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Comparative molecular study based on Loop-mediated isothermal amplification (LAMP) and PCR technique for rapid detection of methicillin-resistant Staphylococcus aureus (MRSA)

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ABSTRACT

Loop-mediated isothermal amplification (LAMP) is a powerful diagnostic tool with various applications that extend beyond clinical settings, including environmental monitoring and food quality control. It is characterized by its reliability, exceptional sensitivity, and specificity in amplifying target DNA, employing up to six primers. The energy-efficient and isothermal nature of LAMP enhances its appeal, making it a cost-effective option for point-of-care diagnostics. In this study, we investigated rapid detection methods for Staphylococcus aureus in dairy products, focusing on the Loop-mediated Isothermal Amplification (LAMP) technique. Our results demonstrate that LAMP is not only one of the fastest but also one of the most cost-effective methods available compared to polymerase chain reaction (PCR). Validation tests revealed that LAMP exhibits high sensitivity relative to PCR, with experimental data showing a strong correlation between the two techniques. Furthermore, we established that mecA-specific LAMP assays can be completed in under an hour while maintaining excellent specificity and sensitivity. These findings suggest that LAMP could be a viable alternative for quick and reliable detection of Staphylococcus aureus in dairy products, potentially enhancing food safety protocols.

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INTRODUCTION:

The Loop-mediated Isothermal Amplification (LAMP) technique utilizes a set of four to six specific primers that target a total of eight distinct sequences on the DNA of interest. This method operates through auto-cycling and strand displacement activity facilitated by DNA polymerase, enabling efficient DNA synthesis. One of the primary advantages of LAMP is its single-step process, which allows for on-site detection and easy integration into any microbiology laboratory. The amplification and detection of the target gene occur at a constant temperature, eliminating the need for a denaturation step or complex instrumentation. This simplicity and efficiency make LAMP a powerful tool for rapid molecular diagnostics (**Wong Y P et al. 2018)** and **(Manar M E et al 2023).**

Nowadays, the LAMP technique has gained widespread application in detecting a variety of bacteria, including pathogenic species responsible for foodborne illnesses, environmental contaminants, and clinical infections. Its ability to amplify DNA rapidly and specifically makes it an ideal choice for identifying bacteria such as *Escherichia coli*, *Listeria monocytogenes*, *Salmonella spp.*, and *Staphylococcus aureus*. The method's adaptability allows researchers to develop LAMP assays tailored to target specific bacterial genes, making it a powerful tool for real-time monitoring and outbreak response in both clinical and food safety settings **(Wang X, Gao YH, Xu XJ et al. 2011)**.

Staphylococcus aureus (S. aureus) is one of the most common bacteria responsible for food poisoning worldwide **(Momtaz B N et al. 2020)**. Since its introduction in the late 1950s, the antibiotic methicillin has been used to treat infections caused by S. aureus **(Peterson LR and Schora DM, 2016).** However, in 1960, a strain of methicillin -resistant Staphylococcus aureus (MRSA) was identified among clinical isolates from hospitalized patients. By the 1980s, MRSA strains had spread to both community and healthcare settings globally **(McCarthy et al. 2015; Okwu et al. 2019).**

The culture-based approach for identifying Staphylococcus aureus (S. aureus) remains a widely accepted method according to **Antonio CG and Irene R. (2020**). The traditional process, as outlined in the Chinese National Food Safety Standard GB 4789.10-2010, involves several steps: enrichment and enumeration in a liquid medium, followed by the recovery and isolation of colonies on a selective culture broth. Further confirmation tests are then performed to accurately identify the pathogen.While there are numerous phenotypic methods available for diagnosing Methicillin-Resistant Staphylococcus aureus (MRSA), these techniques can be time-consuming, often requiring between 18 and 24 hours. To address this issue, molecular biology techniques such as polymerase chain reaction (PCR), real-time PCR, and DNA hybridization have been developed. These methods are now commonly used for the rapid detection of pathogens, offering a faster alternative to traditional culture-based methods **(Sudha haran et al. 2015).**

Even though molecular biology techniques such as PCR, real-time PCR, and DNA hybridization offer faster pathogen detection than traditional methods, they still require several hours to identify even a small number of bacterial cells. According to **Kei et al. (2014**), these technologies involve complex procedures, including the preparation of enzyme reaction mixes, the use of sophisticated equipment, advanced cultural enrichment, and bacterial DNA isolation. Consequently, these methods are often unsuitable for on-site detection.

In contrast, Loop-Mediated Isothermal Amplification (LAMP) assays have been demonstrated to be effective and powerful tools for identifying various *S. aureus* strains. As noted by **Toru Misawa et al. (2014) and Noora S A A et al. (2024)**, LAMP assays offer a more practical and efficient approach, making them a viable alternative for rapid and on-site detection.

