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Original Research Article

Molecular Identification of Genes Resistant to Antibiotics in *Staphylococcus aureus* Isolated from Sub-clinical Animals Affected by Mastitis

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Abstract: ***Background and Aim*:** Mastitis is a prevalent and costly disease that significantly impacts the dairy industry, affecting both sheep's health and milk quality. In order to determine the bacterial causes of subclinical mastitis in Iraq and its susceptibility to antibiotics, the study aims to molecularly detect resistant *Staphylococcus aureus* isolated from sheep infected with mastitis. ***Methods*:** Molecular assay and phylogenetic study to detect *Staph aureus* isolates by using 16 Sr RNA and resistance virulence genes (aac-aph, tetk) by the conventional method of PCR. Two hundred samples were collected from the subclinical mastitis of infected ewes, Samples were reserved on an icebox and transported to the laboratories. Milk samples were cultured on Blood Agar and Mannitol Salt Agar (7.5%) plates. The culture plates were then incubated at 37°C for 24 hours. ***Results*:** The PCR assay revealed amplification of the 16S rRNA gene in all 20 isolates, amplification of the aac-aph gene in 19 isolates, and the tetK gene in all 20 isolates. The PCR products of 20 positive samples for (16 Sr RNA) target genes were sequenced, evaluated, and dropped on the Genbank-NCBI under the accession number, which became a reference to Iraq and the world. According to the current study, *Staphylococcus aureus* is commonly found in milk. To combat drug resistance, antimicrobial drug use should be normalized, and antimicrobial resistance surveillance should be conducted regularly. ***Conclusion*:** PCR-product of 8 positive samples for 16sr RNA target gene were sequenced, analyzed, and deposited on the Genbank-NCBI under the accession number and became a reference to Iraq and the Middle East and the world.

Keywords: *Staphylococcus aureus*, Mastitis, Molecular, Antibiotic, Resistance.

INTRODUCTION

Mastitis is a mammary gland inflammatory infection described by bacteriological changes, physical variations, and chemical deviations of milk and udder tissue abnormalities (Constable *et al.*, 2017). The disease can be evident in both clinical and subclinical forms, with the last being often unobserved due to the nonappearance of visible signs (Halasa *et al.*, 2020). Several pathogens can cause mastitis, but *S. aureus* is the most frequently established causal microorganism of intra mammary infection (IMI) in sheep. (Rainard *et al.*, 2018). *S. aureus* naturally colonizes the teat opening or splintered teat skin. Intramammary contamination caused by *S. aureus* in the dry period continues during lactation, and might pose a potential risk to human health not only because of the danger of transmission pathogens, but nonetheless, also because of the presence of enterotoxins in milk or milk products, particularly in ovine milk (Abdelrahman *et al*., 2015). Polymerase chain reaction (PCR) is a dependable, accurate, and confirmatory method for the identification of pathogens, specifically *S. aureus*, recovered from mastitic milk samples of sheep (Akram, 2015). Salauddin *et al.*, (2020) Isolate *S. aureus* by cultural procedures and biochemical examinations in addition to PCR examination and sequencing of the 16S rRNA specific gene for *S. aureus*, they found that sequencing of 16S rRNA was a great application for the description of *S. aureus* isolated from sheep mastitis. Phylogenetic investigation was used to detect the evolution history of the species, proteins, or genetic factors.

MATERIALS AND METHODS

Collection of Milk Sample

240 milk samples from subclinical mastitis of infected ewes were collected apparent normal in Babylon City from November 2023 to February 2024 from deference area. The samples were processed and transported to the Department of Microbiology at the College of Veterinary Medicine, Al-Qasim Green University, Babylon, Iraq. The milk samples were cultured using blood and Mannitol salt media and incubated at 37°C for 24 hours (Hatem *et al.,* 2013).

Physical Examination of Milk:

The milk samples were inspected visually to detect any abnormal color, odor and consistency.

Media Preparation According to the Company Dirction:

Preparation of Culture Media A group of culture media were prepared according to the manufacturer instructions.

