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Molecular Detection of Methicillin-resistant *Staphylococcus aureus* Isolated From Foods in Germany Using LAMP Assay

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Abstract

THE existence of methicillin-resistant *Staphylococcus aureus* (MRSA) represents a considerable a major danger to the public health, contributing to both foodborne illnesses and hospital-acquired infections. Hundred eighty-nine of *Staphylococcus aureus* (*S. aureus*) isolates were obtained from diverse food items, including poultry (103 isolates), sausage (9 isolates), pork (3 isolates), and minced meat (4 isolates), along with bovine mastitis milk (70 isolates). These isolates were submitted to the University of Veterinary Medicine Hannover, Germany, as part of routine diagnostic procedures for *S. aureus*. This study utilized numerous *S. aureus* isolates from food sources to develop an efficient the loop-mediated isothermal amplification (LAMP) technique enables the swift identification of *S. aureus* and MRSA. Six primers were specifically crafted for each *S. aureus* and MRSA sequence. Results indicated that all presumed *S. aureus* isolates were successfully identified with the *nuc* gene through LAMP and PCR techniques. However, 8.46% of isolates were MRSA which possessed the *mecA* gene. The LAMP assay exhibited high analytical sensitivity (1.49 pg/µL-1, 100% sensitivity) for detecting MRSA DNA with a dilution factor of 10-4. We determined the Limit of Detection (LOD) as 3.6 x 103 CFU/mL, with an 80% detection probability. This method can be seamlessly approved into any microbiology laboratory for *mecA* detection, offering the advantages of simplicity, time efficiency, and accurate results without the need for specialized equipment.

Keywords: MRSA, LAMP assay, food poisoning, meat safety.

Introduction

Ensuring food safety remains a paramount global health concern, and microbe-caused foodborne illnesses are a significant public health issue [1]. *Staphylococcus aureus* has ability to cause the food poisoning which is among the commercially significant foodborne infections and a significant problem for public health programs globally [2 – 6]. *Staphylococcus aureus* may survive in a variety of conditions, including those with pH values between 4.1 and 9.2 and salt chloride concentrations up to 15.5% [7, 8]. These traits make it easier for the organism to contaminate and spread to different kinds of foods, so efficient and quick approach for detecting *S. aureus* should be developed to help minimize the spread of infectious pathogens [9 -11].

Culture and microscopy are essential components of the existing standard clinical protocols for isolating and identifying *S. aureus* [12 – 16]. Despite being reliable, affordable, and capable of providing data on the quantity and kind of microorganism [13, 14], but these technologies are time-consuming and difficult to use, making them unsuitable for rapid diagnosis and therapy. It takes between two and five days to complete the process, the process includes collecting samples, culturing selectively, screening biochemically, and confirming serologically. However, due to improvements in molecular diagnostic technology, the polymerase chain reaction (PCR) assay has greatly improved the detection of bacteria [15, 16], since the detection procedure of this technique requires two to three hours (a short time).

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To rapidly identify *S. aureus*, particularly methicillin-resistant *Staphylococcus aureus* (MRSA), researchers have utilized both traditional PCR and real-time PCR (qPCR) methods [17], requiring specialized machinery. In contrast, Loop-mediated isothermal amplification (LAMP) offers a highly targeted amplification response. This technique utilizes 4-6 primers recognizing multiple sections of the target DNA. Triggered by a strand-displacing DNA polymerase, two specific primers create "loop" structures, facilitating successive rounds of amplification [18]. Unlike PCR's million-fold amplification, LAMP can increase DNA copies by up to a billion in less than an hour. Notably, LAMP can be performed in an apparatus, eliminating the need for sophisticated laboratory equipment [19].

Isothermal amplification, conducted at a constant temperature range from 60 to 65°C, negates the requirement for a thermal cycler, distinguishing it from the PCR method, which involves alternating temperature cycles [20]. Our objective was to isolate methicillin-resistant *S. aureus* from diverse food items using traditional methods. Additionally, we designed to evolve and refine a LAMP technique tailored for identifying MRSA, this involved an oligonucleotide primer set choosing genes (*nuc* and *mecA*). Lastly, the study sought to estimate the sensitivity of the LAMP technique through the analysis of spiked milk samples.