MATERIALS and METHODS

Samples collection: One hundred randomly selected samples were purchased from small grocery stores and supermarkets in Cairo and Elfyoum City. These samples included twenty raw milk samples, twenty Karish cheese samples, twenty beef burgers, twenty beef luncheon samples, and twenty chicken meat samples. The samples were stored in sterilized jars and kept in cooling ice boxes at 4°C before being promptly transported to the laboratory under sterile conditions.

MRSA isolates with the number ATCC 25923 were used as a positive control, in accordance with the guidelines provided by **Mohammad A et al. (2021)** and the Clinical and Laboratory Standards Institute **(CLSI 2017).**

Bacteriological analysis and characterization:

All samples were collected and transported to the laboratory in an ice tank for biochemical or bacteriological analysis. Upon arrival, the samples were first enriched in peptone water (1 ml of milk in 9 ml of sterile peptone water) and incubated for 24 hours at 37°C. After enrichment, the samples were streaked on Mannitol Salt Agar, a selective medium, and incubated for another 24 hours at 37°C. Suspected yellow colonies that developed on the agar were then subjected to a series of biochemical tests to confirm their identity. These tests included urease, coagulase, catalase, and mannitol fermentation tests, which are standard procedures for determining the presence and characteristics of *Staphylococcus aureus*.

Antimicrobial susceptibility testing:

Staphylococcus aureus isolates were examined for oxacillin resistance using the disk diffusion method. Once isolates were confirmed to be oxacillin-resistant, they were further tested against 16 additional antibiotics.

To prepare the inoculum, a few colonies were transferred from a nutrient agar plate to sterile saline, and the turbidity was adjusted to match a 0.5 McFarland standard. A lawn culture was then prepared by spreading a few microliters of the bacterial suspension onto Muller Hinton agar plates.

Antibiotic discs were subsequently applied to the agar surface. The plates

were incubated aerobically at 37°C overnight. After incubation, the inhibition zones around the antibiotic discs were measured, and the results were interpreted according to the guidelines provided by the Clinical and Laboratory Standards Institute (CLSI), as referenced in **Ali et al. (2021).**

DNA extraction:

One gram of each sample was weighed and mixed with 9 mL of sterilized water in a tube. The mixture was incubated for 2 minutes, followed by centrifugation to separate the components. After centrifugation, 1 mL of the supernatant was collected and heated at 100°C for 5 minutes, then immediately chilled on ice to stabilize the DNA.

Following this preparation, the samples were directed for DNA extraction using the Wizrep gDNA Mini kit (Cell/ Tissue), which is specifically designed for extracting genomic DNA from various sample types. LOT NO 5A1018-01. According to the manufacturer's instructions.

PCR amplification and gel electrophoresis:

For the PCR amplification of Methicillin-Resistant Staphylococcus aureus (MRSA), a volume of 5 µL of the sample's extracted DNA was added to a 20 µL PCR mixture. This mixture contained 10 µL of Amplitaq® Gold Fast PCR Master Mix (Waltham, Massachusetts), 1.5 µL of each primer (mecA) at 20 pmol (for both forward and reverse primers), and 3 µL of nuclease-free water.

The PCR reaction protocol involved: An initial denaturation step at 95°C for 10 minutes, Followed by 40 cycles consisting of ,Denaturation at 96°C for 30 seconds, Annealing at 53°C for 45 seconds, and Extension at 68°C for 45 seconds, with A final extension at 72°C for 10 minutes.

The amplified PCR products were then visualized by electrophoresis on a 1.5% agarose gel stained with ethidium bromide. The gel was subsequently observed and documented under a UV transilluminator (UV solo TS® Imaging System, Biometra®, Germany). The optimal annealing temperature was determined based on the methodology described by **Khosravi et al. (2022).**

LAMP amplification:

Optimization of LAMP technique on isolate culture:

LAMP reactions were optimized using a standard positive control (ATCC 25923) to fine-tune the reaction conditions. The LAMP assay was performed in a 25 µL reaction volume, which included the following components:
µL of Warm Start LAMP 2X Master Mix (New England Biolabs, Ipswich, MA, USA), 2.5 µL of LAMP primer mix, which consisted of (0.2 µM of B3 and F3 primers ,1.6 µM each of BIP and FIP primers, 0.5 µL of fluorescent dye ,8.5 µL of distilled deionized water and 1 µL of the DNA template
The optimized reaction was carried out at 63°C for 60 minutes, as determined by **Khosravi et al. (2022**). Sterile water was used as a negative control in place of the bacterial DNA template to ensure the specificity of the assay.