Blood Agar:

This media was used to detect hemolysis around the colony of *S. aureus* isolates. The procedure of preparation by dissolving of 40 mg/l D.W PH shoud be 7.4 then utoclaved at 121°C pound/inch^2 pressure for 15 min. then cool the media for 45C in the water bath adding of sheep blood with 5%, mixed gently by used rod to prevent bubbles and poured into Petri dishes.

Mannitol Salt Agar:

This media was prepared by dissolving 111gm/1L D.W., after being prepared let cooled and poured in Petri dishes in this media *S. aureus* change the color from pink to yellow.

Hi- Chrome Agar:

This media was prepared by suspended 38g/L D.W then dissolved by heating for 1 minute and autoclaved then cooled 45 C and powere into Petri dishes (20 ml) then let it dry after that the dishes were kept in the incubator at 37 C° for 24 hrs. to ensure that the Petri dishes are free from contamination and humidity, the dishes should be used for 7 days.

Brain- Heart Infusion Agar:

This media was prepared by dissolving 52gm/ one litter D.W., then autoclaved and let cooled to 45C° and powered into a sterile petri dish use this media for preserved bacteria for a long time and prevent the death of it.

Brain- Heart Infusion Broth:

After autoclaving at 121 ˚C for 15 minutes, then cooling to 56 ˚C in a water bath, ready prepared brain heart infusion broth medium was used and supplemented with15% glycerol. In sterile test tubes, 5 ml was taken and inoculated by the bacterial colony, then incubated 37 ˚C overnight. The suspension is retained and deeply frozen according to (Tille, 2015). This medium used for long term storage of bacterial isolates.

Microscopic Examination

A drop of water was put on a clean slide, then a single colony from blood agar was spread on it fixed with heat and then stained with gram stain finally examined the bacterial cell under oil immersion (Markey *et al.,* 2014).

Molecular Study:

1. Bacterial DNA Extraction Protocol

The bacterial DNA was extracted from culture S. aureus isolates according to the Geneaid (Korea) genomic DNA isolation kit as directed by the product's instructions.

2. Estimation of DNA quality:

The nanodrop was using apparatus to test DNA concentration (ng/µL). The extracted DNA was calculated, and the DNA concentration was tested by reading the absorbance at 260/280 nm. The technique takes place due to the constructor's instructions.

Primers and Sequences

Table 1: Explained primers name, sequences, and Product size

Gene	Primer sequences (5‑3˚)		(bp)	Reference
16SrRNA	F	AGAGTTTGATCCTGGCTCAG	1500	Miller *et al.,* (2013)
	R	GGTTACCTTGTTACGACTT		
tetK	F	GTAGCGACAATAGGTAATAGT	360	Strommenger *et al.,* (2003)
	R	GTAGTGACAATAAACCTCCTA		
aac-aph	F	CAGGAATTTATCGAAAATGGTAGAAAAG	369	Emaneini *et al.,* (2013)
	R	CACAATCGACTAAAGAGTACCAATC		

3. Preparing for the Primers Suspension

Following the manufacturer's instructions, the lyophilized product primers were liquified to create PCR water (free nuclease water), which was then added to the stock primer to achieve 100 pico mol per micro liter (pmol/µl). After diluting 10 µl of the stock primer with 90 µl of PCR water, a thorough vortex mixing step was performed to create the working primer, which had a concentration of 10 pmol/µl.

4. Polymerase Chain Reaction (PCR) preparation

The Maxime PCR Abm Kit was used to prepare the polymerase chain reaction components, and ABM

Canada's company instructions were followed for the procedure.

5. Polymerase Chain Reaction Thermal Cycling Conditions

In the thermal cycle, PCR tubes were placed, and conditions for correct cycling PCR software parameters were changed according to each primer.

6. Agarose Gel Electrophoresis

The PCR products were analyzed according to the manufacturer's instructions (Plus science / UK) by agarose gel electrophoresis.

7. Agarose Gel:

The process of making agarose gel followed Sambrook and Rusell (2001).

8. Sequencing

Using phylogenetic tree analysis and NCBI-BLAST, the DNA sequencing method was utilized to isolate *S. aureus* pathogenicity. Standard PCR systems were used to confirm this. Following conventional PCR, the amplified products of the 16S rRNA genes were sent to Soul University Corporation in Korea for automatic DNA sequencing.

