



# Phenotypic, Biofilm and Molecular Detection of Methicillin resistance *Staphylococcus aureus* isolated from locally produced Soft Cheese in Diyala Province

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## Abstract

**Background:** one of the most prominent foodborne pathogens that are destructive to dairy animals, and the dairy industry sector is *Staphylococcus aureus*. The isolation and identification of *Staphylococcus aureus* from cheese samples are considered an important way to explore the information about the prevalence of this bacterium from the locally produced cheese. **Aim.** This study aimed to Assess hygienic status of locally produced soft cheese samples, A 100 locally produced cheese samples have been gathered at various regions at Diyala state. **Methods.** The detection of MRSA had been established based on traditional appearance in addition to biochemical properties, biofilm formation, polymerase chain reaction technique was used to diagnose MRSA and to detect the gene which is responsible for resistant antibiotic (Mec A gene). **Results.** The laboratory analysis of the isolate culture across duration of study discovered that there was not significantly difference ( $P < 0.05$ ) in the proportion of MRSA isolated from cheese (9%) but it clinically important. quantitative method of Microtiter plate method (MtP) was used to assess isolates biofilm development. Results of study showed all MRSA isolate (100%) were biofilm producers (8 of them were strong, only 1 was moderate). The PCR study found that all nine isolates (100%) of *Staphylococcus aureus* obtained from 100 specimens taken had products in a molecular weight of about 1250 base pairs, which correlates to the 16SrRNA genes. A total of nine isolates (100%) of the *Staphylococcus aureus* isolates displayed products with an average molecular weight of about 310 base pairs, thus being indicative of an existence of the mecA gene. **Conclusion:** The relatively unhygienic practices and poor sanitation techniques used by farmers in the rural areas were reflected on the bacterial populations in cheese samples, also all these isolates had biofilm and possess Mec A gene which detected in PCR assay.

**Keywords:** MRSA, Soft cheese, Biofilm

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## Introduction:

Milk and its products are widely consumed by humans and considered a proper medium for the rapid growth and multiplication of microorganisms at the proper temperature (1). although warming may kill microorganism in normal cooking temperature, but toxins remain active, Staphylococcal enterotoxins are highly heat resistant (2). *Staphylococcus aureus* is one of the most frequent causes of morbidity and mortality due to an infectious agent. occurring in roughly 25–30% of all healthy individuals. It can cause a range of repercussions from mild to severely life-threatening infections such as sepsis, endocarditis (3)., wound

infections, abscess formation endovascular diseases, toxic shock syndrome (TSS) and Staphylococcal scalded skin syndrome (SSSS) (4). Enterotoxins are generated by *Staphylococcus aureus* during its growth in carbohydrate and protein-rich meals, leading to food poisoning. Vomiting and diarrhea occur after ingesting twenty-five µg of enterotoxin B. The emetic impact of enterotoxin is likely due to central nervous system activation after the toxin's interaction with neural receptors in the stomach (5). Symptoms depend on the enterotoxin and the method of entrance. Staphylococcal enterotoxins are viable and may be spread by sprays or ingesting.

Biofilm generation not merely provides

protection but additionally contributes to the development of resistance to antimicrobial treatments. Bacteria surviving within a biofilm exhibit distinct characteristics compared to planktonic bacteria of the same species, benefiting from the thick and sheltered conditions of the film that facilitate cooperation and interaction. One advantage of this setting is enhanced tolerance to detergents and antibiotics due to the rich extracellular matrix and the outer layer of cells protecting the community's inner. Antibiotic resistance may be amplified by up to 5,000 times in some conditions (6). In fact biofilms can resist antibiotic concentration 10-10,000 fold higher than those required to inhibit the growth of free floating bacteria (7).

Synthesis of penicillin-binding protein (PBP2a) by MRSA that is encoded by *mecA* gene which is carried on a movable cassette named "Staphylococcal Cassette Chromosome *mec*" or SCC*mec*. The gene that codes for an altered PBP called PBP2. The PBP2 does not join methicillin and makes the drug ineffective.

It has also an inhibitory function against Beta-lactam antibiotics by inhibiting them from effectively binding to cell wall proteins (8).

