PHENOTYPIC STUDY OF THE EFFECTS OF ENVIRONMENTAL FACTORS ON THE BIOFILM FORMATION BY *STAPHYLOCOCCUS AUREUS* ISOLATES

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ABSTRACT

The present study was designed to estimate the influence of different stress conditions (temperature, sodium chloride and glucose) on biofilm formation of *Staphylococcus aureus*. Out of 39 *S. aureus* including 22 isolates of methicillin resistant *Staphylococcus aureus* (MRSA) and 17 isolates of methicillin sensitive *S. aureus* (MSSA) obtained from raw milk and subclinical mastitis samples, their phenotypic and genotypic resistance to methicillin were evaluated. Each *S. aureus isolates* was cultured in tryptic soy broth (TSB) with different concentrations of Glucose and Sodium chloride separately (2.5%, 5%, and 10%), incubated at 37° C using a 96-well polystyrene microtiter plate. These isolates also subjected to different degrees of incubation temperatures (25°C, 37°C and 42°C) by using TSB with1% glucose. The results revealed that the increase in the concentrations of sodium chloride or glucose enhanced the biofilm formation in both MRSA and MSSA. On the other hand, the lower incubation temperature enhanced the biofilm formation in all isolates. The results showed that the alterations in growth conditions stimulated the production of extracellular polymeric substances.

Introduction

Staphylococcus aureus is one of an important gram-positive facultative pathogen in both human and veterinary medicine causing a wide-ranging of nosocomial infections [(1)]. MRSA cause hard to treat infections because these are

resistant to most of the antibiotics such as beta lactams, macrolides, and aminoglycosides [2].

Bovine mastitis is the disease that causes most economic losses to the dairy industry worldwide and also possesses a potential health threat for customers [3]. *S. aureus* is one of the chief microorganisms isolated from intramammary infections in dairy cows, and the cases are usually subclinical and hard to treat [4]. *S. aureus* is a well-known major cause of foodborne diseases, and raw milk and dairy products are contaminated by enterotoxigenic strains of this pathogen. Some of these strains constitute a potential risk of food poisoning in human [5].

Biofilm is defined as a community of microorganisms attached to abiotic or biotic surfaces and embedded in a protective extracellular polymeric matrix [6]. Biofilm is considered as a part of the normal life cycle of *S. aureus* in the environment [7]. This structure protects the bacterial community from environmental stresses, from the host immune system, and from antibiotic attacks [8]. Several environmental factors have been reported to affect biofilm formation such as glucose, osmolality, ethanol, incubation temperature, and anaerobiosis, [9].This study aimed to investigate the impacts of many different environmental conditions on the ability of *S. aureus* to the production of biofilm.

MATERIALS AND METHODS

Samples collection

One hundred and seventy-two milk samples were collected directly and indirectly including (102) samples from cows directly and (70) samples from raw milk of local markets during the period from January to June 2018. The samples were collected from cows were screened with California mastitis test (CMT) for subclinical mastitis [10].

Isolation and identification of *S. aureus*

All indirect of raw milk and positive CMT milk samples were subjected to bacteriological analysis by inoculation on mannitol salt agar and blood agar and incubated under aerobic conditions at 37°C for overnight. Primary cultures were analyzed by colony morphology, hemolysis and Gram staining. The suspected colonies were subcultured on mannitol salt agar and tested biochemically by catalase, oxidase, DNase and coagulase [11].

Molecular confirmation of isolates

Genomic DNA extraction

Genomic DNA of suspected *S. aureus* isolates was extracted by using DNA kit (Geneaid, USA) and according to manufacturer's protocol.

Detection of *nuc* gene

The primers from (Bioneer, Korea) were specific for the *nuc* gene which encodes for a specific thermostable nuclease, table (1). The amplification protocol was applied according to [12].

Phenotypic detection of MRSA

All the isolates which identified as *S. aureus* by PCR analysis were tested for antimicrobial susceptibility of Oxacillin and Cefoxitin to detect the phenotype of MRSA [13].

Genotypic detection of MRSA

Molecular confirmation of MRSA isolates was achieved by detection of *mecA* gene, primers sequences illustrated in table (1). The thermal cycling protocol for PCR was comprised according to [14].

