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Molecular detection of MecA , Blaz Genes and phenotypic detection of Antibiotic Sensitivity Pattern For *S.aureus* And MRSA Isolated From Dermal lesions of Sheep In Diyala Governorate - Iraq

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Abstract

Back ground: *S. aureus* is one of the dominant bacterial pathogens among dermal infections in human and animals, which have resistance to different antimicrobial drugs.

Objectives : Isolation and identification of *S.aureus* from skin lesions among sheep by traditional methods, Vitek 2 system and PCR using Staur 4, 6 specific primers for validation, Detection of genes (methicillin resistant (*mec*A), Beta- lactamase gene (blaZ) by conventional PCR ,determine antibiotic sensitivity pattern by kirby bauer disc diffusion method.

Methods:

A total of 75 swaps were collected from sheep suffered from variety of infected skin lesions (wounds, abscesses, dermatitis, abrasion) recording to detect methicillin sensitive and resistant s. aureus by employing traditional methods in addition to confirmatory techniques through fast rapid VETEK2 system and PCR and ,determine antibiotic sensitivity pattern by kirby bauer disc diffusion method.

Results

S. aureus was isolated from 46/75 ,61.33% of sheep samples .MRSA was recovered from 14 / 46,(30.43%), which represent 14/75 , 18.67%) of total sheep samples with positive *mec*A gene using PCR. All sheep *S.aureus* isolates have positive detection for Staur 4, 6 primers (100%), blaZ (100%).

Levofloxacin and Ofloxacin resistance was reported in 16/46,(34.78%). A total of 14/46,(30%) of S.aureus have resistance for methicillin that was confirmed early by detection of MecA gene . Oxacillin and erythromycin resistance was reported in 8/46, (17.39%). Vancomycin resistance was reported in 4/46, (8.69%). Absolute sensitivity was reported for ciprofloxacin ,gentamycin, tetracycline, rifampicin, imipenem and chloramphenicol.

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Non Multidrug resistant S.aureus isolates have resistant for fluoroquinolones 8/46,(17.39%), Penicillins 6/46(13.04%), polypeptide antibiotics, 4/46,(8.69%). Multidrug resistant S.aureus isolates have resistance for Penicillins, Fluoroquinolones, Macrolide ,8/46,(17.39%).

Conclusions : Methicillin sensitive *S.aureus* was more common compared with MRSA isolated from dermal infections of sheep. Blaz gene was predominantly expressed by S.aureus isolates followed by Mec A gene. Levofloxacin and Ofloxacin resistance were higher followed by Oxacillin . erythromycin and vancomycin. Multi drug resistant trait commonly for Penicillins, Fluoroquinolones, Macrolide

Keywords : Staphylococcus aureus, skin ,sheep ,antibiotics, MecA, Beta lactamase

Introduction :

Staphylococcus aureus is the most abundant skin-colonizing bacteria and the most important cause of nosocomial and community-associated skin infections **[1]**. *S. aureus* is an opportunistic pathogen, and its virulence depends on extracellular proteins(enzymes and exotoxins) , that contribute to causing a wide range of diseases in human **[2]**and animals **[3]**. The main cause for the successful distribution is the variability and resistance patterns for many antibiotic **[4]**. *S.aureus* has become resistant to penicillin due to the production of β-lactamases enzymes that hydrolyze β-lactams antibiotics such as penicillin, thereby rendering them biologically inert. Methicillin-resistant *Staphylococcus aureus* (MRSA) possesses reduced affinities for binding to β-lactam antibiotics by producing a specific penicillin-binding protein, PBP2 (or PBP2a), resulting in β-lactam antibiotic resistance **[5, 6]**. The resistance acquired by Methicillin (oxacillin [OX])-resistant *S. aureus* is extended to most of the commonly used antimicrobial agents, including the aminoglycosides, macrolides, chloramphenicol, tetracycline, and fluoroquinolones**[7, 8]** . They are also reported to be resistant to all cephems, cephalosporins, and other β-lactams (such as amoxicillin-clavulanic acid, ticarcillin-clavulanic acid, ampicillin-sulbactam, carbapenems, and the piperacillin-tazobactam) regardless of the *in vitro* test results obtained with those agents **[9]**