#### Method:

##### Isolation and identification of MRSA from Soft cheese :

A total of 100 locally soft cheese samples were collected from different regions located in Diyala province at different times and under hygienic conditions, the samples were transferred to laboratory environment at 4°C. Cheese sample macerated manually ,after that 10 gram from Whole homogenized Cheese were diluted decimally with 9ml (2%) buffered Sodium Citrate solution (emulsifying Cheese lobule to a tiny portion to extract a

hidden cell) then homogenized with stomacher for 3-5 minute ,after that transferring 0.02ml of suspension duplicate on Manitol salt agar with miles & Misra method (mean number of colonies in 5 areas on plate  $\times$  reciprocal of dilution factor  $\times$  50) then incubated at 37 C for 24-48 hr (9).

pure golden yellow single colonies isolated on Manitol salt agar as in Fig (1A) with standard criteria of coagulase and catalase positive, microscopic morphology and Beta hemolysis picked up & re cultured on MRSA chromogenic media then incubated at 37C for 24-48 hr for confirmation to produce blue color colony as in Fig (1B) ,after that inoculated on universal tube in order to complete other confirmation tests such as biofilm formation . MRSA isolates were further identified based on the presence of the *mecA* gene using PCR-based screening method.

##### Biofilm formation assay for MRSA isolates :

According to optical density measurements (OD<sub>570</sub>), bacterial films were divided into the two groups which are biofilm creation or no biofilm making. In the current study, 9 isolates of *Staphylococcus aureus* were screened for their capability to form biofilm via microtitration plate's method as stated by (10).

1 -*Staphylococcus aureus* bacteria harvested from new agar plates were mixed in five mL of brain heart infusion (BHI) provided with 2% sucrose, then incubation this broth to 24 h on 37°C

2- Twenty microliter of bacterial suspension from each isolates (corresponding of solution half McFarland typical) was add to microtiter wells having about 180  $\mu$ l from the Broth Brain Heart Infusion in all wells present in the microplate, which cover and incubation in 37 °C through one day.

3- By normal saline following incubation, this plate washing three times to eliminate non-adherent cells.

4- To fix the adhered germs, quantity about 200 µl of methanol with 99% concentration adding for each well for 15 minutes. The plate was dried for 30 minutes at room temperature .

5-(1% ) crystal violet solution with Approximately twenty microliters placed about fifteen minutes. 6-following eliminating the coloring solution and rinsing by sterile distill water, by ethanol with ninety six % the attached stained can be solubilized. , and the optical density was appointed in a micro-titer plate reader at 570 nm.

Table (1) :Evaluation of biofilm formation by microtiter plate method. according to (10)

Average optical density value	Adherence
$OD \leq OD_c$	Non-adherent
$2OD_c > OD > OD_c$	Weak
$4 OD_c > OD > 2OD_c$	Moderate
$OD > 4 OD_c$	Strong
Cut off value (OD <sub>c</sub> ) = average OD of negative control + (3 *Standard Deviation)	

website and supplied by Bioneer (Korea). Primer sequences used are shown in table (2 A) .These primer amplify 1250 bp region of 16S rRNA gene fragment of *Staphylococcus aureus*, The sequence of Primers ( 16s RNA )used in the study according to (KarmakarA *et al.*, 2016). Also the sequence of Mec A primer used in the study illustrated in table (2B ) . (11).

**Detection MRSA by polymerase chain reaction (PCR) :**

The DNA extraction process was carried out according to DNA Mini Kit protocol, where previously the isolates were purified on MSA and chromogenic Agar, To confirm the results of phenotypic methods for obtained isolates of *Staphylococcus aureus* , using specific primer pairs of 16S rRNA which designed for this study by using primers program on the NCBI

Table (2A ) Primer sequences of 16SRNA gene

Primer	Sequence	Primer sequence	T <sub>m</sub> (°C)	GC%	Size of Product (bp)
16s RNA	F	5'- AGAGTTTGATCCTGGCTCAG- 3'	54.3	50.0	1250bp
	R	5'- GGTTACCTTGTTACGACTT- 3'	49.4	42.1	

Table (2B ) Primer sequences of Mec A gene

Primer	Sequence	Primer sequence	Size of Product (bp)
mecA	F	5'- GTAGAAATGACTGAACGTCGATGA- 3'	310bp
	R	5'- CCAATCCACATTGTTTCGGTCTAA- 3'	

DNA isolation kit according to manufacturer instructions (Bioneer ,Korea ). A specific PCR using primers sequences listed in Table (2). PCR was conducted