Table (1): The primer pair sequences for the amplification of *nuc* and *mec A* genes.

Primer		Sequence	Products bp	Refe.
Nuc	F	5′- GCT TGC TAT GAT TGT GGT AGC C 3'	423	(12)
	R	5′- TCT CTA GCA AGT CCC TTT TCC A 3'		
Mec A	F	5′- AAA ATC GAT GGT AAA GGT TGG C-3'	533	(14)
	R	5′- AGT TCT GGA GTA CCG GAT TTG C-3'		

Biofilm Formation Assay by using slandered conditions

The assay was done according to the method reported by [15]. Briefly, each strain was grown on tryptic-soy broth (TSB), the culture was diluted 1:100 in TSB supplemented with 1 % glucose, 200 µl of 1% inoculum were transferred to each well of microtiter plate (Costar, China) and triplicate wells were used for each isolate. The

negative control wells contained 200 µl of non-cultured (TSB supplemented with 1% glucose). After incubation at 37ºC for 24 h, each well was washed three times with 300µl of sterile phosphate buffered saline (PBS) pH 7.2, then fixed by methanol 150µl for 20 min. The plate was dried for overnight in an inverted position at room temperature, finally the adherent biofilm layer formed in each well was stained with 150µl of crystal violet 2% for 15 min. at room temperature. After that the microtiter plate was washed three times with PBS and dried at room temperature, then150µl of 95% ethanol was added to each well and the wells were read during 30 min. The optical density OD was measured at 570 nm by using microtiter plate reader (Biotek, USA) Optical density cut off were calculated according to [16].

Study the effect of Environmental Conditions on Biofilm Formation

Both MRSA and methicillin sensitive *S. aureus* (MSSA) isolates were applied to study the effect of the following environmental factors on the biofilm formation, the procedure was performed according to [17].

Effect of incubation temperatures

Biofilm formation was investigated at difference incubation temperatures at 25˚C, 37˚ C and 42˚ C by using the same procedure mentioned by [15].

Effect of Glucose concentrations

The biofilm formation ability of S. *aureus* was carried out as mentioned above in biofilm formation assay except the concentrations of glucose by using 200µl of TSB supplemented with 2.5%, 5% and 10% glucose**.**

Effect of NaCl concentrations

The biofilm formation ability of S. *aureus* was carried out as mentioned by [15] using different concentration of NaCl by using 200µl of TSB supplemented with 2.5%, 5% and 10% NaCl.

Statistical Analysis

Results from the analytical determinations were statistically treated with the software package IBM SPSS 22.

RESULTS

Detection of subclinical mastitis

The results of the California mastitis test revealed that 42 (41.17%) out of 102 direct milk samples showed positive CMT including weak-positive were16 (15.68%), trace positive 12 (11.76%), distinct-positive 9(8.82%) and strongly positive 5(4.90%)

were observed(Table 2).The difference among these results was considered to be statistically significant ($p< 0.05$).

Table (2): Percentage of subclinical mastitis according to CMT results

Sample	No. of Samples	Positive results (%)	Trace (%)	Weak (%)	Distinct (%)	Strong (%)
Cow milk	102	42 (41.17)	12 (11.76)	16 (15.69)	9 (8.82)	5 (4.90)

Chi-square: 8,254 degrees of freedom: 3, p-value: 0,04104385

Identification of *S. aureus* by conventional microbiological and PCR techniques

The isolation rate of suspected *S. aureus* was 65(58.03%) by using conventional microbiological techniques, Table (3).

The suspected isolates with positive identified conventional microbiological technique results were subjected to PCR to detect *nuc* gene, figure (1). Out of the 65 samples investigated, 39 (60 %) were characterized as *S. aureus*. The results showed that, the high percentage of *S. aureus* isolates were from subclinical mastitis (64.28%), followed by market samples (56.75%), however, the difference was not considered to be statistically significant ($P>0.05$), Table (3).