Material and Methods

Microorganism strains

To create and assess the mecA- LAMP assay, 189 *S. aureus* isolates from various food products (poultry = 103, sausage = 9, pork = 3, and minced meat = 4) as well as bovine mastitis milk (n = 70) were collected. The University of Veterinary Medicine Hannover, Germany, received these isolates as part of routine diagnoses for *S. aureus*. The *S. aureus* isolates were categorized using the traditional identification methods which includes: morphological colony features, catalase test, hemolytic characteristics, staphaurex latex agglutination test (Oxoid, England), We used the coagulase test on rabbit plasma from Becton, Germany. We also used the API-32 Staph biochemical identification system from bio-Mérieux,. Oxacillin-screening agar (Oxoid) susceptibility testing was used to identify methicillin resistance.

Molecular techniques, including both LAMP and PCR assays, were conducted on all isolates to identify *S. aureus* by detecting the *nuc* and *mecA* genes. We used molecular approaches to verify the *S. aureus* isolates. To make sure our tests were accurate, we included two positive controls: *S. aureus* ATCC 6538 and *S. aureus* (MRSA) DSM 11729.

Genomic DNA extraction and template preparation

DNA isolation from both *S. aureus* and non - *S. aureus* samples was conducted by using the MagVET™Universal Isolation Kit, based on the manufacturer's orders. DNA binding to magnetic beads facilitated isolation, and a KingFisher™ mL Magnetic Particle Processor (Darmstadt, Germany) was employed to separate the beads after each extraction step. We measured the concentration of DNA using a Genova Nano 2000c (Jenway, UK). We stored the DNA at (- 20°C) for later use.

The LAMP reaction happened in the Integrated Cap in (Giessen, Germany). The reaction had a whole volume of (25 µL) as shown in the table 3. For the reaction, we used primer F3 (0.5 µL) and primer B3 (0.5 µL) both at a concentration (25 pmol µL-1), giving a final concentration of 0.5 µM. We also used primer FIP (2 µL) and primer BIP (2 µL) (both at a concentration of 25 pmol μL^{-1}), giving a final concentration of 2 µM. Lastly, we used of primer Loop F (1 µL) and primer Loop B (1 µL) (both at a concentration of 25 pmol μL^{-1}), giving a final concentration of 1 µM.

To perform the reaction, use 13 µL of Isothermal Master Mix Iso-001 (OptiGene, UK). Then, add DNA of isolated (5 µL). Amplification will happen for half hour at 65°C. For analysis of the melting curve, temperatures will range from 98°C to 80°C. The real-time fluorometer (Genie II®) (OptiGene Limited) will ramp at 0.05°C per second, following the manufacturer's instructions.

Identification of MRSA

MRSA isolates were detected through the classical PCR assay utilize the primers targeting the *mecA* genes. The PCR reaction mixtures had a whole volume of 30 μL^{-1}, comprising of 1 μL^{-1} (10 pmol μL^{-1}) of primer 1 and primer 2 from Eurofins Genomics, 15 µL-1 of Mix Master, and 10.5 μL^{-1} double distillate Water (Qiagen). Additionally, 2.5 μL^{-1} DNA of *S. aureus* were mixed in the same tube. To run the PCR, we first heated the mixture to 94°C for 3 minutes. Then, we repeated a cycle 35 times, each cycle consisted of three steps: I. Denaturation (94°C for half a minute), II. Annealing (54°C for half a minute), and III. Extension (72°C for 40 sec.). Finally, we heated the mixture one last time to 72°C for 7 minutes. To see the results, we used a technique called electrophoresis

Analytical sensitivity of the LAMP assay and limit of detection (LOD)

We tested how sensitive the LAMP reaction is by diluting DNA of the *S. aureus* (MRSA) DSM 11729. We utilized a real-time fluorometer to find the amplicons. We made different dilutions of *S. aureus* (MRSA) DSM 11729 DNA (10^{-1} to 10^{-8}) in Tris buffer (TE, pH 8.0). To find the limit of detection (LOD), we added different dilutions (10^{-1} to 10^{-8}) of

the MRSA DSM 11729 reference strain that was grown 24 hours at 37°C on blood agar, to 10 ml of milk. We measured concentration in terms of colony-forming units (CFU). After spinning at 3700 × g for 45 min., we threw away the liquid part. We then mixed the solid part with 180 ml lysis buffer, and isolated the DNA as described before.