**Polymerase Chain Reaction Detection of 16sRNA:**

The identifies of *Staphylococcus. aureus* isolates were confirmed using commercial

was used to assess significant differences among means. Yates' Chi-square was used instead of Chi-square because Use of the chi-square tests is inappropriate if the expected frequency is less than 5 in more than 20% of cells. Expected frequencies less than 5 are usually considered acceptable if Yates' correction is employed (Haviand, 1990)..  $P < 0.05$  is considered statistically significant

**Result:**

**Isolation of MRSA from Soft locally cheese:**

At current research Totally isolates had capability to develop on manitol salt agar which considered as a selective also differential medium for genus *Staphylococcus aureus* . The appearance of colonies characterized by smoothy, rounded, raised, shiny golden and mucoidy .and the bacterial cells exhibited themselves to be Gram-positive cocci grouped randomly in clustered such as the grapes. A catalase test was applied for further recognition, and each of the isolates generated a positive result. For recognizing the isolated bacteria at the species level, a coagulation test has been performed and each isolated indicated the capacity to generate coagulation. On blood agar, it is mainly of the  $\beta$ -hemolysis type. The proportion of Soft Cheese specimens that test positive for MRSA are listed in table (3). Based to the laboratory studies conducted across this investigation time, there has been no statistically significant ( $P < 0.05$ ) fluctuation in the ratio of MRSA among the months( 9% )..

using Thermal Cycler and the cycling conditions utilized were initial denaturation of 94 °C for 5 min; followed by 35 cycles of 95 °C for 45 seconds, 58 °C for 45 seconds, 72 °C for 60 seconds and a final elongation step of 72 °C for 5 min. The PCR products were stored at 4 °C and later separated by agarose gel electrophoresis at 100 V in a 1.5% agarose gels using UV transilluminator. After identification, each *staphylococcus aureus* strain was stored at -80 °C in Brain Heart Infusion broth (BHI), with glycerol (15% v/v).

**Polymerase Chain Reaction Detection of mecA gene :**

The presence of mecA gene in MRSA isolates was confirmed by a PCR assay. primers were used in this study in table (2). The amplification cycles consisted of an initial denaturation of target DNA at 95°C for 5 min was followed by 35 cycles of initial denaturation at 95°C for 40s, 54°C for 40 sec and 72°C for 1 min, ending with a final extension step at 72°C for 5 min, holding step at 4°C. PCR products were seen by 1.5% agarose gel electrophoresis. A 310 bp DNA ladder was used as a size marker. The amplified bands were visualized under ultraviolet trans illumination and photographed.

**Statistical Analysis**

Results expressed as Mean±SE. Data were subjected to analysis using SAS software (Statistical Analysis System - version 9.1). One-way and three-way ANOVA were applied. Least significant difference (LSD)

Month	Number samples	Number isolates	Isolation percentage%	Yates' chi-square	Yates' P-value
January	20	1	5	0.51	0.97 NS
February	20	1	5		
March	20	2	10		
April	20	2	10		
May	20	3	15		

