# Detection of Virulence, Antibiotic Susceptibility and Molecular Characterization of Antibiotic Resistance (blaZ) Gene in Staphylococcus aureus by PCR assay

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*Abstract*—**This study was aimed to examined 59 isolates of Staphylococcus aureus (isolated previously from milk of ewes with mastitis, diagnosed by bacteriological methods and PCR confirmation by using 23SrRNA) for detection of slime producing (as virulence factor) by culturing on modified Congo red agar, antimicrobial susceptibility test to 11 antibiotics and Molecular assay with phylogenetic analysis of antibiotic resistance gene (blaZ) by Conventional PCR technique. The results revealed that all S. aureus isolates were slime producers and showed varying degrees of susceptibility patterns to antibiotic, high resistance to methicillin 100% and penicillin 74.5%, while resistance to Oxacillin, Cefotaxime and Ampicillin showed 50.8%, 47.4% and 42.3% respectively, mild resistance to other antibiotic (Erythromycin, Tetracyclin and Amikacin), while Ciprofloxacin showed Susceptibility 100%. Antibiotic resistance profiles showed multidrug-resistant to two, three and more than three antibiotics. The PCR assay on 59 extracted DNA showed amplification of the blaZ, in 6 isolates only, PCR-product of 6 blaZ gene samples were sequenced, analyzed, and deposited and published on the Genbank (NCBI) under the accession number MZ359750.1, MZ359751.1, MZ359752.1, MZ359753.1, MZ359754.1, MZ359755.1 and it is available for download. Five samples match the global results by 100%, and one sample possess a mutation at the site 1866418, the second at the site 1866425.**

**In conclusion, this study revealed the importance of detection of slime as a virulence factor in all isolates suggests that these isolates were virulent and pathogenic for humans and animals, the widespread use of antibiotics lead to the emergence of multiresistant pathogen, which phenotypically presented as double, triple, and multiple drug resistance. Some isolates were possess the gene blaZ which responsible for resistance to Penicillin.**

*Index Terms*— **Antimicrobial susceptibility test, blaZ gene, PCR, slime producing, S. aureus.**

## I. Introduction

Mastitis is considered as an important factor for culling ewes and has financial noticeable, production, and an animal welfare that related implications (Michael, et al; 2023a). Staphylococcus aureus is a contagious micro-organism causing mastitis due to a combination of the toxin-mediated virulence, invasiveness, and resistance to antibiotic, they are responsible for more than 65% of mastitis cases, the population structure, genomic and phenotypic characteristics of mastitis included in the pathogenicity of S. aureus association with the severity of mastitis is not fully established (Gelasakis et al., 2015; Kotzamanidis et al., 2021).

Many previous studies in Iraq revealed isolation of S. aureus from milk of ewes (Al-Sammari et al., 1985; Hammadi & Yousif, 2013, Hassan & Yousif, 2013, Ahmed and Yousif, 2021a and b).

Staphylococcus species possess many critical virulence factors, including the development of slime layers & biofilms (Götz, 2002). Slime layer (SL) is the term used to refer to Staphylococci's pseudocapsule, which is mainly comprised of polysaccharides (Heilmann et al., 1996). Slime produced by Staphylococcal bacteria is responsible for biofilm formation and is implicated in the pathogenesis of Staphylococcal infections, especially mastitis, during the early stages of infection, when the bacteria adhere to the mammary epithelial cells of the breast, formation of Biofilm is very important virulence factor which may result in persistent or recurrent udder infections and the treatment failure among increased resistancy to antibiotics and the protection against the host defences (Cramton et al., 1999; Schönborn et al., 2017).

Two aspects of biofilm play major roles in the pathogenesis of human and animal infections: first, the adhesion of the bacteria to epithelial cells thus facilitates the insertion and the release of different toxins (Kong et al., 2006). Second, decreased diffusion of antimicrobial molecules into the biofilm matrix limits the effectiveness of antibiotic therapy (Aguilar et al., 2001). Moreover, in the food industry, biofilms on surfaces increase the resistance to disinfectant agents (Abdallah et al., 2014). The most identified pathogen from cases of mastitis in sheep were S. simulans and S. aureus; they found a percentage 65.4% of staphylococci were biofilm-forming (Michael et al 2023a) .

