PHENOTYPIC STUDY ON THE CAPACITY OF BIOFILM PRODUCTION IN STAPHYLOCOCCUS AUREUS ISOLATED FROM BOVINE SUBCLINICAL MASTITIS AND THEIR IMPACT ON RESISTANCE TO ANTIMICROBIALS

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ABSTRACT

The study was intended for identification and characterization of *Staphylococcus aureus* isolated from bovine subclinical mastitis cases. A total of 143 milk samples were collected from apparently normal cows from Basrah province. California mastitis test was used to detect 81 (56.6%) samples as subclinical mastitis. However, by using bacteriological and biochemical tests 36(44.44%) isolates were confirmed as *S. aureus*. Antimicrobials susceptibility assays of isolates revealed that, all of them were completely susceptible to chloramphenicol, gentamycin and vancomycin. Oxacillin and cefoxitin susceptibility illustrated that 22(61.1%) isolates were resistant to methicillin (MRSA) and 14 (38.9%) isolates were methicillin susceptible (MSSA). Phenotypic production of slime and biofilm were evaluated by using Congo red agar and microtiter plate techniques, 31 (86.1%) isolates were slime producer and 29 (80.6%) were biofilm producers from all tested isolates. The production of slime and biofilm of MRSA isolates were 95.5% and 90.9%, whereas, for MSSA were 71.4% and 64.3% respectively. The differences in slime and biofilm production among MRSA and MSSA isolates were statistically significant.

All MRSA isolates were resistant to oxacillin, penicillin, cefoxitin, ampicillin and cefotaxime, and these isolates showed low resistance to erythromycin and lincomycin each (9.1%) and doxycycline (18.2%). However, these isolates were susceptible to vancomycin, gentamycin, nitrofurantoin and chloramphenicol. All MSSA isolates

exhibited resistance to ampicillin and 85.7% of isolates to cefotaxime, however, all isolates were sensitive to azithromycin, cefoxitin, chloramphenicol, gentamycin, oxacillin and vancomycin. The differences in antimicrobial susceptibility between MRSA and MSSA isolates were highly statistically significant.

Results of this study indicate that *Staphylococcus aureus* is the most important agent of bovine subclinical mastitis, isolates which have resistance to methicillin and produce biofilm have propensity to be multiple antibiotic resistant.

INTRODUCTION

Mastitis is the inflammation of the mammary gland mainly due to a bacterial infection and characterized by a variety of local and systemic symptoms. As well, it is a serious concern to both meat and milk producers since the infection can lead to considerable economic losses due to reduction in milk yield, decreased quality of milk and treatment costs (1).

Staphylococcus aureus is the most common etiologic agent of bovine contagious mastitis (2). (3) reported that, *Staphylococcus aureus* is probably the most lethal agent because it causes chronic and deep infection in the mammary glands that is extremely difficult to be cured. The virulence of *S. aureus* is generally considered to be multifactorial and due to the combined action of several virulence determinants (4). The ability of *S. aureus* to form biofilm in vivo is considered to be a major virulence factor influencing its pathogenesis in mastitis (5).

Biofilms are the population of bacteria growing on the biotic and abiotic surfaces and embed themselves in a self-produced extracellular matrix of exopolysaccharide (EPS), proteins and some micro molecules such as DNA (6). On the other hand, it has been estimated that 65% of microbial infections are associated with biofilms (7 and 8). (7) noted that, one possible reason for the persistence of the pathogen in the udder is the formation of biofilms. Biofilm production can be a marker of virulence and, can be detected by phenotypic assays (9).

Susceptibility to antibiotics in bacteria that are protected by biofilm is reduced because drugs are prevented from reaching the bacteria surrounded by biofilm. (6) noted that, bacteria in biofilm are protected from antibiotics due to presence of large amount of exopolysaccharides, expression of biofilm specific resistance genes and having the suitable condition for growing slowly. Furthermore biofilm keeps bacteria out of reach of host immune defense mechanism and often resulting in persistent and difficult-to-treat infections (10).

