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Identification of Antimicrobial – Resistant Genes in *S. aureus* Isolated from Subclinical Mastitis Infected Ewes

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Authors' contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

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ABSTRACT

Mastitis, which is defined as inflammation of the mammary gland, typically causes changes to the udder's anatomy and physiology. The aim of this research was to identification of antibiotic-resistant *Staphylococcus aureus* isolated from sheep with subclinical mastitis by molecular detection. *Staphylococcus aureus* isolates were molecularly detected using 16 Sr RNA and resistance virulence genes (mecA, ermA, ermB, and blaZ) using the traditional PCR method. The results were also validated using VITEK 2 systems. Two hundred samples were collected from the subclinical mastitis of infected ewes from different areas in Babylon Province. Samples were preserved on an icebox and transported to the laboratories. Milk samples were cultured on Blood and Mannitol Salt Agar (7.5%) plates. The culture plates were then incubated at 37°C for 24 hours. The results of milk samples cultured on different media revealed the following: Green, motile colonies of *S. aureus* are produced on selective Hi Chrome agar. The current study found that the percentage of infected

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halves was 19–29 half infected and 5–29 two halves infected, and that the prevalence of subclinical mastitis was 24 (45.83%) based on the CMT and bacterial isolation. The 16S rRNA gene was amplified in all 20 isolates, according to the PCR assay, and the mecA, ermA, ermB, and blaZ genes were amplified in all 20 isolates. Every *S. aureus* isolate that tested positive came from mastitis-infected sheep. Twenty antimicrobial agents were used in the antibiotic susceptibility tests for S. aureus isolates. In summary, the findings indicate that S. aureus has become highly resistant to antibiotics.

Keywords: Molecular; staphylococcus aureus; subclinical mastitis; antimicrobial.

1. INTRODUCTION

An inflammation of the mammary gland is called mastitis. It is a reaction to the harm that bacteria have caused to return to normal function. Many mastitis cases are brought on by microorganisms that enter the udder, grow, and release toxins that are harmful to the mammary gland (Schroeder, 2012). Pathogen type, lactation stage at disease onset, and infection severity all impact milk production in mastitis cases. (Heikkilä et al*.,* 2018). Mastitis is the inflammation of udder tissues caused by physical damage, chemical irritation, or infection (Ruegg et al., 2014). Resistance to antimicrobial agents is regarded as one of the world's main and increasingly global issues, especially among nosocomial pathogens. Staphylococci have been one of the most common causes of nosocomial infections. Multidrug-resistant staphylococci pose a rising alarm for public health. The rise of drug-resistant virulent strains of S. aureus, particularly methicillin resistant S. aureus (MRSA) is a severe problem in the treatment and control of staphylococcal infections (Neamat-Allah and Hend, 2016). Advances in S. aureus gene studies and bioinformatics offer potential for targeting genes. (Land et al., 2015; Everitt et al., 2014). Subclinical mastitis shows a normal mammary gland and milk. The main sign is elevated somatic cell count, with other indicators being higher bacterial levels, decreased milk production, and changes in milk quality. (Bian et al*.,* 2014). Identifying subclinical mastitis is crucial for the successful implementation of effective strategies aimed at controlling and managing mastitis. By detecting this condition early, we can take appropriate measures to ensure the health and productivity of the affected animals, ultimately leading to better overall herd management and milk quality. (Hoque et al*.,* 2015). Laboratory diagnosis is essential for isolating and identifying pathogens. It uses techniques like culturing, microscopy, and molecular methods like PCR to detect infectious agents. Accurate identification is crucial for effective treatment and infection control (Madouasse et al*.,* 2010).

2. MATERIALS AND METHODS

2.1 Collection of Milk Samples

Two hundred forty milk samples were collected from one hundred twenty ewes between November 2023 and February 2024 across various regions of Babylon Province. After cleaning and disinfecting the teats with 70% alcohol and discarding the initial streams, 10 ml samples were taken from cases of subclinical ovine mastitis. All samples were placed into sterile glass vials. (Hatem et al., 2013). The samples were stored in an icebox and transported to the Research Center and Laboratories at Al-Qasim Green University's College of Veterinary Medicine. The milk samples were streaked onto Blood agar and Mannitol salt agar, then incubated at 37°C for 24 hours.

2.2 California Mastitis Test (CMT)

CMT known as screening test which done according to (Kumar et al*.,* 2023), Subclinical mastitis detection was performed using a white plastic paddle with four receptacles, where equal volumes of milk and California reagent (2 ml each) were mixed gently.

2.3 Media Preparation According To The Company Direction

Culture media were prepared following the manufacturer's instructions for Blood Agar, Brain Heart Infusion Agar, Hi-Chrome Agar, and Mannitol Salt Agar, all made by Hi-Media, India.