Table 1. Primers used in LAMP and PCR:

Name	Target gene	Sequence (5`-3`)	Tech-nique	Reference
mecA F	mecA	AAAATCGATGGTAAAGGTTGGC	PCR	Maes,et al2002
mecA R		AGTTCTGCAGTACCGGATTTGC		
F3	mecA	AGAAAAAGCGACTTCACATC	LAMP	*Khosravi* et al. 2022
B3		GCCATCTTTTTTCTTTTTCTCT		
FIP	gyrA	TCCCTTTTTACCAATAACTG-CATCATTATGTTGGTCCCATTAACTCT		
BIP		AAGCTCCAACATGAA-GATGGCCGATTGTATTGCTATTATCGTCAA		

Specificity and Sensitivity determination of PCR and LAMP assays.

to determine the specificity of the LAMP assay, the visual detection was carried out under the conditions described above, using DNA templates extracted from S. aureus, E. coli, and S. enteritidis. The sensitivity of the LAMP assay was assessed using serially diluted DNA templates of the MRSA (ATCC 25923) strain. The process involved performing serial dilutions of S. aureus cells in ddH_2O to achieve final concentrations ranging from 10^7 to 1 CFU/mL., Extracting DNA from 1 mL of each dilution. Then subjecting the extracted DNA to both PCR and LAMP assays to determine the sensitivity of each method.

RESULTS

Specificity and Sensitivity of S. aureus using pcr and LAMP assays

The specificity of the *Staphylococcus aureus* using LAMP (Loop-Mediated Isothermal Amplification) assay was evaluated by testing its amplification performance with DNA isolated from *S. aureus* and other bacterial species. The results demonstrated that only the reaction containing *S. aureus* DNA showed a positive specific band at 530 bp by PCR assay and also green color detected in the visual LAMP assay (as indicated in figure .(١ This indicates that the LAMP assay is highly specific for *S. aureus*, with no cross-reactivity observed with other bacterial species. The high specificity is likely due to the targeted amplification of the mecA and gyrA genes by the LAMP primer set, which is unique to S. aureus, this result suggests that both PCR and LAMP assays are reliable methods for specifically detecting S. aureus without interference from other bacteria.

Specificity of S. aureus using PCR and LAMP assays

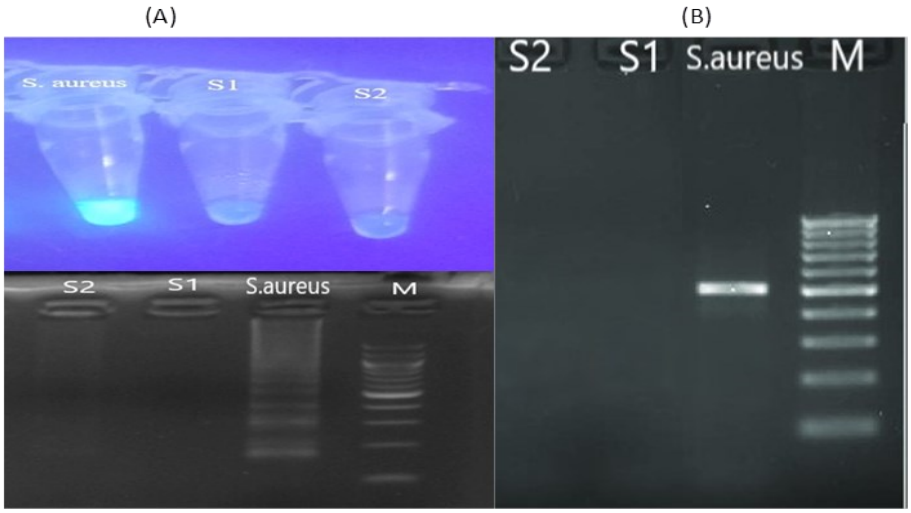

Figure (1): Comparison of specificity detection of MRSA (ATCC 25923) between LAMP and conventional PCR. (A) LAMP specificity assessment results based on Calcein visualization under UV Light followed with agarose gel electrophoresis analysis of the LAMP products. (B) Conventional PCR specificity assessment results. S1: E. coli, S 2: Enterococcus

Sensitivity of S. aureus using PCR and LAMP assays

The sensitivity of both PCR and LAMP assays was evaluated using serially diluted DNA samples of *Staphylococcus aureus*. the limit of detection (LOD) was determined as follows: PCR Assay., The LOD was 1 × 10^2 CFU/Ml while LAMP Assay (both electrophoresis and visual LAMP): The LOD was also 1 × 10 CFU/mL.

These results indicate that both PCR and LAMP assays exhibit comparable sensitivity for detecting S. aureus in DNA samples, with the ability to detect as low as 100 colony-forming units (CFU) per milliliter.

Table 2. Comparison of the sensitivity of LAMP assay and PCR conducted in this study.