Methicillin resistance in *S. aureus* (MRSA) most commonly results from the production of the novel penicillin-binding protein (PBP)-2a, which has a decreased binding affinity for β-lactam antibiotics. PBP-2a requires two to 10 times higher penicillin concentrations for inactivation than PBP-2, and 20 times higher than PBP-1 (11). MRSA infections are life-threatening due to emergence of multidrug resistance strains and also occurrence of isolates that are able to form strong biofilms (12).

This study aimed to determine the isolation rate of *S. aureus* from bovine subclinical mastitis cases, potential of these isolates to produce biofilm and antimicrobial susceptibility of isolates.

MATERIALS AND METHODS

-Samples collection and bacterial isolates

A total of 143 milk samples were collected from apparently normal cows from different parts of Basrah province. The subclinical mastitis was confirmed with California mastitis test, which was done according to (13).

Milk samples were inoculated on mannitol salt agar (Himedia, India) and on blood agar (Himedia, India) the plates were incubated for 24hrs at 37°C. Suspected colonies on mannitol salt agar were identified by Gram's staining, catalase test, oxidase test by using oxidase discs from (Himedia , India) according to (14) and hemolysis on blood agar. The isolates were confirmed by the tube coagulase test with rabbit plasma, DNase production and voges-proskauer test (15).

- Determination of the antibiotic susceptibility of isolates

All the isolates that were identified as *S. aureus* were tested for antibiotic susceptibility according to (16), fifteen antibiotics were chosen for the study. The antibiotic tested were from (Bioanalyse/ Turkey), including; Ampicillin (25µg) ,   Azithromycin (15 µg), Cefotaxime (10 µg) Chloramphenicol (30 µg), Ciprofluxacin (5 µg), Clindamycin (2 µg), Doxycyclin (10 µg), Erythromycin (15 µg), Gentamycin (10 µg), Lincomycin (10 µg), Nitrofurantoin (100 µg), Oxacillin (1 µg), Penicillin (10 µg), Vancomycin (30 µg). Cefoxitin (30 µg) was used as a surrogate for study of Oxacillin resistance.

-Analysis of slime Production by the Congo Red Agar

The production of slime was studied phenotypically by culture of the isolates on Congo Red Agar (CRA) plates as previously described by (17). The medium was comprised brain heart infusion broth [Himedia, India] (37 g/L), sucrose (50 g/L), agar (10 g/L) and Congo Red stain [Sigma, USA] (0.8 g/L). Plates were inoculated and incubated in aerobic environment for 24 h at 37 °C. Under such condition, biofilm producers form black colonies on CRA, whereas non-producers form red colonies. (17).

-Biofilm assay

Biofilm formation was assayed phenotypically by the ability of cells to adhere to the wells of 96-well tissue culture plates made of polystyrene (Tarson, India). The assay was performed as described by (5), *S. aureus* strains were individually grown overnight in tryptone soy broth [TSB] (Himedia, India) at 37 ºC and diluted 1:40 in TSB including 0.25% glucose. 200 µl of the cell suspension was transferred to each well of sterile flat bottom polystyrene microtiter plates, incubated for 24 h at 37 ºC. The wells were washed three times with 200 µl of sterile phosphate buffered saline (PBS, pH 7.4), dried at room temperature and finally stained with 1% crystal violet for 15 min. After rinsing three times with distilled water and subsequent drying, the absorbance of each well was measured at 570 nm by using ELISA reader. The cut-off for the microtiter-plate test was defined as three standard deviations above the mean absorbance of the negative control; the reading was done according to (18).

-Statistical analysis

The statistical calculation was carried out with statistical package Minitab V.14.

RESULTS

Results of this study show that, out of 143 tested milk samples, 81 (56.6%) samples were positive to California mastitis test. The isolation rate of *S. aureus* from subclinical mastitic samples was 36 (44.44%). Resistance of *S. aureus* isolates to methicillin (MRSA) represented 22 (61.1%), while the susceptible isolates (MSSA) constituted 14 (38.9%), Table (1).