Table(3) .The proportion of MRSA at Soft Cheese specimens

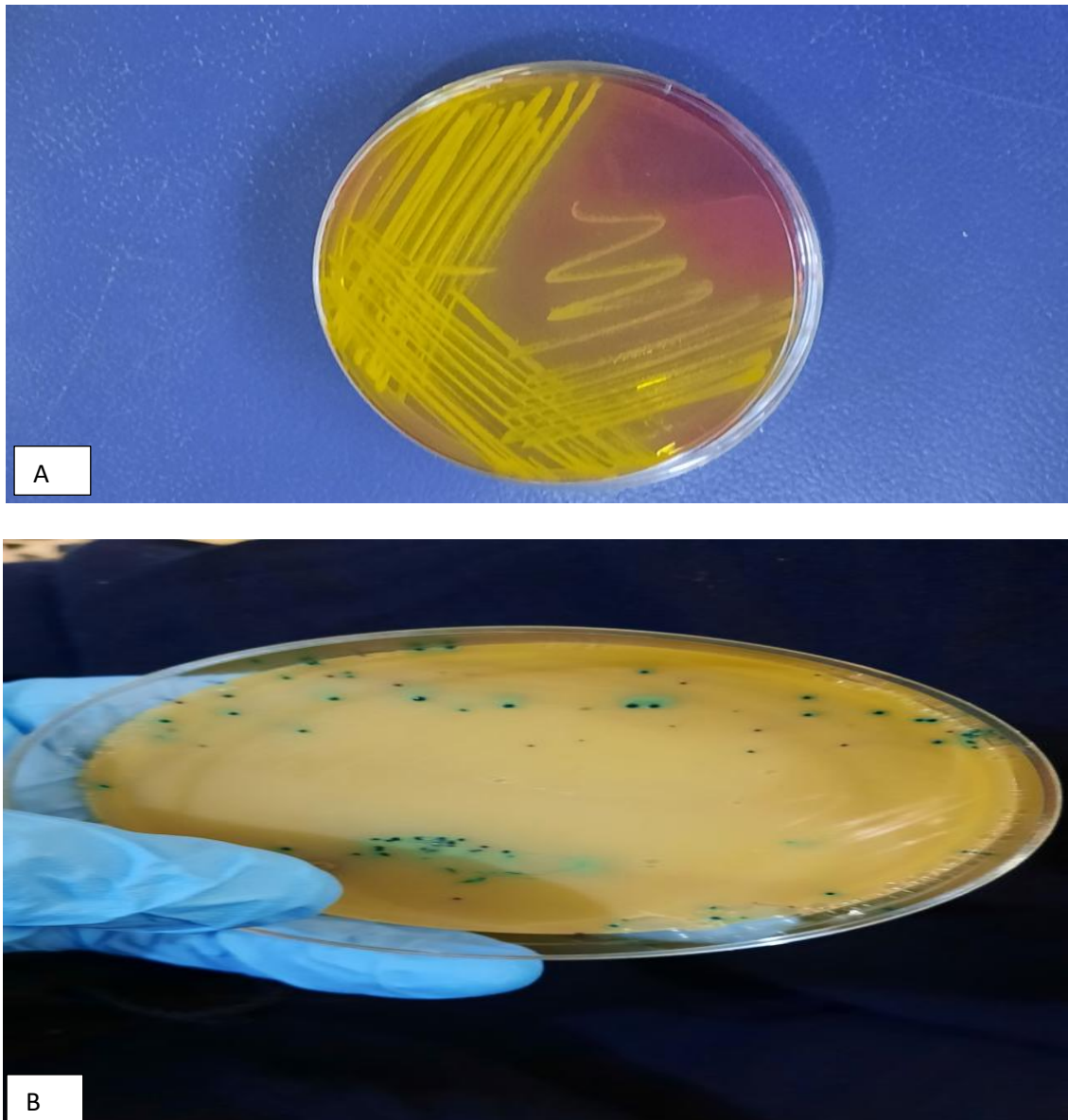


Fig (1) MRSA appearance on Mannitol (A) and Chromogenic agar (B)

accomplished via the evaluated isolates on the micro titer well In accordance with the parameters outlined in Table (4).

**Biofilm assay:**

The readings for absorbance demonstrated the degree of strength of density biofilm

Table (4): The % of strong, moderate and weak isolates according to biofilm formation based on a cutoff value (10).

Sample	Optical density of sample	OD 570 Limits number of isolates	Force of biofilm
1	0.292	$2ODc \leq OD \leq 4ODc$ $0.186 \leq OD \leq 0.372$	Moderate biofilms generator
2	0.785	$\geq 0.372$	Strong biofilms generator
3	0.660	$\geq 0.372$	Strong biofilms generator
4	0.843	$\geq 0.372$	Strong biofilms generator
5	0.836	$\geq 0.372$	Strong biofilms generator
6	1.034	$\geq 0.372$	Strong biofilms generator
7	0.718	$\geq 0.372$	Strong biofilms generator
8	1.195	$\geq 0.372$	Strong biofilms generator
9	1.009	$\geq 0.372$	Strong biofilms generator

\*average of the Negative control at OD<sub>570</sub> equal to (0.072).

