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PHENOTYPIC AND GENOTYPIC DETECTION OF METHICILLIN-RESISTANT *Staphylococcus aureus* (MRSA) ISOLATED FROM LOCAL AND IMPORTED MEAT SOLD IN BAGHDAD CITY.

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ABSTRACT

This study aimed to Isolation and identification with molecular detection of *Methicillin-resistant Staphylococcus aureus* (MRSA) isolated from imported (frozen) and local (fresh) meat using selective media, biochemical tests, VITECK 2 technology, and conventional PCR with sequencing. (100) meat samples were collected from different markets in Baghdad city, (50) samples from each local and imported meat. The results of cultural isolation from frozen meat reported (4/50) 8% as MRSA isolates, while, only (3/50) 6% of MRSA isolates were isolated from the fresh meat samples. All suspected isolates of *Methicillin-resistant Staphylococcus aureus* (MRSA) were positive for VITEK 2 system. The results of PCR assay of (7) positive isolates from both of local and imported meat samples shown 100% (7/7) for *nuc* and *mecA* gene, 71.42% (5/7) for possessed *PVL* gene and 6/7 (85.71%) for *spa* gene. The results of current study concluded that both two types of meat sold in local markets were contaminated with this dangerous pathogenic and the imported meat showed higher contamination rate with (MRSA) than the local fresh meat.

Key words: methicillin-resistant *Staphylococcus aureus*, local meat, imported meat, PCR and sequencing.

الكشف المظهري والجيني عن المكورات العنقودية الذهبية المقاومة للميثسيلين (MRSA) المعزولة من اللحوم المحلية والمستوردة المباعة في مدينة بغداد.

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الخلاصة

هدفت هذه الدراسة إلى عزل وتشخيص المكورات العنقودية الذهبية المقاومة للميثيسيلين (MRSA) من اللحوم المستوردة (المجمدة) والمحلية (الطازجة) باستخدام الاوساط الانتقائية والاختبارات البيوكيميائية وتقنية الفايتك VITECK 2 وتقنية تفاعل البلمرة التقليدي,(PCR)والتسلسل الجيني. جمعت 100 عينة من اللحوم من أسواق مختلفة في مدينة بغداد (50 عينة من كل من اللحوم المحلية والمستوردة). أظهرت نتيجة الزرع البكتيري أن (50/4) 8.% عزلات MRSA من ماركات مستوردة مختلفة مباعة في اسواق مدينه بغداد. بينما عزلت (50/3) 6% من عزلات

[*] **This article is taken from the doctoral dissertation of the first researcher.**

MRSA من (50) عينة من اللحوم المحلية الطازجة, حيث أظهرت جميع العزلات المشتبه بها للمكورات العنقودية الذهبية المقاومة للميثيسيلين (MRSA) تفاعلًا إيجابيًا لنظام الفايتك 2. وكانت نتائج فحص PCR العزلات السبعة المعزولة من عينات اللحوم المحلية والمستوردة (المجمدة) حيث كانت 100% (7/7) من عزلات MRSA إيجابية لجين *nuc* و*mecA* و71.42% (7/5) لجين *PVL* و7/6 (85.71%) لجين *spa*. وخلصت النتائج إلى أن كلتا عينات اللحوم ملوثة بهذه البكتريا الممرضة MRSA حيث عينات اللحوم المستوردة اعلى تلوثا بجرثومة MRSA مقارنة باللحوم الطازجة المحلية .

الكلمات المفتاحية: المكورات العنقودية الذهبية المقاومة للميثسيلين, اللحم المحلي, اللحم المستورد,تقنية تفاعل البلمرة التقليدي والتسلسل الجيني.