Serial dilution	1x10^8 ng/ul	1x10^7 ng/ul	1x10^6 ng/ul	1x10^5 ng/ul	1x10^4 ng/ul	1x10^3 ng/ul	1x10^2 ng/ul	1x10 ng/ul
LAMP Asay	+	+	+	+	+	+	+	+
PCR Method	+	+	+	+	+	+	_	_

Sensitivity of S. aureus using PCR and LAMP assays.

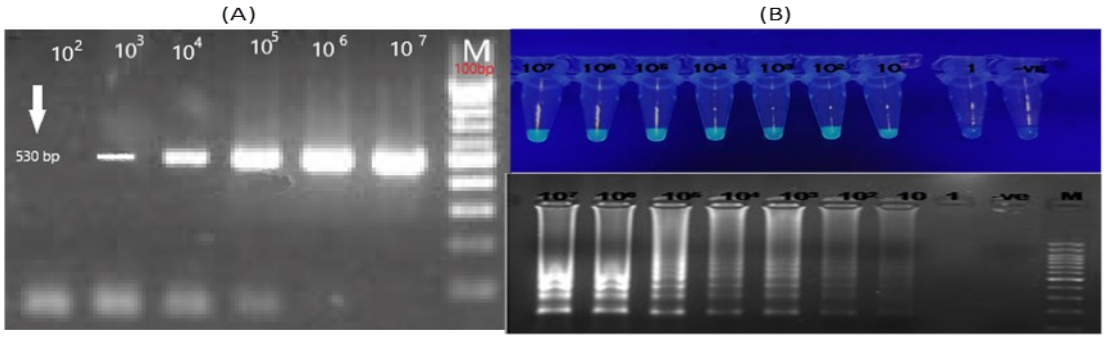

Figure (2) Sensitivity analysis and LOD determination of LAMP assay in comparison with conventional PCR using ten-fold serial dilutions of purified target DNA from MRSA (ATCC 25923). A) Gel electrophoresis of PCR sensitivity analysis products Lane 1-6 (10^2 – 10^7), Lane 7 (DNA ladder 100-bp). B) Visualization of LAMP assay sensitivity analysis products by Calcein fluorescence dye using under UV light lift-to-right (10^7-10^0). LAMP products migrated in agarose gel electrophoresis lane1-8 (10^7-10^0), lane 9 (+VE), lane 10 (DNA ladder 100-bp).

Bacterial isolates

In the study, a total of 59 Staphylococcus aureus isolates were characterized using standard microbiological tests. The isolates were obtained from various food sources as follows: -11 isolates from Raw milk, 7 isolates from Beef burger,15 isolates from Karish cheese,11 isolates from Beef luncheon, and 15 isolates from Chicken meat.

Based on phenotypic resistance to Methicillin, 26 out of these 59 S. aureus isolates were identified as Methicillin-resistant Staphylococcus aureus (MRSA). The distribution of MRSA among the different food sources will be detailed in the next **table. (3)**

Table 3. culture& sensitivity with Methicillin-resistant Staphylococcus aureus of all samples

samples	Culture Isolation (S. aureus)	Phynotipc resistance (Methicillin)
Raw milk	(11/20) 55%	(5/11) 45.4%
Beef burger	(7/20) 35%	(2/7) 28.5%
Karish cheese	(15/20) 75%	(11/15) 73.3%
Beef luncheon	(11/20) 55%	(4/11) 36.3%
Chicken meat	(15/20) 75%	(4/15) 26.6%

This information underscores the prevalence of MRSA in various food products, highlighting the importance of monitoring and controlling antibiotic resistance in foodborne pathogens.

Detection of S. aureus in collected samples by PCR and LAMP assays.

Positive PCR results are detected in 19 samples from specimens, including raw milk (20%, n = 4), Karish cheese (55%, n = 9), Beef luncheon (15%, n = 3) and Chicken meat (15%,n 3), while positive results using LAMP assay detected in 26 samples from specimens, including raw milk (25%, n = 5), Karish cheese (٦٥ ،%n = 11 Beef luncheon (20%, n = 4), Chicken meat (20%, n =4) and Beef burger (10%, n=2) as presented in the next **table 4.**

Table 4. Results between the LAMP technique and conventional PCR of food samples

Samples	LAMP (F3, B3, FIP, and BIP genes)	PCR (MecA gene)
Raw milk	(5/20) 25%	(4/20) 20%
Beef burger	(2/20) 10%	(0/20) 0%
Karish cheese	(11/20) 65%	(9/20) 55 %
Beef luncheon	(4/20) 20%	(3/20) 15%
Chicken meat	(4/20) 20%	(3/20) 15%

Raw milk samples: -

Raw milk Samples 1, 2, 3,4, and 5 were determined to be S. aureus positive by LAMP methods. but conventional PCR assays determined four S. aureus (1,2,3 and 4) **(Fig. 3)** The LAMP method, with its visual detection capability, is practical and user-friendly, particularly for food safety testing.

