Plant Archives Volume 20 No. 2, 2020 pp. 5121-5129 **e-ISSN:2581-6063 (online), ISSN:0972-5210**

BACTERIOLOGICAL STUDY OF *STAPHYLOCOCCUS AUREUS* ISOLATED FROM SKIN INFECTED SHEEP

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Abstract

The study was conducted to diagnosis *Staphylococcus aureus* from infection skin of sheep and its susceptibility to important antibiotic, in addition to identifying some virulence factors activity. Eighty skin swabs were collected from infected sheep in various areas from Baghdad Governorate from period (October 2019 to January 2020). The isolates were initially recognized depending on their morphological shape, size, color, depending on Grams stain, biochemical test, and confirmed by Vitek2 system. Isolates were phenotypically investigated for some virulence factors (hemolysin, lipase, amylase and Detection of Protein A (Spa) activity). Further, the isolates were studied for their antimicrobial susceptibility patterns using 11 antibiotics commonly used. It was found that *S. aureus* isolates were evaluated for hemolysis production on sheep blood agar. Alpha hemolysis 4(17.77%), beta-hemolysis 8(35.55%) and non-hemolytsis *S. aureus* 6(26.66%). While 18(100%) estimated for lipase test whereas showed 3(13.33%) positive for amylase test and 15(66.66%) negative for amylase test then could not detected of Protein A activity. Results of Antimicrobial susceptibility test expression all isolates were appearance resistance18(100%) to Penicillin, Methicillin, Amoxicillin/Clavulanic acid, Cefixime and Aztronam while Variations in a result for Vancomycin between resistance to Vancomycin11(48.88%) and 7(31.11%) consider as Intermediate resistance to Vancomycin. on other hand most of isolates were sensitive to (Netilimicin18(100%), Chloramphenicol 16(71.11%), Tetracycline 15(66.66%), Clindamycin 14(62.66%), oxacillin 7(31.11%).

Key words : *Staphylococcus aureus,* Lipase, Amylase, Hemolysin, protein A.

Introduction

Staphylococcus aureus (*S. aureus*) is a Gram-positive bacteria known to be considered as a major public concern. And called a 'Janus-faced' bacterium, *S. aureus* usages complex regulatory networks to sense various signals that enable it to adapt to diverse environments and modulate virulence. (Balasubramanian *et al.,* 2017), It was a commensal organism, Its remain one of the furthermost intensively studied bacterial species in human and animal pathogen, it is an adaptable, opportunistic pathogen with capabilities to persist and multiply in a variability of environments and causes a extensive scale of diseases (Cucarella *et al.,* 2004). The skin is a common site of staphylococcal contamination in both humans and animals (Misic *et al.,* 2014). The skin and mucous membrane provide excellent barriers against *S. aureus* invasion of local tissue. However, the pathogen causes a wide-range of infections including those of skin and soft tissues extending from superficial skin infections to serious and life-threatening deep tissue infections (Guerra *et al.,* 2017). The power of *S. aureus* to causes infections is related to appearance of various virulence features like surface proteins, biofilm, exoenzymes, exotoxins and exfoliative toxins. All of these countenance bacteria to adhere to tissues producing pathogenesis and invade the immune system causing toxic effect (Costa *et al.,* 2013).*S.aureus* is also infamous for its ability to withstand the arsenal of antibiotics currently available and for the dissemination of various multidrug-resistant, *S. aureus* clones limits therapeutic possibilities for a *S. aureus* infection. (Kong *et al.,* 2016). Administration of *S. aureus* is complicated by the development of 'super bugs' that have become resistant to multiple antibiotics, as in the incident of methicillin-resistant and vancomycin resistant *S.aureus* (MRSA and VRSA, respectively). (Balasubramanian *et al.,* 2017). Lipase enzymes contribute to bacterial pathogenicity due to their importance in bacterial lipid breakdown. It is supposed