INTRODUCTION

Meat from the nutrition point is important due to the higher quality protein, essential, non-essential amino acids, minerals and vitamins (**Smith *et al.*, 2022**). It represents an ideal medium for the growth and multiplications of spoilage microorganisms and serious pathogens as *Bacillus cereus, Escherichia. coli O157:H7, Salmonella spp., Listeria monocytogenes and Staphylococcus aureus* (**Saini *et al.*, 2021**). Many foods as dairy products can be contaminated by *Staphylococcus aureus* bacteria through different stages of milk production, transmission and processing (**Khudhir & Ahmed, 2017; Flaiyih & Khudhir., 2018**) Meat is a vehicle for methicillin-resistant *Staphylococcus aureus* (MRSA) transmission to consumers during the slaughtering processes, butchers' shops by contaminated equipment tables and knives**. (Mohamed and Hafez, 2016).** Hospital-acquired infections is causes by *Staphylococcus aureus* ranges from mild skin to dangerous invasive infections including sepsis, deep surgical site lesion and life-threatening bacteremia, (**Chung, 2023**). *Staphylococcus aureus* is a well-known as opportunistic pathogen, widely distributed in a broad range of hosts, including humans and animals (**Alzubaidy, 2022**). *Staphylococcus aureus* have the ability to cause *staphylococcal* food poisoning (SFP) by production the heat–stable enterotoxins, in (1972), Methicillin-resistant *Staphylococcus* (MRSA) was confirmed as the first causative agent of cow mastitis (**Cvetni´c, *et al.*, 2021; Al-Rasheed, *et al.*, 2022**). **Khudhir, (2019)** reported that isolation of *Staph. aureus* from the local Iraqi soft cheese samples was (90%), where out of (50) locally produced buffalos soft cheese samples examined (45) samples were positive with mean log counts of 6.930 *Staphylococcal.* food poisoning (SFP), was originate from animal causes of food poisoning mainly by *S. aureus* and very occasionally by other Staphylococcus species, **(Szczuka *et al.*, 2022)**. *S. aureus* Panton-Valentine leucocidin (PVL), are the most of virulence factors are involved in the characterization of colonization, bacterial adherence and invasion the host tissue (**Abbas & Rady.,2023**). for both *mecA* and *mecC* genes were amplified by Polymerase chain reaction technique as a confirmatory accurate method for the *S. aureus* detection from mastitic milk (**Ahmed & Yousif., 2021**). The main objective of this study was isolation and identification of (MRSA) isolated from local and imported meat to Highlight the contamination level of this pathogenic isolated from different kind of meat sold in Baghdad city.

MATERIALS AND METHODS

All culture and biochemical materials were prepared according to the Manufacturer Company and Markey, *et al*. (2013).

Samples collection

A total of (100) meat samples including (50) samples from each of local fresh and imported beef meat were obtained from the local shops of butchers and markets in Baghdad city, during the period extended from March 2022 to January 2023.

1. Frozen sample:

Frozen meat samples packaging with the vacuumed polyethylene sealed bags weighted (500) g

2. Fresh sample:

Fresh beef (500) g of minced meat were rapidly transported in the sterile polyethylene bags in a cooling icebox to the veterinary public health lab for the bacteriological analysis.

Processing of samples

1. Direct plating:

Twenty-five grams of fresh beef meat and minced frozen samples were homogenized in the 225 ml of sterile peptone water, well mixed using a laboratory blender for (2-5) minutes, and serially diluted (up to 10^{-5}), inoculated onto mannitol salt agar and Baird–parker agar with egg yolk and incubated at 37ºC for 24 hrs, pure colonies of the tested bacterial isolates were identified based on the colony, morphology, Gram staining, biochemical characteristics, serological and PCR technique (**Mohamed & Hafez, 2016).**

2. Indirect plating:

Isolation and identification of *S. aureus* were performed by pre-enrichment broth culture prepared as (25) g portion of meat homogenized with (225) mL of sterile buffered peptone water, incubated at 37 °C for 18–24 hrs. After the period of pre-enrichment18 hrs in sterile buffered peptone water, 1 mL of the culture was uniformly mixed with 5 mL of sterile nutrient broth and incubated for 24 hrs at 37 °C. A loopful of broth culture was streaked onto Mannitol Salt Agar and Baird–parker agar with egg yolk in triplicate, incubated at 37 °C for 18-24 hrs. Three to five presumptive of *S. aureus* yellow color colonies from selective agar plate were picked and sub cultured to obtained a pure culture for further biochemical tests such as Gram staining, catalase, coagulase Oxidase, DNase test serological (agglutination test) and PCR technique (**Thaker *et al.* 2013**).