2.4 Molecular Study

2.4.1 Bacterial DNA extraction protocol

Executed as instructed by the company, Anatolia Canada.

2.4.2 Polymerase Chain Reaction (PCR) preparation

The components of the Polymerase Chain Reaction were used (Maxime PCR Abm Kit) and the process was carried out according to company instructions.

2.4.3 Polymerase chain reaction thermo cycling conditions

The PCR tubes were positioned in the thermal cycler and the conditions of the correct PCR cycling software parameters were changed according to each prime.

2.4.4 Agarose gel electrophoresis

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

2.4.5 Ethical management of the study

The current study was carried out in compliance with guidance issued by College of Medicine, University of AL Qasim Green. No banned biological materials or genetically modified organisms were included in the report.

3. RESULTS AND DISSECTIONS

3.1 Results of California Mastitis Test (CMT)

The results of examining of 240 milk samples from 120 apparent ewes with or without clinical signs on udder by California mastitis test (CMT) revealed that there were only 55 ewes (240 samples) positive to CMT (Table 2). The chance of finding *S. aureus* in milk samples is increased by the use of screening testing like the California Mastitis Test, which marks positive samples for additional culturing. The CMT score that is still in use for ewe is still well-known. For the diagnosis of subclinical mastitis, a CMT score of (+) is advised, while for the diagnosis of infectious mastitis in sheep, a maximum score of (+++) is advised (Barbosa et al*.,* 2004).

Due to the high rate of false-positive and false-negative reactions shown in diagnostic testing, healthy and sick ewes are mistakenly recognized, delaying the acceptance of preventive and therapeutic involvements.

The prevalence of sub-clinical mastitis in the present study was 24 (45.83%). In Iraq, while many veterinarian scientific researchers recorded a different percentages of Incidences of the Sub-clinical mastitis in ewes and such results were performed previously by (Al-Hamamy, 1977) ; (Al-Judi, 1979);and (Yousif, 1982) was 18.51%; (Karim, 1988) and (Sulaiman, 1989) was 3.5%; (AL-Kubaysi, 2000) was 36.90%; (Al-Obaidy, 2010) was 25.0%; (Hammadi, 2013) was 27% ; (Al Muhammady, 2013) was 33.87% ; (Abed, 2014) and (Hatem, 2014) was 13.7%. In the current study, it was found that high percentage of ewes infected with sub-clinical mastitis (45.83%), and this high percentage could be attributed to many reasons such as most ewes with Sub-clinical mastitis were contracted during two time periods (first few weeks post weaning and last 2 weeks pre-lambing up to 2–3-day post lambing). These were the periods of the highest susceptibility to Sub-clinical mastitis in ewes, as well as dairy cattle and probably other mammalian species (Timms, 2007).

Primers:-

Table 1. Explained primers name, sequences, annealing temperature and Product size

Gene	Primer sequences (5‒3˚)	(bp)	Reference
16SrRNA	F AGAGTTTGATCCTGGCTCAG	500	Miller et al*.,* (2013)
	R GGTTACCTTGTTACGACTT		
ermA	F GTTCAAGAACAATCAATACAGAG		
	R GGATCAGGAAAAGGACATTTTAC	421	Lina et al., (1999)
ermB	F CCGTTTACGAAATTGGAACA	359	
	GGTAAAGGGC		Lina et al., (1999)
	R GAATCGAGAC TTGAGTGTG		
	F TGAGTTGAACCTGGTGAAGTT	855	
mecA	R TGGTATGTGGAAGTTAGATTGG		Zarei et al*.,* (2016)
blaZ	F GCTTAATTTTCCATTTGCGA	303	
	R GATGATATAGTTGCTTATTC		2022442

Table 2. Occurrence of subclinical mastitis based on california mastitis test

No. of examined ewes	No. of collected milk samples	CMT + ve samples		CMT - ve samples	
		NO.	%	NO.	%
120	240	110	45.83%	130	54.16%

Table 3. Number and percentage of subclinical mastitis based on examined ewes and halves

No. of infected ewes	No. of halves	No. of Subclinical Mastitis			
		Based on infected ewes		Based on infected halves	
55	110	24\55	45.83%	29\110	26.36%

Table 4. Number and percentage of subclinical mastitis based on infected halves

No. of infected halves	No. of Subclinical Mastitis Based on infected halves			
	One half		Two halves	
29	19\29	65.51%%	10\29	34.48%%

According on CMT and bacterial isolation, the occurrence of subclinical mastitis in this study was (24/55). Comparable findings were reported by Al-Graibawi et al*.,* (2002), who discovered that 12% of ewes had SCM. Our results, however, were less than those of Gebrewahid et al*.,* (2012), who used CMT positive milk samples and bacterial isolation to find the occurrence of subclinical mastitis in ewes (28.14%). A comparable isolation rate of 12.22% was previously documented (Hassan and Yousif, 2013).