9. Phylogenetic Molecular Analysis

A set of nucleotides was used to obtain a degree of similarity between this isolate and other reference strains in the world by using the MEGA11 version and NCBI. After that, the construction of the phylogenetic tree was analyzed using the neighbor contraction tree method with the MEGA11 version program (Tamura *et al.*, 2013).

10. Ethical Management of the Study

The present study was approved in agreement with guidance issued by the College of Veterinary Medicine, University of Al-Qasim Green. No banned biological materials or genetically modified organisms were included in the report.

RESULTS AND DISCUSSION

Detection of *S. Aureus* by 16srRNA gene:

The PCR amplification of the 1500 bp fragment was successfully demonstrated in all 20 isolates of *S. aureus* whose DNA was extracted and treated with primers specific to a positive for the 16SrRNA gene (Figure 1).

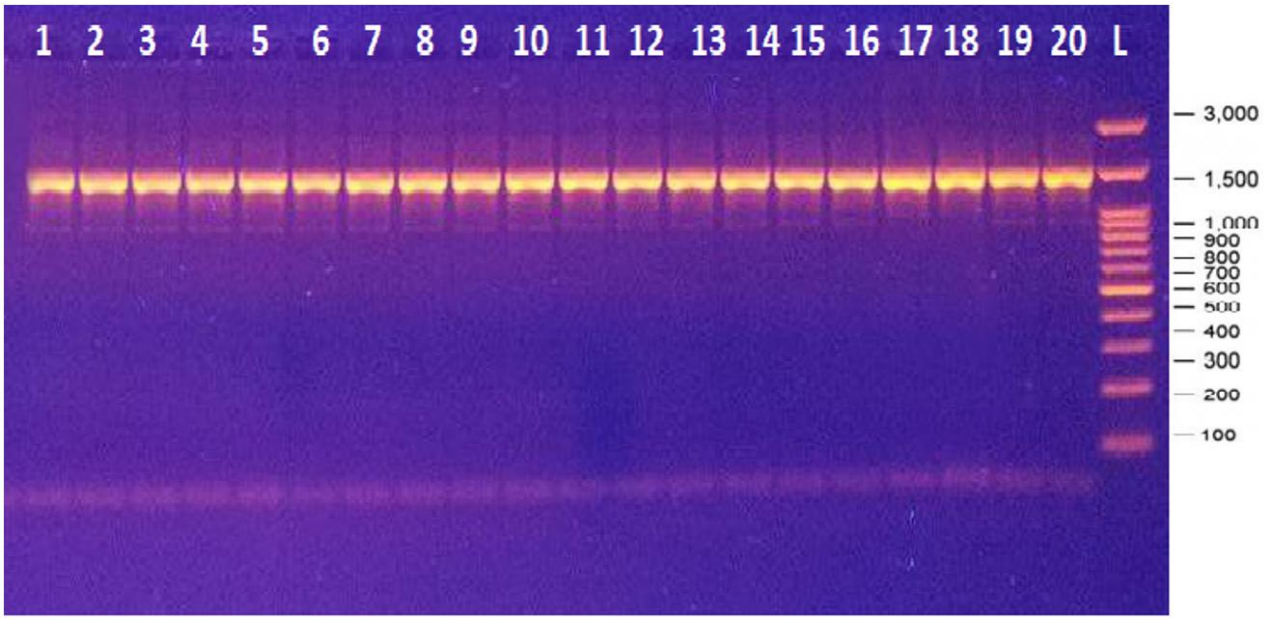

Figure 1: agarose gel electrophoresis for the PCR amplification *16SrRNA* gene of *S. aureus* isolates show partial amplification of 1500bp of the above gene

In the current study, the PCR assay was used to detect *S. aureus* by amplifying the 16S rRNA gene. Previous research has not explored the use of the 16S rRNA gene for detecting clinical mastitis in ewes, except for a study by Fournier *et al*., (2008) which used 16S-23S rRNA/intergenic spacer RS-PCR to genotype *S. aureus* isolated from bovines in Switzerland.