Table (3) Identification of *S. aureus* identified by using conventional microbiological techniques and molecular detection of *nuc* gene

No.	Samples	Total No. of tested sample	No. of *S. aureus isolates*	
			By Con. micro. tech No. %	by *nuc* gene %
1.	Subclinical mastitis	42	28 (66.67%)	18/28 (64.29%)
2.	Raw milk from local markets	70	37 (52.86%)	21/37 (56.76%)
3.	Total	١١٢	65 (58.03%)	39/65 (60%)

Chi-square:0.376, degrees of freedom:1, p-value: 0.53975178) calculation of identification by using *nuc* gene.

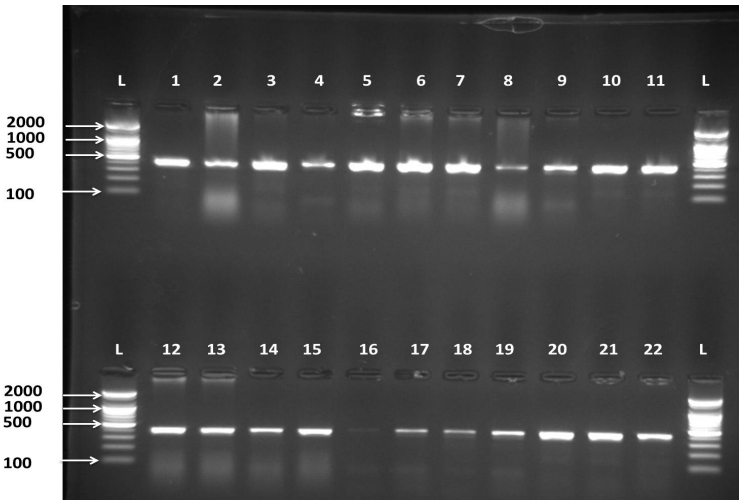

Fig (1): Amplification of *nuc* gene from bacterial isolated showed by agarose gel electrophoresis using in 1.5 % agarose gel containing ethidium bromide Lane L: 1 Kb molecular weight DNA ladder, Lane 1-22: the product size (423) bp,

Detection of MRSA by phenotypic and genotypic methods

All the isolates which identified as *S. aureus* by PCR analysis were tested for antimicrobial susceptibility by using Oxacillin and Cefoxitin. Table (4), showed that the total resistant rate against Oxacillin was (66.66%) followed by Cefoxitin (٥٦.٤١%). While the results of the PCR detection the presence of *mec A* gens was (53.84%).

The resistant to cefoxitin was observed in highest percentage of MRSA (66.66%) were observed in subclinical mastitis, followed by the percentage (47.61%) of market milk table (4). The difference between the conventional microbiology tested antibiotic and PCR results was considered to be statistically significant (P >0.05).

Table (4): Phenotypic and Genotypic detection of MRSA isolates

Sample	No. of *S. aureus isolates*	No. of resistant isolates to Oxacillin %	No. of resistant isolates to Cefoxitin %	No. of isolates carried of *Mec A* %
Subclinical mastitis	**18**	**14 77.78%**	**12 66.67%**	**11 61.11%**
Raw milk from local markets	**21**	**12 57.14%**	**10 47.62%**	**10 47.62%**
Total	**39**	**٢٦ ٦٦.٦7%**	**٢٢ ٥٦.٤١%**	**٢١ ٥٣.٨5%**

(Chi-square :28٠.79, degrees of freedom:5, p-value: 0.00003513)

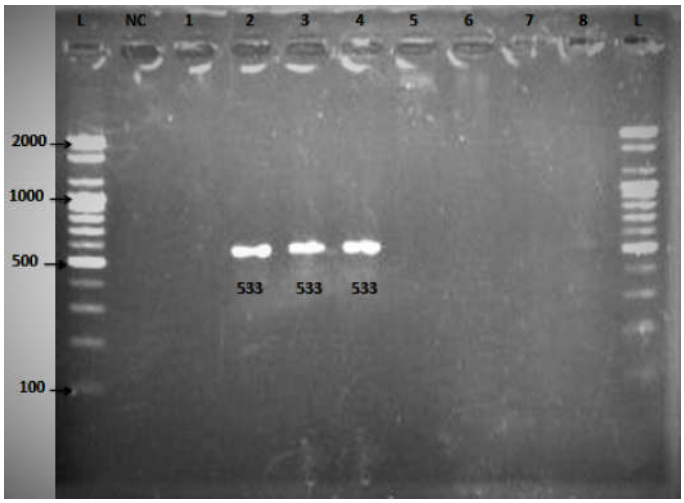

Fig (2): Amplification of of *mecA* gene from bacterial isolated showed by agarose gel electrophoresis using in 1.5 % agarose gel containing ethidium bromide Lane L: 1 Kb molecular weight DNA ladder, Lane 2-4: the product size (533) bp, NC: Negative control.