Antimicrobial resistance is a problem obstructing treatment of an ever-rising range of infectious agents such as resistance of Staphylococci for antimicrobials (Ayis and Fadlalla, 2017; Ceniti et al 2017). The appearance of resistant bacteria in food manufacture may lead to transmit the resistance gene to the

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indigenous microbiota to the human digestive system (Lee, 2003). Michael et al (2023b)detect Antibiotic resistance in 57 of the 179 staphylococcal isolates from subclinical mastitis (31.8%)and recorded the resistance against eleven antibiotics, 63.2% of isolates were resistant to ampicillin, 63.2% to penicillin and 47.4% to tetracycline Isolates resistant to penicillin and ampicillin were showed in 12 sheep farms, 11.7% were multidrug-resistant isolates.

According to the pattern of Antibiotic sensitivity and Antibiotic resistance genes, Penicillin resistance genes was the most important resistance mechanism to penicillin is the production of beta-lactamase which inactivates penicillin by the hydrolysis of the beta-lactam ring, blaz gene is also involved in the resistance to penicillin of Staphylococcus bacteria which encoded the β-lactamase (Zapun et al., 2008; Akram, 2015). The resistance of these microorganisms to β-lactam antibiotics may also be related to the presence of certain genes responsible for regulating the synthesis of enzymes (e.g., beta-lactamases) mediated by the blaZ gene (Macori et al., 2017).

McMillan et al., (2016) record in their study that only the antibiotic resistance to S. aureus isolated from bovine and ovine milk samples was penicillin; and the isolate contained penicillin resistance gene blaZ.

Polymerase reactive chain (PCR) is a reliable, accurate & confirmatory technique for the identification of pathogens, especially Staph. aureus recovered from milk samples of sheep Akram (2015). Salauddin et al., (2020); Ahmed and Yousif, (2021a) Isolate S. aureus by cultural methods and biochemical tests as well as PCR assay and sequencing of the 23S rRNA specific gene for Staph.

Phylogenetic analysis was conducted to detect evolutionary history of species, proteins or the genes. The phylogenetic relationships between the organisms is a prerequisite of any evolutionary studies, as spp. Contemporary, Moreover, the Phylogenetic analysis was important because of the wide range applications which includes genomic organization, protein functions, epidemiological studies, and deciding the genes analyzed in the comparsion studies (Frey et al., 2013; Som, 2014, Ahmed and Yousif, 2021a and b).

The aims of this study were to detect slime producer staphylococci isolated from ewes mastitis, susceptibility to antimicrobial agents with detection of multidrug resistance and to detect the blaZ gene of Staphylococcus using PCR technique with phylogentic analysis.

## II. MATERIALS AND METHODS

### *A. Staphylococcus aureus isolates:*

Fifty nine isolates of S. aureus were obtained from pervious study done in Dept. of Internal and Preventive Vet. Medicine laboratory by (Ahmed and Yousif 2021a). these isolates were characterized by Bacteriological and molecular as follows: Four hundred and fourteen milk samples from ewes found in the field at different places in Baghdad city, all bacterial examination were done according to (Markey et al 2013) by culturing on blood agar and Mannitol salt agar, then incubated at 37ºC / 24 hours, Gram stain finally suspected colony sub-cultured on different selective media, Staph-110 agar and Chrome agar (Biomedia company). Then Biochemical tests were done (Catalase test, Oxidase test, Urease test, Coagulase test, Gelatin medium and O/ F glucose test) were done to confirm the diagnosis of S. aureus. All 59 isolates were positive and possess 23SrRNA for S. aureus by PCR (Ahmed and Yousif2021b)

Detection of slime production by Congo red agar methods:

According to Freeman et al., (1989) who described an alternative method for screening of slime producer (biofilm formation) by Staphylococcus isolates by using brain heart agar with 5% sucrose & Congo red stain as follow: This medium was consisted of:- Brain heart agar 37 / gms/1L., Sucrose 50 / gms/1L., Agar no.1 10 / gms/1L.Congo red stain 0.8 / gms/L. Then Mixing of Brain heart agar, sucrose and agar no.1 in liter and autoclaved, While Congo red stain was autoclaved separately after preparation of concentration; aqueous solution constituents, then added when the media had cooled to 50°C. S. aureus colonies (slime producer) appeared with black center with dry crystalline in consistency while other bacteria (Non-slime production) remain with the pink center after inoculation and incubation aerobically for 24 hours at 37°C.