that these enzymes must be present for *staphylococci* to attack cutaneous and subcutaneous tissues and for superficial skin infections like furuncles (boils), and carbuncles to mature (Xie *et al.,* 2012). Amylase is one of the enzymes that has been secreted by *S. aureus* which catalyzing the breakdown of complex sugars into monosaccharides required to colonize and survive this pathogen in any anatomical locales. (Lakshmi *et al.,* 2013).There are several types of hemolysin including α, β, γ and δ-hemolysis (Kong *et al.,* 2016). α- hemolysis was a beta-barrel that produces toxins that are secreted as a water-soluble monomer(Oliveira *et al.,* 2018). Also plays an important role in the pathogenesis of *S. aureus* skin and soft tissue infections (SSTIs) and especially encourages dermonecrosis in animal infection models (Kobayashi *et al.,* 2015). β-hemolysin is non-pore-forming and has been described as a sphingomyelinase, in addition to its well-known hemolytic function for erythrocytes,it plays an significant role in skin colonization by destroying keratinocytes. (Katayama *et al*., 2013). δ-hemolysis has been Identified as a phenol-soluble modulin (PSM) which does not need a receptor for its hemolytic activity (Kong *et al.,* 2016). Hemolysine affects red blood cells, it forms a trans-membrane pore that lyses the cell membrane at high concentrations (Verdon *et al.,* 2009). g-hemolysis are bi-component and made up of polypeptides (Kong *et al.,* 2016).protein A (Spa) has a number of immunosuppressive traits and is one of *S.aureus* most important mechanisms of immune evasion. SpA binds to the Fc portion of IgG, resulting in the bacteria becoming coated in IgG bound in the incorrect orientation, leading to decreased recognition by neutrophils and consequently evasion of phagocytosis (Lacey *et al.,* 2016). Interestingly, protein A was identified even in the intercellular space of keratinocytes by immunoelectron microscopy suggesting that *S. aureus* colonizing skin is internalized with protein A and causes local skin inflammation. (Iwamoto *et al.,* 2019).

The present study was identify and characterize *S. aureus* isolates collected from sheep skin infection swabs focus on detection of some virulence factors produced by *S. aureus* isolates including(hemolysin activity, lipase activity,amylase activity and detection of Protein A (Spa) activity. Also in this study we determined the antimicrobial patterns of the isolates of *S. aureus* isolated from skin infection swabs which is a cornerstone in the control of *S. aureus* infection.

Materials and Methods

Sample collection and there sources

A number of 80 swab samples of sheep skin infections were collected randomly on moistened sterile cotton tipped applicator from infected skin of sheep in 5ml of freshly prepared tryptic soya broth (TSB) in a test tube under complete hygienic condition from different areas in Baghdad Governorate, from October 2019 to January 2020 and transferred immediately to the laboratory.

Isolation and identification of *Staphylococcus aureus*

Skin infections swabs were suspended in 5ml of freshly Tryptic Soya Broth (TSB) in a test tube and grown at 37°C for 24 hrs. The inoculum was than streaked on *Staphylococcal* 110 (STAPH 110) agar, plates were incubated at 37°C for 24 hrs. And sub-cultured on Mannitol salt agar (MSA) plates and incubated for 24 hrs. at 37°C.

Gram stain

Each colony was examined by microscope. *Staphylococcus* Gram positive bacterium (coccus) occurs in pairs, or grapelike clusters (Cheesbrough, 2006).

Catalase test

This was used to differentiate *Staphylococci* from *Streptococci* which are both gram positive. According to (Cheesbrough, 2006).

Coagulase test

Bound coagulase (clumping factor): Coagulase production was determined by slide test (Cheesbrough, 2006). Free coagulase Test**:** Coagulase production was determined by tube test (Macfaddin, 2000).

Deoxy ribonuclease activity (DNase test)

DNase test according (MacFaddin,1985).

Phosphatase Test

According to (Quinn *et al.,* 2011).

Gelatin hydrolysis test

Nutrient gelatin tube method Accoraing to **(**Anacarso *et al.,* 2013)

Capsule stain

Various types of methods are available for the demonstrate of the presence of capsule here used Anthony's staining method (Hughes and Smith, 2007).

VITEK2 system diagnosis

The Vitek2 system with biochemical tests in table 1 to increase the confirmation of the diagnosis of *Staphylococcus aureus*.

Phenotypic detection of virulence factors

Detection of hemolysin activity

Sheep blood agar (SBA) was streaked with a pure culture of bacterial isolate to be tested and incubated at