Statistical analysis

The significant variations among each element were assessed using the Chi-square at $P \leq 0.01$ [21].

Results

Using PCR and LAMP assay, *nuc* and *mecA* genes were examined in all 189 *S. aureus* isolates (Fig. 1, 2, and 3). The results declared that all presumptive *S. aureus* were confirmed the presence of *nuc* gene using LAMP and PCR techniques. However, only 16 (8.5%) *S. aureus* isolates were identified as MRSA due to they carried *mecA* gene. Furthermore, a significant percentage of MRSA was detected in S. aureus isolated from pork (33.3%/1/3), minced meat (25.1%/1/4), and fowl (13.6% (14/103). However, MRSA was not detected in S. aureus isolated from bovine mastitis milk and sausage (Table 3).

As predicted, there was a considerable rise in fluorescence in the chambers of LAMP, indicating the presence of gene amplification in the different samples (Fig. 1 and 2). Furthermore, the outcomes of conventional PCR showed the presence of the *mecA* gene in MRSA (Fig. 4).

Analytical Sensitivity of the LAMP Assay and Limit of Detection (LOD)

In the serial dilution, DNA concentrations extended from 1.49 ng μL^{-1} (10^{-1}) to 0.149 pg μL^{-1} (10^{-5}). The LAMP assay exhibited an analytical sensitivity of 0.149 pg µL-1 for detecting MRSA DNA, resulting in 0.0745 pg per reaction (5 µL of DNA/reaction) commensurable subsequent isothermal amplification until 21:30 minutes (Table 4). The probability of detection at the 10^{-5} concentration was 16.7% (one out of six was positive), while the detection probability for other concentrations was 100%.

S. aureus (MRSA) DSM 11729 reference strain detected utilized the LAMP assay in spiked milk. The limit of detection is $3.6\times10^{\wedge 3}$ CFU mL^{-1} in a 10 ml milk sample. The detection probability is 80%, with five out of seven samples testing positive. The mean detection time is 14:30 (± 02:40) minutes (Table 5).

Discussion

A widespread food-borne pathogen in the globe nowadays, *S. aureus* has drawn a lot of attention and concern because of its low detection level and link to antibiotic resistance [22]. Identification of microorganisms via conventional methods requires a number of sequential processes and long time and expert personnel. By sequencing the genomes of common pathogens and identifying resistance genes, significant insights into microbial resistance mechanisms can be gained, PCR and multiplex PCR have been utilized in recent decades to quickly recognize infections and detect antibiotic resistance [23, 24]. PCR-based techniques have great sensitivity and specificity, but they are not suitable for employ in point-of-care testing (POCT) because they require complex thermocyclers and multiple temperature controls [25]. Since 2000, the LAMP assay has emerged and solidified its presence. These primers recognize 6 - 8 distinct areas of the target sequence. This enables swift nucleic acid amplification in under an hour, maintaining both high sensitivity and specificity [29, 30].

LAMP assays demonstrate lower sensitivity to restrained materials found in a samples compared to PCR [29, 30]. This characteristic reduces the time and cost associated with the relatively stringent requirements for DNA extraction and purification. These advantages of LAMP technique also include ease of use, reduced time consumption, and cost effectiveness [31]. In current work, we have planned a LAMP technique that targets the *nuc* and *mecA* gene to identify *S. aureus* and predict their methicillin resistance.

Our study revealed that 8.46% of *S. aureus* carried the *mecA* gene, aligning with the PCR results. In terms of Limit of Detection (LOD), the findings indicated that higher DNA template concentrations required less time for the reaction. These findings align with previous studies that have established a correlation between time and template concentration [32 – 34]. Moreover, both LAMP and PCR assays exhibited 100% specificity, with the LAMP assay showcasing greater sensitivity, given its detection limit of 1.49 pg/µL.

Lastly, our findings aligned with several research [35 – 37] that demonstrated the 10^3/ml microbial culture concentration had good sensitivity (80%) of the LAMP assay for five runs, while the culture concentration of 10^4 had the highest sensitivity (100%). Increasing the sample volume may contribute to further enhancing analytical performance, even if the limits of detection (LODs) are already sufficient for clinical sample detection [38].