VITEK 2 Compact system:

This test was used for the fast and accurate bacterial identification with the minimal training time and increased productivity (**Wrenn, 2015**).

Molecular detection of (MRSA):

DNA extraction from the bacterial isolates was done by using SaMag ™ bacterial colony DNA extraction kit, and SaMag-12™ automatic nucleic acid extraction system for the extraction of

the genomic DNA (SaMag , Cepheid, Italy). The QuantusTM Fluorometer (Promega, USA) was used to measure the concentration of extracted nucleic acid to detect the quality of the samples for further application (**Hussein, *et al.*, 2021).**
Four primers were obtained from Bioneer, Korea were used to detect *Methicillin-resistance Staph. aureus*
***Spa* F** (5_-AGACGATCCTTCGGTGAGC-3_)
R (5_-GCTTTTGCAATGTCATTTACTG-3_) **(shopsin, *et al.*,1999).**
mec A F-AAAATCGATGGTAAAGGTTGGC
R-AGTTCTGCAGTACCGGATTTGC (**Bühlmann, *et al.*, 2008).**
PVL F,ATCATTAGGTAAAATGTCTGGACATGATCCA
R, 5′ GCATCAAGTGTATTGGATAGCAAAAGC **(McClure, *et al.*, 2006).**
nuc F-GTGCTGGCATATGTATGGCAATTGT R-TACGCCGTTATCTGTTTGTGATGC **(Hegde *et al.*, 2013)**

Detection of genes by using the PCR single gene:

The PCR amplification mixture was used for detection the genes include master mix 12.5 µl, 3 µl of template DNA, 2 µl of each forwarded and reversed primers and 5.5 µl of nuclease free water to complete the amplification mixture to 25µl.

Phylogenetic tree analysis:

The phylogenetic tree with the branch lengths in the same units as those of the evolutionary distances used for explain the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (MEGA6) (Tamura and Kumar, 2004).

Sequencing analysis:

The analysis of phylogenetic tree involved 22 nucleotide sequences; the codon positions were included the 1st+2nd+3rd+Noncoding. The Evolutionary analyses were conducted in the MEGA6 (Tamura, *et al*., 2013).

Statistical analysis

Statistical analysis of data was performed using SAS (Statistical Analysis System - version 9.1). One-way ANOVA and Least significant differences (LSD) post hoc test were performed to assess significant differences among means whereas dependent and independent t tests were used in case we have two groups. Chi-Square was used for proportions comparisons. $P < 0.05$ is considered statistically significant.

RESULTS AND DISCUSSION

1. Laboratory identification of MRSA by Cultural and Biochemical characteristics:

The suspected colonies were appeared as mucoid, smooth and green to greenish blue in color on chromagar^{TM} (MeReSa Agar Base), yellow (gold) on the mannitol salt agar and dark gray to black on Baird–parker agar with clear zone around the colonies and golden colonies on

Muller-Hinton agar as shown in Table 1 and Figure 1. Gram staining of suspected colonies MRSA colony showed typical gram-positive cocci under light microscope. The biochemical tests of the isolates give positive identification as *S. aurous* (MRSA), as oxidase negative results, positive for dry spot (Staphytect Plus), latex agglutination, DNAse, catalase and coagulase testes.

Table (1): Biochemical and Cultural characteristics of *Methicillin resistance staphylococcus aureus (*MRSA*)* isolated from fresh and frozen meat.

Cultural and biochemical characteristics of *Methicillin resistance staphylococcus aureus* (MRSA)			
Cultural characteristics	Media	Cultural characteristics	
	MeReSa Agar Base	green to greenish blue colonies	
	Mannitol salt agar	yellow (gold) colonies	
	Baird–parker agar	dark gray to black colonies	
	Nutrient agar	White colonies	
	Mueller-Hinton agar	golden colonies	
	Blood agar	beta hemolysis (incomplete hemolysis)	
Biochemical characteristics	Biochemical tests	Result	Characteristics
	Gram stain	Positive	Violate cluster
	Oxidase	Negative	Violate color
	Coagulase	Positive	Fragmentation
	DNAse	Positive	Hemolysis (hydrolyze DNA)
	Catalase	Positive	Bubbles formation
	Dry spot (Staphytect Plus)	Positive	Clumping factor
	Latex agglutination test	Positive	Agglutination
	Biochemical tests	Result	Characteristics
	Gram stain	Positive	Violate cluster

Figure (1): Growth of *Methicillin resistance staphylococcus aureus* (MRSA) on chromagar^{TM} (MeReSa Agar Base), Baird–parkeragar and Dry spot (Staphytect Plus).

