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MOLECULAR DETECTION OF STAPHYLOCOCCUS AUREUS ISOLATED FROM RAW MILK SAMPLES WITH COW MASTITIS.

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ABSTRACT

Staphylococcus aureus is among the bacterial pathogens responsible for the global occurrence of cow mastitis. This study used the Vitek II system, morphological, cultural, biochemical, and identification of SpA and rpoA genes by polymerase chain reaction (PCR) to isolate and identify pathogenic bacteria S. aureus from raw milk samples. The study included collecting fifty milk samples from mastitis-affected cows from various sites within the Baghdad governorate included:(Abu Ghraib,Mahmudiyah and Radwaniyah) .The samples were streaked on a plate of Mannitol Salt Agar (MSA) to detect the presence of S. aureus, and the plates were then incubated at 37°C for a whole day. 12 out of 50 samples tested positive for S. aureus, according to the data. The results showed the isolates had the highest resistance rate (100%) to Benzylpenicillin, Amoxicillin , Ampicillin/SulbactamPiperacillin/Tazobactam , Cloxacillin ,Oxacillin, Azithromycin ,Clarithromycin, Erythromycin , and Clindamycin, and showed the prevalence of antimicrobial sensitivity to Ciprofloxacin , Moxifloxacin , Norfloxacin, Linezolid Teicoplanin, Vancomycin, Rifampicin, Trimethoprim/ Sulfamethoxazole. The SpA sequence analysis demonstrated 99% and 100% identity of the samples, confirming the results of the biochemical and culture characterisation. Additionally, the SpA and rpoA genes were present in all isolates.

Key words: SpA, rpoA genes, Conventional PCR ,Antimicrobial.

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INTRODUCTION

Mastitis is an inflammatory condition of the mammary gland mostly caused by a bacterial infection. It is characterized by a variety of local and systemic symptoms (Jamali et al., 2018). Worldwide, milk is an important part of the human diet, and although important to humans, it may pose a health concern to consumers because it contains residues of antimicrobial and zoonotic anti-inflammatory drugs and also serves as a suitable medium for the growth of a variety of germs, such as bacteria harmful, especially *Staphylococcus aureus* (Sayhood et al., 2022). There are: coagulase-positive *Staphylococcus aureus* and non-*Staphylococcus aureus* (NAS). Positive coagulase *S. aureus* is well recognized as a significant pathogen, while NAS is classified as a minor pathogen (Khasapane et al., 2024; Markey et al., 2013). The predominant coagulase-positive staphylococcal species found in bovine mastitis, accounting for roughly 95% of cases, are *S.* aureus (Hassan Beg et al., 2024). Conversely, coagulase-negative bacteria, comprising over 50 species, constitute approximately 15-20% of mastitis cases. However, they can also infect humans and, in certain instances, lead to mastitis. Additionally, there are other coagulase-positive bacteria, including *S. hyicus*, which is a significant pathogen in pigs but also causes mastitis in dairy cows. Another variable is *S. agnetis*, which is known to cause mastitis in dairy cows(Guliev et al., 2020). Staphylococcus, Monitoring the epidemiology, prevalence, and incidence of bacteria responsible for bovine mastitis, with a specific focus on Staphylococcus species, is of utmost importance(Grazul et al., 2023). This monitoring is crucial for the development of programmes and strategies aimed at safeguarding human health in alignment with