Conclusion

The outcomes of this study lead to the conclusion that the new LAMP-based MRSA detection assay, characterized by efficiency in labor and time, can successfully identify MRSA from culture isolates with 100% sensitivity at a concentration of 10^4, providing the benefit of prompt and accurate results. Furthermore, this approach is simple to employ in

any lab for detecting the *mecA* gene since it works without the requirement for specialized equipment. The LAMP reaction amplifies DNA at constant temperatures in a single tube and this feature of the LAMP technique eliminates the requirement for thermal cyclers equipment.

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Conflict of interest

The manuscript researcher confirm that they wrote and analyzed the data without any conflicts of interest.

TABLE 1. The primer sequences of the LAMP assay are used to specifically amplify the *nuc* and *mecA* genes

Primer	sequence	Molecular weight (bp)	Melting temperature
***nuc* gene**			
nuc-F3	5`-GAAGTGGTTCTGAAGATCCAA-`3	21	55.9°C
nuc-B3	5`-CCAAGCCTTGACGAACTAA-`3	19	54.5°C
nuc-FIP	5`-AGGATGCTTTGTTTCAGGTGT CGATTGATGGTGATACGGTTA-`3	42	71.4°C
nuc-BIP	5`-AATATGGTCCTGAAGCAAGTG CGCTAAGCCACGTCCATAT-`3	40	72.5°C
nuc-Loop F	5`-TCTGAATGTCATTGGTTGACCT-`3	22	56.5°C
nuc-Loop B	5`-GAAGTCGAGTTTGACAAAGGT C-`3	22	58.4°C
***mecA* gene**			
mecA-F3	5`-GCAATACAATCGCACATACA-`3	20	53.2°C
mecA-B3	5`-TCTTGTAACGTTGTAACCACC-`3	21	55.9°C
mecA-FIP	5`-TGAAGGTGTGCTTACAAGTGCTA ATGATTATGGCTCAGGTACTG-`3	44	72.2°C
mecA-BIP	5`-TGTATGGCATGAGTAACGAAGA TTGAGTTGAACCTGGTGAAG-`3	42	71.4°C
mecA-Loop F	5`-ACCTGTTTGAGGGTGGATAG-`3	20	57.3°C
mecA-Loop B	5`-AAGAACCTCTGCTCAACAAGT-`3	21	55.9°C
PCR			
mecA -1	5`-GTGAAGATATACCAAGTGATT-`3	21	46.5 °C
mecA -2	5`-ATGCGCTATAGATTGAAAGGAT-`3	22	49.2 °C

TABLE 2. The LAMP reaction mixture composed to detect the *nuc* and *mecA* genes

Components	Volume (µL) / reaction	Final concentration (µM)	Require vol/reaction µL
2× Master Mix	13		13
Primer F3 (25 pmol/µL)	0.5	0.5	0.5
Primer B3 (25 pmol/µL)	0.5	0.5	0.5
Primer Loop F (25 pmol/µL)	1	1	1
Primer Loop B (25 pmol/µL)	1	1	1
Primer FIP (25 pmol/µL)	2	2	2
Primer BIP (25 pmol/µL)	2	2	2
Template DNA (52 ng/ μL^{-1})	5		5
Total reaction volume	25 µL		25 µL

TABLE 3. Prevalence of *S. aureus* and MRSA isolates from mastitis milk and different food commodities

Source	No. *S. aureus* isolated	No. (%) MRSA isolates
Bovine mastitis milk	70	0
Poultry	103	14 (13.6%)
Sausage	9	0
Pork	3	1 (33.3%)
Minced meat	4	1 (25%)
Total	189	8.5%