Colonies of *staphylococcus aureus* appeared as yellow (golden) due to the fermenting the mannitol salt and change the phenol red to the golden color and the bacteria could be resist the high salts concentration in Mannitol salt agar which used as selective medium **(Moura, *et al.*, 2018).**While on the Baird-Parker agar supplemented with egg yolk and potassium tellurite the colonies appeared as bright black surrounded by 2-4 mm clear zones after 24 hrs of incubation due to action of lecithinase which caused breakdown the egg yolk and reduced the tellurite at 35°C for 24 hrs, these were in agreement with **(Kanaan & AL-Shammary, 2013)**. Chromogenic medium with supplements aid to inhibited all *Methicillin susceptible staphylococcus aureus* (MSSA) isolates and allowed the growth and multiplication of *Methicillin resistance staphylococcus aureus* MRSA isolates, which are developed the bluish green color due to the action of the chromogenic mixture of chromogenic substance, inhibitor mixture, carbonaceous, nitrogenous, vitamin B complex and chromogenic agar promotes the detection of MRSA from the primary isolation plates during 24hrs after direct plating (**Abd Zaid & Kandala, 2021).**

The biochemical tests were mannitol positive due to fermentation of mannitol, negative reaction for oxidase, positive results for catalase through the detoxifies hydrogen peroxide and breaking it down into water and oxygen, positive reaction for both coagulase and DNase test by hydrolyze the DNA and produced a clear zone around the bacterial growth, dry spot (Staphytect Plus) give agglutination as result for detection the clumping factor, Protein A and certain polysaccharides that found in the MRSA **(Abd Zaid & Kandala, 2021; Kadhum & Abood, 2022).**

Detection by the VITEK 2 system:

All the isolates showed positive reaction for MRSA as shown in Table 2.

Table (2) results of Vitek 2 system for detection the *Methicillin-resistant Staphylococcus aureus* (MRSA) that isolated from local (fresh) and imported (frozen) meat.

Source	NO. of samples	No. of Positive MRSA isolates	No. of Positive Vitek 2 isolates	Percent %
Frozen meat	50	4	4/4	57.14 %
Fresh meat	50	3	3/3	42.86%
Total	100	7	7	100%

VITEK 2 system represents a smarter way for identification and antimicrobial susceptibility test. The results showed, that the tested isolates were highly resistant to different antibiotics, especially cefoxitin and methicillin, as these are considered the indicator and strong evidence for diagnosing the tasted bacteria (**Farhan, *et al.*, 2020).**

Isolation of MRSA from imported and fresh (local) meat:

The results revealed to presence four isolates of MRSA isolated from (50) frozen meat samples, while, only three isolates isolated from (50) fresh meat samples that collected from different markets in Baghdad city as shown in Table (Tables 3 and 4).

Table (3): Number and percentage (%) of *Methicillin-resistant Staphylococcus aureus* (MRSA) isolated from imported (frozen) meat.

Brands	N0. 0f samples	Positive (%) *S. aureus* isolates	Positive (%) MRSA isolates
Brand 1	13	3/ 13 (23.07%)	1/13(7.69%)
Brand 2	13	4/13 (30.76%)	2/13 (15.38%)
Brand 3	12	0 /12 (00.00%)	0/12(00.00%)
Brand 4	12	3 /12 (25.00%)	1/12(8.33%)
Total	50	11/50 (22.00%)	4/50(8.00%)

Table (4): Number and percentage (%) of *Methicillin-resistant Staphylococcus aureus* (MRSA) isolated from imported (frozen) and local (fresh) meat.