Table 1: Result of Vietek2 System of *Staphylococcus aureus.*

No.	Test Name	Abbreviation	Results
1	D-AMYCDALIN	AMY	-
2	Ala-Phe-Pro ARYLAMIDASE	APPA	-
3	Leucine ARYLAMIDASE	LeuA	(-)
4	Alanine ARYLAMIDASE	AlaA	-
5	D-RIBOSE	dRIB	+
6	NOVOBIOCIN RESISTANCE	NOVO	(+)
7	D-RAFFINOSE	dRAF	-
8	OPTOCHIN RESISTANCE	OPTO	+
9	PHOSPHATIDYLINOSITOL	PIPLC	-
10	CYCLODEXTRIN	CDEX	-
11	L-porline ARYLAMIDASE	ProA	-
12	Tyrosine ARYLAMIDASE	Tyra	-
13	L-LACTATE alkalinization	ILATk	+
14	GROWTH IN 6.5% NaCl	NC6.5	+
15	O/129 RESISTANCE	O129R	+
16	D-XYLOSE	dXYL	-
17	L-Asparatate ARYLAMIDASE	AspA	-
18	BETA- GLUCORONIDASE	BGURr	-
19	D-SORBITOL	dSOR	-
20	LACTOSE	LAC	-
21	D-MANNITOL	dMAN	+
22	SALICIN	SAL	-
23	ARGININE DIHYDROLASE1	ADH1	+
24	BETA-GALACTOPYRAANOSIDASE	BGAR	-
25	ALPH-GALACTOSIDASE	AGAL	-
26	UREASE	URE	+
27	N-ACETYL-D-GLUCOSAMINE	NAG	+
28	D-MANNOSE	dMNE	+
29	SACHHAROSE/SUCROSE	SAC	+
30	BETA-GALACTOSIDASE	BGAL	(-)
31	ALPH-MANNOSIDASE	AMAN	-
32	L-PYrrolidonyl-ARYLAMIDASE	PyrA	+
33	POLYMIXIN RESISTANCE	POLYB	+
34	D-MALTOSE	dMAL	+
35	METHYL-B-GLUCOPYRANOSIDE	MBdG	+
36	D-TREHALOSE	dTRE	+
37	ALPH-GLUCOSIDASE	AGLU	-
38	PHOSPHATASE	PHOS	+
39	BETA- GLUCORONIDASE	BGUR	-
40	D-GALACTOSE	dGAL	+
41	BACITIRACIN RESISTANCE	BACI	+
42	PULLUIN	PUL	-
43	ARGININE DIHYDROLASE 2	ADH2s	+

37Cº for 24hrs. α-Hemolysis generates a wide zone of complete hemolysis with blurred edges on sheep blood agar (SBA). β-Hemolysis produces a wide zone of incomplete hemolysis with sharp edges. δ-Hemolysis is a narrow zone of incomplete hemolysis with blurred edges γ-Hemolysis cannot be seen on SBA, as it is inhibited by agar. (Wang *et al.,* 2020).

Detection of amylase activity

Nutrient Agar plates complemented with 2% starch were used. Colonies of the isolates were transferred to the plating of Starch Agar plates and incubated at 37Cº for 48 hour. Plates was then flooded with Gram's iodine reagent (Hemraj and Avneet, 2013).

Detection of lipase activity

Isolates were tested for lipolytic activity by inoculation of 30 ml/L lipoidal emulsion in a nutrient agar plates (1ml tween 80 and 100 ml olive oil per 500 ml water (Atlas, 2010). The plates were incubated at 37Cº for 48 hrs. and then flooded for 15 min with saturated solution of copper sulphate solution; greenish blue color around growth indicates positive lipase enzyme activity.

Detection of protein A (Spa) activity

Observation of Spa activity on dog-serum-containing agar plate around the colonies. To detect producibility of Spa on heart infusion agar containing 1% natural dog serum (Hwang *et al.,* 1989). After overnight incubation on the plates at 37Cº, the Spa producibility of these strains was investigated in to Protein A halos around grown masses of different *S. aureus* isolate.

Antimicrobial susceptibility testing

The Antimicrobial susceptibility testing by using disc diffusion method were determined by (Kirby-Bauer,1966) method on Muller-Hinton agar and the results were compared according to the Clinical laboratory standards Institute (CLSI, 2017). Penicillin P(10U), Methicillin ME (10µg), Amoxicillin/Clavulanic acid AMC (30 µg), CefiximeCFM (5mcg), Aztronam ATM(30mcg), VancomycinVA(30mcg), Oxacillin OX(5µg), Netilimicin NET (30mcg), Chloramphenicol C (30mcg), Tetracycline TE (30mcg), Clindamycin DA (10mcg). The results of sensitivity against antibiotic discs (zone of inhibition) were categorized as sensitive (S), intermediate (I), resistant (R).

Results and Discussion

Isolation and Identification of *S.auerus*

Out of 80 swabs 18(22.5%) were identificated as *Staphylococcus aureus* according to Grams stain,culturing on(staph110,MSA) agar,biochemical test and then comfirm by vitek2 system.