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the "One Health" policy, as well as mitigating financial losses for dairy producers(Madhaiyan et al., 2020). Antibiotic-resistant bacteria are becoming a global public health problem that impacts both veterinary and human medicine (Ahmed, 2017). The *SpA* gene encodes the *Staphylococcus aureus* protein-A, which is thought to be one of the key virulence factors in the onset and severity of mastitis (Al-Dujaily & Mahmood, 2022). The bacterial cell wall product known as staphylococcal protein A binds immunoglobulin G and hinders opsonization by serum complement and phagocytosis by polymorohonuclear leukocytes (Boero et al., 2022). When protein A levels on the surface of *S. aureus* decreased, complement C3b's free receptor sites increased and phagocytosis increased (Duong et al., 2015). Transcription is a process strictly controlled in all life forms. This is because the correct expression of genes is necessary for survival(De Campos et al., 2023). RNA polymerase (RNAP), dependent on DNA, is responsible for maintaining all of the transcriptional activity within a cell. This multiprotein complex is physically and functionally comparable across distant forms of life, displaying only small differences in composition, such as the presence or lack of specific subunits at different times (Weiss et al., 2017). (β') It is the largest subunit and is composed of the active center, which is responsible for the synthesis of RNA. (β) The subunit is the second largest and is composed of the remaining portion of the active center. (α) It is the third largest subunit in two copies (ϑI and ƑII). (ω) It is the subunit that is the smallest and helps facilitate the assembly of the RNA Pol enzyme. Through the interaction of the RNA Pol with the σ factor, which is a transcription initiation factor, the RNAP holoenzyme is formed (Borukhov & Nudler, 2003). which is capable of specific promoter recognition and efficient initiation of transcription (Kireeva et al., 2013). This study set out to isolate and characterize the pathogenic *S. aureus* from raw milk samples with cow mastitis infections and also to study the variation and the production of the *SpA* gene and the *rpoA* amplicons in *S. aureus* isolates using a particular primer.

MATERIALS AND METHODS

Collection of milk samples: Fifty unpasteurized milk samples were procured from various sites throughout the province of Baghdad, Iraq. Samples were collected from local breed cows diagnosis with mastitis by California mastitis test (CMT) in sterile tubes between February to July of 2023 and submitted straight to the laboratory in a refrigerated environment.

Preparation of milk samples: Sample preparation was done by using laminar air flow serial dilutions. For every sample, five sterile test tubes were obtained, labeled, and marked. Added 1 ml sample from milk was added to a first tube containing 9 ml of peptone water. After serial dilution to (10^5), 0.1 ml from the diluted sample (10^5) was inoculated on Mannitol Salt Agar (MSA) then incubated for 24 hours at 37˚C in an incubator.

Identification of *S. aureus*

Identification was conducted using phenotypic and biochemical identification by using a standard protocol.

Culture Characteristics: The identification of *S. aureus* was accomplished through the utilisation of selective media such as blood agar, brain heart infusion (BHI) medium, and Mannitol Salt Agar (MSA) (Subhi et al., 2017).

Microscopic identification: Gram׳s stain was utilized for microscopic investigation and identification of bacterial cells (Trimmel, 2017).

Biochemical characteristics: Oxidase, Catalase, Coagulase were among the biochemical tests used to identify and describe the isolates (Kareem et al., 2020).

Bacterial identification using the Vitek II compact system: *S.aureus* identification by Vitek II compact system according to the manufacturer (bioMérieux, France), was employed by inoculating bacteria on blood agar plates and then incubating them at (37 ºC /24 hours).

DNA extraction: The extraction of bacterial DNA was performed using the FavorPrep™ Genomic DNA extraction Mini Kit, manufactured by Favorgen Biotech Corp in Korea. The quantities and quality of eluted DNA were assessed and thereafter kept at a temperature of -20 VIIC for subsequent analysis. The DNA samples that were obtained were visualized by the process of electrophoresis on a gel containing 1% agarose.

Primers: The amplification of protein A was performed by using the following primers , F (5’-CTTATATCTGGTGGCGTAACA -3’) and R (5’-CATGTACTCCGTTACCATCTT-3’) ,and for RNA polymerase α subunit gene (*rpoA*) the following primers F(5’-

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AGAACGTGGCTACGGTAC -3') and R (5'-CTGCTAATGCGTAACCTCT -3') were used

PCR amplification: The PCR reaction mixture consists of 12.5µl of pre-Master Mix, 1.5µl of DNA, 1µl of each forward and reverse primer and 25µl of deionized water. The temperature cycling parameters consisted of the following: an initial denaturation period of three minutes at a temperature of 95 °C, followed by thirty seconds of denaturation cycles at the same temperature, sixty seconds of annealing at 52 °C, ninety seconds of extension at 72 °C, and finally, a final extension period of seven minutes at 72 °C. The PCR products were separated using 1.5% agarose gel electrophoresis.

