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Phylogenetic analysis and genotypic characterization of coagulase-negative *Staphylococcus aureus* isolates from sheep subclinical mastitis milk in Nineveh governorate, Iraq

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Abstract

Staphylococcus (S.) aureus is a major microorganism that causes a subclinical inflammation of the mammary glands in animals. The current study aimed to isolate and identify coagulase-negative *Staphylococcus aureus* (CNSA) in non-clinical inflammation of the mammary glands and to detect the *nuc*, *mecA*, *clfA*, *clfB*, *coa*, and *16S rRNA* genes, along with constructing a phylogenetic tree. Samples of the non-clinical milk of sixty sheep were gathered from various regions. The standard bacteriological methods were used to isolate and identify the CNSA isolates, while the PCR method was utilized to confirm and find the specific genes. Based on the results, the occurrence rate of CNSA was initiated in sheep asymptomatic mastitis at a proportion of 11.7 (7/60), and a high prevalence of CNSA in non-clinical inflammation of the mammary glands of sheep was 20% (3/15) in the Al-Nimrod district. Nevertheless, Hawe Al-Kaneisa doesn't have any isolated CNSA. The results of the PCR method showed that all CNSA isolates possessed the *nuc* gene 100 (7/7). Additionally, all isolates were methicillin-resistant coagulase-negative *S. aureus*, which has the *mec A* gene 100%, and they possessed the *clfA*, *clfB*, and *16S rRNA* genes 100%. No one in CNSA has the *coa* gene. In addition, this study showed that only one of the gene profiles was 100%. According to the *16S rRNA* gene, seven unique strains of *S. aureus* sequences have been registered in GenBank. The phylogenetic tree showed the relationship between the CNSA isolated in this study and the relationship with the CNSA isolates worldwide.

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Introduction

Intramammary infections (IMI) are a prevalent infection in dairy farms that affect animals, especially sheep. Mastitis is a major illness that results in substantial significant financial losses, both through direct costs and indirect consequences. (1). Mastitis-related costs are closely related to veterinary care and escalate the requirement for labor (2). The major economic loss resulting from clinical and asymptomatic mastitis is poor milk quality and quantity (3). The annual incidence of dairy sheep with symptomatic IMI typically remains below 5%. However, in rare instances, the incidence may range from 30% to 50% of the mammaries that live in a herd, potentially resulting in up to 70% culling or mortality due to gangrenous mastitis (4). Despite the reality that ruminant mammary glands contain over 100 different species of bacteria, only some of these microorganisms may cause mastitis (5). Contagious bacteria and environmental bacteria are two distinct groups into which the bacteria that cause bovine mastitis can be categorized based on their source, reservoir, and manner of transport (6). The most frequently bacterial isolates from asymptomatic mastitis (SCM) are staphylococci (7). Coagulase-positive (CPS) and coagulase-negative (CNS) staphylococci can be distinguished by their capacity to coagulate rabbit plasma in the diagnosis of mastitis.

Although coagulase-negative strains of Staphylococcus aureus do exist, the predominant pathogen-caused mastitis in ruminants is typically coagulase-positive (8). Several previous studies have indicated that the most frequently discovered bacteria in sheep and goats with non-clinical forms of mastitis are CNS, with prevalence rates ranging from 25% to 93% (9-11). CNSA can cause mastitis in animals, and it is regarded as a non-contagious coagulase-negative Staphylococci, thereby leading to insufficient infection therapy (12). The most causative agent isolated from milk samples in dairy goats with SCM, 44.7% to 95.9%, are CNS, and *S. aureus* comprises up to 4.1 to 18.0% of SCM agents and is usually considered to have greater pathogenicity (13). Coagulase-negative staphylococci (CNS) are categorized as opportunistic microorganisms because they can cause mastitis through direct or indirect contact with contaminated surfaces such as the environment, equipment, and skin (14). These microbes can induce chronic infections, leading to an escalation in the count of somatic cells, modifications in milk composition, and a decline in milk production (15), and they will subsequently result in escalated death rates and reduced lamb growth (16). The identification of CNS primarily relies on phenotypic biochemical reactions, and misidentification may happen due to the varying presentation of various phenotypic characteristics (17). Staphylococcus species can be characterized using a variety of studies of biology on molecular level-based procedures, including polymerase chain reaction (PCR) (18) and internal transcribed spacer (ITS)-PCR (19). These technologies reveal promise in expanding our knowledge of the distribution and potential variations in the asymptomatic features of mastitis (20).