Detection of *aac-aph gene:*

The results showed DNA of 19 isolates of *S. aureus* from 20 isolates possess the gene *aac-aph* at amplification 369bp fragments (Figure 2).

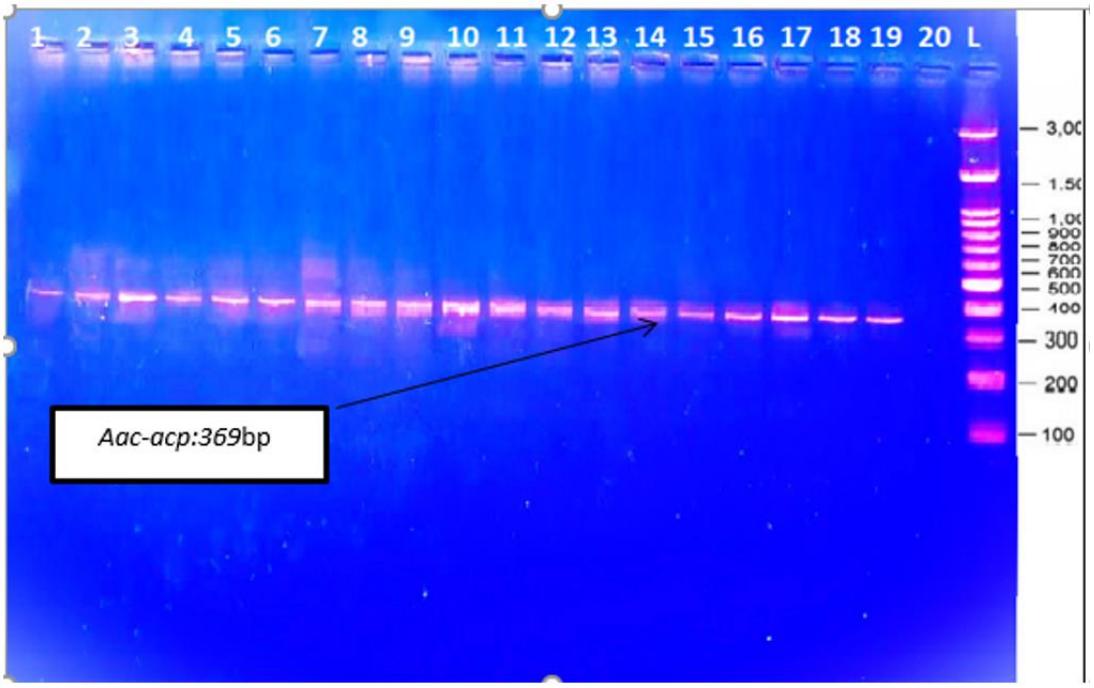

Figure 2: DNA amplification of a bp for of staphylococcus aureus. detecting gene aac-acp using PCR. Lane1: 1 adder, lane1-19, positive results., lane 1: 3000bp marker (Ladder)

No previous study detects the presence of *acc–aph* genes in ewes affected with clinical mastitis. But there were studies about that gene in mastitic cow, In China Qu *et al.,* (2019) observed presence of the *aac-aph* genes in (23%) of *Staph. aureus* isolates from clinical mastitis in cows between years 2014 and 2017. In addition, Turutoglu *et al.,* (2009) detected the gene in 3 isolates of *Staph. aureus* and the aac-aph one isolate of *Staph. aureus* from cows' mastitis in Turkey.

Detection of *tet-k gene:*

The *tet-k* used for confirm resistant gene against tetracycline drug give a positive result at 369bp in all DNA of 20 *S. aureus* isolates (figure 3).