Biofilm formation by using standard conditions

The results of biofilm production by using microtiter plate assay of all *S. aureus* isolates were illustrated in table (5). Different levels of biofilm production were detected as none, weak and moderate in both MRSA and MSSA. Among the MRSA isolates,31.8% of the isolates were biofilm producers, whereas 68.2% did not produce biofilm. 57.14% of the biofilm producer isolates were found to be weak, and 42.85% were moderate producer, whereas among the MSSA isolates, 41.2% were able to produce biofilm and 58.8% unable to produce biofilm, moreover, 57.14% of the biofilm producer isolates were moderate and 42.85% were weak. There were no significant differences (P >0.05) between the MRSA and MSSA concerning the biofilm formation.

Table (5) Results of Microtiter plate assay of Biofilm formation by *S. aureus* using standard conditions

Samples	Total No. of isolates	Methicillin resistant and sensitive isolates	No. of Isolates	Biofilm production			
				No. of Non producer (%)	No. of biofilm producer (%)	No. of weak	No of moderate
Cow milk	18	MRSA	12	6(50)	6(50)	4	2
		MSSA	6	2(33.3)	4(66.7)	2	2
Cow milk of market	21	MRSA	10	9(90)	1(10)	0	1
		MSSA	11	8(72.7)	3(27.3)	1	2
Total	39	MRSA	22	15 (68.2)	7 (31.8)	4	3
		MSSA	17	10 (58.8)	7 (41.2)	3	4

The chi-square = 1.906, df = 1 p-value 0.16740826. The result is *not* significant at $p < 0.05$

Biofilm formation under different environmental conditions

Effect of Incubation temperature

Table (6) illustrates the biofilm production results of microtiter plate assay of all *S. aureus* isolates cultivated at different incubation temperatures (25 ˚C, 37 ˚C and 42 ˚C).

There were significant differences (p <0.05) between MRSA isolates biofilm production at different incubation temperatures. While there were no statistically significant differences (p <0.05) between MSSA isolates in biofilm production.

The highest production of biofilm of MRSA isolates were observed at temperature 25 ˚C (68.2 %), followed by 42 ˚C (45.5%). While the lowest biofilm production was at temperature 37 ˚C (31.8 %).

Also, approximately similar results were obtained from MSSA isolates, 52.9%, 47.1% and 41.2% at incubation temperatures 25˚C, 42˚C and 37˚C respectively (figure 3).

The difference between MRSA and MSSA was statistically significant. figure (3), the highest ability to produce biofilm was shown by MRSA isolates incubated at 25˚C and 42˚C.

Table (6) Effect of incubation temperatures on biofilm production by *S. aureus* isolates using the microtiter plate assay

Incubation Temperature	Methicillin resistant and sensitive isolates	No. of Isolates	Biofilm production				
			No. of none producer (%)	No. of biofilm production (%)	No. of weak	No of moderate	Strong
25° C	MRSA	22	٧ (31.8)	15 (68.2)	9	3	3
	MSSA	17	٨ (47.1)	9 (52.9)	7	1	1
37 ° C	MRSA	22	15 (68.2)	7 (31.8)	4	3	0
	MSSA	17	10 (58.8)	7 (41.2)	3	4	0
42 ° C	MRSA	22	١٢ (54.5)	10 (45.5)	7	2	1
	MSSA	17	9 (52.9)	8 (47.1)	5	3	0

Chi-square 27.064, df = 2 p-value ::0.00000133 for MRSA at different incubation temperatures.

Chi-square : 2.747 df = 2, p-value : 0.25321914 for MSSA different incubation temperatures.