The goals of the current project are to isolate and identify the pathogenic CNSA in milk samples obtained from sheep affected by subclinical mastitis, to detect the *nuc, mecA, clfA, clfB, coa,* and *16S rRNA* genes in CNSA isolates, and to analyze the relationship among the CNSA in this study with CNSA isolates from other studies.

Materials and methods

Ethical approval

The Institutional Animal Ethics Committee at the University of Mosul, College of Veterinary Medicine approved the ID count for all samples was UM. Vet. 2024.065. All samples were obtained with the consent of their owners and were utilized in accordance with the aforementioned ethical standard.

Samples collection

Between November 2023 and March 2024, sixty samples of sheep with asymptomatic mastitis were collected from various regions across the governorate based on the California Mastitis Test. These locations included Al-Nimrod, Hawe Al-Kaneisa, Al-Qassra, and Al-Shamsiiat. The sheep milk samples were collected in sanitary containers and sent immediately to the laboratory. Subsequently, the peptone water container was placed in an incubator at 37°C for 18-24 hours (21). One loop of milk samples was sprayed onto medium plates containing 7.5% mannitol and blood, and then they were incubated at 37°C for a whole day.

Coagulase-negative *S. aureus* isolation and characterization

To examine the characteristics of CNSA colonies using classical methods such as coagulase test, catalase test, and morphology to identify them through phenotypic characterization (22).

Isolation of DNA

To facilitate the extraction of the genomic DNA of CNSA, positive isolates were cultivated for 24 hours at 37°C on mannitol salt agar. The Gram-positive bacteria's DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Germany). A Biodrop spectrophotometer (UK) was used to evaluate the DNA concentration of CNSA.

Reaction of PCR

The *nuc* gene has a molecular weight of 166 bp (23), *mec A* is 147 bp (24), *clf A* is 288 bp (25), *clf B* is 203 bp (25), *coa* is 674 bp (26), and 16S rRNA is 1403 bp (27). A 200 µl tube was used to prepare the mixture for the PCR reaction, which required a total volume of 50 µl (Biozym, Germany). In the reactant combination, there was 8 µl of the parent strand of DNA, 25 µl of GoTaq Green Mix Master (2×) (Promega, USA), 2 µl of each nucleic acid primer F and R, and 13 µl of double - distilled water (Addbio, Korea). The resultant amplicons were subjected to an examination by gel electrophoresis on a 2% agarose gel (Peqlab, Germany), with a 100 bp ladder acting as a reference (Table 1).

DNA sequencing

All amplicons were delivered to Macrogen, a commercial sequencing business in the Republic of Korea so that **six** PCR amplicons that were taken from isolates of milk sheep suffering from non-clinical mastitis and had all earlier been determined to be positive for CNSA using the traditional PCR method could be purified and sequenced. The target genes for sequencing were supposed to be the 16S rRNA gene. The NCBI BLASTn program was subsequently utilized to compare the 16S rRNA gene sequences that had been acquired and accessible from previously disclosed GenBank-accessible CNSA sequences. The online multiple sequence alignment tool CLUSTALW is available in MEGA11. The identical amplicons Net tool CLUSTALW and the DNA star software were employed to generate phylogenetic trees. This comprehensive approach was targeted to improve knowledge of the phylogenetic context of the amplicons and make available the genetic lineage between the CNSA isolates via refinement, sequencing, and later data interpretation.