While subclinical mastitis (SCM) may not cause visible changes in the milk or udder, it is economically more significant than clinical mastitis (CM) due to its higher prevalence. SCM has a considerable impact on both animal welfare and the dairy industry. It is associated with a decrease in milk yield and can alter the quality and physico-chemical properties of the milk. Additionally, SCM is one of the primary factors affecting the composition of milk. (Chiaradia et al*.,* 2013).

The present study showed the percentage of infected halves was 19\29 one half infected and 10\29 two halves infected. Several studies were referred to higher incidence of sub-clinical mastitis of right halves than that of the left halves, in spite of the fact that some studies were referred to higher incidence of Ovine Sub-clinical mastitis in right halves (Al Muhammady, 2013; Abed, 2014 and Hatem, 2014).

On the other hand, it was concluded that the right halves were more susceptible to the pathogens as compared to the left halves because of laying of ewes on the right side due to the presence of rumen on left side, also the rainy weather when the ground was muddy assisted for dissemination of environmental bacteria. The current result disagreed with Las Heras et al*.,* (1999) who demonstrated that most of the mammary infections were unilateral with only of the animals having a bilateral infection.

Also disagreed with Fthenakis (2004) who found that the prevalence rate of bilateral mastitis was more dominant, also with (Tormod and Steinar, 2007) who found that Sub-clinical mastitis was present in one gland in ewes and in both glands in ewes. The current results were in accordance to the finding of Ergun et al*.,* (2009) who recorded a non-significant result between left and right halves infection results rates, and with Yasar et al*.,* (2009) who revealed that the mammary infections were unilateral and only of the animals had bilateral infection.

3.2 Isolation, Identification and Molecular of *S. aureus*

Bacteriological examination of milk samples for isolation of *Staph. aureus* was confirmed by the following steps.

3.3 Cultural Characteristics

The results of milk samples cultured on different media revealed the following; On selective Hi Chrome agar *S. aureus* produce move, green colonies (Fig. 1a). On selective mannitol salt agar appeared mucoid, round, convex and change the color of media to yellow color (Fig. 1b), while on blood agar the colonies

appeared round and opaque colonies (Fig. 1c). Brain heart broth used for transported of media (Fig. 1d).

Cortimiglia et al*.,* (2015) discovered that *S. aureus* was the most common species in sheep milk (20.14%), and that the prevalence of isolated *S. aureus* was 43% in bulk tank milk from sheep. However, Hammadi and Yousif (2013) discovered that the isolated *S. aureus* bacterium had a higher proportion 26 % of SCM in ewes. When milk samples were collected and tested for the presence of *S. aureus*, Ateba et al*.,* (2010) discovered that every sample had *S. aureus* infections. Most udder inflammation is sub-clinical, leading farmers to overlook the disease. Consequently, infections can linger, allowing subclinical mastitis (SCM) to progress to clinical mastitis (CM), negatively impacting dairy farm profits. Many dairy farmers in developed countries have implemented dry cow antibiotic therapy to manage mastitis in their herds, often finding it cost-effective and satisfactory (Dingwell et al*.,* 2002). SCM can occur due to improper treatment of CM, leading to the absence of clinical signs while the infection persists. The rate of SCM may be high, as infections can go undetected for extended periods.

3.4 Molecular Study

3.4.1 DNA extraction

The total genomic DNA of *S. aureus* isolates was successfully extracted, and this DNA produced sharp, clear and pure bands (Fig. 2).

3.4.2 Detection of *S. aureus* by 16SrRNA gene

All of the DNA extracted from 20 isolates of *S. aureus* treat with primers specific a positive for the 16SrRNA gene which amplified by PCR showed successful amplification of 1500 bp fragments (Fig. 3).