Table (1): Antibiogram of MRSA and MSSA against fifteen antimicrobials.

Antimicrobials	MRSA (22)			MSSA (14)			Total (36)		
	Resistant (%)	Intermediate (%)	Susceptible (%)	Resistant (%)	Intermediate (%)	Susceptible (%)	Resistant (%)	Intermediate (%)	Susceptible (%)
Ampicillin	100%	0%	0%	100%	0%	0%	100%	0%	0%
Azithromycin	0%	18.2%	81.8%	0%	0%	100%	0%	11.1%	88.9%
Cefotaxime	100%	0%	0%	85.7%	14.3%	0%	94.5%	5.5%	0%
Cefoxitin	100%	0%	0%	0%	0%	100%	61.1%	0%	38.9%
Chloramphenicol	0%	0%	100%	0%	0%	100%	0%	0%	100%
Ciprofloxacin	0%	27.3%	72.7%	0%	28.6%	71.4%	0%	27.8%	72.2%
Clindamycin	0%	18.2%	81.8%	14.3%	14.3%	71.4%	5.5%	16.7%	77.8%
Doxycycline	18.2%	18.2%	63.6%	0%	14.3%	85.7%	11.1%	16.7%	72.2%
Erythromycin	9.1%	18.2%	72.7%	0%	14.3%	85.7%	5.5%	16.7%	77.8%
Gentamycin	0%	0%	100%	0%	0%	100%	0%	0%	100%
Lincomycin	9.1%	9.1%	81.8%	0%	28.6%	71.4%	5.5%	16.7%	77.8%
Nitrofurantoin	0%	0%	100%	14.3%	0%	85.7%	5.5%	0%	94.5%
Oxacillin	100%	0%	0%	0%	0%	100%	61.1%	0%	38.9%
Penicillin	100%	0%	0%	14.3%	14.3%	71.4%	66.7%	5.5%	27.8%
Vancomycin	0%	0%	100%	0%	0%	100%	0%	0%	100%

X^2 = 479.974; DF = 65; P-Value = 0.000

Table (1) and figure (1) illustrate the susceptibly of *S. aureus* isolates against fifteen antimicrobials.
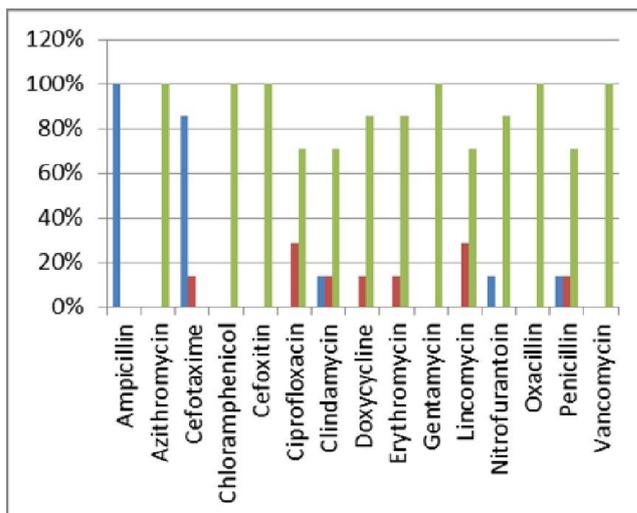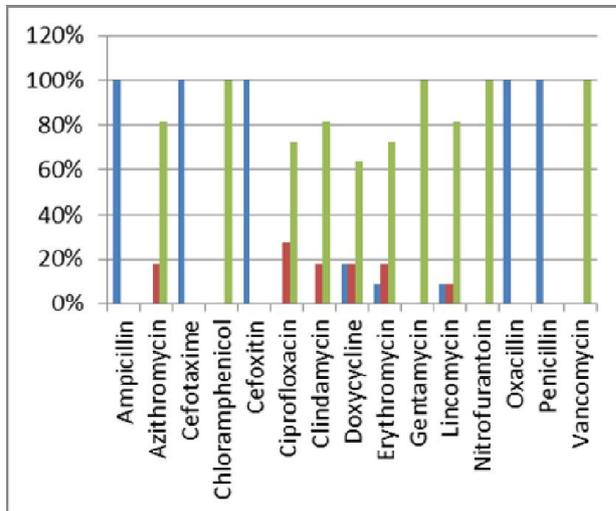
A   B