Source of meat sample	Total number and percentage of *Methicillin-resistant Staphylococcus aureus* (MRSA		
	No. of samples	No. and percentage of *S. aureus*	No. and percentage of MRSA
Imported meat	50	11/50 (22.00%)	4 / 50 (8.00%)
Local meat	50	16/50 (32.00%)	3 / 50 (6.00%)
Total	100	27/100 (27.00%)	7 / 100 (7.00%)
P-value		0.26	0.69

This study showed that (MRSA) that isolated from frozen meat showed variable significance according to brands and locations, contaminations of food by *S. aureus* depended on the several factors including the contaminated carriers, poor hygiene practices as handling of the food by infected workers and bad transport system between locations. **Esemu *et al* (2023)** who reported that foods regarded as vehicles for the transmission of antimicrobial-resistant bacteria.

Meats contamination can occur through the different stages of food preparation such as production, distribution and storage in the retailing supermarkets, during improper refrigeration temperature that allowed the bacterial growth and productions of enterotoxins and food handlers are implicated in the meat contamination by the dangerous pathogen *(***Bhatia & Zahoor, 2007; Ismail, *et al*., 2016)**.

The current results are in agreement with **Mohammed & Alwan, (2017)** who pointed that bovine meats are considered a main food borne disease in Karbala city. The bacterial isolation from frozen meats may indicate that imported bovine meat could be an important food vehicle for food borne infection by *S. aureus* **(Saleh *et al*., 2016)**.

Results of this study are in agreement with **Waters *et al*., (2011)** who reported that the high prevalence of *S. aureus* isolated from fresh bovine meat. In the current study contamination of local and imported meat can be regarded as indicator of bad hygiene practices

used in the local markets that may be attributed to the abuse storage conditions, contaminated from the animals themselves during the slaughtering process, worker handling with meat who carried this bacterium as normal flora, contaminated water, air and dusts, workers, handler and the dirty environment.

Genotyping Detection of MRSA by Conventional PCR:

The genomic DNA was extracted and visualized by gel electrophoresis using gel concentration 1-2 %. The purity of the DNA extracted from (7) isolates ranged from (1.8-2.1).

PCR for *nuc* and *mecA* genes:

The results of PCR assay for the (7) isolates isolated from local (fresh) and imported (frozen) meat samples showed that 100% (7/7) of the *S. aurous* (MRSA) possess the *nuc* and *mecA* as shown in Table (5) and Fig. (2).

PCR for *PVL* gene and *spa* gene:

The results of PCR assay for the (7) isolates isolated from local (fresh) and imported (frozen) meat samples showed that 71.42% (5/7) of MRSA possess the PVL gene and (6/7) 85.71% of the (MRSA) possess the *spa* gene as shown in Table (5) and Fig.3

Table (5): Number and percent of *Methicillin-resistant Staphylococcus aureus* (MRSA) with relation to the *nuc. mecA, PVL* and *spa* gene encoding in fresh and frozen isolates.

sources	Genes			
	nuc	***mecA***	***PVL***	***spa***
Fresh meat	3/3 (42.86%)	3/3 (42.86%)	1/3 (14.28%)	2/3 (28.57%)
Frozen meat	4/4 (57.14%)	4/4 (57.14%)	4/4 (57.14%)	4/4 (57.14%)
Total	7/7 (100%)	7/7 (100%)	5/7 (71.42%)	6/7 (85.71%)

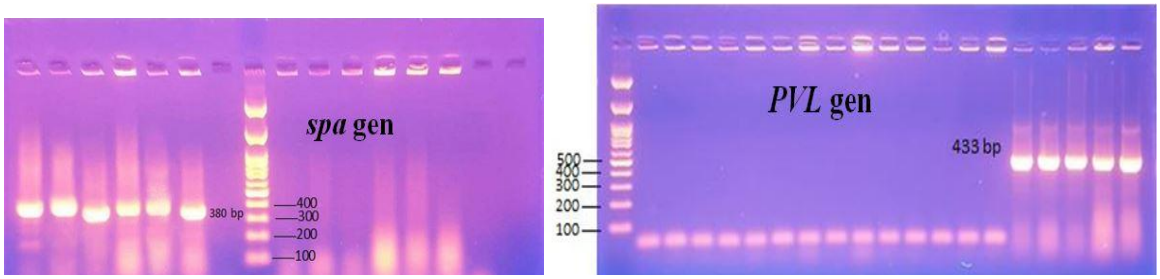


Figure (2): Gel electrophoresis (2%) stained with ethidium bromide (2µl) running of (530) bp fragment for *mecA* gene and (181) bp for *nus* gene detection in *S. aurous* (MRSA) isolates.