Table 1: Primer sequences and PCR conditions for CNSA gene analysis

Gene	Primer	Sequence (5- 3)	Amplicon Size [bp]	Program *	Reference
nuc	nuc-1	5-CCTGAAGCAAGTGCATTTACGA-3	166	I	(23)
	nuc-2	5-CTTTAGCCAA GCCTTGACGAACT-3			
mecA	MEC A-1	5-GTGAAGATATACCAAGTGATT-3	147	II	(24)
	MEC A-2	5-ATGCGCTATAGATTGAAAGGAT-3			
clfA	clfA-1	5-ATTGGCGTGGCTTCAGTGCT-3	288	I	(25)
	clfA-2	5-CGTTTCTTCCGTAGTTGCATTTG-3			
clfB	clfB-1	5-ACATCAGTAATAGTAGGGGCAAC-3	203	III	(25)
	clfB-2	5-TTCGCACTGTTTGTGTTTGCAC-3			
coa	coa-1	5-ATAGAGATGCTGGTACAGG-3	674	I	(26)
	coa-2	5-GCTTCCGATTGTTCGATGC-3			
16S rRNA	16S-1	5-AGTCGAGCGAACAGATAAGGA-3	1403	IV	(27)
	16S-2	5-AAATGGTTACTCCACCGGCTT-3			

PCR program: I: 35 times (94°C – 30s, 55°C – 30s, 72°C – 30s), II: 35 times (94°C – 30s, 54°C – 30s, 72°C – 30s), III: 35 times (94°C – 30s, 60°C – 30s, 72°C – 30s), IV: 35 times (94°C – 30s, 54°C – 30s, 72°C – 30s).

Results

The CNSA colonies that yielded excellent results displayed a pink-red color on Mannitol salt agar. Additionally, various biochemical tests, which included the coagulase assay, yielded negative results, while the catalase assay revealed positive results, verifying the existence of CNSA isolates. According to our analysis, the incidence rate of the CNSA isolates was 11.7% (7/60). In the region of Al-Nimrod, the highest percentage of CNSA isolated from asymptomatic mastitis in sheep was 20% (3/15). Consequently, the incidence rate of CNSA in Al-Qassra and Al-Shamsiiat was 13.3% (2/15). However, no CNSA isolated was found in Hawe Al-Kaneisa (Table 2).

Table 2: Frequency and proportion of CNSA isolates from sheep milk samples

Region	Samples (No.)	Positive *CNSA* (No.)	Percentages (%)
Al-Nimrod	15	3	20%
Hawe al-Kaneisa	15	0	0%
Al-Qassra	15	2	13.3%
Al-Shamsiiat	15	2	13.3%
Total	60	7	11.7%

The results of the PCR experiment confirmed the conclusions reached from more traditional techniques, demonstrating that 100% (7/7) of the isolates of CNSA carried the *nuc* gene (Figure 1). The CNSA isolates were characterized as methicillin-resistant coagulase-negative *Staphylococcus aureus* (MRCoNSA), with all isolates 100% (7/) found to carry the *mec A* gene (Table 3 and Figure 2). Moreover, the results showed that all CNSA carried the *clf A, clf B,* and *16rRNA* genes 100% (7/7) (Figures 3-6). Notably, the current investigation revealed that not all CNSA isolates contained the *coa* gene (Figure 5). Furthermore, the results indicated that CNSA exhibited a unique genetic profile determined by the presence of specific genes in all CNSA. All CNSA isolates 100% (7/7) most commonly exhibited gene profile I (*nuc + mec A + clf A + clf B + 16S rRNA*). None of the CNSA isolates contained just a single gene.

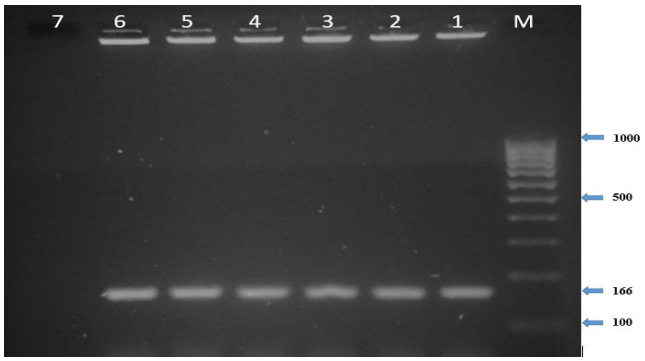

Figure 1: Relative molecular mass of the *nuc* gene in CNSA was 166 bp based on Agarose Gel Electrophoresis (2%). The DNA amplification results in a banding pattern resembling a ladder.