Figure (1): Antibiogram of MRSA and MSSA against fifteen antimicrobials.
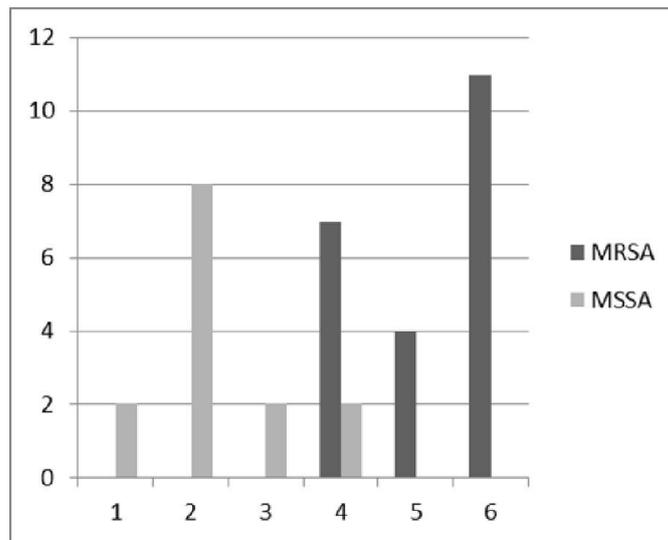
   A- Antibiogram of MRSA against fifteen antimicrobials.

   B- Antibiogram of MSSA against fifteen antimicrobials.

Note: blue represent the resistant, red represent the intermediate and green represent the susceptible.

Table (2) and figure (2) demonstrate the number of antimicrobials which did not have pronounced effects on the *S. aureus* isolates.

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Table (2): Distribution of multiple antibiotic resistance among MRSA and MSSA.

Number of antimicrobials which became ineffective	MRSA		MSSA	
	No. of isolat es	%	No. of isolat es	%
1	0	0	2	14.3
2	0	0	8	57.1
3	0	0	2	14.3
4	7	31.8	2	14.3
5	4	18.2	0	0
6	11	50	0	0
Total	22	100	14	100

Figure (2): Distribution of multiple antibiotic resistance among MRSA and MSSA.

X axis represents number of antimicrobials which became ineffective and Y axis represents number of isolates which are resistant.

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Table (3) shows number and rates of *S. aureus* isolates which produce biofilm and slime. Figure (3) explicates the appearance of slime producer and non-producer colonies of *S. aureus* on CRA.

Table (3): Biofilm production by MRSA and MSSA strains

	MRSA total number (22)		MSSA total number (14)		Total number (36)	
	No.	%	No.	%	No.	%
Biofilm producer	20	90.9	9	64.3	29	80.6
Non-biofilm producer	2	9.1	5	35.7	7	19.4
Slime producer	21	95.5	10	71.4	31	86.1
Non-slime producer	1	4.5	4	28.6	5	13.9

$X^2 = 8.001$; DF = 3; P-Value = 0.046

A   B

Figure (3): Appearance of colonies on Congo Red Agar, A: slime producer colonies, B: non-slime producer colonies.

DISCUSSION

Mastitis is one of the major problematic diseases of dairy animals throughout the world which is associated with different etiological agents; however, *Staphylococcus aureus* is one of the major causes which are responsible for this dairy scourge (19). The pathogenesis of *S. aureus* is attributed to the combined effect of extracellular factors and toxins, together with the invasive properties of the strain such as adherence, biofilm formation, and resistance to phagocytosis (20).