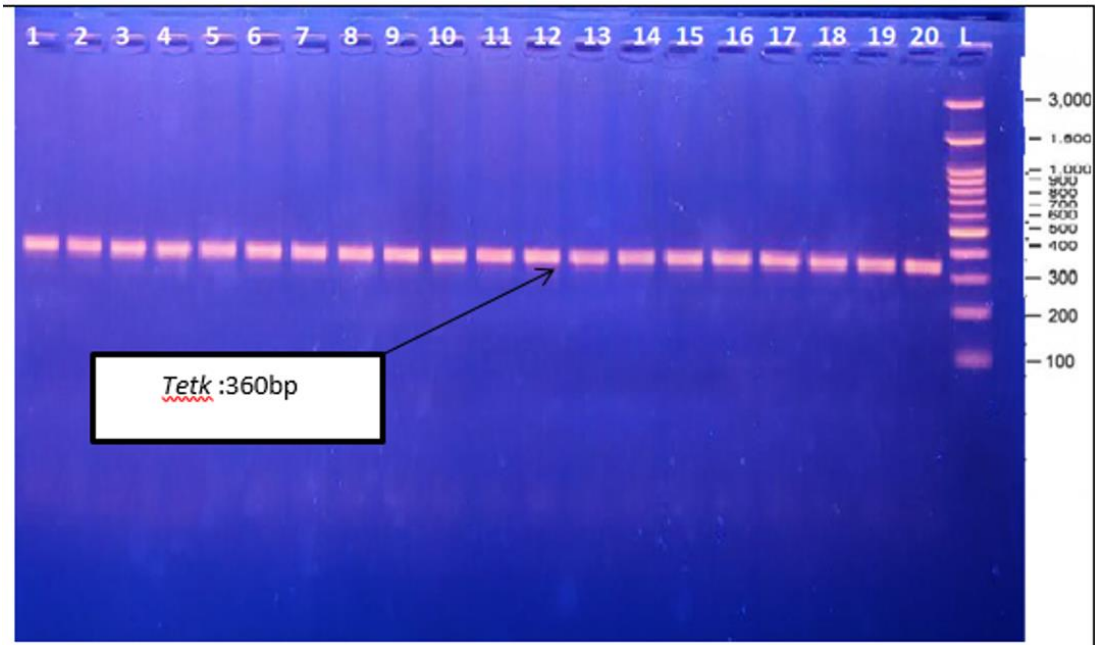

Figure 3: Agarose gel electrophoresis for the PCR amplification *tetk* gene of *S. aureus* isolates show partial amplification of 369bp of the above gene

This study focuses on the molecular characterization of *S. aureus* isolated from ewe milk in Babylon City. Our findings indicated high rates of *S. aureus* resistance to common veterinary antibiotics. We identified two mecA-positive *S. aureus* isolates that were susceptible to cefoxitin and three tetK-positive isolates

that were susceptible to tetracycline, likely due to non-expression of the corresponding genes, which may lead to an underestimation of resistant strains in the food chain (Capurro *et al*., 2010). Notably, 52% (13/25) of the penicillin-resistant isolates did not show the blaZ gene, possibly due to mutations at the primer-annealing site inhibiting amplification (Schmidt *et al*., 2015). The presence of other resistance genes was consistent with the observed phenotypic resistance, indicating they are significant contributors to the antibiotic resistance noted in our context. Our tetracycline resistance rate for *S. aureus* was 56.7%, aligning with findings from Akanbi *et al.*, (2017), while lower rates of 31.2% and 33% were reported by Nwankwo and Nasiru (2011) and Naimi *et al.*, (2017), respectively. Conversely, Gitau *et al*., (2018) reported a higher prevalence of tetracycline resistance 98.2%.

After sequencing of 16S rDNA gene, a partial fragment was obtained. This nucleotide sequence was analyzed via BLASTN algorithm of NCBI, the accession number of the *Staphylococcus aureus* is: PP883587, PP883588, PP883589, PP8835890, PP883591, PP883592, PP883593, PP883594, PP883595, PP883596, PP883597, PP883598, PP886135, PP886136, PP886137, PP886138, PP886139, PP886140, PP886141, PP886142.

Phylogenetic Tree Analysis:

The results of the multiple sequence alignment are a crucial component of the phylogenetic analysis in this study. They illustrate the relationships between sets of genomes despite the changes that have occurred over time. The alignment of multiple sequences can be obtained using the Clustal W2 online tool, which arranges three or more sequences in a computationally efficient manner (Goujon *et al.,* 2010).