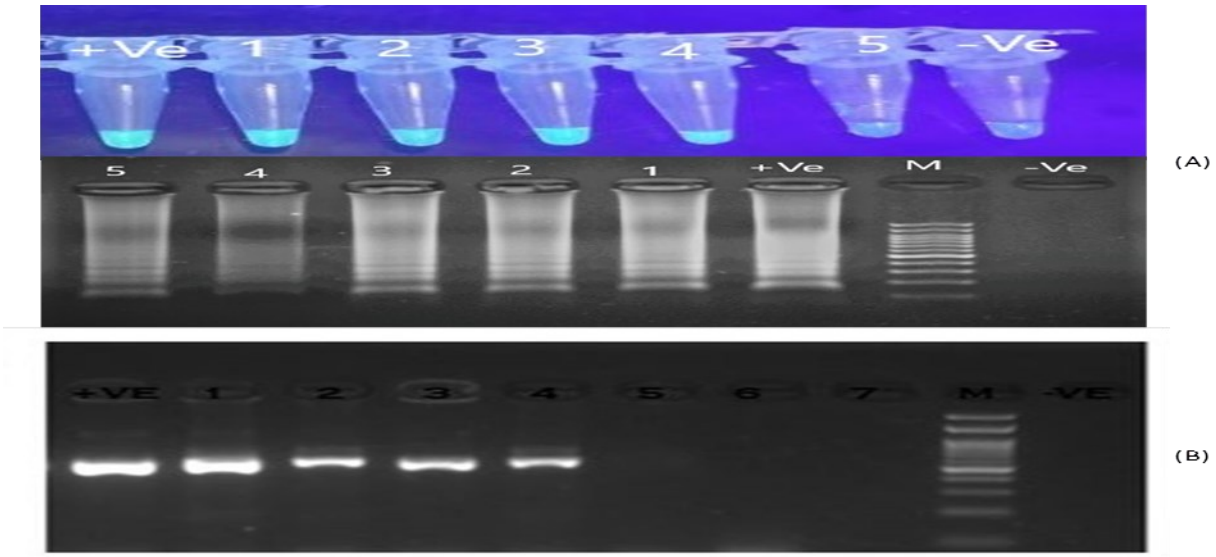

Figure (3) Results of 5 milk samples, The LAMP products detected by Calcein fluorescence dye visualization under UV Light. results of LAMP products by agarose gel electrophoresis(A). The 4 real milk samples detected by PCR conventional are amplified at 530 bp Lane 1: from left to right positive control; lane 2 to 5 positive samples (from 1 to 4); Lane 9: marker DNA 100 bp and Lane 10: negative control (B).

2-Beef burger samples: -

Tow samples (3 and 6) were determined to be S. aureus positive by LAMP method. but by conventional PCR assay, no positive results were detected as shown in the next **(Fig. 4).**

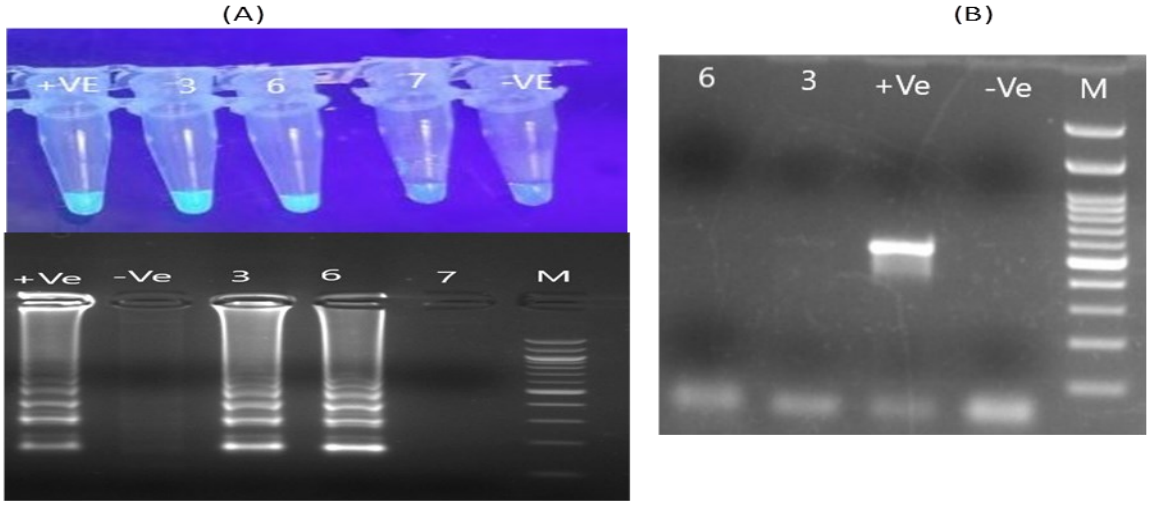

Figure (4): Results of 2 beef burger samples (3 and 6), The LAMP products detected by Calcein fluorescence dye visualization under UV Light, results of LAMP products by agarose gel electrophoresis (A). Result of PCR Lane 1 sample 6; lane2: sample 3: lane3: positive control; lane4: negative control; Lane 5 marker DNA 100 bp (B).