Chi-square : 29.959 df 5, p- value 0.00001503for total of MRSA and MSSA.

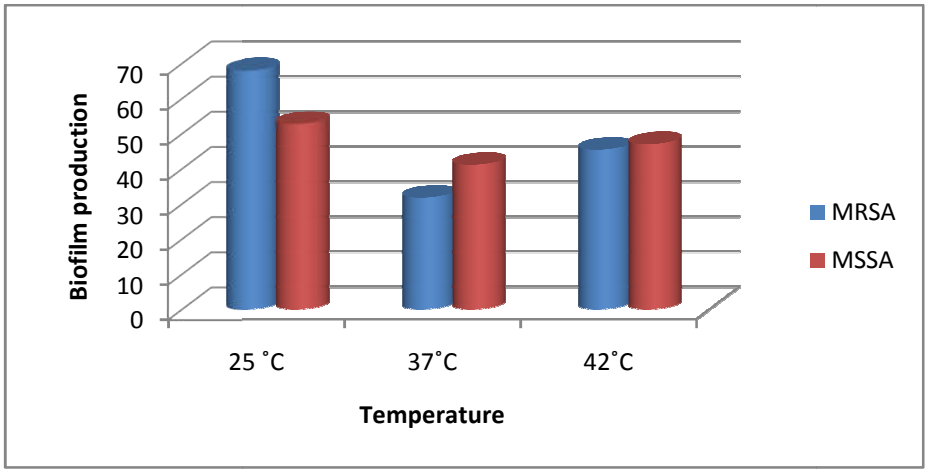

Figure (3): Effect of incubation temperatures on biofilm production by MRSA and MSSA isolates.

Effect of glucose concentrations

Table (7) illustrate the results of microtiter plate assay of all *S. aureus* isolates by using different concentration of glucose. The MRSA isolates showed the highest percentage of biofilm production (72.7%) at glucose concentration 10%, followed by (50%) and (31.8%) at 5% and 2.5% glucose concentration respectively, while the

results among MSSA isolates were (70.6%), (58.8), and (35.3) at 10%, 5%, and 2.5% glucose concentration respectively (figure 4).

The isolates of MRSA and MSSA was different statistically in production of biofilm at different concentrations($P<0.05$).

Table (7) Result of glucose concentration on biofilm production by *S. aureus* isolates by using the microtiter plate assay

Glucose concentration	Methicillin resistant and sensitive isolates	No. of Isolates	Biofilm production				
			No. of none producer %	No. of biofilm production %	No. of weak	No of moderate	Strong
Glucose 2.5%	MRSA	22	١٥ (68.2)	7(31.8)	٦	1	0
	MSSA	17	١١(64.7)	6(35.3)	٦	0	0
Glucose 5%	MRSA	22	١١(50)	11(50)	٧	٤	0
	MSSA	17	٧ (41.2)	10 (58.8)	٨	1	1
Glucose 10%	MRSA	22	٦ (27.3)	16(72.7)	١٤	2	0
	MSSA	17	٥ (29.4)	12 (70.6)	٩	2	1

Chi-square 33.8242 , df :2, p-value: 0.00001 for MRSA at different concentrations.

Chi-square: 26.085, df:2, p-value: 0.00000217 for MSSA at different concentrations.

Chi-square: 60.366, df:5, p-value:0.03532 for total reading as producer and non- biofilm producer