\*Standard Deviation of Negative control equal to 0.007

\*Cut off value (OD<sub>c</sub>)= 0.093

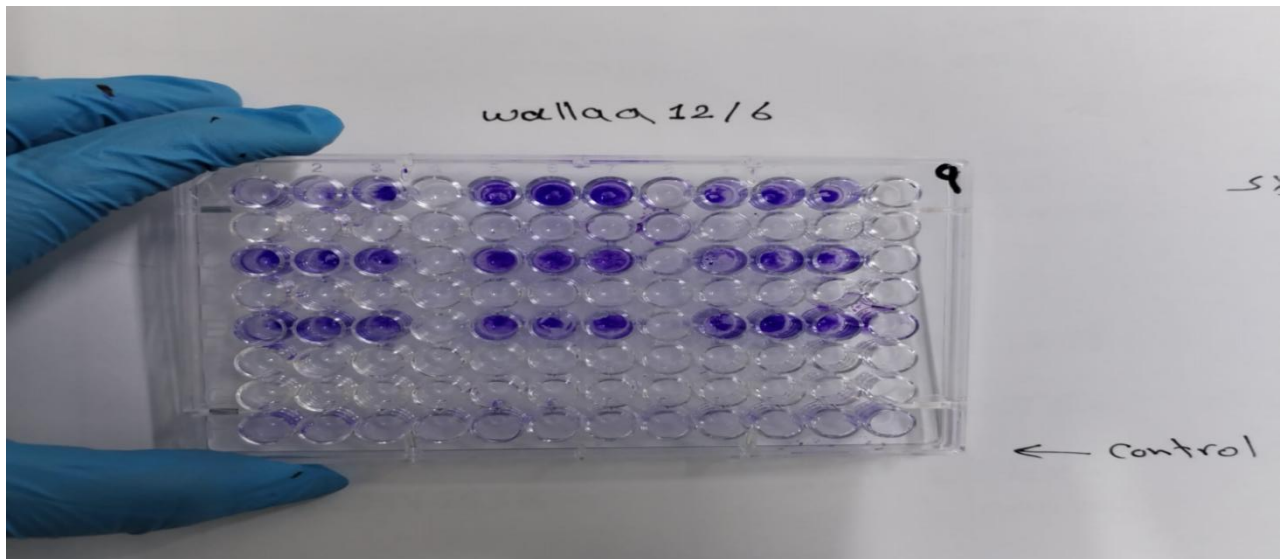


Figure (2) Detection biofilms of MRSA isolates with Micro titter plate

isolates exhibited products with a molecular weight of around 310 bp, which corresponds to the *mecA* gene, as illustrated in the figure(3).

DNA from MRSA was extracted”, “amplified in a thermo cycler (14) for “detection of genes, identification, product size (bp) and virulence *MecA* “ methicillin resistant gene”.

#### Detection of the *16S rRNA*, *mecA* Genes by PCR Assay:

The PCR analysis demonstrated that all 9 (100%) isolates of *S.aureus* out of the 100 samples generated products with a molecular weight of around 1250 base pairs, matching to the 16S rRNA gene, as depicted in Figure (2) Furthermore, all 9 positive isolate 100% of the *S. aureus*