Karish cheese samples:

Karish cheese samples (numbered 1 through 11) were found to be positive for S. aureus by the LAMP technique. While nine samples are only positive by PCR (from samples no 3 to 11) as shown in **(Fig. 5).**

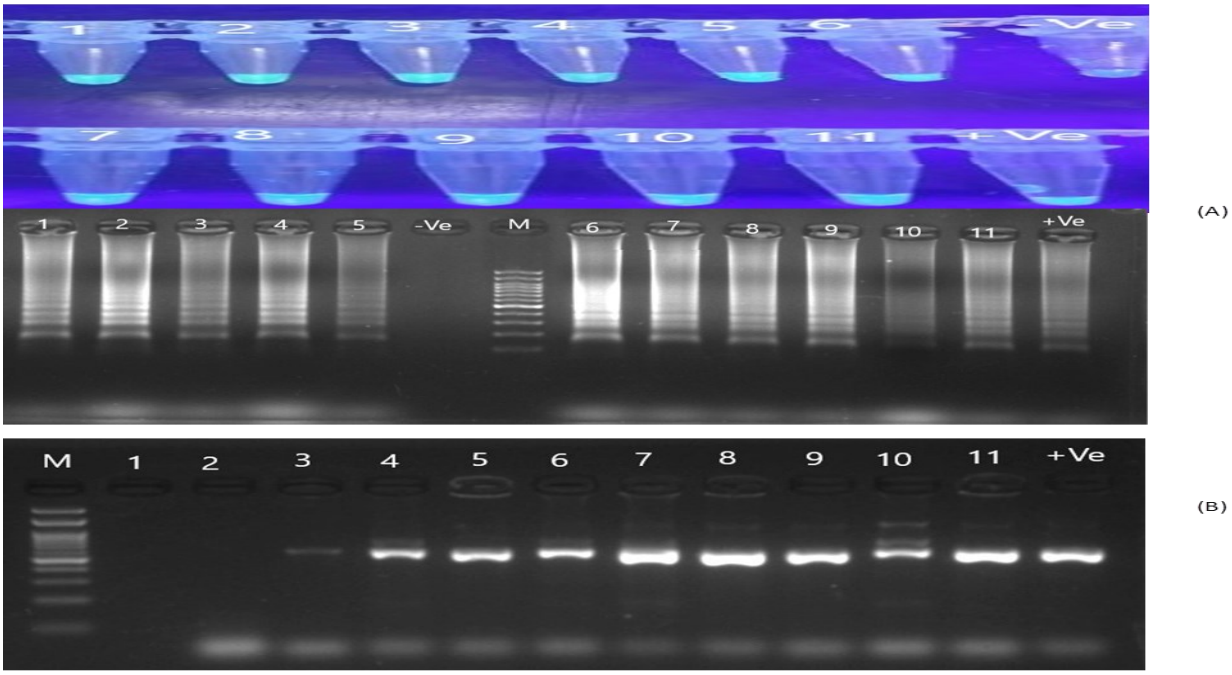

Figure (5): Results of 11 Karish cheese samples, The LAMP products detected by Calcein fluorescence dye visualization under UV Light. Results of LAMP products by agarose gel electrophoresis (A). 9 samples were only detected by PCR-specific bands at 530 bp Lane M: DNA 100 bp; lanes 2 and 3: are negative samples; lanes 4, to 12) positive samples; Lane 12 positive control (B).

Beef luncheon and Chicken meat samples

Four beef luncheon and chicken meat samples (from 1 to 4) were positive for S. aureus by the LAMP technique. Only 3 (from 1 to 3) samples were positive by PCR as shown in **(fig.6 and 7).**