Sequencing of *SpA* gene: Confirming the amplification of *SpA* gene by conventional PCR, 25µl from PCR reaction was sent to Macrogene Company, for sequencing.

RESULTS AND DISCUSSION

Bacterial isolation and identification: Out of 50 milk samples from cows suffering from mastitis in various parts of the Baghdad governorate, 12 (24%) samples had *S. aureus*, and 76% of the raw milk samples tested positive for others bacteria. (Figure 1). Our results confirmed with reports by Emeru *et al.* (2019) and Girmay *et al.* (2020) that *S. aureus* was isolated at varying rates from mastitis-positive cows.

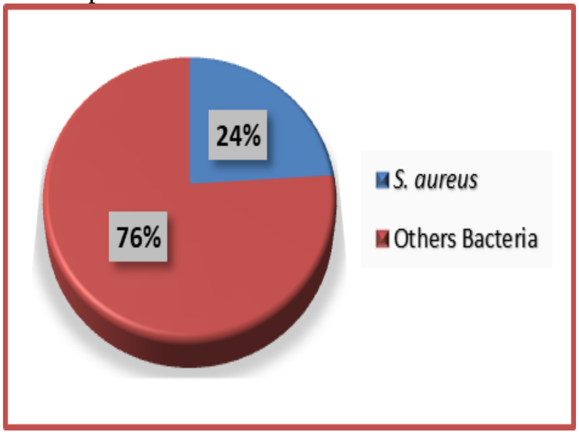

Figure 1. Percentage of *S. aureus* isolation from milk samples

Cultural and phenotypic properties

In the current study, three different commonly used culture media were studied including BHI, MSA, and blood agar . The growing of bacteria was identified in BHI , exhibiting a spherical shape with a smooth surface. The colonies were observed to be glistening, smooth, intact, elevated, and translucent(Rajab et al., 2014) this agreement with study conducted by Uma Maheswari (2019). The morphological appearance of *S. aureus* colonies on mannitol salt agar that have the capacity to ferment mannitol is regarded as a presumptive tool in the identification of *S. aureus*. small colonies surrounded by yellow zones (Schneewind & Missiakas, 2008), this agreement with study conducted by Ariyadi *et al.* (2023) and Khudiar *et al.* (2016) as seen in figure (2). The analysis of the Blood agar media revealed that the bacterial colonies exhibited hemolytic characteristics, specifically referred to as β-hemolysis (Vos et al., 2011) due to their ability to produced hemolysin enzyme, also were white, round shape, soft and convex this agreement with study conducted by Turista *et al.* (2021).

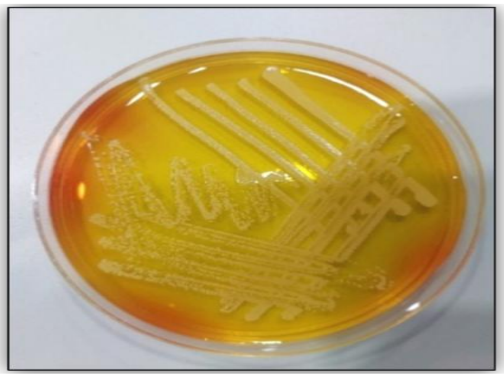

Figure 2. Mannitol Salt Agar (MSA) for the isolation *S. aureus* at 37°C for 24 h

Microscopic identification

Microscopic examination appearance of *S. aureus* after staining with Gram stain showed spherical clusters resembling clusters of grapes positive bacteria for Gram stain, as seen in figure (3), this agreement with study conducted by Shams Al Arefin (2021).