### *B. Antimicrobial susceptibility test:*

This test was carried out according to Markey et al.,(2013)as follow: The bacterial colony was transformed to test tubes that contain PBS then diluted until reach top tube number two of McFerland solution that bacterial number in it is 6 X 108, then the bacterial suspension was spread by using a sterile cotton swab on Muller Hinton agar as a net shape then the Petri dishes left to dry for 3-5 minute, by using a sterile forceps the antibiotic disc was fixed on the agar surface and to avoid overlap between the inhibition area of the antibiotic disc we left 24mm between one disc and another and about 1cm between the disc and the margin of the Petri dish, then incubated at 37 C°/24 hrs. Inhibition zone diameter for each antibiotic disc (mm) (the clear area that surrounds the antibiotic disc including a diameter of the disc itself which is free of the bacterial growth) was measured by using Vernia and then compared the result with the standard diameter of the antibiotic inhibition zone as mention in (CLSI,2017).

### *C. PCR Assay for detection of blaZ gene in isolated S.aureus:*

DNA extraction: The isolation of Genomic DNA from bacterial growth done according to ABIOpure protocol Extraction.

Primer: The oligonucleotide primers for blaZ gene were:

**F: 5`-AAGAGATTTGCCTATGCTTC-3`**and **R: 5`-GCTTGACCACTTTTATCAGC-3`**. The product size was 571 pb were designed by (Vesterholm-Nielsen et al., 1999).

DNA Quantitation: QuantusFluoro meter was used to detect the concentration of extracted DNA. For 1 µl of DNA, 199 µl of diluted QuantyFlour Dye was mixed. After 5min incubation at room temperature.

Primer preparation.

The primers used in this study were supplied by Macrogen Company in a lyophilized form .Lyophilized primers were dissolved in a nuclease free water (300µl) to reach a final concentration of 100pmol/µl as a stock solution. Then a working solution of the primers was prepared by adding 10µl of the primer stock solution which stored at freezer (-20 C°) to

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90µl of the nuclease free water to gain a working primer solution of 10pmol/µl.

D. *Gel electrophoresis:*

This step was done to complete PCR assay, to check the extracted DNA by loading the eluted DNA by agarose gel electrophoresis.

E. *Preparation of PCR Master Mix:*

All required reagents were thawed completely and put them on ice, and reagent was mixed well by inversion and spins them down prior to pipetting. PCR master mix reaction was prepared by using GoTaq® Green Master Mix from Promega, USA. The PCR tubes containing an amplification mixture were transferred to thermal-cycler (BioRad/ USA) and started the program for amplification as shown in the (Table 1).

TABLE I
STEPS OF PCR CYCLE FOR BLAZ PRIMER.

| | **Temperature** | **minute: second** | **Cycles** |
|---|---|---|---|
| Initial Denaturation | 95 | 05:00 | 1 |
| Denaturation | 95 | 00:30 | 30 |
| Annealing | 55 | 00:30 | |
| Extension | 72 | 00:30 | |
| Final extension | 72 | 07:00 | 1 |
| Hold | 10 | 10:00 | |

*F. Sequencing:*

Product was sent for Sanger sequencing using ABI3730XL and the automated DNA was sequences by Macrogen Corporation (Korea). The results were analyzed by using generous software. 9

## III. RESULTS

*Virulence factor (slime production) of isolated Staph.aureus:*

The results of this study revealed that all isolated S. aureus (59) were positive to Congo red test showed black or brown colony, this results indicate that these isolates were slime producer (Figure1).