Table 3: Gene prevalence in CNSA isolates: Quantitative analysis

Gene	CNSA n(%)
1. *nuc*	7 (100%)
2. *mec A*	7 (100%)
3. *clf A*	7 (100%)
4. *clf B*	7 (100%)
5. *coa*	0 (0%)
6. 16S rRNA	7 (100%)

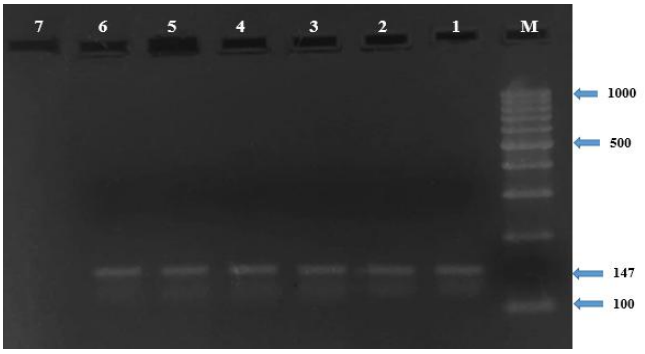

Figure 2: Relative molecular mass of the *mec A* gene in CNSA was 147 bp based on Agarose Gel Electrophoresis (2%). The DNA amplification results in a banding pattern resembling a ladder.

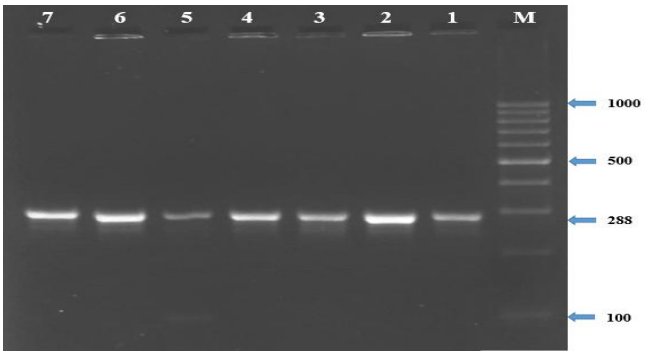

Figure 3: Relative molecular mass of the *clf A* gene in CNSA was 288 bp based on Agarose Gel Electrophoresis (2%). The DNA amplification results in a banding pattern resembling a ladder.

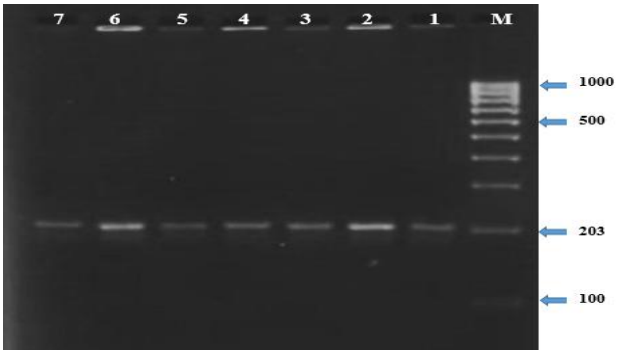

Figure 4: Relative molecular mass of the *clf B* gene in CNSA was 203 bp based on Agarose Gel Electrophoresis (2%). The DNA amplification results in a banding pattern resembling a ladder.

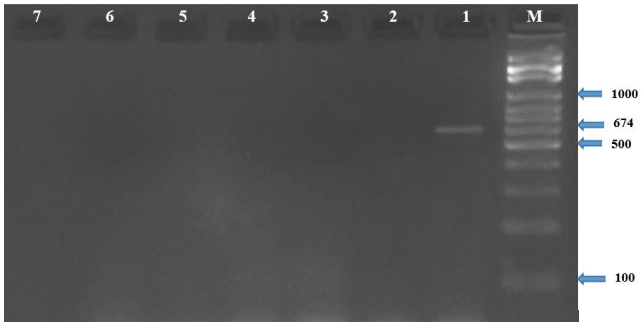

Figure 5: Relative molecular mass of the *coa* gene in CNSA was not detected based on Agarose Gel Electrophoresis (2%). The DNA amplification results in a banding pattern resembling a ladder.