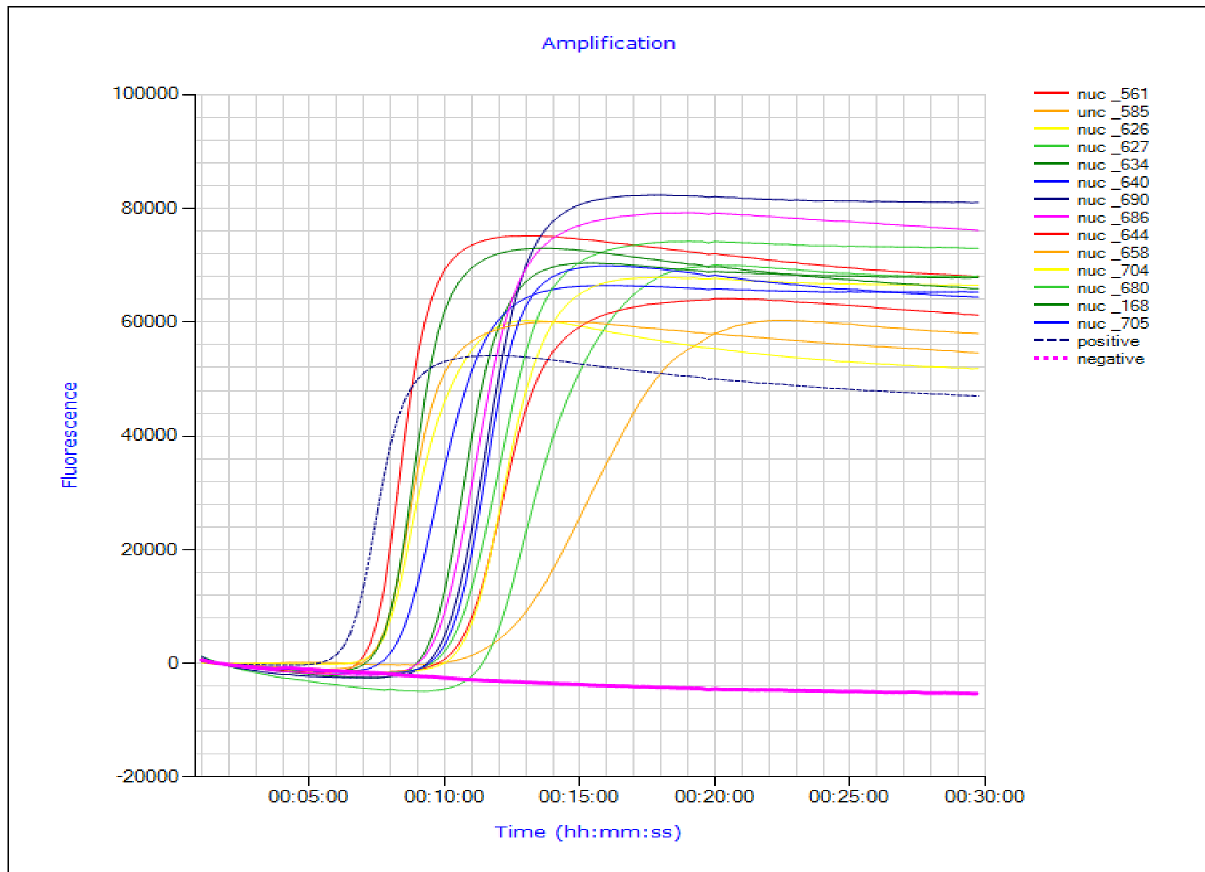

Fig. 1. Typical amplification curves of *S. aureus* isolated from different food for detecting the *nuc* gene using LAMP assay