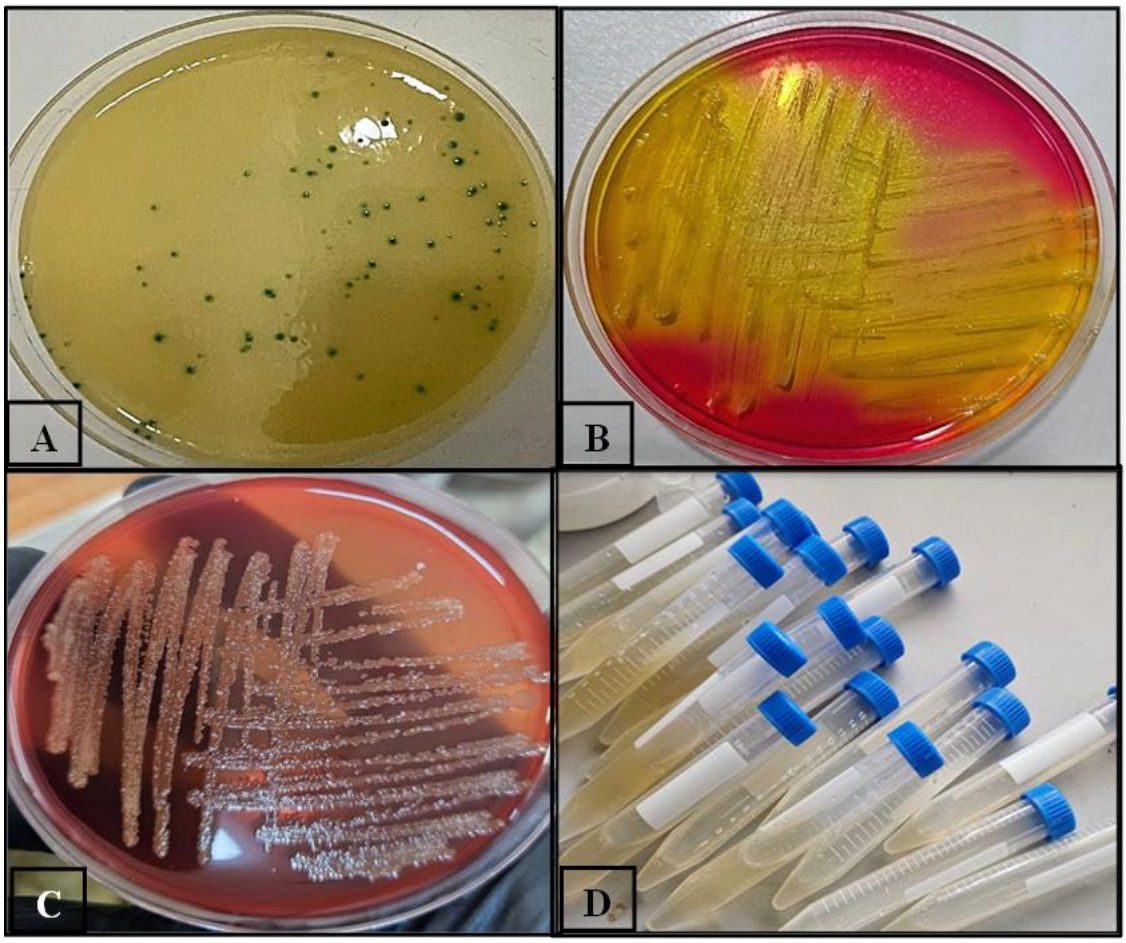

Fig. 1. A-Hi chrome *Staph*. Agar. B-Mannitol salt agar. C-Blood agar. D-brain heart broth

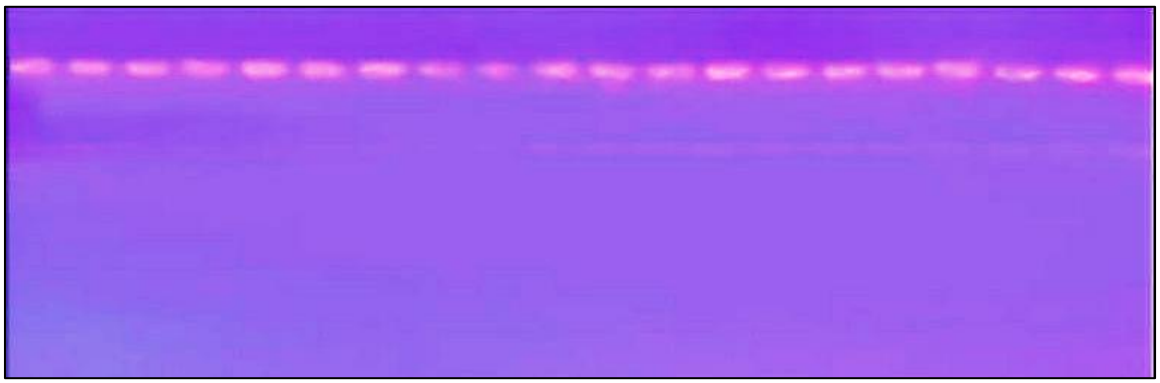

Fig. 2. Total genomic DNA extracted from isolates using 0.7 % agarose gel electrophoresis purified DNA of Staphylococcus aureus