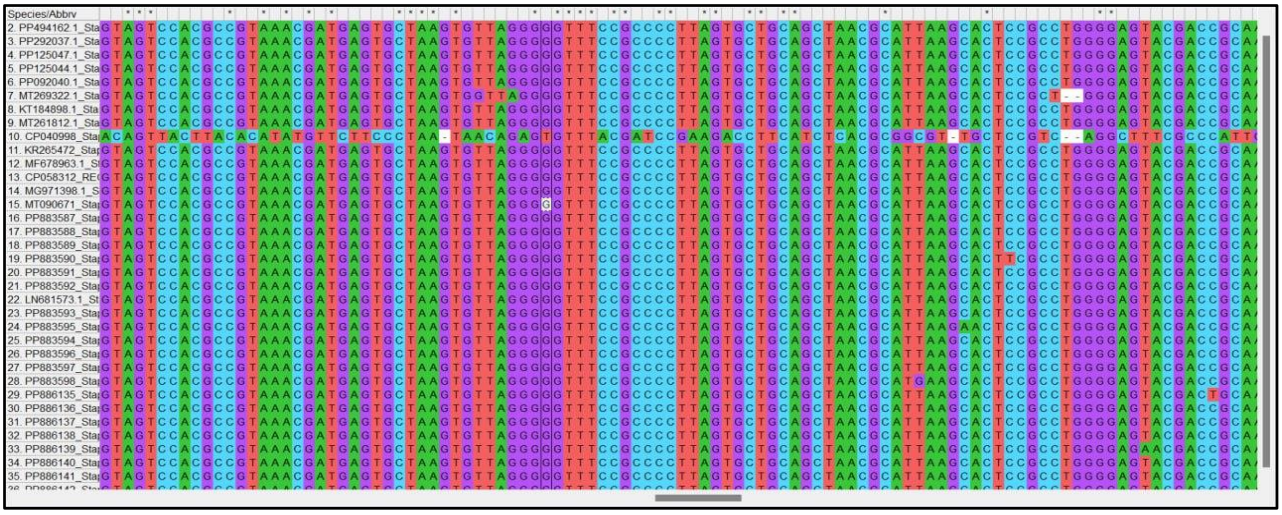

Figure 4: Using the MEGA 11 multiple alignment analysis tool, a multiple sequence alignment analysis of the partial 16S ribosomal RNA gene was conducted. The 16S ribosomal RNA gene exhibited similarities (*) and differences according to the multiple alignment analysis

The results from arranging the partial DNA sequences of 20 Staphylococcus spp Iraqi isolates of the 16S rRNA gene (ranging from 1500-350 bp) with selected reference sequences from around the world showed high similarity and identity at specific locations in the sequences. These results were obtained using BLAST to determine the percentage of identity, which ranged from 99-100% between the Iraqi isolates and the reference strains, as shown in (figures 4).

These findings align with previous studies in Iraq that identified *S. aureus* isolates in dogs (Abbas and Rady, 2023) and identified the 23S rRNA gene in ewe's milk samples (Ahmed and Yousif, 2021). The sequencing was conducted in Korea by Macrogen, and the evolutionary tree was constructed using Molecular Evolutionary Genetic Analysis (MEGA) (Tamura *et al.*, 2013).

The phylogenetic tree analysis was based on a limited sequence of the 16s rRNA gene (1500-350 bp) for typing recognition of Staphylococcus spp. The phylogenetic tree was constructed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA tree) in the MEGA 11 version. The local Staphylococcus spp. isolates from the current study are shown in (figure 5). After comparing the samples with those from around the world, it was found that the samples matched the results of the mentioned countries by 100%, and one Iraqi isolate was 99% compatible. Our phylogenetic analysis results showed that all *S. aureus* was 100% compatible with other strains from Japan (Ishihara *et al.*, 2010).