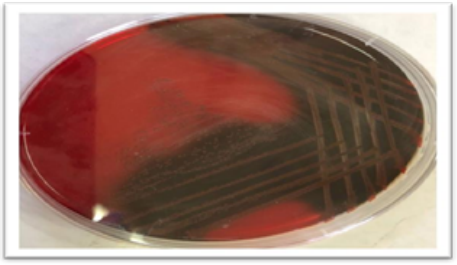

Fig. 1 Slime production of S. aureus on Congo red agar

Many studies used CRA test as an indication for the virulence factor of S. aureus, they found different percentage of positivity This study showed all bacteria positive 100% to the CRA test, this incompatible with study by Vasil et al., (2017) who determined ability of S. aureus isolated from milk samples of ewes with clinical mastitis, for their ability to formation of biofilms by using growth on CRA which showed a percentage 79.2%. A study by Achek et al., (2020) revealed a percentage of 64.3% of S. aureus strains that isolated from mastitic sheep were slime producer. Hammadi and Yousif, 2015) recorded (11.76%) of S.aureus isolated from ovine mastitis strong positivity to CRA test and (Krukowski et al.,2008) were reported a percentage of (42.37%) of slime producing Staph.aureus isolated from mastitic milk of sheep. Only study of Vasudevan et al., (2003) revealed that a high percentage (91.42%) of Staph.aureus mastitis isolates produced slime material which compatible with our study.

The present study showed that all isolates (59) producing slime on Congo red agar, this describe the importance of this as a virulence factor as explained by Dubravka et al., (2010) that the slime was a thick extracellular polysaccharide coating which recognized as a potent virulence factor because have the ability to enhance the bacterial adherence to the mammary- epithelial cells & get rid bacteria from the opsonization and phagocytosis. Some strains of these pathogens are resided in the tissue of udder as a biofilm that account for frequent treatment failures and a prolonged infection course

*Antimicrobial Susceptibility test.*

The results of antimicrobial susceptibility testing to 11 antibiotics against isolated S. aureus revealed varying degrees of susceptibility patterns. S. aureus were resistant as 100% and 74.5% to methicillin and penicillin respectively, while a moderate resistance to Oxacillin, Cefotaxime and Ampicillin as 50.8%, 47.4% and 42.3%, mild results to Erythromycin, Tetracyclin and Amikacin as 15.2%, 13.5% and 10.1%respectively, while Ciprofloxacin revealed Susceptibility 100% to S. aureus (Table2 ; figure 2 ) .

Antibiotic resistance profiles of isolates showed multidrug resistance to different antibiotic. From 59 isolates, 8 single, 7 double, 15 triple and 29 revealed multiple resistances for more than three antibiotics (Table 3).

TABLE II
STAPHYLOCOCCUS AUREUS AGAINST ANTIMICROBIAL DISC

| Antimicrobial | Type of Antimicrobial disc | Resistant % | Susceptible % |
|---|---|---|---|
| Aminoglycosides | Neomycin (NE) | - | 59(100%) |
| | Amikacin (AK) | 6 (10.1%) | 53(89.8%) |

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| | | | | |
|---|---|---|---|---|
| B-lactam | Cephalosporin | Cefotaxime (CFM) | 28(47.4 %) | 31(52.5%) |
| | Synthetic Penicillin | Oxacillin (OX) | 30(50.8 %) | 29(49.1%) |
| | | Ampicillin (AM) | 26(42.3 %) | 34(57.6%) |
| | | Methicillin (ME) | 59(100 %) | - |
| | Penicillin | Penicillin (P) | 44(74.5 %) | 15 (25.4%) |
| Tetracycline | | Tetracycline | 8 (13.5%) | 51 (86.4%) |
| Macrolides | | Erythromycin (E) | 10(15.2 %) | 49 (83.1%) |
| Flourquinlone | | Ciprofloxacin (CIP) | - | 59 (100%) |
| Glycopeptide | | Vancomycin (AV) | - | - |