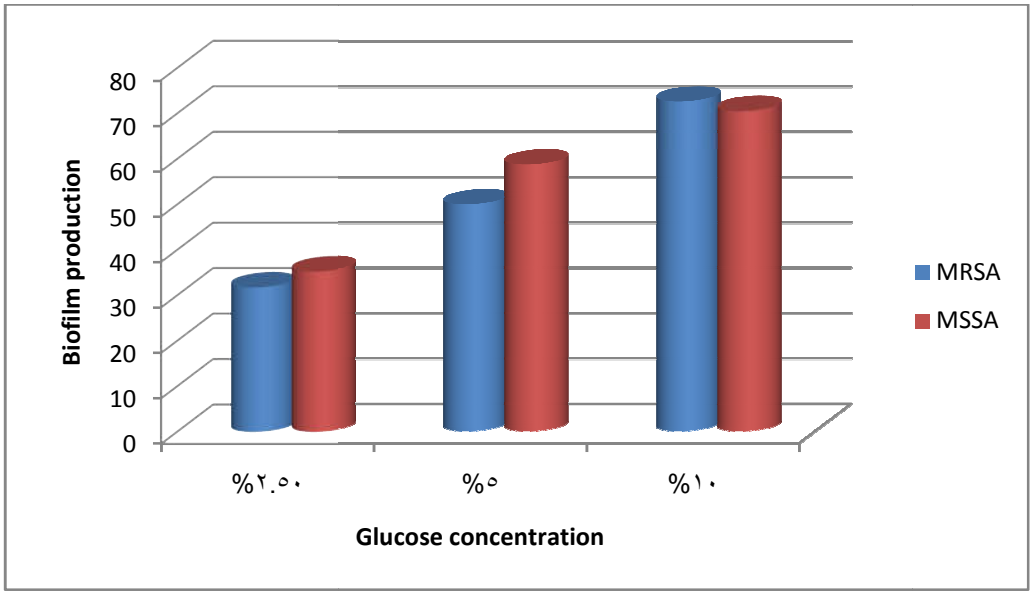

Figure (4): Influence of glucose concentration on biofilm production by MRSA and MSSA isolates

Effect of sodium chloride

Table (8) and figure (5) illustrate the results of microtiter plate assay of all *S. aureus* isolates by using different concentrations of sodium chloride. The MRSA isolates, showed the highest percentage of biofilm production (٦٣.٦) at 10%, followed by (٤٥.٥%) at 5% and the lower percentage of biofilm production (٣٦.٤%) at 2.5% . The MSSA isolates, apparently highest percentage of biofilm production was (٧٠.٦) % at 10% , followed by (٥٨.٨%) at 5% with lower percentage ٥٢.٩% at 2.5%. The MSSA biofilm producers were relatively more than MRSA producers in the different concentrations of NaCl. There were significant differences ($p < 0.05$) between MRSA isolates in biofilm production at different concentrations, also, calculations of differences between the MSSA biofilm production were statistically significant ($P < 0.05$). The difference between MRSA and MSSA in biofilm production at different concentrations was statistically significant.

Table (8) Effect of NaCl concentration on biofilm production by *S. aureus* isolates using the microtiter plate assay

NaCl concentration	Methicillin resistant and sensitive isolates	No. of Isolates	Biofilm production				
			No. of none producer %	No. of biofilm production %	No. of weak	No of moderate	Strong
NaCl 2.5%	MRSA	22	14(63.6)	8(36.4)	6	2	0
	MSSA	17	8(47.1)	9(52.9)	3	5	3
NaCl 5%	MRSA	22	12(54.5)	10(45.5)	3	5	2
	MSSA	17	7(41.2)	10(58.8)	5	3	2
NaCl 10%	MRSA	22	8(36.4)	14(63.6)	4	4	8
	MSSA	17	5(29.4)	12(70.6)	2	2	5

Chi-square :15.351df 2 p-value 0.00046406for MRSA at different concentrations

Chi-square 6.814, df:2, p-value: 0.03314047for MSSA at different concentrations.

Chi-square:34.863, df:5, p-value:0.00000883 for total reading as producer and non- biofilm producer.