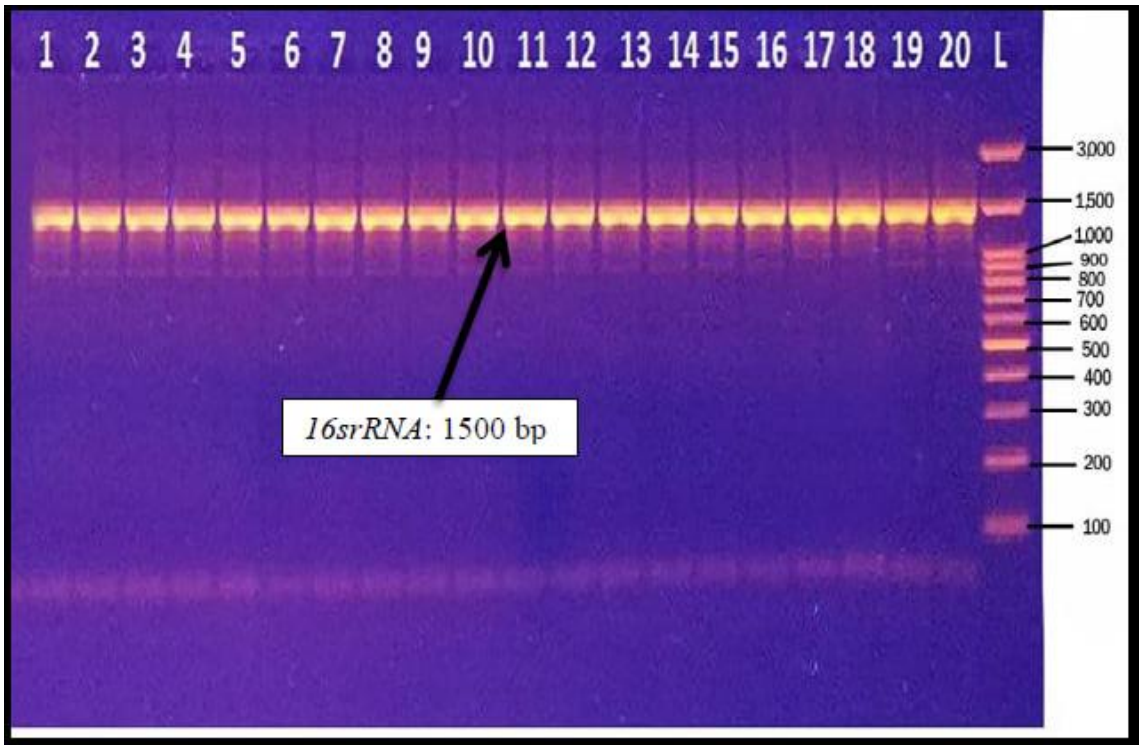

Fig. 3. Agarose gel electrophoresis for the PCR amplification *16SrRNA* gene of *S. aureus* isolates show partial amplification of 1500bp of the above gene

Genetic identification methods, such as 16S rRNA gene sequencing, effectively identify various bacterial pathogens. However, these techniques remain labor-intensive and costly, preventing their integration into routine veterinary diagnostics. As a result, the identification of bacterial pathogens primarily depends on phenotypic criteria, with many mastitis samples analyzed under strict financial constraints. (Fida et al*.,* 2021, Hamzah et al*.,* 2020) The 16S ribosomal RNA (16S rRNA) gene sequence has recently been utilized for the detection, identification, and taxonomic classification of bacteria (Sulo and Šipková, 2021) This sequence was chosen for several reasons, including that the bacterial 16S rRNA gene consists of about 1,500 nucleotides and contains several highly conserved regions (Liu et al*.,* 2022). "Universal, broad-range primers can be designed from the conserved regions of 16S rRNA gene sequences." (Kumar et al., 2023). Universal primers, used alongside species-specific primers or probes from the 16S rRNA gene sequences, allow for the taxonomic identification of pathogens. (Liu et al*.,* 2022). The 16S rRNA gene sequences in the Staphylococcus genus

are highly similar, complicating the creation of a species-specific probe for S. aureus identification. Our laboratory also conducted an analysis comparing PCR-amplified 16S rRNA genes to identify human pathogens. (Fournier et al*.,* 2014)

3.4.3 Detection of *blaZ gene*

The results showed DNA of 20 isolates of *S. aureus* from 20 isolates possess *blaZ* gene at amplification 303 bp fragments. And these 20 isolates appeared with isolates resistant to Penicillin (Fig. 4).

Detection of the blaZ gene (encoding β-lactamase) and the mecA gene (encoding alternative penicillin-binding proteins) is the gold standard for identifying penicillin resistance and finding is consistent with those of (Gooraninejad et al*.,* 2007).

3.4.4 Detection *ermA gene*

The *erm-A* used for detection of resistance gene in *S. aureus* against macrolide group (erythromycin drug) gave 20 positives from 20 isolates at amplification 421 bp fragment, this isolate appeared within the 20 isolates resistant to Erythromycin (Fig. 5).

The production of penicillinase, which degraded the beta-lactam ring in penicillin, was the first indication that S. aureus was resistant to beta-lactam drugs. However, the mecA gene promoted the development of methicillin-resistant S. aureus (MRSA) strains once methicillin was added to replace penicillin resistance. The methicillin antibiotic sensitivity test was performed on all recovered S. aureus strains, and the PCR assay was used to detect the mecA and mecC genes, which are thought to be a genetic marker utilized for quick and straightforward confirmation of MRSA. Eight (16.32%) of the 49 S. aureus isolates from ewes' milk that were subjected to genotypic investigation had mecA genes.