This study aimed to Isolation and identification of *S.aureus* from skin lesions among sheep by traditional methods, Vitek 2 system and PCR using Staur 4, 6 specific primers for validation, Detection of genes (methicillin resistant (*mec*A), Beta- lactamase gene (blaZ) by conventional PCR ,determine antibiotic sensitivity pattern by kirby bauer disc diffusion method**.**

Methods :

Samples collection :

A total of **75** dermal swap samples collected from sheep flock with infected skin . sample collection was extended from 1st October 2021 to the end of February 2022. All collected samples

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were sent to microbiology laboratory at the college of veterinary medicine – university of Diyala, Iraq in a cool box for initial isolation on mannitol salt agar for 18-24 h. A golden yellow colonies were selected for further investigation; (Gram staining, Nigrosin capsule staining, catalase test, coagulase test, DNase), identified *S. aureus* and (MRSA) through Vitek system 2 and PCR**[10]** .

Molecular diagnosis for S. aureus and virulence genes :

PCR based detection was applied according to the instructions of references as illustrated in table (1)

Table(1): primers used for PCR based detection of S.aureus and virulence factors

Gene	Primers	Base pairs	Sequence (5'- 3')	PCR Protocol			Reference
				Denaturation	**Annealing**	**Extension**	
S.aureus 23srRNA	Staur4	1250 bp	5'-ACGGA GTT AC A AAGGAC GAC-3'	94 oC/ 45 sec	64 oC / 60 sec	72 oC / 2min	**[11]**
	Staur6		5'-AGCTCAGCCT TAAC GAG TAC-3'				
Methicillin Resistant Gene A	*mec*A-F	162 bp	5-TCCAGATTACAACTTCAC CAGG-3	94 oC / 45sec	50 oC / 30sec	72 oC / 30sec	**[11]**
	*mec*A-R		3-CCACTTCATATCTT-GTAACG-5				
Beta lactamase gene	Blaz -F	517 bp	5'-AAGAGATTTGCCTAT GC TTC-3'	94oC /4min	94oC /60 second	94oC /60 second	**[11]**
	Blaz-R		5'-GCTTGACCACTTTTAT C A GC-3'				
	IcD-R		5'- GCTTGACCACTTT TATC AGC-3'				

Antimicrobial Susceptibility Test:

All positive samples cultured on mannitol agar were submitted for antimicrobial susceptibility testing on Mueller- Hinton agar as stated by Clinical and Laboratory Standards Institute

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[12]. Susceptibility was tested to antibiotics illustrated in table(2). As stated by CLSI guidelines, *S.aureus* isolates were classified to (susceptible, intermediate, or resistant), as shown in table(3) .

Table(2) : Antibiotic Discs utilized throughout the study

Antibiotic	Abbreviation	Weight
Methicillin	MET	5 µg
Oxacillin	OX	5 µg
Levofloxacin	LE	5 µg
Ofloxacin	OF	5 µg
Erythromycin	E	15 µg
Gentamicin	GEN	10 µg
Tetracycline	TE	30 µg
Rifampicin	RIF	5 µg
Imipenem	IPM	10 µg
Vancomycin	VA	30 µg
Ciprofloxacin	CIP	5 µg
Chloramphenicol	C	30 µg

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Table(3): Criteria For Antibiotic Sensitivity OF S.aureus

Antimicrobial category	Antibiotic	Sensitive more than (mm) ≥	Intermediate (mm)	Resistant (less than) (mm) ≤
Penicillins	Methicillin (5µg)	22	17-22	17
	Oxacillin(5µg)	13	11-12	10
Fluoroquinolones	Levofloxacin (5µg)	19	16-18	15
	Ofloxacin (5µg)	18	15-17	14
	Ciprofloxacin (5µg)	21	16-20	15
Macrolides	Erythromycin(15µg)	21	18-20	18
Aminoglycosides	Gentamycin(10µg)	15	13-14	12
Tetracyclines	Tetracycline(30µg)	19	15-18	14
Ansamycins	Rifampicin(5µg)	20	17-19	16
Carbapenems	Imipenem (10µg)	16	14-15	13
Polypeptides	Vancomycin (30µg)	21		17
phenicols	Chloramphenicol (30µg)	18	13-17	12

Ethical consideration:

This study conducted according to the principles of Helsinki declaration. A full explanation of the purpose of this study to all owners before starting. Dully filled consent form obtained from all owners who agree to participate in the study. Approval of an ethical review committee of pathology department, college of veterinary medicine, Diyala University, Iraq, taken before initiation into the work**[13-24]** .