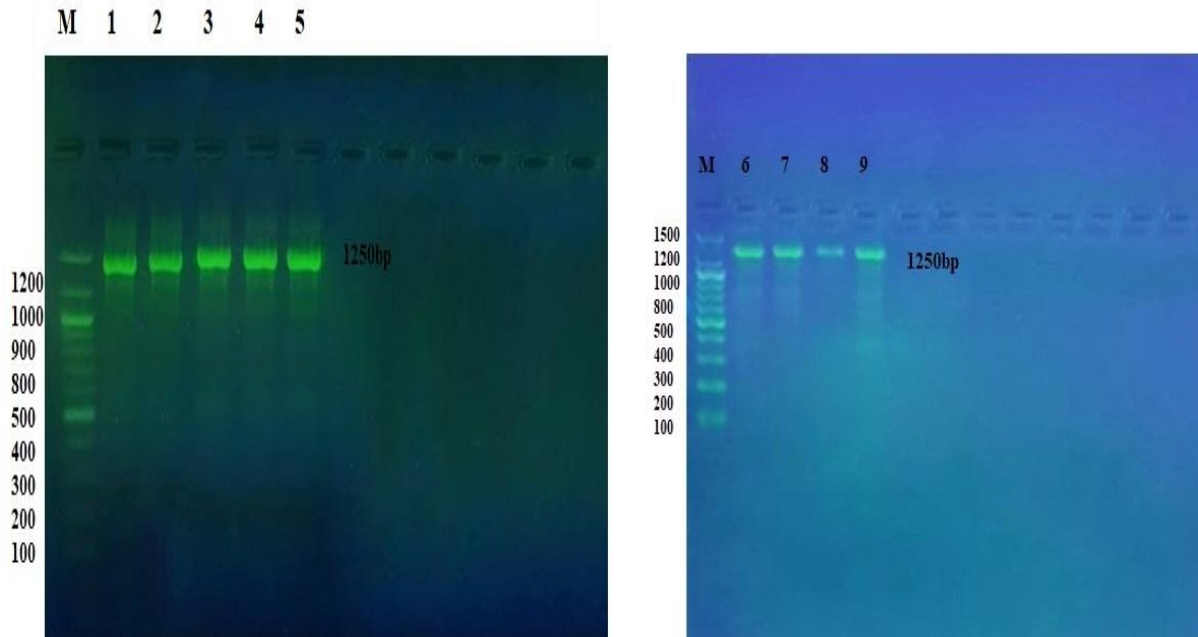


Figure (2) PCR product the band size. The product was electrophoresis on 1.5% agarose at 5 volt/cm<sup>2</sup>. 1x TBE buffer for 1:30 hours. M: DNA ladder (100).

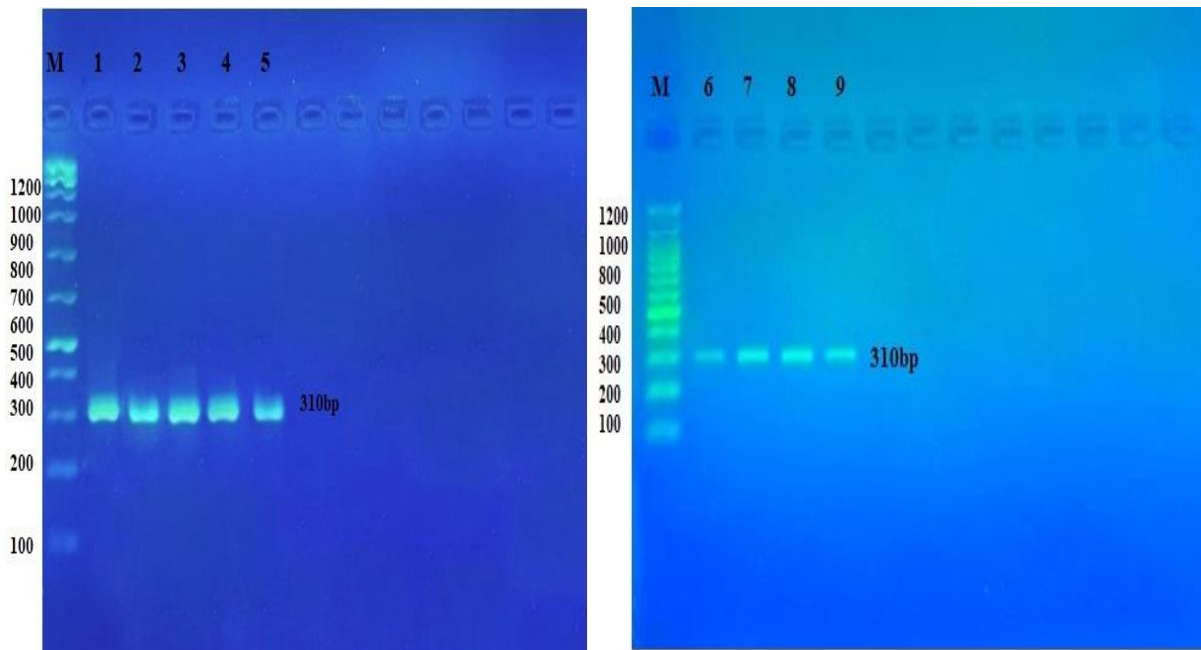


Figure 3) PCR product the band size . The product was electrophoresis on 1.5% agarose at 5 volt/cm<sup>2</sup>. 1x TBE buffer for 1:30 hours. M: DNA ladder (100). (*MecA* gene 310 bp)

One of the reliable methods for identifying MRSA strains is by detecting the *mec A* gene, which is commonly regarded as the term "gold standard." Because of its extraordinary sensitivity and rapidity (15).