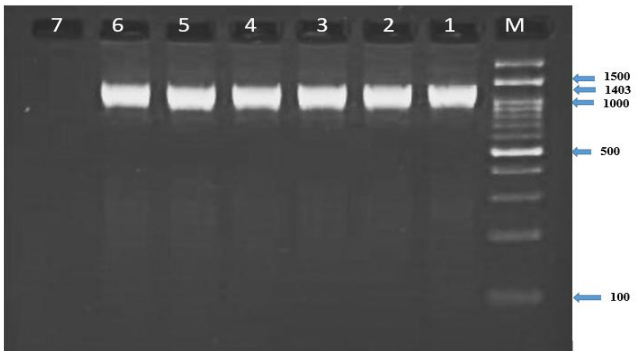

Figure 6: Relative molecular mass of the 16S rRNA gene in CNSA was 1403 bp based on Agarose Gel Electrophoresis (2%). The DNA amplification results in a banding pattern resembling a ladder.

A BLASTn analysis was shown on seven novel 16S rRNA gene sequences found from milk samples of sheep with asymptomatic mastitis. These sequences were analyzed individually and corresponded to the sequencing results presented in this research. Table 4 indicates that NCBI GenBank contains *S. aureus* sequences indexed beneath reference numbers such as PV034830, PV034831, PV034832, PV034833, PV034834, PV034835, and PV034836. Furthermore, Phylogenetic analysis using the maximum likelihood method in MEGA11 software demonstrated that local gene sequences exhibited substantial genetic similarity compared to earlier GenBank-deposited reference sequences. Moreover, the sequence types PV034830, PV034831, and PV034836 appeared to have a strong 100% connection between the *S. aureus* sequence kinds reliant on the 16S rRNA gene with the sequence types FJ463832.1, PQ721095.1, PQ721090.1, PQ721089.1, PQ721088.0, and JQ975895.1 from China. Nevertheless, the sequence types PV034832.1 showed a high relationship of 94% with a sequence type from USA KF600372.1, China MZ198261.1, Germany KT153203.1, and China

HM352415.1. In addition, the sequence types PV034833.1 declared similarities 79% with a sequence type from USA KF600372.1, China MZ198261.1, Germany KT153203.1, and China HM352415.1 of *S. aureus* based on the 16S rRNA gene (Figure 7).

Table 4: NCBI accession numbers for *16S rRNA* gene sequences of *S. aureus*

Reference number	Bacteria	Gene
PV034830	*S. aureus*	*16S rRNA*
PV034831	*S. aureus*	*16S rRNA*
PV034832	*S. aureus*	*16S rRNA*
PV034833	*S. aureus*	*16S rRNA*
PV034834	*S. aureus*	*16S rRNA*
PV034835	*S. aureus*	*16S rRNA*
PV034836	*S. aureus*	*16S rRNA*

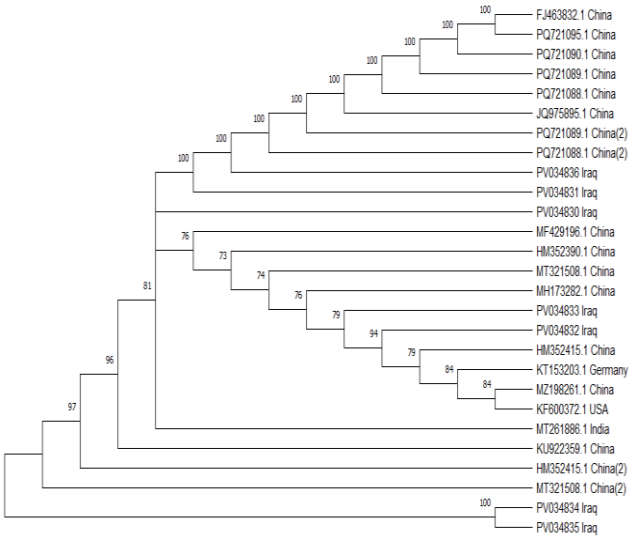

Figure 7: Hierarchical clustering of *S. aureus* gene sequences retrieved from NCBI GenBank, with accession numbers indicated in Parentheses