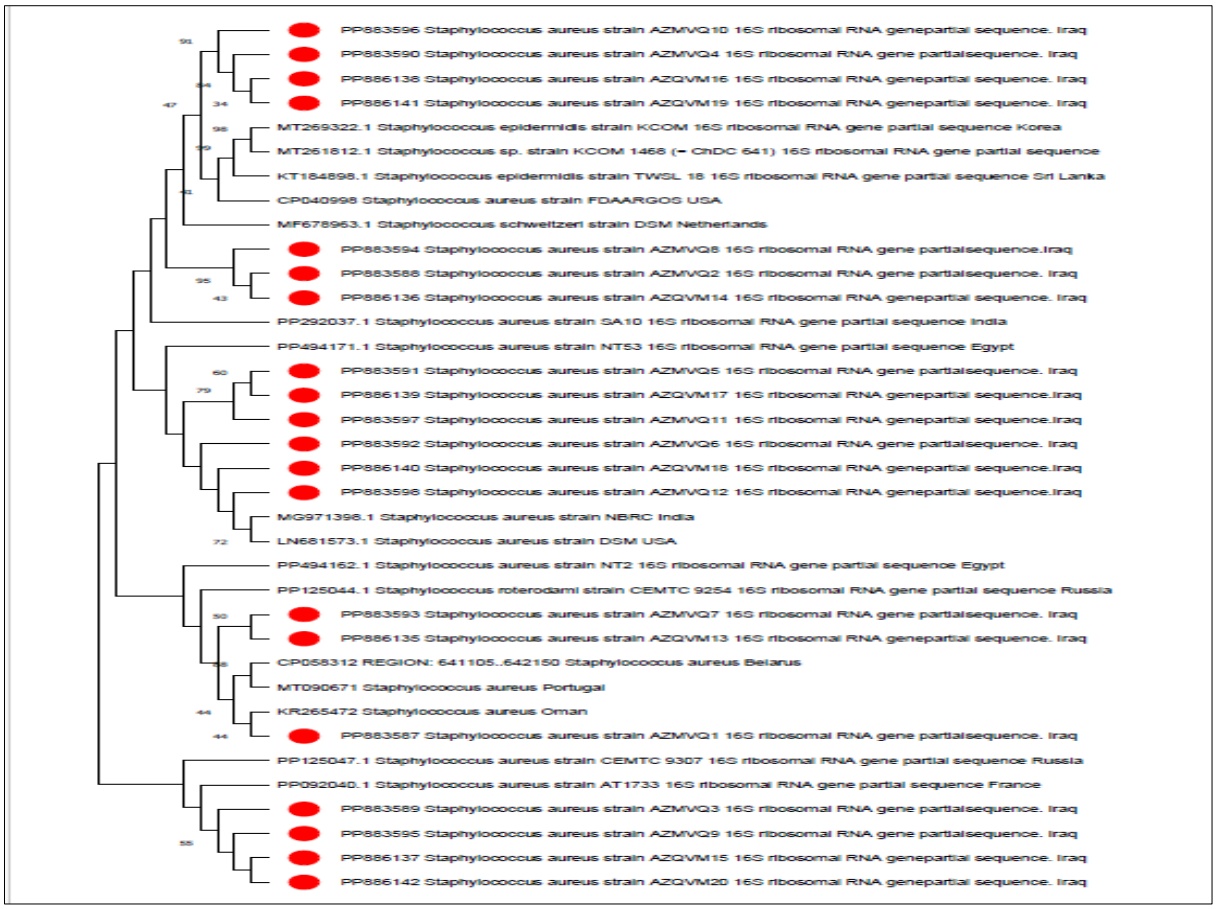

Figure 5: Phylogenetic tree analysis based on the 16S ribosomal RNA gene partial sequence that used for detection of local *staphylococcus* species isolates. that show closed related to NCBI-Blast *staphylococcus auraus*

The results of phylogenetics are consistent with Saleh *et al.*, (2018), who conducted a study on the phylogenetic tree of *Staphylococcus aureus* based on 16S rRNA sequencing.

CONCLUSION

1. The result of the current study demonstrated the importance of Staph. aureus as the causes of sheep subclinical mastitis.
2. Molecular assay and phylogenetic study to detect Staph aureus isolates by using 16 Sr RNA.
3. Antimicrobial resistance genes aac- aph and tet K.

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Conflict of Interest: The authors have confirmed that they do not have any conflicts of interest.

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Authors' Contribution: All authors contributed equally.

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