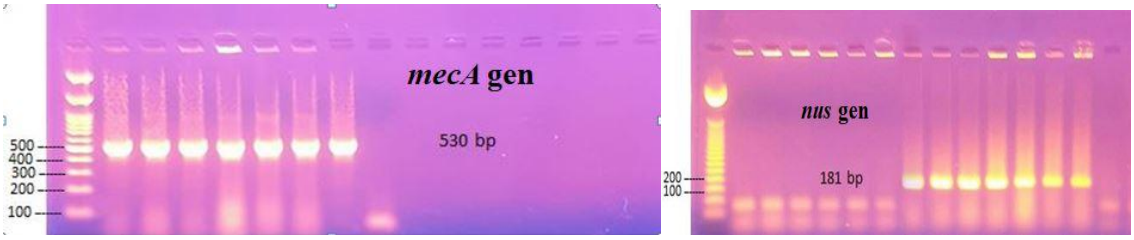


Figure (3): Gel electrophoresis (2%) stained with ethidium bromide (2µl) running of (433) bp fragment for *PVL* and (380) bp for *spa* gene detection in *S. aurous* (MRSA) isolates.

Molecular assays is accurate method for the detection of the *PVL* genes, either alone or in combination with other marker genes including *mecA*, *spa* and *nuc* **,** the PCR is well-suited for diagnostic purposes as they are easy to perform with high sensitivity and specificity **,** The *pvl* gene is the most potent staphylococcal leukotoxin capable of resisting neutrophils as a result by destruction of the polymorph-nuclear cells; this gene contributes to resistance and the pathogenicity against the host (**Zhang, *et al.*, 2018**). **Roshan, *et al.* (2022**) reported that bovine mastitis caused by MRSA harboring the virulence *spa* gene with identification percentage 92.8% and the detection rate (39/42) of the collected samples.

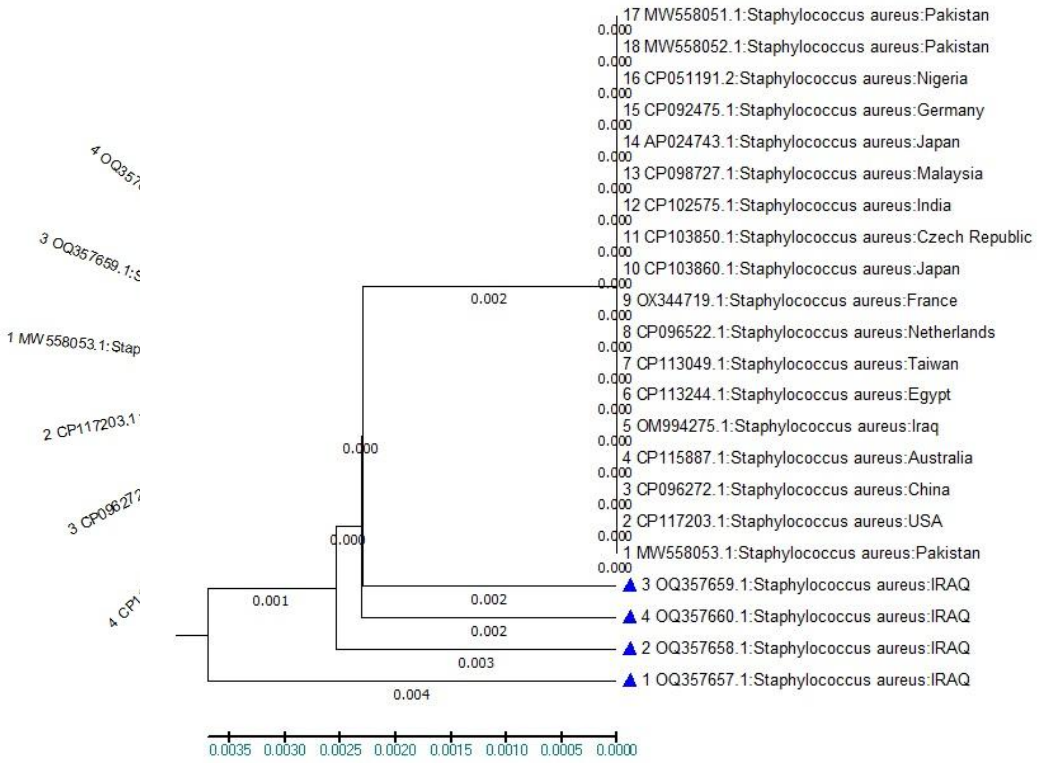
Sequence ID for *Methicillin-resistant Staphylococcus aureus* (MRSA):