Discussion

Dairy farming worldwide is greatly affected economically by mastitis. This is particularly important because non-clinical mastitis patients do not exhibit outward signs of infection (28). Research has shown that *Staphylococcus aureus*, including CNS infections, is linked to escalated inflammation (29), udder tissue damage (30), and declined milk production in dairy sheep (31). The study intended to find the percentage rate of CNSA and MRCoNSA in sheep milk of asymptomatic mastitis and to recognize the genes encoding virulence factors of CNSA isolates. The prevalence of CNSA isolated from sheep with non-clinical mastitis was 11.7% (7/60). Many previous studies reported that CNSA was identified in mastitic milk from ruminant farms (32,33). In Egypt, the prevalence of CNSA in milk collected from cows and buffaloes was 5.3% (2/38) (34). Classical methods are used to isolate and identify CNSA from samples and provide the phenotypic characterization of CNSA isolates (35,36). Biochemical tests used to identify CNSA cannot determine the bacterial species and subspecies or provide comprehensive information about the genes present in CNSA (37). Molecular methods, such as PCR assays, are used to differentiate coagulase-positive *S. aureus* (CPSA) from CNSA based on possessed of the *coa* gene (38). Consequently, studies on CNSA in ewe milk remain extremely limited compared to the extensive research conducted on other pathogenic bacteria that cause mastitis worldwide. Several causes play a role in the spread of CNSA in milk and its products, including unhygienic conditions during milking on dairy farms, transportation, processing in dairy plants, and storage (39).

In addition, the PCR assay showed that all CNSA isolates possessed the *nuc*, *mecA*, *clfA*, *clfB*, and 16S rRNA genes 100%, while none of the CNSA isolates had the *coa* gene. Previous studies revealed that the *nuc* gene was found in *S. aureus*, including CPSA and CNSA, while the *mecA* gene was present only in methicillin-resistant isolates (40-44). The prevalence of the *mecA* gene in *S. aureus* isolated from sheep milk in Iran was 41.4% (45), in Turkey was 17.2% (46), and in Spain was 99.5% (47). In this investigation, *S. aureus* isolates from milk samples displayed genes such as *clfA* and *clfB*; these genes have played an important part in udder lesions related to mastitis; worldwide studies have found high levels of virulence genes including *clfA* and *clfB* (48). Additionally, the *clfA* and *clfB* genes found in *S. aureus*, which isolated from the milk of cow and goat in Brazil was, 76 and 76.67%, respectively (49); in Iran, the prevalence *S. aureus* possessed the *clfA* and *clfB* was 84% and 65.3%, respectively (50). According to numerous studies, the *clfA* gene was detected in 19-100% of *S. aureus* isolated from cow mastitis infections, while the *clfB* gene was identified in 91.8-92.9% of isolates (51,52). Moreover, the absence of the *coa* gene was detected in all CNSA isolates, highlighting its significance as a genetic symbol for distinguishing between coagulase-positive *S. aureus* (CPSA) and coagulase-negative *S. aureus* (CNSA). The results of this study are in agreement with earlier research indicating that CNSA isolates do not possess the *coa* gene, a characteristic that distinguishes them from coagulase-positive *S. aureus* (CPSA) (53-58).

Conclusion

In conclusion, CNSA is responsible for causing asymptomatic mastitis in sheep, leading to significant economic losses worldwide. The current investigation confirms the identification of CNSA isolated from ovine

udders through genotypic characterization using the PCR assay. The owners treated the infected ewes with antibiotics without consulting a veterinarian, leading to the development of CNSA resistance to various antibiotics and the emergence of MRSA isolates carrying the *mecA* gene. The isolation of CNSA and MRCoNSA in milk from sheep with non-clinical mastitis indicates the use of unhygienic veterinary practices on farms. Genetic variation in the *16S rRNA* gene of CNSA isolated from sheep with asymptomatic mastitis differs based on geographic distribution worldwide. Ongoing research and monitoring are crucial for tracking and assessing CNSA and MRCoNSA strains, as well as for devising effective strategies to mitigate their effects on public health and the dairy industry.