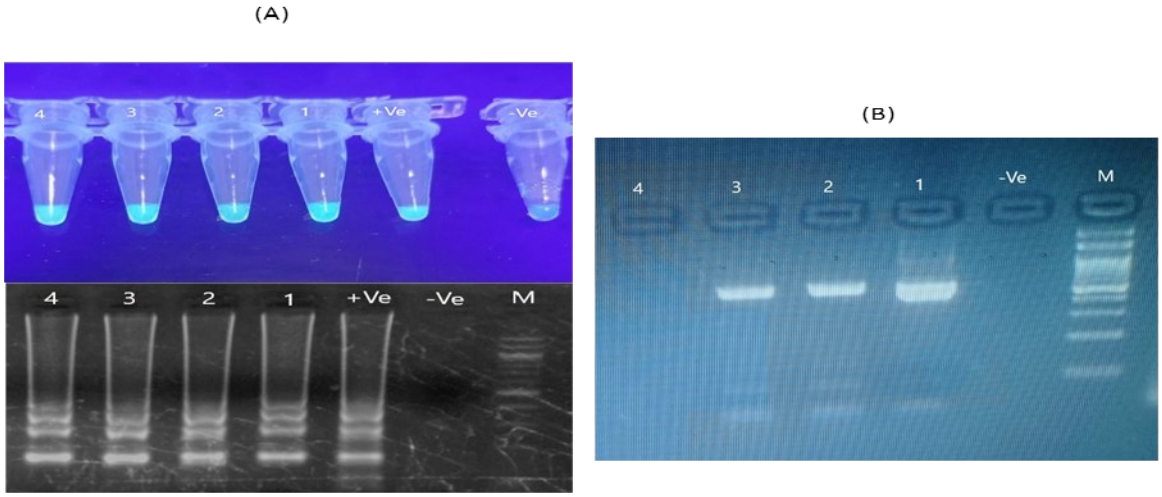

Figure (6): Results of 4 beef luncheon samples, The LAMP products detected by Calcein fluorescence dye visualization under UV Light. LAMP products detected by agarose gel electrophoresis (A). The 3 beef luncheon samples were detected by PCR amplified product at 530 bp Lane 1: negative sample; lanes 2 to 4 positive samples; lane 5: negative control; Lane 6: M, DNA 100 bp (B).

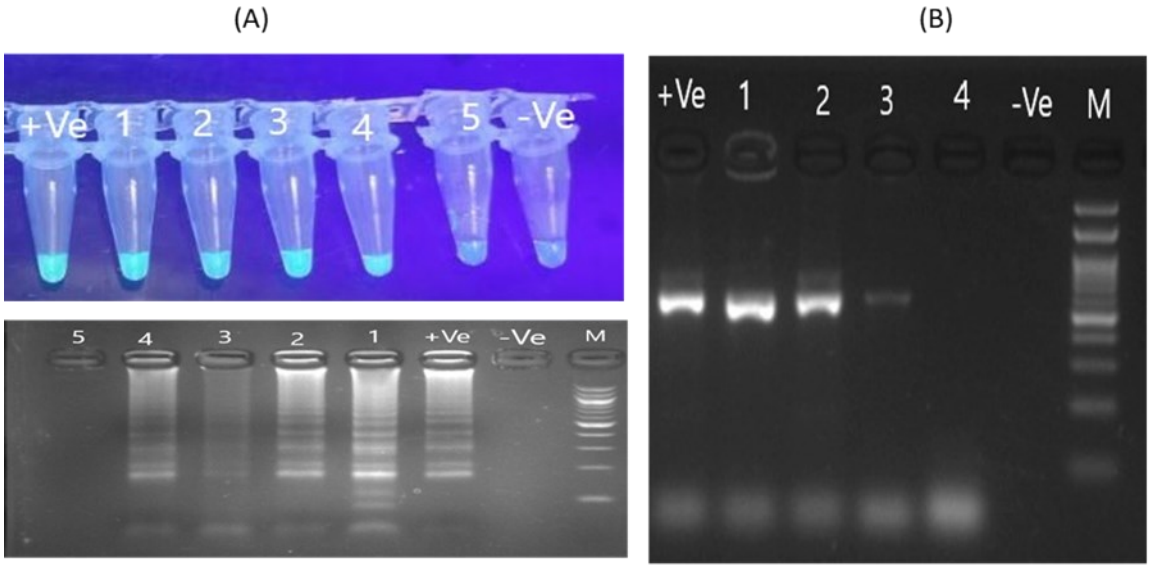

Figure (7): results of 4 chicken meat samples, The LAMP products detected by Calcein fluorescence dye visualization under UV Light. LAMP products detected by agarose gel electrophoresis (A). The 3 chicken meat samples detected by PCR amplified product detected at 530 bp Lane 1: positive control; lanes 2 to 4 positive samples; Lane 5: negative sample; Lane 6: negative control; lane 7: M, DNA 100 bp (B).

DISCUSSION

Staphylococcus aureus is a major cause of foodborne illness, typically transmitted through food contaminated by individuals with S. aureus infections or skin lesions. These bacteria can be spread when contaminated food is prepared or handled by infected individuals, leading to potential outbreaks. Proper hygiene practices and food handling are essential to prevent contamination and safeguard public health **(Gundogan et al. 2005)**.

In current study, a high percentage of contamination was observed, with 59% of dairy products (including milk, Karish cheese, beef luncheon, beef burgers, and meat) and chicken meat testing positive for S. aureus, this finding aligns with similar studies conducted in Turkey, which reported S. aureus contamination rates, 61% in raw and pasteurized milk, and 53% in beef and chicken **(Gundogan et al. and Loncarevic et al. 2005).** These results highlight the widespread presence of S. aureus in various food products and the importance of maintaining stringent hygiene practices to prevent contamination.