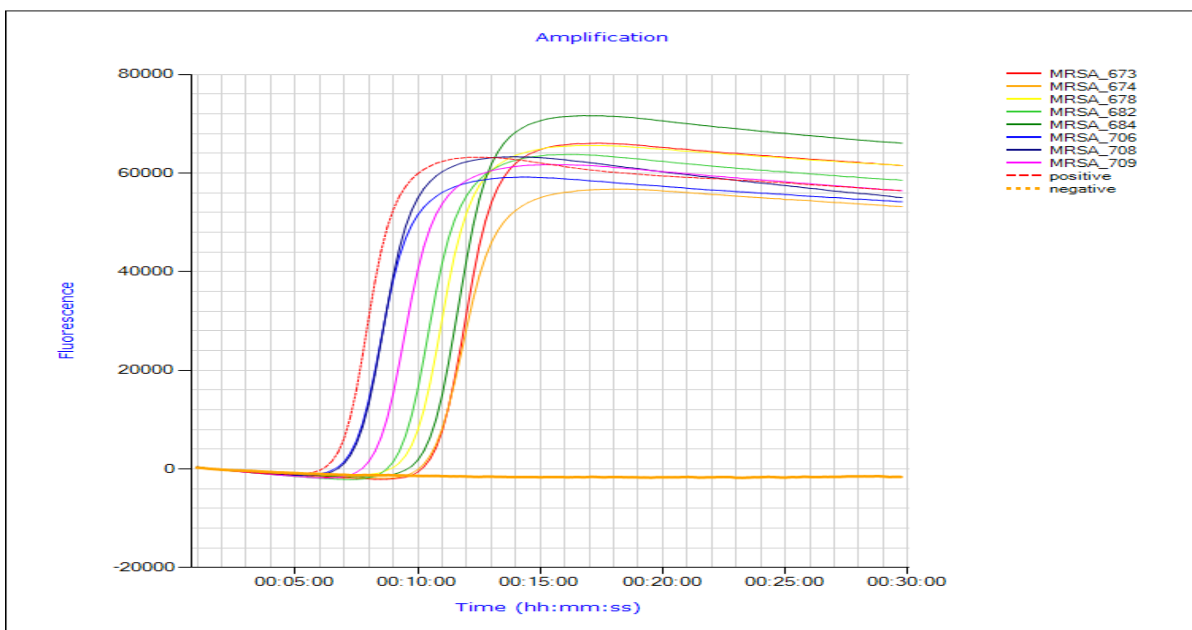

Fig. 2. Typical amplification curves of MRSA isolated from different food for detecting the *mecA* gene using LAMP assay

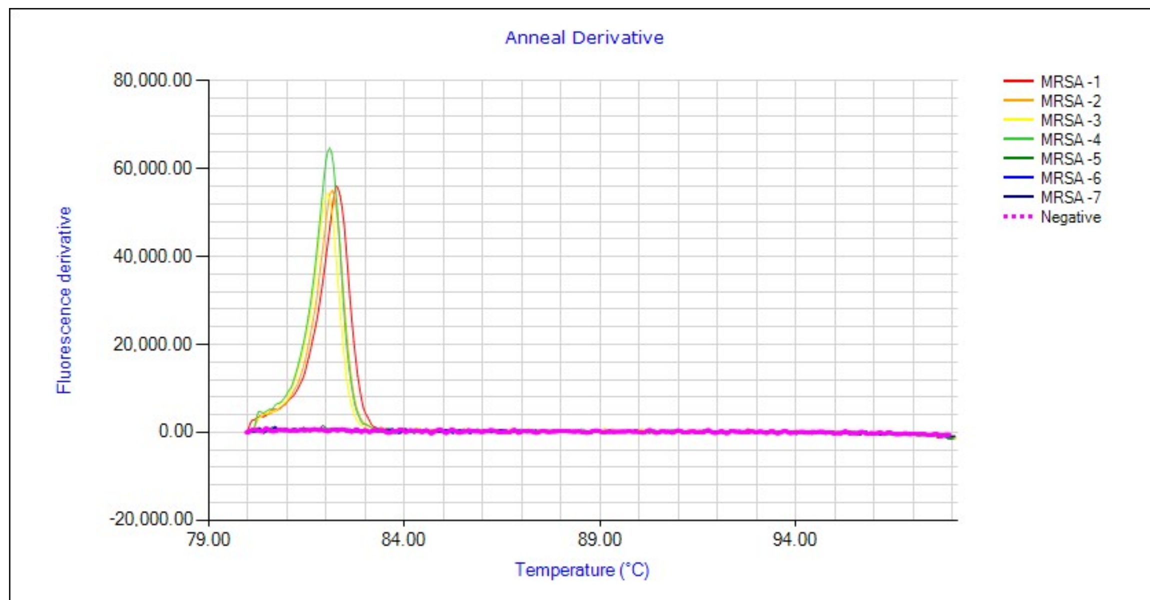

Fig. 3. The melting curve (anneal reaction) of the characteristic expansion curves of MRSA DNA for identifying the *mecA* gene using LAMP assay