These findings concur with those of (19), who discovered that mecA was not present in all S. aureus. Additionally, this result is consistent with results from (20) in Turkey, where 16 isolates (17.2%) were MRSA and 93 isolates were Staph. aureus. All 49 MRSA isolates had negative mecC values; these findings differed from the research by (Khan et al*.,* 2020, Mahmood et al*.,* 2020) Diagnostic laboratories should be aware of looking for the mecC gene because they represent the detection of mecC-positive in MRSA isolation and would confirm that sheep might be a mecC MRSA reservoir. They believed that mecC-positive MRSA is difficult to confirm by routine diagnostic methods that are employed for mecA positive MRSA. However, depending on the sequencing segment, the mecA and mecC negative MRSA in the current study could potentially be a factor in the existence of additional ß lactam resistances, including the blaZ gene.

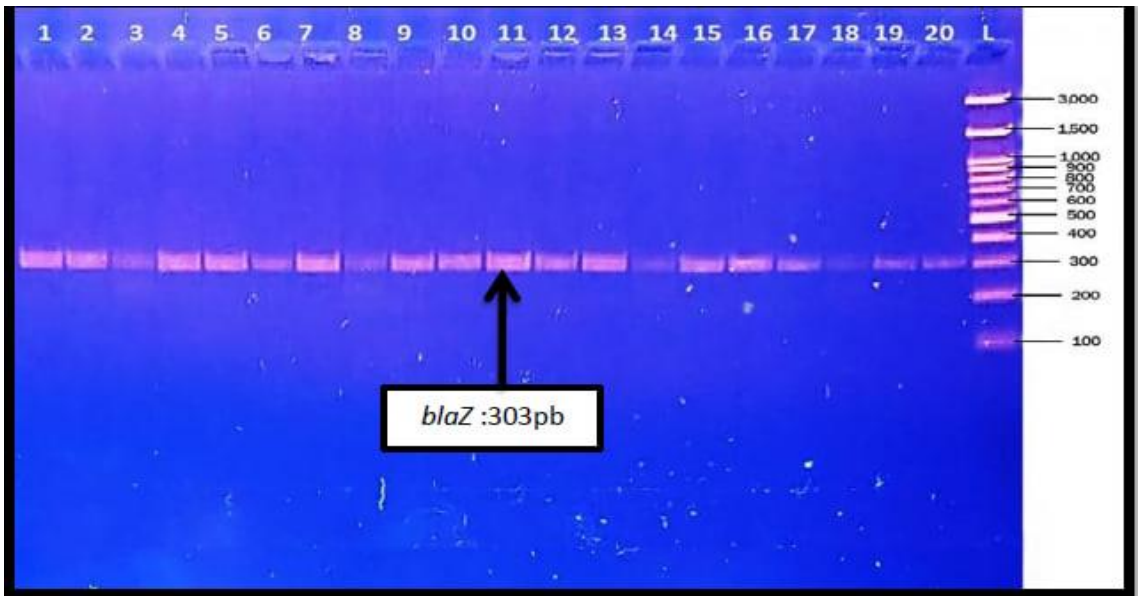

Fig. 4. Agarose gel electrophoresis for the PCR amplified *bla* gene of *S. aureus* showing at 303bp give 20 positive results M: DNA marker (100bp-3000) 20 lanes reveal positive samples