Statistical Analysis

Calculation down by the Statistical Package of the Social Sciences for windows version 17 (SPSS, Armonk, NY: IBM Corp) **[25, 26]**. Pearson's chi-square and Pearson's correlation coefficient was utilized for the correlation between the changeable of 2 test. P value of ≤ 0.05 and ≤ 0.01(2-tailed) were set to be statistically important**[27, 28]** .

Results :

Table (4); shows that 75 sheep submitted in this study whom suffered from different skin lesions. *S. aureus* involved among only 46 of them rated (61.33%) of collected samples from skin lesion which grew positively on mannitol salt agar, valid by Vitek 2 system and conventional PCR by using *S. aureus* 23s RNA gene sequence specific primer (staur4, 6) as shown in (Figure **1**). While a total of 14/46, (30.43%) were (MRSA), represents (18.67%) of total samples 14/75 according to methicillin resistance on Muller Hinton medium and results of conventional PCR by using *S. aureus* (*mec*A gene) as shown in (Figure

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2).Beta lactamase gene primers was detected in all S.aureus isolates as shown in table(5) and figure (3).

As shown in table (6) figure (4), Levofloxacin and Ofloxacin resistance was reported in 16/46,(34.78%). A total of 14/46,(30%) of *S.aureus* have resistance for methicillin that was confirmed early by detection of MecA gene . Oxacillin and erythromycin resistance was reported in 8/46, (17.39%). Vancomycin resistance was reported in 4/46, (8.69%). Absolute sensitivity was reported for ciprofloxacin ,gentamycin, tetracycline, rifampicin, imipenem and chloramphenicol.

As shown in table (7), Non Multidrug resistant *S.aureus* isolates have resistance for fluoroquinolones 8/46,(17.39%), Penicillins 6/46(13.04%), polypeptide antibiotics, 4/46,(8.69%). Multidrug resistant *S.aureus* isolates have resistance for Penicillins, Fluoroquinolones, Macrolide ,8/46,(17.39%).

Table (4): Isolation Rate Of *S. Aureus* & MRSA From skin of Sheep

Source of skin swaps	Total no. of samples	No.(%) Of S. aureus Isolates	No.(%) Of MRSA from positive samples	No.(%) Of MRSA from total samples
Sheep	**75**	**46(61.33%)**	**14/46,(30.43)**	**14/75,(18.67%)**

Table (5): Conventional PCR based Detection of Staur4,6, mecA and BlaZ genes Of S. aureus isolated From skin of Sheep

Source	Staur 4,6	Mec A	Blaz
Sheep	**46/46 (100%)**	**14/46 (30.43%)**	**5/5 (100%)**

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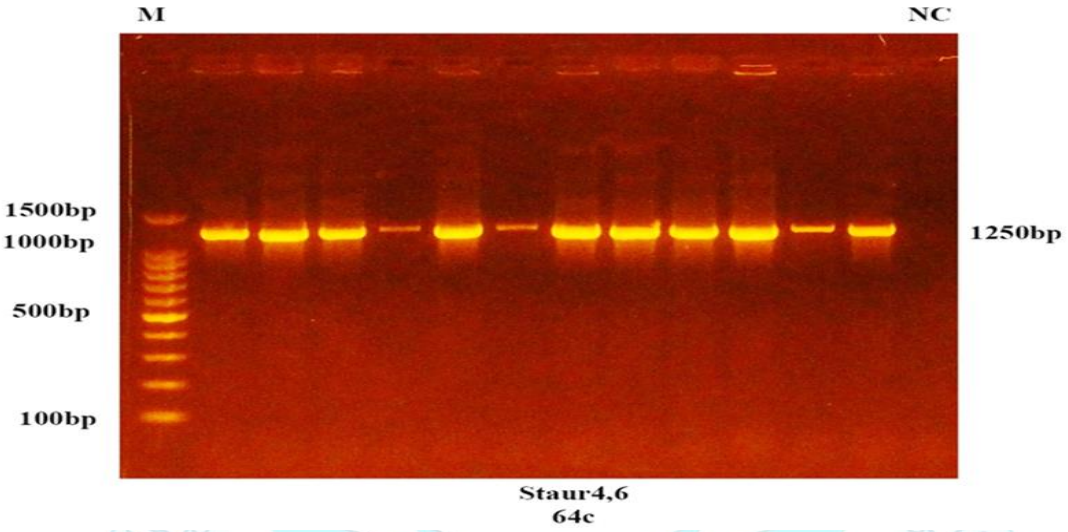

Figure (1): Amplification for staur primers 4&6 (1250bp) by conventional polymerase chain reaction for S. aureus recovered from skin lesions of sheep breeder.NC: Negative control.