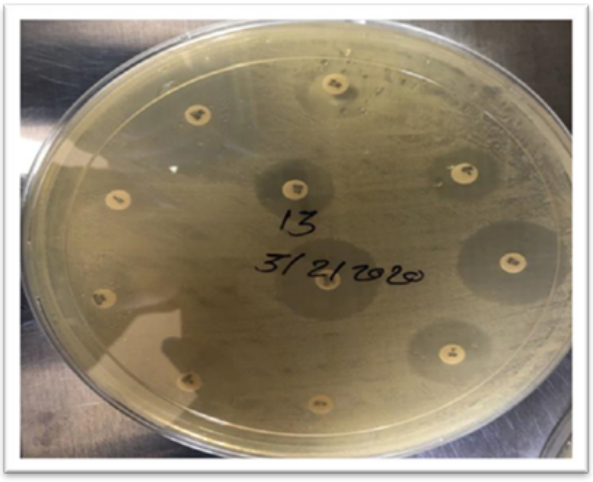


Fig. 2 Multidrug resistance S. aureus to antimicrobial drugs

TABLE III
COMPARISON OF ANTIBIOTIC RESISTANCE PROFILES OF ISOLATES

| Number of *S. aureus* | Single Resistance (%) | Double Resistance (%) | Triple Resistance (%) | Multiple Resistance (%) |
|---|---|---|---|---|
| 59 % | 8 (13.5%) | 7 (11.86%) | 15 (25.42%) | 29 (49.15%) |

The present results were similar or different from other researchers in the sensitivity of Staph aureus to antimicrobial drugs, Hammadi and Yousif (2013) mention that S. aureus isolates from ewes milk were sensitive to Cefotaxime, Erythromycin and Ciprofloxacin, Amikacin, Ceftriaxone, Oxacillin, Cephalexin, while bacteria were less sensitive to Penicillin G, Ampicillin, Amoxicillin.

Our study showed the high resistance of S.aureus isolates to Ampicillin and Penicillin may be attributed to improper and continuous uses of these antibiotics as a systemic and in local treatment, this may lead to the development of resistancy due to produce of the Pencillinase Enzymes from this bacteria. On the other hand, Ciprofloxacin and Erythromycin, didn't use consciously may lead to a decrease in resistance of S. aureus isolates to these antibiotic as recorded by (Hammadi and Yousif (2013).

Ampicillin is a beta-lactam antibiotic and evidence have suggested that resistance to beta-lactamase sensitive penicillin is extensive among S. aureus regardless of animal origin (Agrawal et al., 2013). Another study revealed a high percentage of resistance to penicillin that recorded (90.9%) from samples isolated from cattle, goats and sheep (Mia-Siyama et al., 2014).

Previous studies recorded that Staphylococcus pathogen isolated from milk samples of small ruminants revealed a low percentage of resistance than those of cow milk samples (Pengov and Ceru, 2003; Martins et al., 2015). Vasileiou et al (2019) report a resistance of Staphylococcal isolates from ewes subclinical mastitis, to penicillin, tetracycline and ampicillin, 5.6% of isolates appeared as multidrug-resistant

According to Lianou et al., (2021) S. aureus was found to be resistant more frequently to ampicillin, penicillin at 12.9% and tetracycline at 11.11% that different from our study this may be due to differences in the protocol of treatment or differences in climate or environment from Iraq.

Our results demonstrates that all Staph. aureus isolated from ewes showed high resistance to the methicillin antibiotic(100%). This agrees with Papadopoulos et al., (2018) recorded that 99.5% of Staph. aureus isolates from sheep farms were resistant to methicillin. And disagree with study by Macori et al., (2017) found that 53% of S. aureus from the ewes milk showed resistance to methicillin, but Giacinti et al.,(2016) recorded a low percentage of methicillin-resistant S. aureus among sheep revealed only 0.7%.

The current results revealed multiple resistance to more than 4 antibiotics, Penicillin, Oxacillin, Methicillin and Erythromycin. This result was compatible with study of França et al., (2012) who report that Staphylococcus isolated from ewes mastitis are characterized by multi- drug resistancy. And Azara et al., (2017) record a resistance and susceptibility of Staph. aureus isolated from sheep milk to different antibiotics but only 2 isolates were shown multidrug-resistant to 7 antibiotics including Oxacillin & Erythromycin. Multidrug resistance of Staph aureus is globally emerging which is concerned by overuse of antimicrobials and inadequate selection (Stevens et al., 2018).