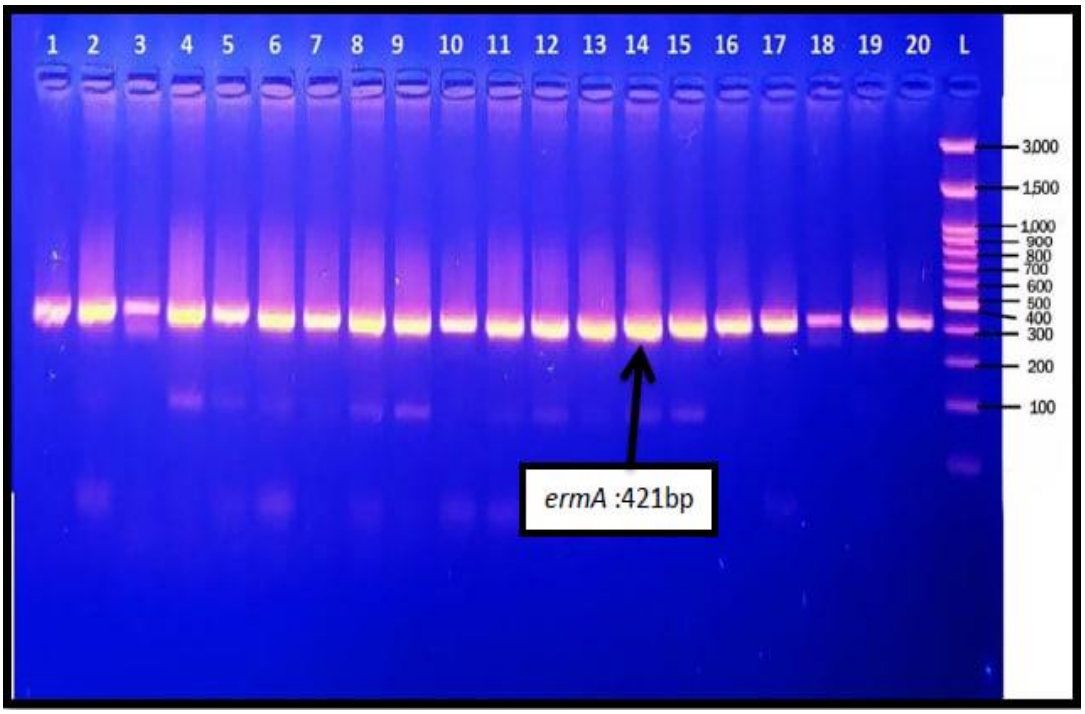

Fig. 5. Agarose gel electrophoresis for the PCR amplified *ermA* gene of *S. aureus* showing at 421bp give 20 positive results M: DNA marker (100bp-3000) 20 lanes reveal positive samples

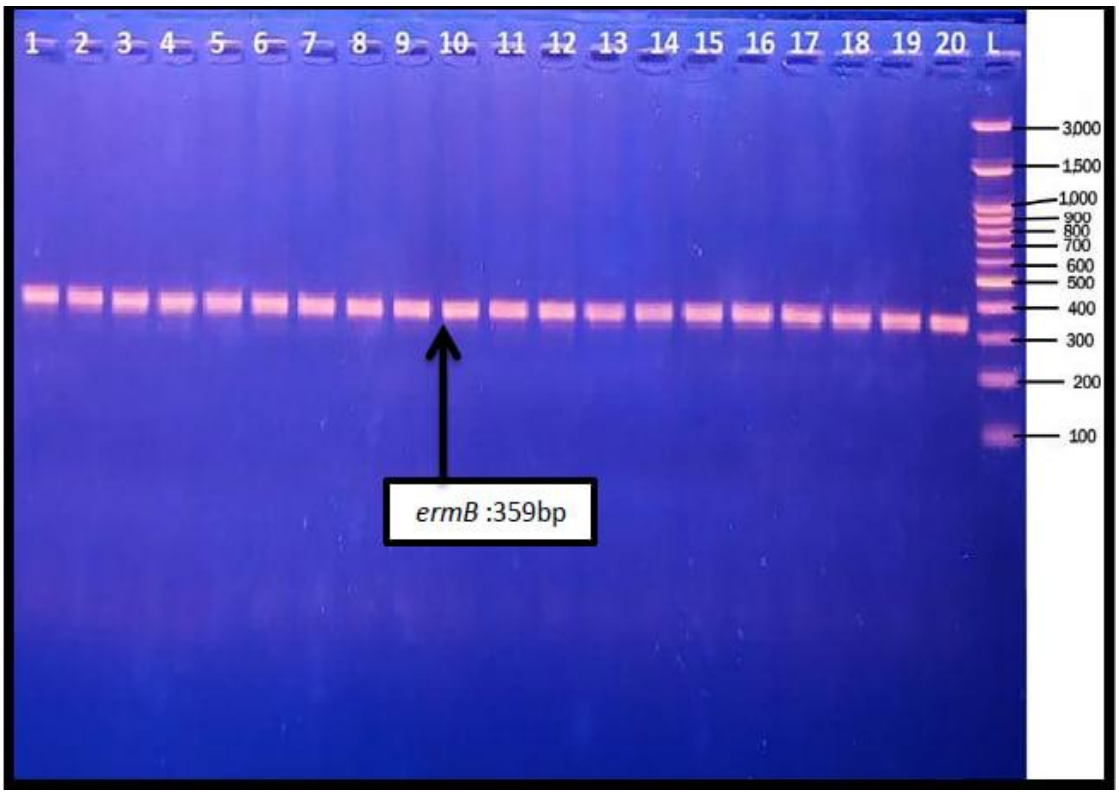

Fig. 6. Agarose gel electrophoresis for the PCR amplified *ermB* gene of *S. aureus* showing at 359bp give 20 positive results M: DNA marker (100bp-3000) 20 lanes reveal positive samples