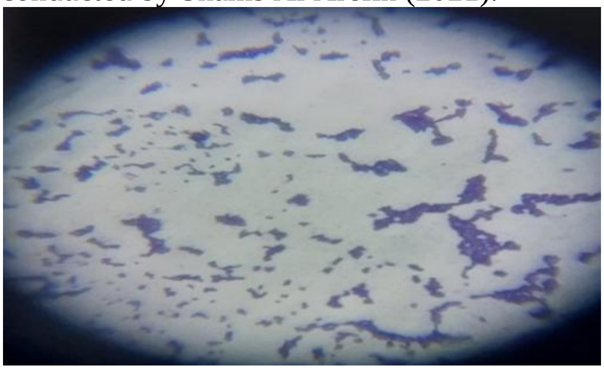

Figure 3. Microscopic *S. aureus* by using Gram Stain

Biochemical characteristics

The biochemical identification revealed that *S. aureus* generates enzymes like catalase, coagulase but not oxidase(Schleifer & Bell, 2009). This is consistent with a study done by Micheal et al. (2022). The traditional screening technique for identifying *S. aureus* is biochemical testing. As the gold standard, it has to be verified using genotypic

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techniques and species-specific gene amplification (Jamil Al-Obaidi et al., 2018).

Antibiotic sensitivity

The determination of the antimicrobial susceptibility of the *S. aureus* isolates under investigation was conducted using the Vitek II system, as presented in Table 1.The findings indicated that all isolates of *S. aureus* resistance 100% to Benzylpenicillin, Amoxicillin, Ampicillin/ Sulbactam Piperacillin/ Tazobactam, Cloxacillin, Oxacillin, Azithromycin, Clarithromycin, Erythromycin, and Clindamycin, and showed the prevalence of antimicrobial Rifampicin, Trimethoprim/ Sulfamethoxazole by sensitive Ciprofloxacin , Moxifloxacin , Norfloxacin, Linezolid Teicoplanin, Vancomycin, (100%).

Table 1. Antibiotic tests used in this study

Antibiotics	Resistant (No. and%)	(No. and%)	Sensitive (No. and%)
Amoxicillin(AMX)	30(100%)	0 (0%)	0 (0%)
Ampicillin(AMP)	30(100%)	0 (0%)	0 (0%)
Amoxicillin/Clavulanic Acid(AMC)	30(100%)	0 (0%)	0 (0%)
Ampicillin/Sulbactam(FAM)	30(100%)	0 (0%)	0 (0%)
Carbenicillin(CB)	30(100%)	0 (0%)	0 (0%)
Ticarcillin(TIC)	30(100%)	0 (0%)	0 (0%)
Ticarcillin/Clavulanic Acid(TCC)	30(100%)	0 (0%)	0 (0%)
Azlocillin	30(100%)	0 (0%)	0 (0%)
Piperacillin(PIP)	30(100%)	0 (0%)	0 (0%)
Piperacillin/Tazobactam(TAZ)	30(100%)	0 (0%)	0 (0%)
Cefsulodin	30(100%)	0 (0%)	0 (0%)
Ceftaroline(CPT)	30(100%)	0 (0%)	0 (0%)
Cefaclor(CEC)	30(100%)	0 (0%)	0 (0%)
Cefalotin(CEF)	30(100%)	0 (0%)	0 (0%)
Cefazolin(CZ)	30(100%)	0 (0%)	0 (0%)
Cefradine(CED)	30(100%)	0 (0%)	0 (0%)
Cefamandole(MA)	30(100%)	0 (0%)	0 (0%)
Cefotiam(CTF)	30(100%)	0 (0%)	0 (0%)
Cefuroxime(CXM)	30(100%)	0 (0%)	0 (0%)
Cefotetan(CTT)	30(100%)	0 (0%)	0 (0%)
Cefoxitin(FOX)	30(100%)	0 (0%)	0 (0%)
Cefcapene	30(100%)	0 (0%)	0 (0%)
Cefditoren(CDN)	30(100%)	0 (0%)	0 (0%)
Cefixime(IX)	30(100%)	0 (0%)	0 (0%)
Cefmenoxime (INN)	30(100%)	0 (0%)	0 (0%)
Cefoperazone(CFP)	30(100%)	0 (0%)	0 (0%)
Cefotaxime(CTX)	30(100%)	0 (0%)	0 (0%)
Ceftazidime(CAZ)	30(100%)	0 (0%)	0 (0%)
Ceftriaxone(CRO)	30(100%)	0 (0%)	0 (0%)
Cefepime(CEF)	30(100%)	0 (0%)	0 (0%)
Cefozopran	30(100%)	0 (0%)	0 (0%)
Cefpirome	30(100%)	0 (0%)	0 (0%)
Imipenem(IPM)	4(13.4%)	26(86.6%)	0 (0%)
Meropenem(MEM)	4(13.4%)	26(86.6%)	0 (0%)
Amikacin(AN)	30(100%	0 (0%)	0 (0%)
Gentamicin(GEN)	4(13.4%)	26(86.6%)	0 (0%)
Tobramycin(TOB)	4(13.4%)	0 (0%)	26(86.6%)
Ciprofloxacin(CIP)	30(100%	0 (0%)	0 (0%)
Levofloxacin(LVX)	30(100%	0 (0%)	0 (0%)
Minocycline(MNO)	30(100%	0 (0%)	0 (0%)
Colistin(COL)	30(100%	0 (0%)	0 (0%)
Trimethoprim(SXT)Sulfamethoxazole	4(13.4%)	0 (0%)	26(86.6%)