Isolation rate of *S. aureus* from total number of samples was 36/143 (25.17%), almost similar ratios were reported by (18) and (21) who noted that, among 320 raw milk samples, *S. aureus* were detected in 88 (27.5%) samples, and in the 350 dairy products, 87 samples (24.8%) were positive for *S. aureus*. Higher ratios were detected by (22) in Turkey who found the rate of *S. aureus* in raw milk and dairy product was 56% and (23) recorded 75% in Bangladesh; these results are significantly higher than the present study.

This study showed that *S. aureus* was the agent of 44.44% of subclinical mastitis cases. The isolation rates of *S. aureus* in this study lower than that reported by others. (24), in India reported the isolation rate (54.87%) of *S. aureus* from mastitic samples. Moreover, (23) reported that, 70% of mastitic milk contained *S. aureus*.

Reaction with the components of exopolysaccharides may lessen the penetration of many antibiotics through the biofilms resulting in reduced exposure of biofilm bacteria to the antibiotics and consequently an apparent decrease in antibiotic efficacy (25). The transport limitations may be an important factor in the antimicrobial resistance of biofilm bacteria (26).

Regarding resistance to methicillin, result of present study showed that 61.1% of *S. aureus* isolates were MRSA, this result higher than 43.2% reported by (27). However, (28) found that (82%) of *S. aureus* isolates which producing biofilm were resistant to methicillin.

All MSSA isolates were resistant to ampicillin and 85.7% of which were resistant to cefotaxime, however, all isolates were sensitive to azithromycin, cefoxitin,

chloramphenicol, gentamycin, oxacillin and vancomycin. Similar results were recorded by (29) and (30).

All isolates of MRSA were resistant to at least four antibiotics, and half the number of strains was resistant to at least seven of the fifteen antimicrobials, in addition to oxacillin, all MRSA were resistant to penicillin, cefoxitin, ampicillin and cefotaxime. The differences in antimicrobial susceptibility between MRSA and MSSA were statistically significant. Similar data were recorded by (31). Also, (32), noted that in addition to resistance of MRSA to almost all types of beta-lactam antibiotics, also these strains show resistance to a wide range of other antimicrobial agents used to treat or prevent mastitis.

Low resistance showed by MRSA isolates to erythromycin and lincomycin each (9.1%) and doxycycline (18.2%). All MRSA were highly susceptible to vancomycin, gentamycin, nitrofurantoin and chloramphenicol. These results are in agreement with (33), who recorded that, the efficacy of erythromycin, doxycycline, gentamicin and ciprofloxacin are moderately affected by the exopolysaccharide matrix of Staphylococci. On the other hand, many earlier studies have also reported that *S. aureus* slime remarkably decreases the activity of glycopeptides including vancomycin (34 and 35).

The ability of *Staphylococcus aureus* to produce biofilm is an important factor affecting the long-term persistence of the bacteria in the mammary gland and can result in chronic mastitis and decreased efficacy of antibiotic therapy (36). The growth on Congo red agar and the microtitre plate test are methods that could be used to determine whether an isolate has the potential for biofilm production. (36). Moreover, (37) suggested that, the CRA and microtitre plate tests showed results that were significantly correlated with the molecular analysis. Results of this study illustrated that 80.6% of total *S. aureus* isolates were phenotypically biofilm producer and (86.1%) slime producers. These included MRSA and MSSA with (90.9%) and (64.3%) for biofilm production, however, the slime production was (95.5%) and (71.4%) respectively. These results are similar to that reported by (5), (38), (39), (40) and (41) who found the biofilm production among MRSA and MSSA isolates were 97.5% and 60%, respectively. However, results of other researchers are not in accordance with this study, they detected a lower rate of CRA method for biofilm formation than the quantitative methods. (36), found 85% of the

isolates tested by using Congo red agar produce slime; while, 98.9% of the isolates produced biofilms by using microtitre plate test.

Methicillin resistant isolates which produce biofilm have propensity to be multiple antibiotic resistant (MAR), all MRSA isolates were resistant to at least four antibiotics in ratio (31.8%). While, MSSA isolates were resisting at maximum to four antibiotics at ratio (14.3%). Moreover, MRSA have the ability to resist five and six antibiotics at ratios (18.2%) and (50%) respectively. These results are in agreement with (31) and (32).