**Discussion:**

A prominent food-borne organism that is destructive to dairy animals and the dairy industry sector is *Staphylococcus aureus*

(16). The isolation and identification of *Staphylococcus aureus* from cheese samples are considered as an important method to investigate the information about the prevalence of this bacterium from the locally produced cheese. *Staphylococcus aureus* is a significant contributor to foodborne poisoning in many food products suitable of social

ingesting. The transmission of *Staphylococcus aureus* food poisoning occurs commonly due to inadequate storage, distribution, and management of conventional dairy products. In numerous nations, *Staphylococcus aureus* is a highly prevalent pathogen that causes outbreaks of food poisoning when raw dairy products are consumed (17). In order to investigate the prevalence rate of *Methicillin resistance staphylococcus aureus* in the cheese samples, a total of 100 cheese samples were randomly collected from the locally shops in diyala province. 9% of the 100 samples tested positive for *Staphylococcus aureus* contamination. The findings suggested that the cheese samples were frequently polluted with *Staphylococcus aureus*, which coincides with previous research indicating the presence of enterotoxigenic *Staphylococcus aureus* in commodities made from raw milk (18). Standard method based on the cultural properties, rapid biochemical and PCR tests were used for the isolation and identification of MRSA from Cheese. The collected samples were cultured on the Manitol salt agar to form golden yellow colonies was regarded as a positive result (19). The colonial morphology was detected according to (20). *Staphylococcus aureus* was Gram positive –cocci bacteria, facultative anaerobes, pure colonies are circular, convex with 1to 1.5 mm in diameter, under the microscopic examination appeared similar to grapes collection of cells with unbalanced arrangement. Haemolysis on the blood agar enriched with 5% (v/v) sheep blood, and both the catalase and coagulase reactions were recognized. *Staphylococcus aureus* produced the coagulase enzyme which clots the plasma into gel in both tube or slide coagulase tests and the mechanism of this test including the alteration of the fibrinogen to fibrin that leading to clotting of the plasma. The coagulase test is very important differentiating the coagulase positive

*Staphylococcus aureus* from the other coagulase –negative staphylococci (21). At current study ,the catalase test was performed for all the *Staphylococcus aureus* isolates and all of them produced catalase enzyme and this result was in agreement with (22) who reported that this test was used for the differentiation of *Staphylococcus aureus* from the Streptococci species where the positive reaction appeared bubbles as a result of converting the hydrogen peroxide to water and oxygen .According to (23), hemolysis upon blood agar had been believed to constitute an essential feature to quick detection of staphylococcus aureus bacterium. Our findings corroborated their findings, that's show that  $\beta$ -hemolysis on blood agar was still thought to be a vital portion during the quickly identifying of *Staphylococcus aureus* organisms. Utilizing chromogenic agar, that generates a blue colony as illustrated in figure. (1), every presumed *Staphylococcus aureus* isolated were proven to be *Staphylococcus aureus*. An earlier investigation carried out in the city of Al-Basrah by (24) sought to ascertain the molecular identification of MRSA in bovine and buffalo-derived cheese and milk. According to the results, *Staphylococcus aureus* isolates were detected at a prevalence of fifty percent in buffalo's cheese, forty-four percent in buffalo's milk, thirty-six percent in cow's milk, and thirty-three percent in cow's cheese. With a complete prevalence rate, all isolates were identified as Methicillin-Resistant *Staphylococcus aureus* (MRSA). Another study conducted by (25) for analysis the enterotoxin genes and MRSA isolated from goat milk and its products revealed that MRSA was 9 of the total samples (9%), 2 of 25 (8%) cream samples, 4 of 50 (8%) milk samples, 3 of 25 (12%) cheese samples. The milk industry continues facing problems with eliminating contaminating by spoiled germs and restricting the development of survival organisms following processing. As a result, it is critical to give preference

to microbiological testing of milk and other dairy products. It is essential to highlight that milk must be thoroughly processed, and appropriate sanitary procedures must be observed when producing dairy-based products. The results we obtained are actually lesser than the results of a prior investigation performed in 2013 within the Veterinary Public Health department of the University of Baghdad. The study showed the isolation, differentiation, and identification of eighteen (30%) MRSA isolates coming from a total of sixty samples of unprocessed milk from cows (30 samples) and soft cheeses (30 samples). Particularly 8 (26.66%) isolates found have been identified in untreated milk and 10 (33.33%) isolates were observed in soft cheese (26). Another investigation done by (27), their goal was to identify the frequency of MRSA within personnel acting as food service employees at the city of Duhok. From the two hundred food handling cultures, 74 of them, constituting 37% of the total, have been identified to be populated in *Staphylococcus aureus*. Out of the total, 53 (26.5%) were found in the nose, 8 (4.0%) were found on the hands, and 13 (6.5%) were found in both locations. The production of all isolates to biofilm were evaluated through the quantified Micro titter plate assay (MtP),(28) and (29) reported that *Staphylococcus aureus* has the ability to form biofilms as a protective mechanism. The biofilm represents a collection of microorganisms that stick to an external surface. Microorganism cells are often surrounded by a matrix of extracellular polymerized ingredients that they generate spontaneously. Previous study revealed the significant relationship among the forming of biofilm as the resistance towards antibiotics in *Staphylococcus aureus* microbes that were obtained via untreated milk. (30). The mechanisms that are responsible for the enhanced biofilm production at temperatures below optimum levels are not yet understood. Other