Biochemical Characteristics

All 18(22.5%) *Staphylococcal* isolates were showed positive results for Catalase, Coagulase, phosphatase, gelatinase test and DNase test.

The results of isolation of *Staphylococcus aureus* in this study was agree with other studies (Hatem*et al.*, 2013;Rizk*et al.*, 2019:Al-Harbi,2011)who isolated (20.8%)(15.75%)(12.37%) respectively.

Detection of virulence factors

Lipase activity

A variation of bacterial lipases is capable to hydrolyze triglycerols for acquisition of nutrients. It was postulated that lipase improves adhesion to host surface. Undoubtedly, lipase plays a key role in the severity of infection (El-baz *et al*., 2016). Lipases hydrolyze triglycerides and diglycerides that release free fatty acids as substrates for FAME (The exoprotein Fatty Acid-Modifying Enzyme) is produced by *S. aureus* and *S. epidermidis* and esterifies lipids with cholesterol or primary alcohols to reduce their cellular toxicity), FAME activity is inhibited by triglycerides and diglycerides, which could explain why most skin isolated staphylococci have lipase activity (Coates *et al*., 2014). Positive18(100%) results for all sheep isolates in this study agreed with a number of studies which confirmed that lipase might contribute to virulence by enabling the bacteria to persist in fatty secretions of mammalian skin (Rosenstein and Götz, 2000; El-baz *etal*., 2016) and agree with study

Fig. 1: Positive result for lipase test.

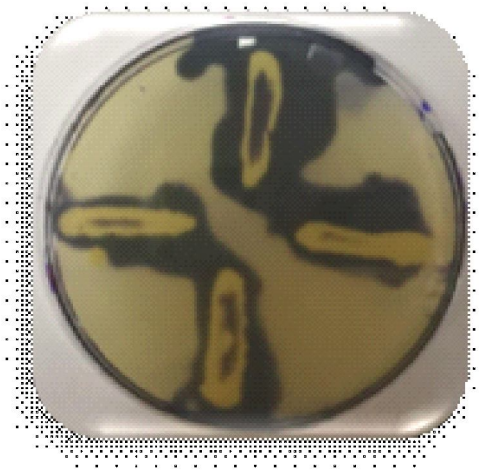

Fig. 2: Positive result for amylase test.

(Hasan *et al.*, 2014) who reported (96.1%.).

Amylase activity

In this study only three isolates 3(13.33%) showed positive results for the amylase activity detected and 15(66.66%) negative for amylase test. As an explanation of these results, we believe that the amylase enzyme is not important in the severity of skin infections in sheep. These results nearby with (Hasan *et al.*, 2014)who reported negative results for amylase test.

Protein A (Spa) activity

When antibiotic susceptibility test the results presented all isolates were 18(100%) *Staphylococcus aureu*s resistant to Methicillin and resistant to Oxacillin 11(48.88%) in addition all isolates 18(22.5%) possess capsular when Anthony's staining method was used to demonstrate presence. all of these reasons could not detected Spa activity on heart infusion agar containing 1% normal dog serum according to many studies explain why not detected Spa activity, Here review to some studies to confirm:-

Some methicillin-resistant strains of *Staphylococcus aureus* (MRSA) have shown to express undetectable levels of Protein A (Ruane *et al.*, 1986; Roberts *et al.*, 1987; Wanger *et al.*, 1992) .However, it has been shown that all these strain have capsular polysaccharide (Fournier, *et al.*,1989) The capsule is capable of masking Protein A and thus preventing agglutination.

More than 90% of the *S. aureus* clinical strains have been revealed to own capsular polysaccharides (Thakker *et al.*, 1998). A predominance of capsular serotype 5 was also identified among oxacillin-resistant *S. aureus* isolates (Fournier *et al.*, 1987).