This study showed the susceptibility and resistance of *S. aureus* to antimicrobial agents confirm previous findings. Antimicrobial resistance has emerged as a result of incorrect use of antimicrobial medications, which poses serious risks to human and animal health. Numerous studies have demonstrated that *S. aureus* is resistant to one or more antimicrobial treatments (Argaw-Denboba et al., 2016) .This study was agreement with study conducted by Jafar Alwash and Abed Aburesha (2021). Multidrug-resistant strains of *S. aureus* in humans and animals are a critical control point for the spread of this pathogen and the associated zoonotic hazard. This is because of the widespread use of antimicrobial agents in the production of food animals, where they are frequently applied sub-therapeutically for growth production and routine disease prevention (Guliev et al., 2020).

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DNA extraction: DNA extraction methods are used to isolate DNA from samples for applications PCR. Gel electrophoresis can be used after DNA extraction to confirm the presence of DNA, assess its quality, and determine its size. A band was found in isolates, as shown in (Figure 4).

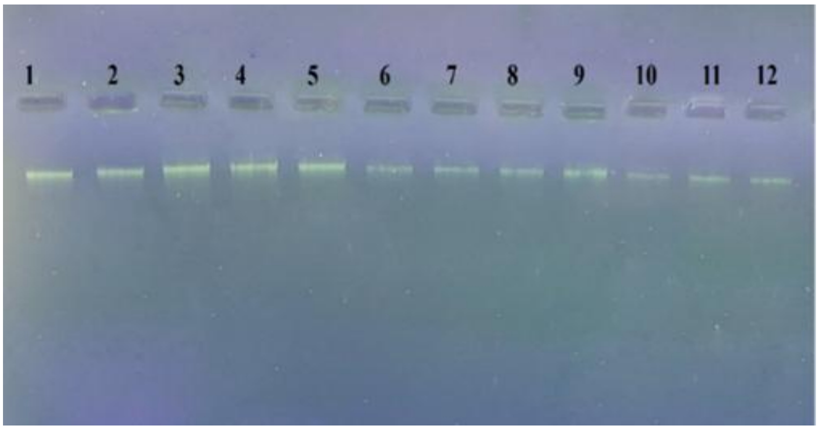

Figure 4. Total DNA extraction from *S. aureus* 1% agarose gel electrophoresis

PCR amplification

All 12 isolates of *S. aureus* out were positive for the *SpA*, *and rpoA* genes, with PCR products of 1200bp and 450bp, respectively, as seen in figures (5,6).