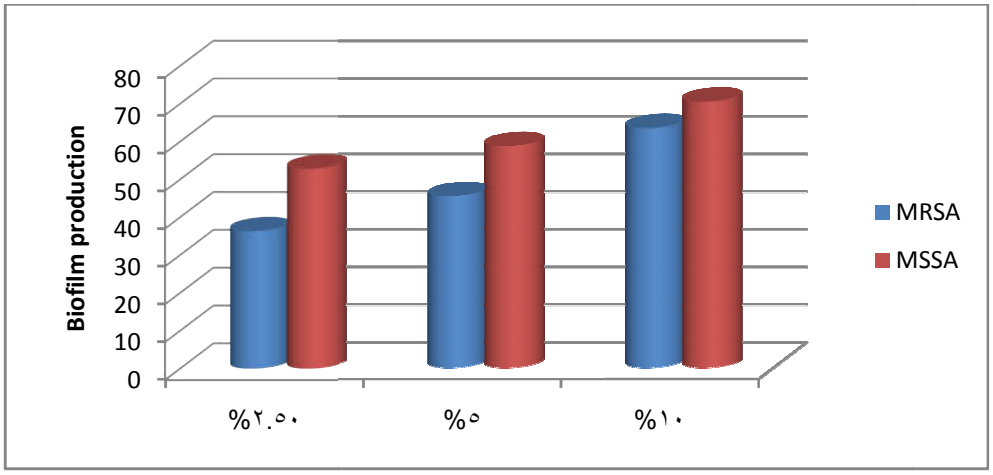

Figure (5): Effect of NaCl concentration on biofilm production by MRSA and MSSA isolates

DISCUSSION

Subclinical mastitis is a major problem because it lacks the visible symptoms [18]. The present results of California mastitis test revealed that 42 (41.17%) milk samples were positive (table 2) this result is in line with several studies. In Iraqi studies, recorded that the prevalence of subclinical mastitis among the sampled cattle was 38.89% in Al Sulaimaniyah province [19]. In Diyala province, 68% of cows revealed subclinical mastitis [20]. In comparison with the study of [21] a higher percentage (68.44%) were diagnosed as subclinical mastitis in cows, which diagnosed by CMT in Basrah province. Other studies conducted in Basrah revealed that the detection rate of SCM by using CMT ranged from 38% to 56.6% [22 , 23].

The present study displays that, the occurrence of *S. aureus* was 60% (Table 3). These results were in the same line of many studies such as [24 , 25] who isolated *S. aureus* in these ratio (64% and 60% respectively). The results of this study are higher than that observed in several studies, especially in Basrah province where isolation rates were recorded about48.61% and 36.84% by [26, 27] respectively. In contrast, higher ratios of isolation were observed by [28, 29], who isolated *S. aureus* in ratios, 70.4% and 80% respectively. These different results may attributed to the differences in the sample size, seasons and differences in detection methodologies.

During the last period, MRSA strains have developed as serious nosocomial pathogens and spread in many regions of world because of its ability to getting resistance to antimicrobial chemotherapy [30]. thus, rapid recognition of these

pathogens and detection of methicillin resistance are necessary for encouragement effective therapy, and preventing distribution of infection [31].

In this study the rate of MRSA detected by Oxacillin disc diffusion method was (66.66%) as compared with cefoxitin disc which was 56.41% (table 4). These results are in line with the results recorded by local studies dealing with mastitis milk of dairy animals [23, 32] who reported that the percentage of methicillin resistance in *S.aureus* isolated from mastitis milk of dairy animals was 61% , 60%, respectively. Also [33] found that the resistance to methicillin was 55%. However, higher results were obtained by [34], who recorded the occurrence of MRSA was 88% and lower results were detected by [35] who found only 10 % of *S. aureus* as MRSA.

The cefoxitin diffusion method was used as an alternative marker for the detection of methicillin resistance [13], Such testing obviously distinguished methicillin-resistant strains of *S. aureus* from methicillin-susceptible strains [13]. Many of investigators have reported that the results of cefoxitin disk diffusion method compares better with the presence of *mecA* than oxacillin disk diffusion method(ODD) [36, 37]. Based on other studies, ODD test usually demonstrations false negative results and its sensitivity is low, especially for the strains with heterogeneous resistance [38]. Findings of this investigation indicate that the ODD method can also be related with false-positive results, as reliable with other studies [39].

The present study reveals that there were no significant differences (P>0.05) in the percentage of weak and moderate biofilm formation between MRSA and MSSA isolates) this result agree with [33].

Effects of environmental conditions on biofilm formation

Effect of incubation temperature on biofilm formation

A temperature is commonly used in biofilm experiments with staphylococci is 37 °C [40]. This is due to the relevance of this temperature in optimal temperature of hosts. However, in hospital environments and also in the food production environment , temperatures both below and above 37 °C are appropriate. The results of this study indicated that biofilm production was the highest at 25˚C and 42˚C of both MRSA (68.2% and 45.5% respectively) and MSSA (52.9% and 47.1% respectively) in comparison with optimal incubation temperature at 37˚C of growth in both MRSA and MSSA. This result was in line with [41] who reported a higher cell count of *S. aureus* growth in TSB at 25˚C than at 37˚C.