Protein A does not produce an anti-phagocytic effect toward anti-capsule specific antibodies, in strains

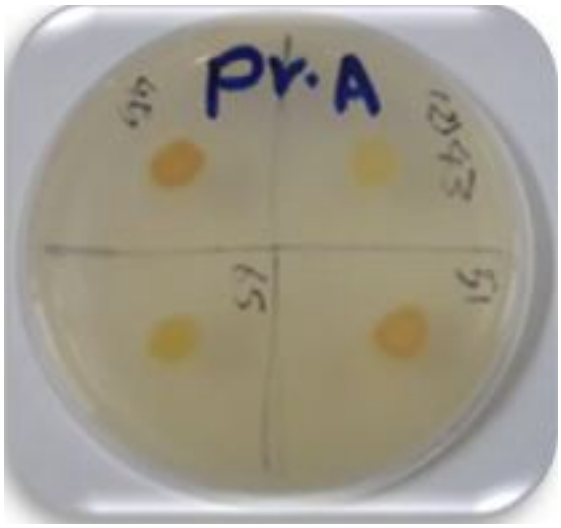

Fig. 3: Negative result for protein A (Spa) test.

expressing both capsular polysaccharide and Spa, suggesting that capsule may interfere with Spa functions.(Nanra *et al.,* 2013) In addition, multiple examples of adaptive responses have been reported where Spa and capsule expression are inversely modified for example, lower Spa expression and higher capsule production are common phenotypic characteristics in Vancomycin Intermediate S. aureus (VISA) strains (Kuroda *et al.,* 2000; McAleese *et al.,* 2006; Howden *et al.,* 2008; Gardete *et al.,* 2012; Jansen *et al.,* 2013; McGuinness *et al.,* 2017).

Hemolysin activity

Hemolysis activity results showed that alpha hemolysis was detected 4(17.77%) isolates while beta-hemolysis 8(35.55%) isolates and non-hemolytic(g-Hemolysis) 6 (26.66%) isolates. High results for beta-hemolysis detected in skin lesion isolates in this study underscore the fact that this type of skin infection virulence confirms that beta-hemolysis plays an important role in skin colonization by damaging keratinocytes (Katayama *et al.,* 2013).

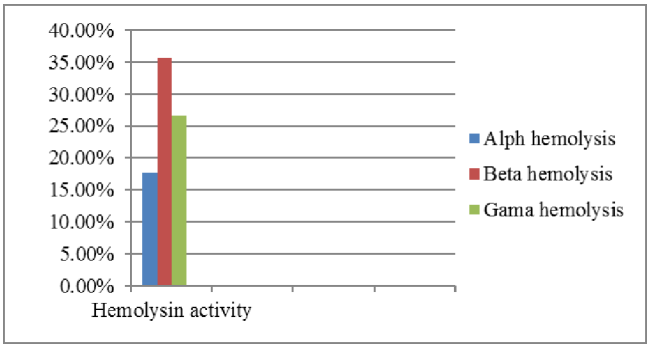

Fig. 4: Hemolysis activity.

Antimicrobial susceptibility for *S. aureus* isolates

Antibiotics widely used in farm animals to treat various diseases, and for prophylaxis and growth purposes (Waters *et al.,* 2011), Misuse of antibiotics has led to a wild world problem due to resistant development among bacterial populations (Shryock and Richwine, 2010). Determination the levels of antibiotics resistant in *S. aureus* strains could serve to character this pathogen and could be used to limit the dangers associated the deteriorating in treatment of diseased conditions (Acar and Rostel, 2001). Facing with the risk of animal origin released from MRSA, the studies showed the widespread occurrence of MARS in animal species and transmissions in both directions were shown in human with several studies. MRSA was therefore of great concern in both veterinary and human medicine as it serious disease in both sets of populations (Saleha and Zunita, 2010). Carrier animals may in themselves serve as a reservoir for sickness, and may transmit MRSA to other animals or people (Ali *et al.*, 2017). Methicillin–resistant *Staphylococcus aureus* (MRSA) has become significant acquired pathogen in hospital and likewise livestock (LA-MRSA) in recent years. MRSA associated with (LA-MRSA) have been described worldwide in several species (Persoons *et al.,* 2009).

Antimicrobial susceptibility in this study was observed *S. aureus* isolates from skin infection in sheep that all isolates appearance resistance 18(100%) to Penicillin, Methicillin, Amoxycillin/Clavulanic acid, Aztronam and Cefixime.

Resistance to penicillins (PRSA) in this study was found commonly among *S. aureus* isolates tested and is closely related to many studies (Katayama *et al.,* 2000; Lee, 2003; Merlino *et al.,* 2002; Khudaier *et al.,* 2013). Also in recent years, *Staphylococcus aureus* has become one of the most dangerous pathogens due to its increased resistance to b-lactam antibiotics and vancomycin (Kuehnert *et al.,* 2006; Friães *et al.,* 2015).

Table 2: Detection of virulence factors (Amylase, Lipase and Spa activity) in sheep isolates.