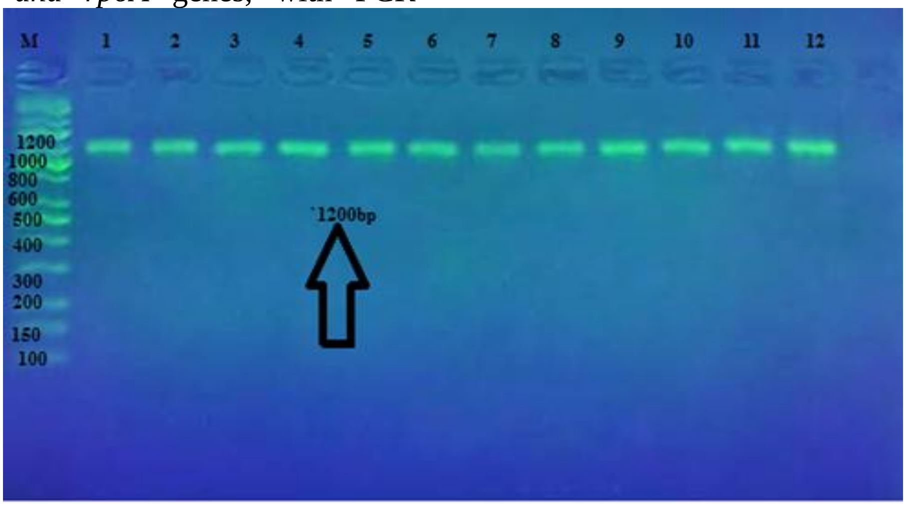

Figure 5. Agarose gel electrophoresis for SpA (1200bp). Bands were fractionated by electrophoresis on a 1.5% agarose gel , and visualized under U.V. light after staining with red stain. Lane: M (M: KAPA DNA Ladders 100bp.

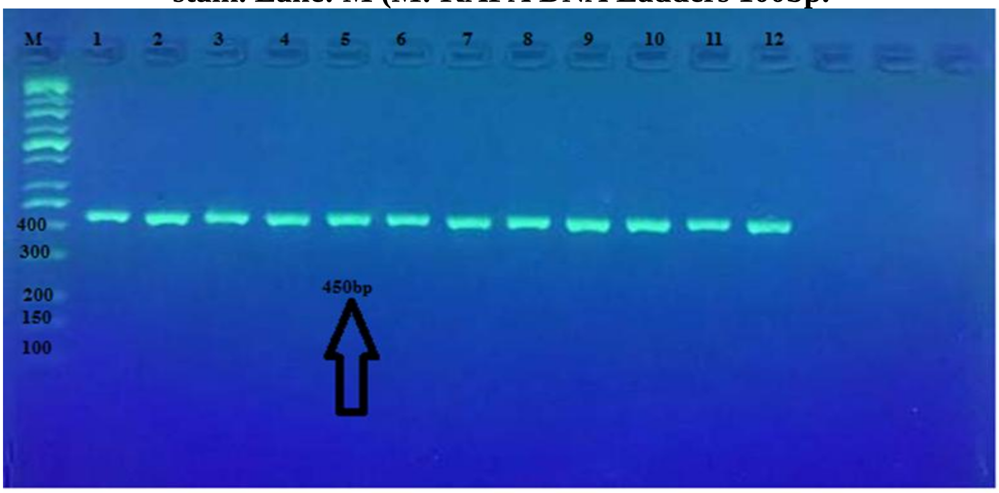

Figure 6. 450bp *rpoA* gene agarose gel electrophoresis. Bands were electrophoretically fractionated on 1.5% agarose

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Strommenger *et al.* (2008) showed multiple bands siz due to *SpA* diversity this study was agreement Salem-Bekhit *et al.,* (2010), however,our work and related study carried out by Shakeri *et al.*, (2010) are in accord. A related study carried out in India revealed an amplicon of the *SpA* gene in MRSA isolates; likewise, earlier research found isolates of *S. aureus* that lacked the *SpA* gene (Sadiq et al., 2020). The study by Abbas et al. (2016) agrees with O'Neill et al. (2006). According to the study by O'Neill et al. (2006), The RNA polymerase complex was subjected to DNA sequencing in order to identify any potential mutations that could confer resistance to rifampin. There were no strains that possessed coding polymorphisms in rpoA in comparison to the sequence of S. aureus, and there were also no genes that encoded the other subunits of the RNA polymerase complex (rpoA and rpoC).