The percentage of similarity or identity between all the 4 isolates sequences with GenBank sequence (MW558053.1) detailing the type, location of mutations and their conferred amino acid change (Table 6 and Fig 4), the GenBank isolates that are the most related to isolates of the current study with their accession number, country, isolation source and percent identities as shown in (Table 7). Molecular phylogenetic tree in the current study and their relatedness of 18 Gen-bank sequences that have the highest percent identity as shown in the Fig.4

Table (6): Comparison of the 4 isolates sequences with GenBank sequence (MW558053.1) detailing the type, location of mutations and their conferred amino acid change.

No. Of sample	Type of substitution	Location	Nucleotide	Nucleotide change	Amino acid change	Predicted effect	Sequence ID with Submission	Percent Identities
1	Transvertion	318	T\G	TTT\TTG	Phenylalanine\ Leucine	Missense	OQ357657.1	99%
	Transvertion	413	A\T	TAT\TTT	Tyrosine \Phenylalanine	Missense		
	Transition	448	G\A	GAA\AAA	Glutamic acid\ Lysine	Missense		
2	Transition	298	T\C	TCA\CCA	Serine\ Proline	Missense	OQ357658.1	99%

	Transvertion	318	T\G	TTT\TTG	Phenylalanine\ Leucine	Missense		
3	Transition	295	G\A	GAA\AAA	Glutamic acid\ Lysine	Missense	OQ357659.1	99%
	Transition	398	G\A	AGT\AAT	Serine\ Asparagine	Missense		
4	Transition	295	G\A	GAA\AAA	Glutamic acid\ Lysine	Missense	OQ357660.1	99%
	Transition	650	T\C	GTC\GCC	Valine\ Alanine	Missense		



Genetic mechanism of the bacterial resistance is crucial for controlling the infection, whole-genome sequencing-based analysis was performed with combination *mecA* role onto regulation and stringent like response that have important activity in the acquisition of β-lactam resistance in the MRSA (**Boonsiri, *et al.*, 2020).** In order to determine the similarity, relatedness and areas of mutation, the current sequences were aligned with the deposited on

GenBank. The results showed the (18) GenBank sequences are the most identical to current isolates with their percent identity, accession numbers and the source of isolates, are shown in (Table 7). Those sequences included three isolates from Pakistan, two isolates from Japan, one isolate from Iraq, America, China, Egypt and Australia.

The present results emphasized the importance of genetic diversity and confirm the presence of continuous sequence alterations. Through comparing the present sequences to each other and found nucleotide mutations in each of isolates compared with others, genome comparison between the mutants and their respective parent strains identified a total of (40) mutations in 1 gene and 9 intergenic regions as showed in Table 6, the mutations are frequently found in *mecA* genes (**Mohameed & Khudhir, 2023**).

Conclusion

This study concluded that the isolation of MRSA bacteria from local and imported meat can be regarded as indicator of bad hygiene practices used in markets that may be due to abuse storage conditions. Genotyping results show that all isolates of *Methicillin resistant S.aureus* holding the *mecA* gene and other virulence genes *PVL, Spa and nuc* that regarded as indicator of pathogenicity of this dangerous bacterium .

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