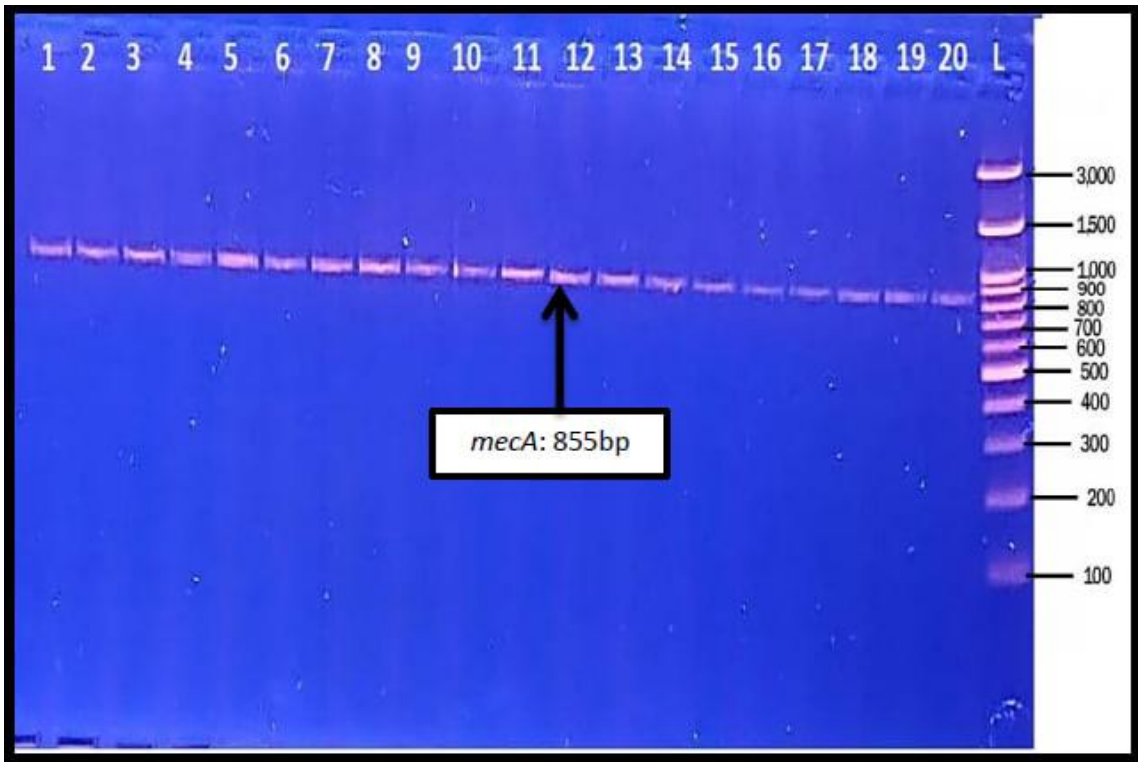

Fig. 7. Agarose gel electrophoresis for the PCR amplified *mecA* gene of *S. aureus* showing at 855bp give 20 positive results M: DNA marker (100bp-3000) 20 lanes reveal positive samples

3.4.5 Detection *ermB gene*

The *erm-B* used for detection of resistance gene in *S. aureus* against macrolide group (erythromycin drug) gave 20 positives from 20 isolates at amplification 359 bp fragment, this isolate appeared within the 20 isolates resistant to Erythromycin (Fig. 6).

The erythromycin ribosome methylase family of genes (ermA, ermB, and ermC) includes the ermA and ermB genes, which are connected to resistance to macrolides, lincosamide, and streptogramin. These genes are mostly found on plasmids and are broadly dispersed in human and animal isolates of *Staphylococcus* species (Hauschild et al., 2007, Mosa et al*.,* 2022). The potential for these genes to spread to other bacteria or possibly contaminate other animals, including humans, with multiresistant germs makes their existence worrisome.

3.4.6 Detection *mecA gene*

The *mec-A* used for detection of resistance gene in *S. aureus* against macrolide group (erythromycin drug) gave 20 positives from 20 isolates at amplification 855 bp fragment, this isolate appeared within the 20 isolates resistant to Erythromycin (Fig. 4).

Antibiotic treatment of staphylococcal infections is getting more difficult as a result of the frequency of Staph. spp. strains that are resistant to various drugs. Conventional MRSA control strategies contribute to the situation. Drug-resistant infectious illnesses demand antibiotic alternatives, and phages may be a viable strategy to replace, curtail, or encourage responsible antibiotic usage in farm animals (Jassim and Limoges, 2014).

4. CONCLUSION

This study was suggested that Every *S. aureus* isolate that tested positive came from mastitis-infected sheep. Twenty antimicrobial agents were used in the antibiotic susceptibility tests for *S. aureus* isolates. In summary, the findings indicate that *S. aureus* has become highly resistant to antibiotics.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of this manuscript.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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