Sequencing of *SpA* product

The *SpA* gene was amplified using the PCR technique and delivered to Macrogen Company Korea for sequencing. The results of the *spA* gene sequencing for *S. aureus* indicate that there is one Transition A\G in location (753 nucleotide) code CAA\CAG of amino acid glutamine\ glutamine and predicted effect Silent; additionally, there is one Transversion in location A\T (852 nucleotide) code GAA\GAT amino acid change Glutamic acid to Aspartic acid with the effect Missense; and there is one Transition A\G in location (75614 nucleotide) code GAA\GAG of the same amino acid glutamine\ glutamic acid. The Gene Bank found that part of the *spA* gene has 99% compatibility with the subject of the *spA* gene in NCBI, and another part of sequencing shows 100% compatibility under sequence ID: LC386138.1 and ID: CP038229.1 no recorded change noticed from the Gene Bank in spa gene as seen in table (2).This study was agreement with Kareem *et al*.,(2020) which showed the *SpA* variation significantly higher among clinical isolates**.**

Table 2. Type of *SpA* gene polymorphism

Source: Staphylococcus aureus ; spa gene								
No.	**Type of substitution**	**Location**	**Nucleotide**	**Nucleotide change**	**Amino acid change**	**Predicted effect**	**Sequence ID with compare**	**Identities**
Sp1	**Transition**	**753**	**A\G**	**CAA\CAG**	**Glutamine\ Glutamine**	**Silent**	**ID: LC386138.1**	**99%**
	Transversion	**852**	**A\T**	**GAA\GAT**	**Glutamic acid\ Aspartic acid**	**Missense**		
SP2	---------	--------	--------	--------	--------	------	**ID: CP038229.1**	**100%**
SP3	**Transition**	**75614**	**A\G**	**GAA\GAG**	**Glutamic acid\ Glutamic acid**	**Silent**	**ID: CP038229.1**	**99%**
SP4	**Transition**	**753**	**A\G**	**CAA\CAG**	**Glutamine\ Glutamine**	**Silent**	**ID: LC386138.1**	**99%**
SP5	---------	--------	--------	--------	--------	------	**ID: LC386138.1**	**100%**
SP6	---------	--------	--------	--------	--------	------	**ID: LC386138.1**	**100%**

Phylogenetic tree

The phylogenetic tree diagrammatic produced by MEGA (Molecular Evolutionary Genetics Analysis) version 6.0 program is shown in Figure 9. Using a neighbor-joining tree, a phylogenetic study was conducted. These alignments showed that the strains of *S. aureus* from Iraq and other worldwide strains shared a partial sequence in the *SpA* gene, which is responsible for translating a particular area. The following clusters are identified by hierarchical cluster analysis: *S. aureus* Iraqi isolates that are similar in 99.88 and 99.89% and are located near Ghana (ID: CP043914).1 the same 99.77 percent.