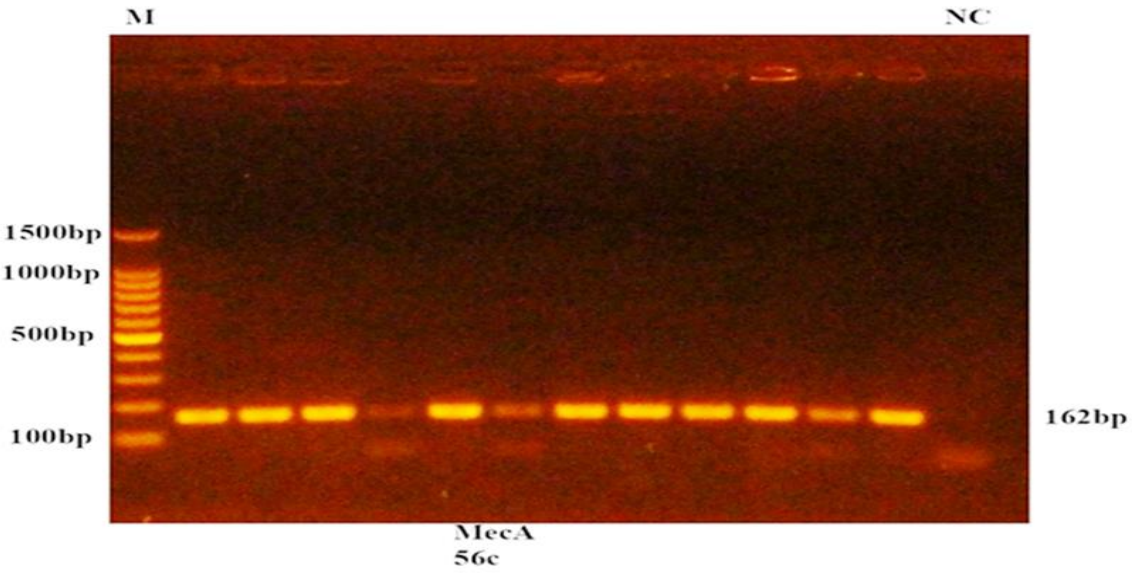

Figure (2): Amplification MecA (162bp) by conventional polymerase chain reaction for S. aureus recovered from skin lesions of sheep breeder.
NC: Negative control.

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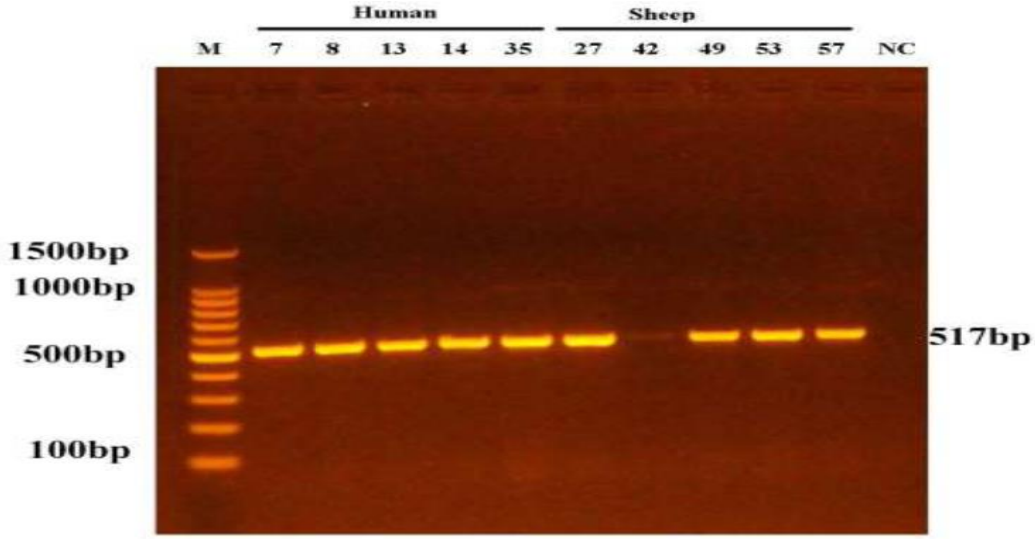