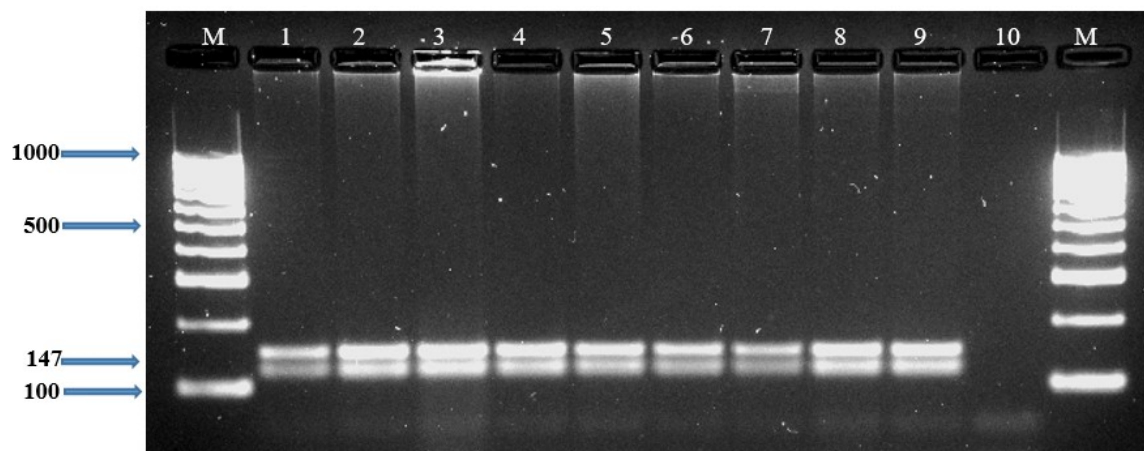

Fig. 4. The characteristic amplicon of the *mecA* gene product from *S. aureus* isolates

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الكشف الجزيئي عن جراثيم المكورات العنقودية الذهبية المقاومة للمثيسلين المعزولة من الاغذية في ألمانيا باستخدام طريقة LAMP

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الملخص

يمكن اعتبار جراثيم المكورات العنقودية الذهبية المقاومة للميثيسيلين مصدر قلق كبير على صحة الإنسان لأنها يمكن أن تسبب التسمم الغذائي والتهابات المستشفيات. في هذا الدراسة، تم الحصول على 189 عزلة من جراثيم المكورات العنقودية الذهبية من مواد غذائية متنوعة، بما في ذلك الدواجن (103 عزلات)، والسجق (9 عزلات)، ولحم الخنزير (3 عزلات)، واللحم المفروم (4 عزلات)، بالإضافة إلى حليب التهاب الضرع البقري (70 عزلة).). تم تقديم هذه العزلات إلى جامعة الطب البيطري في هانوفر بألمانيا، كجزء من إجراءات التشخيص الروتينية للمكورات العنقودية الذهبية. تم استخدام عدد كبير من عزلات المكورات العنقودية الذهبية من عينات الطعام على نطاق واسع لتطوير نهج تضخيم متساوي الحرارة (LAMP) للكشف بسرعة عن عترات المكورات العنقودية الذهبية وجراثيم المكورات العنقودية الذهبية المقاومة للميثيسيلين. لكل عترة من المكورات العنقودية الذهبية و جراثيم المكورات العنقودية الذهبية المقاومة للميثيسيلين، تم تصميم ستة بادئات خصيصًا للتعرف على هذه العزلات. وأظهرت النتائج أن جميع جراثيم المكورات العنقودية الذهبية تمتلك جين *nuc* باستخدام تقنيات LAMP وتفاعل البلمرة المتسلسل. ومع ذلك، تم تحديد ١٦ عزلة فقط من جراثيم المكورات العنقودية الذهبية (٨,٤٦□) على أنها جراثيم المكورات العنقودية الذهبية المقاومة للميثيسيلين لأنها تمتلك جين *mecA*. بلغت الحساسية التحليلية لاختبار LAMP للكشف ١,٤٩ بيكوغرام/مايكرولتر لجراثيم المكورات العنقودية الذهبية المقاومة للميثيسيلين مع عامل تخفيف $10^{٤}$ (حساسية ١٠٠□). كان الحد من الكشف لاختبار LAMP هو ٣,٦× $10^{٣}$ وحدة تشكيل المستعمرة/ مل، مع احتمال الكشف ٨٠□. هذه التقنية سهلة الاستخدام في أي مختبر الاحياء المجهرية للكشف عن جين *mecA* لأنها تعمل دون الحاجة إلى معدات متخصصة بالاضافة الى توفير الوقت اللازم لاجراء الفحص ونتائجها الدقيقة.

الكلمات المفتاحية: المكورات العنقودية الذهبية المقاومة للمثيلين، طريقة LAMP ، التسمم الغذائي ، سلامة اللحوم.