The *Staphylococcus aureus* is the most important agent of bovine subclinical mastitis. The differences between MRSA and MSSA in susceptibility against fifteen antimicrobials and in biofilm and slime production were statistically significant. These data confirm that the biofilm producer more resistant than the non-producer. Similar conclusion was proposed by (33) who noted that the slime producer isolates had more resistance to antibiotics as compared to those of non-slime producer. Moreover (42) suggested that, the methicillin resistant *S. aureus* (MRSA) that have the ability of biofilm formation can become resistant to the most currently use antibiotics.

دراسة مظهرية حول قدرة انتاج الغشاء الحيوي للمكورات العنقودية الذهبية المعزولة من التهاب الضرع تحت السريري البقري وتأثيرها على مقاومتها للمضادات الميكروبية

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الخلاصة

تهدف الدراسة الى تحديد وتوصيف المكورات العنقودية الذهبية المعزولة من حالات التهاب الضرع تحت السريري في الابقار. جمعت 143 عينة حليب من أبقار سليمة ظاهريا من محافظة البصرة. عند استخدام اختبار التهاب الضرع كاليفورنيا تم اكتشاف 81 (56.6٪) حالةالتهاب ضرع تحت السريري. جرى التأكد من وجود 36 (44.44٪) عزلة مكورات عنقودية ذهبية عن طريق الاختبارات الجرثومية والكيميائية الحيوية. اظهرت فحوصات الحساسية للمضادات الميكروبية ان كل العزلات كانت حساسة تماما للكلورامفينيكول، الجنتاميسين والفانكومايسين. الحساسية للأوكساسيلين والسيفوكسيتين اوضحت أن 22 (61.1٪) من العزلات كانت مقاومة للميثيسيلين (MRSA)و 14 (38.9٪) عزلة كانت حساسة للميثيسيلين (MSSA) . تم التقييم المظهري لإنتاج الوحل والغشاء

الحيوي باستخدام أجار الكونغو الأحمر و طبق التغيير الميكروي ، اظهر ان31 (86.1٪) عزلة كانت منتجة للوحل و 29 (80.6٪) عزلة منتجة للغشاء الحيوي من جميع العزلات المختبرة. كان إنتاج الوحل والغشاء الحيوي من العزلات المقاومة للميثيسيلين 95.5٪ (MRSA) و 90.9٪، في حين للحساسة للمثيسيلين MSSA كانت 71.4٪ و 64.3٪ على التوالي. كانت الفروق في إنتاج الوحل والغشاء الحيوي بين عزلات MRSA و MSSAذات دلالة إحصائية.

جميع عزلات MRSA كانت مقاومة للأوكساسيلين، البنسلين، سيفوكسيتين، الأمبيسلين والسيفوتاكسيم، وأظهرت هذه العزلات مقاومة منخفضة للإريثروميسين واللينكومايسين (9.1٪) والدوكسيسيكلين (18.2٪). و كانت هذه العزلات حساسة للفانكومايسين، الجنتاميسين، نتروفورانتوين والكلورامفينيكول. جميع عزلات MSSA أظهرت مقاومة للأمبيسلين و 85.7٪ من العزلات لسيفوتاكسيم، و كانت كل العزلات حساسة للأزيثروميسين، سيفوكسيتين، الكلورامفينيكول، جنتاميسين، أوكساسيلين وفانكومايسين. الفروق في الحساسية للمضادات الميكروبية بين عزلات MRSAو MSSAكانت إحصائيا هامة جدا.

استنتج من هذه الدراسة أن المكورات العنقودية الذهبية هي عامل مهم جدا في التهاب الضرع البقري تحت السريري ، العزلات المقاومة للميثيسيلين والتي تنتج الغشاء الحيوي يكون لديها الميل لأن تكون مقاومة للمضادات الحيوية المتعددة.

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