The widespread of antibiotics use, randomly and incorrect doses on the treatment of mastitis resulted in the emergence of multi-resistance bacteria

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*Molecular study:*

*A. DNA extraction*

The 59 Staph. aureus isolates was successfully extracted to obtain DNA and this DNA which loaded on the1.2% agarose produce a sharp, clear and a pure bands (Figure3).

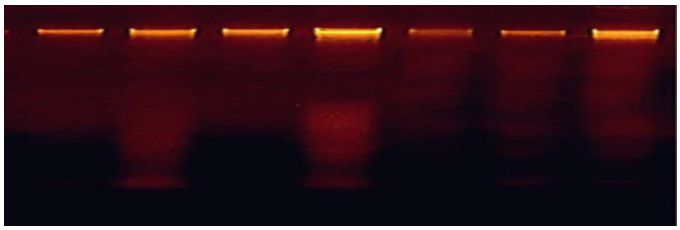

Fig. 3 DNA bands

The results showed DNA of 7 (11.86%) isolates of Staph aureus from 59 isolates were possess blaZ gene at amplification 571 bp fragments. And these 7 isolates appeared within the 44 isolates resistant to Penicillin (figure 4)

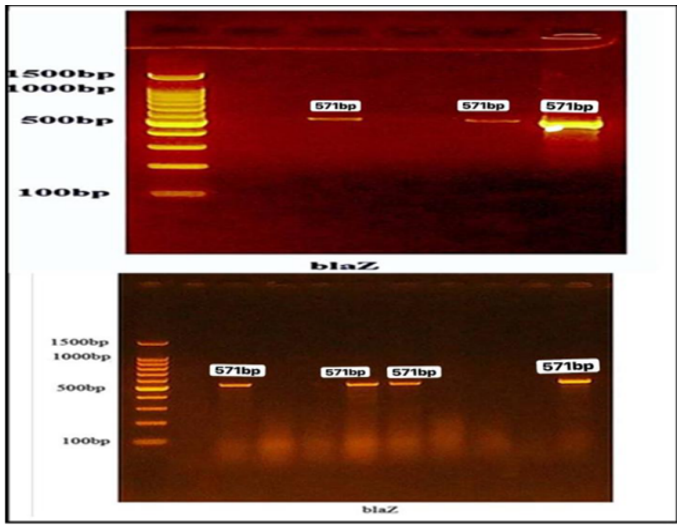


Fig. 4 agarose gel electrophoresis for the PCR amplified bla-Z gene of S. aureus showing amplification at 571bp give 7 positive results M: DNA marker (100bp) 7 lanes reveal positive samples.

The present study showed a percentage of 11.85% S aureus isolates possessed blaZ gene. These results were disagreed with other studies,. France et al (2012) found the percentage of the blaZ gene in S. aureus in small ruminant mastitis was (20.6%). Achek et al., (2020) report that the antibiotic resistance genes (blaZ, erm B, and tet K) genes were detected in Staphylococci and consider the Staphylococci which possessed these genes were potential hazards for sheep, consumers, and farmers. The resistance of these microorganisms to β-lactam antibiotics may also be related to the presence of certain genes (Macori et al., 2017) responsible for regulating the synthesis of enzymes (e.g., beta-lactamases) mediated by the blaZ gene.

The beta-lactamase production encoded by the blaZ gene was the principal mechanism associated with the resistance to (beta-lactams) in Staphylococci pathogen which causes mastitis in sheep and goats in Brazil (Franca et al., 2012). McMillan et al., (2016) record in their study that only the antibiotic resistance to Staph aureus isolated from bovine and ovine milk samples was penicillin; and the isolate contained penicillin resistance gene blaZ

*B. Sequencing for blaZ gene*

Six samples were send for analysis of blaZ gene, and the result showed that Five samples were globally matched 100%, and one sample contains the mutation at the site (1866418), the Nucleotide C\T, Nucleotide changed CCT\CTT, the Amino acid change Proline\ Leucine and Predicted effect Missense; the second was showed at the site 1866425, Nucleotide C\T, Nucleotide change ATC\ATT, Amino acid change Isoleucin\ Isoleucine and Predicted effect Silent (table). The ID NO. (Accession NO.) of S. aureus blaZ gene were recored in NCBI as showed in (Table 4) .