Figure (3): amplification *blaZ* (517 bp) By conventional polymers chain reaction for *S. aureus* recovered from skin lesion of Sheep breeders and

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Table(6): Antibiotic Sensitivity Pattern For *S.aureus* Isolated From Sheep

Antibiotic	Minimum inhibition zone Diameter (mm)	Maximum inhibition zone Diameter (mm)	Mean± SE Inhibition zone Diameter (mm)	Sensitive No.(%)	Inter-ter-medi-ate No.(%)	Re-sistant No.(%)	Total No. of isolates
Methicillin	**10**	**24**	**18.02± .630**	**4(8.69%)**	**28(60.86%)**	**14(30.43%)**	**46(100%)**
Oxacillin	**11**	**24**	**18.77± .583**	**38(82.60%)**	**0(0%)**	**8(17.39%)**	
Levofloxacin	**10**	**28**	**20.35± .850**	**30(65.21%)**	**0(0%)**	**16(34.78%)**	
Ofloxacin	**10**	**26**	**19.44± .797**	**30(65.21%)**	**0(0%)**	**16(34.78%)**	
Ciprofloxacin	**23**	**30**	**26.12± 0.346**	**46(100%)**	**0(0%)**	**0(0%)**	
Erythromycin	**13**	**29**	**23.14±.608**	**34(73.91%)**	**4(8.69%)**	**8(17.39%)**	
Gentamycin	**18**	**28**	**23.79±0.386**	**46(100%)**	**0(0%)**	**0(0%)**	
Tetracycline	**19**	**30**	**22.00± 0.420**	**46(100%)**	**0(0%)**	**0(0%)**	
Rifampicin	**22**	**34**	**28.51± 0.379**	**46(100%)**	**0(0%)**	**0(0%)**	
Imipenem	**27**	**37**	**30.26± 0.302**	**46(100%)**	**0(0%)**	**0(0%)**	
Vancomycin	**17**	**28**	**20.49± 0.281**	**20(43.47%)**	**22(47.82%)**	**4(8.69%)**	
Chloramphenicol	**19**	**27**	**23.23 ± 0.367**	**46(100%)**	**0(0%)**	**0(0%)**	

