LAMP assay can detect mecA in clinical dental plaque samples with sensitivity, specificity, PPV and NPV of (100%) each. Additionally, **Choopara et al.** [18] and **Khosravi et al.** [2] reported that the result of LAMP assay for mecA gene detection in both laboratory and blood samples was (100%) specific and sensitive compared to Real-time PCR findings. **Panda et al.** [45] reported that oxacillin E.test failed to identify 1 MRSA isolate among 62 confirmed isolates by PCR with sensitivity, specificity, PPV, NPV and accuracy of (98.3%), (100%), (100%), (99.2%) and (99.5%), respectively. Interestingly, LAMP was (100%) sensitive and specific compared to PCR in the study of **Fiore et al.** [46] for detection of *Listeria monocytogenes* and the study of **Hassan et al.** [47] for detection of antimicrobial resistance genes (mcr-1, KPC, OXA-48, blaOXA-23 and VIM) in Gram-negative bacteria. The accuracy of LAMP for detection of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) in the study of **Nuchnoi et al.** [48] was (99.64%).

On the other side, **Nawattanapaiboon et al.** [49] in their study on blood samples reported that the sensitivity of LAMP assay for mecA gene detection was (100%), while the PCR assay was (98.1%) in comparison with phenotypic methods. The NPV of LAMP and PCR were (100%) and (95.2%), respectively with (100%) PPV for both. LAMP has a high sensitivity since it can identify relatively few copies of nucleic acid in a sample and is resistant to various PCR inhibitors, such blood (50). Also, **Abdel Sameea et al.** [51] in Benha University, Egypt found that LAMP assay showed (AUC) 0.917 with (100%) sensitivity, (83.3%) specificity, (86.7%) (PPV), and (100%) (NPV) in detecting MRSA colonies compared to traditional microbiological method. The discrepancies in the previous study are due to the use of only mecA gene to detect MRSA. mecA gene is present also in CoNS [14].

This is the first study reporting the application of LAMP as a simple, sensitive, specific and cost-effective alternative to PCR in the detection of MRSA in Tanta University Hospitals.

The limitations of our study are that LAMP is not tested for direct detection of MRSA from clinical samples and different organisms isolated from more samples are needed to assess the efficiency of LAMP.

Conclusion

According to the findings of this study, LAMP assay has potential as an simple, effective, rapid, less costly diagnostic alternative and should be developed for POCT of infectious organisms in the near future.

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Nil

Conflict of interest

The authors have no conflicts of interest.

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