Studies on Staphylococcus aureus contamination in hamburgers have revealed varying levels of infection across different regions In Iran, a significant 39% of handmade and packaged hamburgers were found to be contaminated with S. aureus **(Momtaz B N et al. 2020).**

In Brazil, S. aureus contamination was reported in 14% of sandwiches and 68% of raw hamburgers **(Contreras et al. 2015). Shahraz et al. (2012)** reported a 25% contamination rate in hamburgers in Tehran, Iran.

Antibiotic-resistant *Staphylococcus aureus* strains pose a significant threat to healthcare settings due to their ability to cause both nosocomial and community-acquired infections. Reports indicate that resistant strains of *S. aureus* can spread through contaminated meat, dairy products, and retail chicken **(Gündoğan et al. 2006)**. Oxacillin is commonly prescribed for treating infections in both humans and animals; however, its overuse can contribute to the development of resistance **(Al-Zu'bi et al. 2004**). This highlights the importance of judicious use of antibiotics and robust infection control measures to manage and prevent the spread of resistant strains.

In the present study, it was found that 26% of *Staphylococcus aureus* isolates were Methicillin-Resistant *Staphylococcus aureus* (MRSA). This finding is consistent with previous studies, including those by **Sobhy et al. (2012), Hend and Rasha et al. (2014), and Hala AM et al. (2015**). The high rate of MRSA in Egypt may be attributed to the widespread colonization of MRSA in the community, which contributes to the increased prevalence of resistant strains. This emphasizes the need for effective surveillance and control measures to address MRSA colonization and infection.

The main drivers of antibiotic resistance include the use of antibiotics with low activity, their administration to food-producing animals for medical purposes, and improper dosage. In this study, the resistance to oxacillin among Staphylococcus aureus strains varied across different food products: 25% resistance in milk ,55% resistance in Karish cheese ,20% resistance in beef luncheons ,10% resistance in beef burgers and 20% resistance in chicken meat samples

These findings are consistent with the results reported by **Contreras et al. (2015) and Momtaz B N et al. (2020),** reflecting the variability in antibiotic resistance across different types of food products.

Culture-based techniques for identifying MRSA typically involve plating on blood agar and performing various biochemical tests, which usually takes 1-2 days for results. In contrast, PCR-based amplification techniques, have been developed to diagnose MRSA more rapidly. Despite their simplicity and accuracy, PCR techniques require specific tools such as a gel documentation system, a heat cycler, and an electrophoresis set to perform and analyze the tests **(French G. 2009; Su J et al. 2014; Chen C et al. 2017).**

In this study, the LAMP method was uti-

lized to identify MRSA, with specific primers for the mecA gene. LAMP was compared with PCR, which served as the standard reference for identifying MRSA isolates. The LAMP assay successfully detected 100% of mecA genes **(Elnomrosy S M et al. 2022; Noora S. A. A et. al. 2024)**

The LAMP method proved to be highly specific for identifying the target gene compared to conventional PCR across all food samples, aligning with findings from **Mehran Khan et al. (2018**). The four inner loop primers (F3, B3, FIP, and BIP) used in the LAMP assay enhanced DNA amplification by generating loop amplicons from multiple-sized LAMP amplicons, as described by **Grittaya S et al. (2020).**

The LAMP technique demonstrated high sensitivity and specificity for detecting the mecA gene, with results obtained in less than an hour. It offered notable advantages over PCR in terms of speed and simplicity, operating at a constant temperature and allowing for visual inspection. The sensitivity and specificity of the LAMP method were superior to those of conventional PCR, as supported by **Lim KT et al. (2013) and Mehran Khan et al. (2018).**

In conclusion, conventional PCR is more costly and time-consuming compared to the LAMP technique, making LAMP a preferred method for detecting MRSA due to its lower cost and faster results. However, it's crucial to emphasize the need for improved hygiene practices to reduce the risk of contamination in milk, milk products, meat, and meat products. Enhanced hygiene can help mitigate the spread of antibiotic-resistant bacteria and ensure food safety.

Author contributions

Sara M. Elnomrosy designed the study, Mohamed I. AbdAllah, Nahla Hussien AbouEl Ela and Naglaa M. Hagag analyzed the data with Sara M. Elnomrosy and contributed to writing. Naglaa M. Hagag and Momtaz A. Shahein critically reviewed the manuscript, Samah F. Ali, Hala R. Ali, and Esraa G. Hefny are cultured and isolated of bacteria with, analyzed data and also contributed to the paper publication. All authors performed the study and wrote the first draft of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest:

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest

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