The mechanisms that cause increased biofilm formation at suboptimal temperatures are not recognized. Other researchers proposed causes for interpretation of this phenomenon as following, low temperature increase production of fimbriae, hence biofilm production, moreover decrease of temperature enhance hydrophilic properties of bacteria and its ability to adhere to hydrophobic materials like polystyrene [42, 43].

Effect of Glucose concentration on biofilm formation

Overall biofilm producer *S. aureus* isolates showed a significant ($p < 0.05$) increase in their biofilm production with increase of glucose concentration (table 7).

There was a significant increase ($p < 0.05$) of biofilm production by MRSA and MSSA isolates with increasing glucose concentration (figure 4).This result was in agreement with [44] who found that glucose is considered a restrictive factor of biofilm formation due to its requisite during the production of the extracellular matrix components, and [45] Who reported the rise in glucose concentration increases the ability of pathogenic and non –pathogenic staphylococcal isolates to develop biofilm.

Effect of sodium chloride on biofilm formation

Because *S. aureus* is poorly competitive in microbiota and has salt tolerance (10-15%) %) [46], food borne outbreaks are usually linked to salted foods, such as dried fermented sauages and hams, rather than raw foods [47].The present study showed that the biofilm production by MRSA and MSSA, were increased when NaCl concentration increased , this result was in the line with many studies showed that NaCl could promote bacterial aggregation, and enhanced the stability of biofilms in polystyrene [17] who noted that, the expression levels of *ica A* are approximately 9-fold and 20- fold at concentration of 4% and 6% NaCl, respectively as compared with 0% NaCl.

In conclusion, according to the results obtained in this study, *S. aureus* showed high ability to bind to polystyrene surfaces and to form biofilms under different stress environmental factors. The use of the conventional routes for food preservation may support the staphylococcal biofilm formation, therefore, food preservation needs to be decontaminated to apply good food safety.

دراسة مظهرية لتأثيرات العوامل البيئية على تكوين الأغشية الحيوية بواسطة عزلات المكورات العنقودية الذهبية

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الخلاصة

تم تصميم هذه الدراسة لتقييم تأثير ظروف الإجهاد المختلفة (درجة الحرارة ، كلوريد الصوديوم ، والجلوكوز) على تشكيل الاغشية الحيوية من قبل المكورات العنقودية الذهبية. من أصل ٣٩ من المكورات العنقودية الذهبية كان هناك ٢٢ عزلة من المكورات العنقودية الذهبية المقاومة للميثيسيلين (MRSA) و ١٧ عزلة من المكورات العنقودية الذهبية الحساسة للميثيسيلين (MSSA) التي تم الحصول عليها من الحليب الخام وعينات من التهاب الضرع تحت السريري ، تم تقييم المقاومة المظهرية والوراثية للميثيسيلين. تم استزراع كل عزلة من المكورات العنقودية الذهبية في مرق الصويا التريبتي (TSB) بتركيزات مختلفة من الجلوكوز وكلوريد الصوديوم بشكل منفصل (٢.٥ ٪ ، ٥ ٪ ، و ١٠ ٪) ، وحضنت في ٣٧ درجة مئوية باستخدام لوحة المعايرة الدقيقة ذات ٩٦ حفرة. هذه العزلات عرضت أيضًا لحضن بدرجات حرارة مختلفة (٢٥ درجة مئوية ، ٣٧ درجة مئوية و ٤٢ درجة مئوية) باستخدام TSB مع ١ ٪ الجلوكوز. أظهرت النتائج أن الزيادة في تركيزات كلوريد الصوديوم أو الجلوكوز عززت تكوين الغشاء الحيوي في كل من MRSA و MSSA ، من ناحية أخرى ، فإن الحضن بدرجة حرارة منخفضة عزز تكوين الغشاء الحيوي في جميع العزلات. أظهرت النتائج أن التغيرات في ظروف النمو شجعت على إنتاج المواد البوليمرية خارج الخلية.

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