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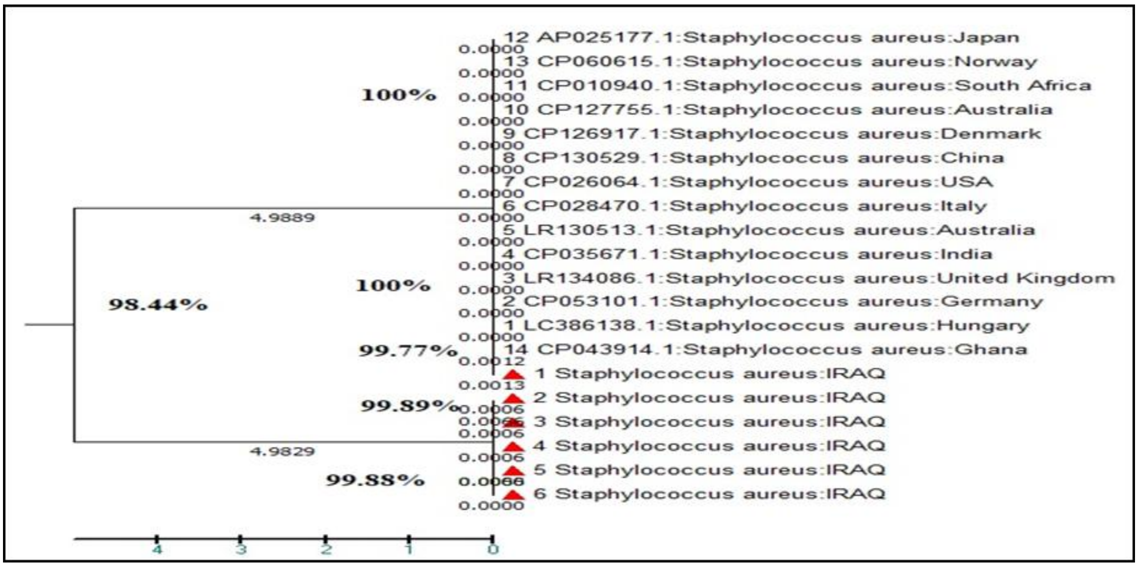

Figure 7. phylogenetic tree produced by MEGA 6 program showed that the strains of *S. aureus* from Iraq and other worldwide strains shared a partial sequence in the SpA gene, which is responsible for translating a particular area

CONCLUSION

S.aureus is a major pathogen of mastitis in cows. Conventional PCR was suitable method for detection of pathogen by amplification of both genes *spA* and *rpoA* genes.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

DECLARATION OF FUND

The authors declare that they have not received a fund.

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التحري الجزيئي عن بكتريا المكورات العنقوديه الذهبيه المعزوله من عينات حليب خام في الابقار المصابه بالتهاب الضرع

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المستخلص

تعد المكورات العنقودية الذهبية من بين مسببات الأمراض البكتيرية المسؤولة عن حدوث التهاب الضرع في البقر على مستوى العالم .الهدف من هذه الدراسة هي عزل وتشخيص البكتيريا المسببة للأمراض *S. aureus* من عينات الحليب الخام باستخدام الفحوصات المظهريه، الأوساط الزرعية، الفحوصات الكيميائية، وجهاز Vitek II، وتم تشخيص جينات *SpA* و *rpoA* بواسطة تفاعل البوليميراز المتسلسل (PCR). هذه الدراسه تتضمن جمع خمسين عينة من حليب الأبقار المصابة بالتهاب الضرع من مواقع مختلفة في محافظة بغداد ومن ضمنها (ابو غريب ، المحموديه والرضوانيه) وتم اختبارها للكشف عن المكورات العنقودية الذهبية بواسطة آجار ملح المانيتول (MSA)، وحضنت في درجة حرارة 37 درجة مئوية لمدة 24 ساعة. وأظهرت النتائج أن 12 من أصل 50 كانت إيجابية لبكتيريا *S. aureus*. أظهرت العزلات أعلى معدل مقاومة للمضادات الحياتية بنزيل بنسلين، الأموكسيسيلين، الأمبيسلين/سولباكتام، بيبراسيللين/تازوباكتام، كلوكساسيلين، أوكساسيلين، أزيثروميسين، كلاريثروميسين، إريثرومايسين، وكليندامايسين، واكثر حساسية سيبروفلوكساسين، موكسيفلوكساسين، نورفلوكساسين، لينزوليد تي. إيكوبلانين، فانكومايسين، ريفامبيسين – تريميثوبريم / سلفاميثوكسازول بنسبة (100%). أكد تحليل PCR للجين *SpA* نتائج الفحوصات المظهريه، الأوساط الزرعية، الفحوصات الكيميائية من خلال Sequencing – PCR بنسبة تطابق 99% و100%، كما كانت جينات *SpA* و*rpoA* إيجابية لعينات المكورات العنقودية الذهبية.

الكلمات مفتاحية: جينات *SpA*، *rpoA*،تفاعل تسلسل البوليميراز التقليدي، مضادات الميكروبات.