researchers have given several explanations for the interpretation of those phenomena. Decreased temperature promotes the formation of fimbriae, in consequently encourages the establishment of biofilm. Additionally, lower temperatures raise the hydrophilic features of microorganisms and their capacity to attach to hydrophobic surfaces such as polystyrene (31). Furthermore, milk offers an ideal environment for the growth of *Staphylococcus aureus*, a bacterium that can persist in dairy products for extended periods. The conclusion coincides with other research that has also revealed a similar finding, indicating that most of the milk samples contained *Staphylococcus aureus* strains that exhibited the ability to generate biofilms, as reported by (32). The findings of (33) indicate that a significant proportion of *Staphylococcus aureus* strains gained from uncooked milk have ability to generate biofilms. The presence of glucose in the milk had a beneficial effect on the production of biofilm. Dietary MRSA, especially those that are prevalent in milk, has the ability of making biofilm, facilitating them to survive & spreading in dairy operations (34) the primary determinant that enhances the *Staphylococcus aureus* pathogenicity its capacity for biofilm creation (35). According to (36), A polymerase chain reaction, or PCR, is a technique which permits the amplifying of particular DNA sections (genes) by utilizing tiny DNA primers that are unique to the target sequence. Amplification by PCR technology possesses the ability to convert a small number of specified target nucleic acid particles into a substantial amount of DNA, reaching up to a microgram. One of the reliable methods for identifying MRSA strains is by detecting the *mecA* gene, which is commonly regarded as The term "gold standard." Because of its extraordinary sensitivity and rapidity (37). This study involved the method of agarose gel electrophoresis to assess the *mecA* gene. The outcome of the analysis

possessed an average size of 310 base pairs (bp). It had been identified within 104 among the 105 isolates of *Staphylococcus aureus*. representing a detection rate of 99.04%. Conclusions had been in conformity with what our result when determined MRSA via an identical technique to detect the *mecA* gene, as well as the dimensions of the end result was 310bp. Molecular strategies offer extremely reliable ways to identify both species and genus of *Staphylococcus aureus*. These techniques have an accuracy level of one hundred percent for diagnosing MRSA, in comparison to standard detection method. The MRSA represent substantial threat to a public health in many countries including Iraq due to staphylococci's capacity to develop antibiotic resistance over time, MRSA will persist as a future concern (38) . The results of our research agree with the findings of a study conducted by (24) in Al Basra. This study specifically investigated the molecular identification the MRSA strains. *Staphylococcus aureus* has been detected in the milk and cheese of both cows and buffaloes in Basra, governorate. The *Staphylococcus aureus* strains were evaluated using PCR analyses. The DNA of *Staphylococcus aureus* isolates derived

from the cheese and milk samples extracted via purification kits to verify the existence of the 16S rRNA, *nuc*, *mecA*, and *femA* genes and they found in their study *Staphylococcus aureus* isolates were give products with molecular weight approximately (310 bp) corresponding to *mecA* gene. (39) declared which 90.7% of the confirmed *Staphylococcus aureus* isolates could be verified by amplified the 16S rRNA gene, according to their research findings. The analysis also revealed that all *Staphylococcus aureus* strains recovered in both buffaloes and cows have the gene 16S rRNA. existing investigation corroborated the findings of (40) , which demonstrated that all the *Staphylococcus aureus* that were successfully recovered were confirmed to be MRSA strains using the molecular identification of the *mecA* gene. **Conclusion:** monitoring of locally produced cheese that made inside village area by educating milkers, distributors and retailers on good hygiene practices during milking and sailing of milk also, Adequate heat treatment for raw milk with little initial contamination with *Staphylococcus aureus* are regard an important step for reduction pathogen to reduce food poisoning.

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