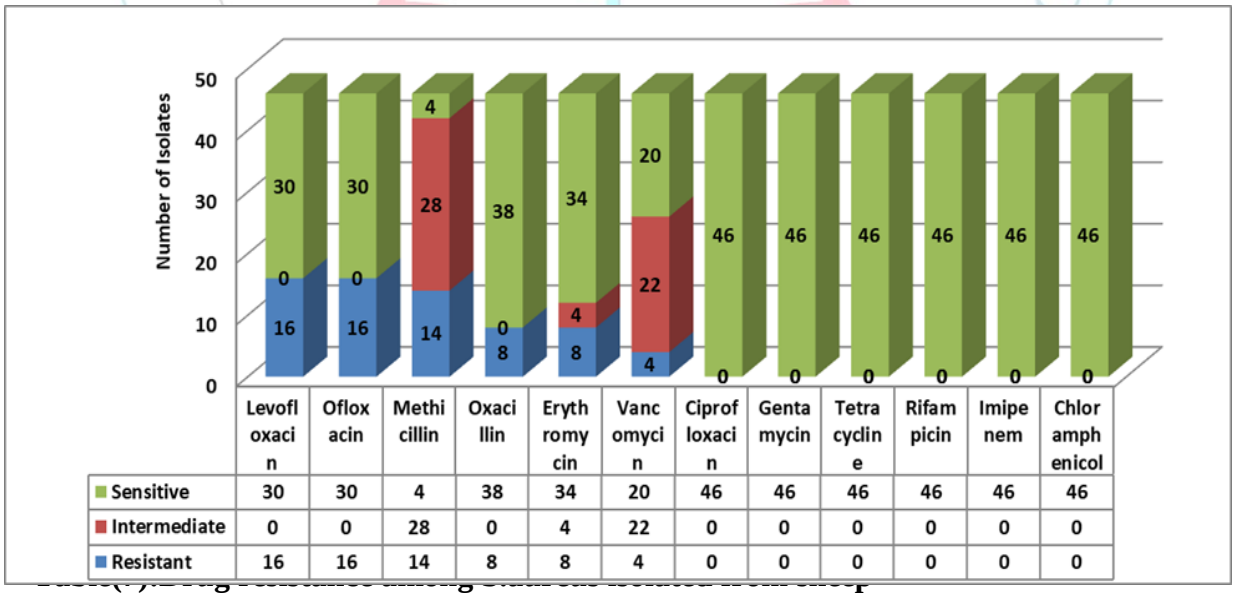

Figure (4):Antimicrobial susceptibility of S.aureus Isolated From sheep

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Classification of *S.aureus* according to antimicrobial drugs resistance	Resistance for Antimicrobial class	No.(%)
Non Multidrug resistant isolates	Fluoroquinolones	8/46,(17.39%)
	Penicillins	6/46(13.04%)
	polypeptide antibiotics	4/46,(8.69%)
Multidrug resistant isolates	Penicillins, Fluoroquinolones, Macrolide	8/46, (17.39%)
Pan Drug resistant isolates	None	0(0%)
Total		15,(100%)

Discussion :

S.aureus one of the dominant pathogenic bacteria among skin infections, the aim of this study isolation and identification of S. aureus and MRSA in skin lesions of sheep in addition to molecular detection through PCR assay upon genes (staur4,6, MecA, blaz). on A 75 skin lesion samples collected from sheep cultured on Blood agar isolated and mannitol salt agar (MSA) which is a medium encouraging growth of certain bacteria while inhibiting the growth of others, short time distinguishes. Contains a high concentration of salt7% + Mannitol sugar1% + Agar (solidifying agent) + Enzymatic digest of casein + Enzymatic digest of animal tissue + Beef extract + Phenol red indicator) which inhibit most bacteria that makes MSA selective against most gram negative and selective for some gram positive bacteria that tolerate high salt concentrations. It is also a differential medium for mannitol-fermenting staphylococci**[29]** containing carbohydrate mannitol and the indicator phenol red, a pH indicator for detecting acid produced by mannitol-fermenting staphylococci. *S. aureus* produces yellow colonies, by acidic byproduct formation that causes the phenol red in the agar to turn yellow. It is used for the selective isolation of presumptive pathogenic (pp) Staphylococcus species corresponding **[30]**, verified by Gram stain, Nigrosin stain, biochemical tests (Catalase, Coagulase, DNase), confirming technique by Vitek2 system then evaluation of antibiotic sensitivity, 10 pure positive samples (five for each) referred to conventional PCR, same result in this study finds on a VITEK® 2 System of bioMérieux Inc automated machine confirm matching 96%, 94% at different incubation periods according to the manufacturers procedures **[31]**

This study finds *S. aureus* sheep skin infection rates 61.33% and among those infected with MRSA rates 30.43 % in Diyala Iraq. In north-western Greece

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[32] found Of 367 samples tested, 57.8% were *S. aureus* positive and only 3% MRSA positive. While in china **[33]**,reported that *S.aureus* was isolated from 32/74, (43.24%).In Bangladesh S.aureus was isolated from skin of sheep was 70% **[34]**. In Italy **[35]** recorded infections rate of *S. aureus* among dairy sheep farms 53.5% and MRSA rates 7% among the hall flock. French farms study showed nasal carriage of *S. aureus* in 29% of dairy ewes **[36]**, in Norway **[37]**recoded S. aureus was (32.6%) in sheep.

In current study S.aureus 23srRNA gene sequence specific primers (staur4, 6,) for S.aureus was detected in all *S.aureus* isolates by Conventional PCR which come in line with that reported by **[11]** .