The evolutionary tree was drawn by using mega and program of NCBI (Figure 5) for the analysis and comparing of blaZ gene, seven samples were matched the results that mentioned in different countries by 100%, and one Iraqi isolate was 99%.

TABLE IV
ID NO. (Accession NO.) of S. aureus blaZ gene.

| Samples | ID NO. (Accession NO.) |
|---|---|
| **A** | MZ359750.1 |
| **B** | MZ359751.1 |
| **C** | MZ359752.1 |
| **D** | MZ359753.1 |
| **E** | MZ359754.1 |
| F | MZ359755.1 |

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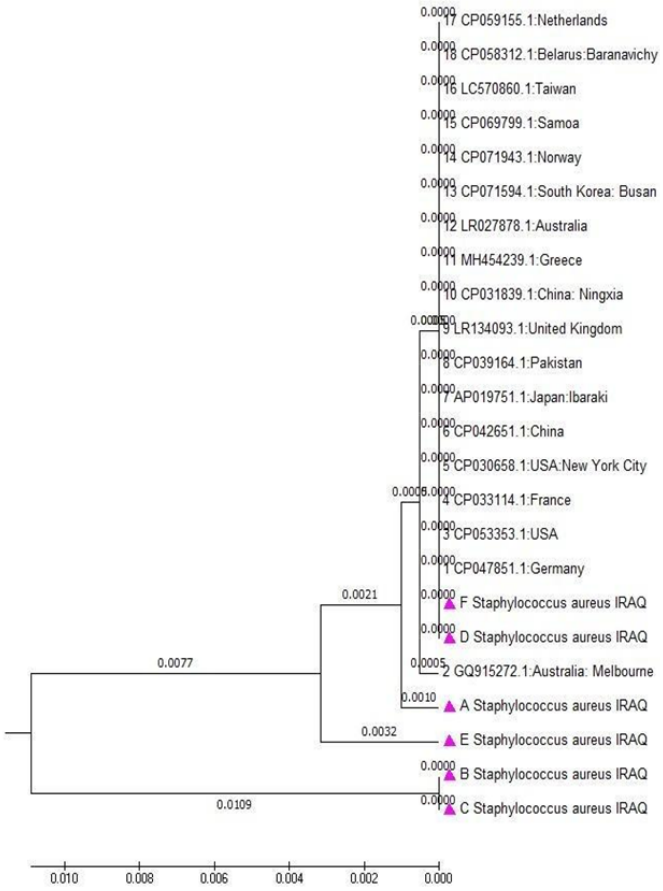


Fig. 5 Neighbor-joining tree of S. aureus (blaZ gene)

The current results of phylogenetic analysis of the six S. aureus isolates with ID: ( MZ359750.1; MZ359751.1; MZ359752.1; MZ359753.1; MZ359754.1; MZ359755.1). Which resistant to antibiotic Penicillin revealed the compatibility 99% with other rescuers: Germany (ID: CP047851.1), Australia: Melbourne ID: GQ915272.1, USA ID: CP053353.1, France ID: CP033114.1, USA: New York City ID: CP030658.1, China ID: CP042651.1 , Japan: Ibaraki ID: AP019751.1, Pakistan ID: CP039164.1, United Kingdom ID: LR134093.1, China: Ningxia ID: CP031839.1, Greece ID: MH454239.1 , Australia ID: LR027878.1 , South Korea: Busan ID: CP071594.1, Norway ID: CP071943.1 , Samoa ID: CP069799.1 , Taiwan ID: LC570860.1), Netherlands ID: CP059155.1 , Belarus: Baranavichy ID: CP058312.1.

## CONCLUSION

The finding of this study demonstrate the importance of S. aureus in the causes of mastitis in ewes. All isolates of S. aureus was resistant to methicillin antibiotic but only 7 strains possess blaZ gene. However, the detecting of blaZ A in some isolates and the absence in other resistant isolates to penicillin in antibiotic succebitbilty test requires detecting the alternative genetic possibilities related to the resistance profile and fragment of gene used.

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