Total & type isolates	Amylase test		Lipase test		Spa activity		Hemolysins activity	
	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
Sheep18(22.5%)	3 (13.33%)	15 (66.66%)	18 (100%)	0.00	0.00	0.00	18 (100%)	0.00
Chi-Square (X^2)	11.85 **		15.00 **		0.00 NS		15.00 **	

** ($P \leq 0.01$).

Table 3: Results of Antimicrobial susceptibilities test for S. aureus isolates.

Antibiotics	Conc. Microgram/disk	No. of resistance isolates	Intermediate	No. of sensitive isolates	Intermediate resistance	Mean ± SE
Penicillin	P 10U	18(100%)				23.44 ± 0.57
Methicillin	ME 10µg	18(100%)				0.00 ± 0.00
Oxacillin	OX 5 µg	11(48.88%)		7(31.11%)		19.00 ± 0.00
Amoxicillin/Clavulanic	18(100%)				15.78 ± 0.78	
acid AMC 30 µg						
Vancomycin	VA 30mcg	11(48.88%)			7(31.11%)	15.28 ± 0.61
Tetracycline	TE 30mcg	3(13.33%)		15(66.66%)		25.96 ± 1.17
Netilimicin	NET 30mcg			18(100%)		20.31 ± 0.47
Chloramphenicol	C 30mcg	2(8.88%)		16(71.11%)		23.68 ± 1.22
Clindamycin	DA 10mcg	1(4.44%)	3(13.33%)	14(62.66%)		23.37 ± 0.96
Cefixime	CFM 5mcg	18(100%)				0.00 ± 0.00
Aztronam	ATM 30mcg	18(100%)				0.00 ± 0.00
LSD value	.....	.....		.....		6.022 **
		** ($P \leq 0.01$).				

S.aureus with reduced susceptibility to vancomycin is not limited to humans. (McGuinness *et al*., 2017). Recently, VRSA and/or VISA have been secluded from pigs, goats, and cattle, the livestock isolates VRSA and VISA were reported to be resistant to several antibiotics, including â-lactams,It also suggested that isolates originated either from humans or that resistance developed as a result of the continuous animals exposure to other of antibiotics such as the use of antibiotics as a feed supplement. (Bhattacharyya *et al*., 2016; Moreno *et al*., 2016).

The results in this study revealed that resistance to both Vancomycin (VRSA) and oxacillin (ORSA) showed 11(48.88%) while 7(31.11%) consider as Intermediate resistance to Vancomycin (McGuinness, *et al.*, 2017). This resistance diversity due to MRSA strains are also multi resistant to other antibiotics and leaves a limited choice for their control (Pesavento *et al.,* 2007).

There are several reasons for the result variations in resistance to tetracyclines, erythromycins, aminoglycosides, and other drugs because the frequent plasmids are mediated in *staphylococci*, and these plasmids can be moved by conjugation or transduction from one strain to another. Hence, plasmid-borne resistance spread rapidly amongst many different strains and genera (Haaber *et al*., 2017). Although there are several studies describing the resistance to tetracyclines, erythromycins, aminoglycosides, carrying on the mecA of chromosome MRSA strains (Cauwelier *et al.*, 2004; Skov *et al.,* 2003; Al-Mohana *et al*., 2012). Antibiotic resistance between isolates of *S. aureus* in this research was comparable with reports from other parts of the world, which also recorded multiple drug resistance. (Merlino *et al.*, 2002;Appiah *et al*., 2020).

On other hand most of isolates were sensitive to (Netilimicin100%, Chloramphenicol 71.11%, Tetracycline 66.66%, Clindamycin 62.66%, oxacillin 31.11%) this results agree with(Hasan *et al.*, 2016). Sensitively18(100 %) to Netilimicin making it the drugs of choice for the treatment of skin disease caused by *S. aureus* in sheep.

Also recorded the same results in other study (Ali *et al*., 2017) who show resistant to methicillin(MRSA), Pencillin (PRSA), Amoxycillin/ Clavulanic acid, Oxacillin.

Finally the results of Antimicrobial susceptibility patterns in the present study nearly agree with results of study that show Prevalence of vancomycin resistant *Staphylococcus aureus* (VRSA) in methicillin resistant *S. aureus* (MRSA) strains (Hasan *et al.,* 2016).

In this study high prevalence of MRSA and VRSA in sheep has been detected raising concerns about the role of these animals as MRSA reservoirs, which are considered to be of great concern for both veterinary and human health. The highest percentage of MRSA reported from these outcomes is due to the fact that *S. aureus* is frequent commensal bacteria on the skin and mucous surfaces.

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