Molecular Detection of Methicillin Resistant Gene in *S. aureu* pure isolate of sheep by conventional PCR for (*mecA*) that was detected among (3/5), 60% positive of *S. aureus* which is lower than that reported by **[38]** In Italy ,they stated that MecA was detected among 58/65(89%) of S.aureus isolates.on the other hand **[38]**,clarified Staphylococcal cassette chromosome mobile element complex gene inserted to the chromosome gene, the SCC*mec* harbors the single determinant for methicillin resistance, namely the *mecA* or *mecC* gene, encodes PBP2a (Penicillin binding protein 2a) enzymes that have low affinity for all β-lactams. Acquisition of *mec*A renders β-lactams useless against MRSA and alternative therapies. HA-MRSA compared to CA-MRSA strains by SCCmec type IV, V, or VI, which were susceptible to macrolides and fluoroquinolones antibiotics. In Italy,**[39]** ,they reported that in a dairy sheep farms , sheep isolate might act as a mecC-MRSA reservoir in LA-MRSA SCCmec type III, IV, XI thus, recommends laboratories to search for the *mecC* gene in all the *mecA*-negative isolate. Less percentage done by **[40]** Of 146 *S. aureus* isolates, 24 (16.4%) carried *mec*A genes and identified as MRSA strains. In a study by**[41]** ,they counted mecA and its new homologues (mecB, mecC, and mecD) on thirteen types in more than ten Allele. Resistance bestowed by the mecA gene product is demonstrated via a reduced rate of β-lactam-mediated enzyme acylation and decreased affinity for β-lactams compared to that of native PBPs. The crystal structure of the mecA gene product (i.e., PBP2a) provided the structural basis for this resistance. PBP2a is an elongated protein with a transpeptidase domain, a transmembrane domain, and a non-penicillin-binding domain, which possesses an allosteric site **[42]**. Compared to the active sites of native PBPs, the active site of PBP2a is less accessible to β-lactams, as it is located in a narrow extended cleft. Hence, it does not affect the synthesis of peptidoglycan,

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given the antibiotic strength reached in vivo **[41]**

Molecular Detection, Beta-Lactamase Gene in *S. aureus* isolate of Sheep On Conventional PCR, gen *(blaZ)* detected among 5 pure isolate of sheep was (5/5) 100% of *S. aureus.* In a study by **[43]** ,stated most strains of S. aureus possess ability to produce beta-lactamases, an enzyme that can open beta-lactam rings in Cephalosporin and Penicillin. Some acquire resistance genes from the environments and/or from other bacteria and thus may exhibit resistance to antibiotics in other classes produced on plasmid encoded as class A β-lactamase (penicillinase)**[44]**. hence, its hydrolytic activity against oxacillin, cephems, and carbapenems. Additionally, site-directed mutagenesis within amino acid sequences showed that an alanine at position 112 of *BlaZ* plays an important role in the hydrolysis of oxacillin**[44]**. They are two mechanisms for resistance of beta-lactam antibiotics. One is production of beta-lactamases; enzymes hydrolytically destroy beta-lactams. Other is expression of penicillin-binding protein (PBP 2a), which is not susceptible to inhibition by beta-lactam antibiotics. *S. aureus* either beta-lactamase or PBP 2a-directed resistance (or both)**[41]**.

This study detect Antibiotic Sensitivity Pattern for *S. aureus* and MRSA Isolate in Sheep infected with *S. aureus* 30.43% resistant to Methicillin that confirmed by detection of (mecA) gene which records resistant for Oxacillin, Erythromycin, 17.39%, and Levofloxacin, Ofloxacin, resistant 34.78% and Vancomycin resistant 8.69%, but absolute sensitivity was reported for Ciprofloxacin, Gentamycin, Tetracycline, Rifampicin, Imipenem, and Chloramphenicol, while **[45]**recorded that MRSA was determined by PCR and resistance to cefoxitin. Although **[46]** stated that antimicrobial resistance of MRSA detected by penicillin 93.4%, ampicillin 88.9%, and cloxacillin 83.3%, whereas .In Palestine **[47]** claimed that MRSA isolates identified by cefoxitin disc diffusion and all were vancomycin sensitive and Gentamicin. In Italy **[35]** reported that MRSA Susceptibility, 60.58% were susceptible to all the antimicrobials tested, and 39.42% were resistant to at least 1 antimicrobial. In particular, 22.12% were resistant to tetracycline, 15.38% to sulfonomides, 13.46% to trimethoprim and sulfa methoxazole, and 8.65% to ampicillin, however only one isolate was resistant to both Fluoroquinolones and aminoglycosides, *S. aureus* isolates displaying resistance to oxacillin, cefoxitin, or both. Resistant to all the β-lactams tested and to erythromycin, streptomycin, kanamycin, and tetracycline.

Conclusions : Methicillin sensitive *S.aureus* was more common compared with MRSA isolated from dermal infections of sheep. Blaz gene was predominantly expressed by S.aureus isolates followed by Mec A gene. Levofloxacin and Ofloxacin

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