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Conflict of interest

No conflicts of interest occurred during the writing or data analysis process.

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التحليل التطوري والتوصيف الجيني لجراثيم المكورات العنقودية الذهبية السلبية لإنزيم الكواجولاز المعزولة من حليب التهاب الضرع تحت السريري في الأغنام في محافظة نينوى، العراق

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الخلاصة

تعد جراثيم المكورات العنقودية الذهبية إحدى اهم الكائنات الحية الدقيقة الرئيسية التي تسبب التهاب الضرع تحت السريري في الحيوانات. أجريت هذه الدراسة وذلك لتحقيق اهم الأهداف ومنها عزل وتشخيص جراثيم المكورات العنقودية الذهبية السلبية لإنزيم الكواجولاز في عينات حليب الأغنام المصابة بالتهاب الضرع تحت السريري واكتشاف بعض جينات التي تشفر عوامل الضراوة، إضافة الى ذلك استخدام شجرة النشوء والتطور لمعرفة مدى العلاقات بين العزلات. تم جمع ستون عينة حليب من الأغنام المصابة بالتهاب الضرع تحت السريري من أماكن مختلفة في مدينة الموصل. وقد تم استخدام الطريقة التقليدية التي تشمل الأوساط الانتخابية والاختبارات الكيمياوية الحيوية لعزل وتشخيص جراثيم المكورات العنقودية الذهبية السلبية لإنزيم الكواجولاز، في حين تم استخدام طريقة تفاعل البلمرة المتسلسل للكشف عن الجينات المحددة في هذه الدراسة. أظهرت نتائج الدراسة بان معدل عزل جراثيم المكورات العنقودية الذهبية السلبية لإنزيم الكواجولاز في حليب الأغنام المصابة بالتهاب الضرع تحت السريري كانت ١١,٧ (٦٠/٧) وكان اعلى معدل انتشار لجراثيم المكورات العنقودية الذهبية السلبية لإنزيم الكواجولاز بلغ ٢٠٪ (١٥/٣) في منطقة النمرود. بينما لم يتم عزل جراثيم المكورات العنقودية الذهبية السلبية لإنزيم الكواجولاز في منطقة حاوي الكنيسة. أظهرت نتائج طريقة تفاعل البلمرة المتسلسل أن جميع عزلات جراثيم المكورات العنقودية الذهبية السلبية لإنزيم الكواجولاز تمتلك جين *nuc* ١٠٠% (7/7). بالإضافة إلى ذلك، كانت جميع العزلات من المكورات العنقودية الذهبية مقاومة للميثيسيلين والتي تمتلك جين *mecA* وبنسبة ١٠٠٪، وكذلك تمتلك جميع العزلات على جينات *clfA* و *clfB* و *S rRNA16* بنسبة ١٠٠٪. في حين لا تمتلك أي من جراثيم المكورات العنقودية الذهبية السلبية لإنزيم الكواجولاز على جين *coa*. وكان نمط الجينات الوحيد الذي تم تحديده في جميع العزلات هو (*nuc + mecA + clfA + clfB + 16S rRNA*) وبنسبة ١٠٠٪. إضافة الى ذلك فقد تم تسجيل سبع سلالات جديدة من سلالات جراثيم المكورات العنقودية الذهبية السلبية لإنزيم الكواجولاز في بنك الجينات بالاعتماد على جين *S rRNA16*. وأظهرت شجرة النشوء والتطور وجدود علاقة بين جراثيم المكورات العنقودية الذهبية السلبية لإنزيم الكواجولاز في هذه الدراسة مع جراثيم المكورات العنقودية الذهبية السلبية لإنزيم الكواجولاز المعزولة من جميع أنحاء العالم.