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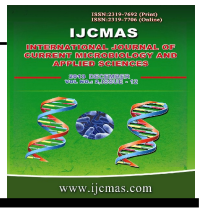

Original Research Article

Detection of Biofilm Producer *Staphylococcus aureus* and its Susceptibility against Antibiotics

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A B S T R A C T

In this study, 159 milk samples were collected from Abu-Ghraib, Al-Mahmodia and Khanaqeen city from cows suffering from acute mastitis and apparently normal cows, cultured on blood agar and mannitol salt agar, and according to biochemical tests and API Staph System, results showed that 56 isolates out of 68 which grown on mannitol salt agar were *Staphylococcus aureus*. Results showed that 46 out of 56 *S. aureus* isolateswere produced biofilm at rate (82.14%) with different thickness ranged between (0.2-1.5)mm, while 10 isolates did not have the ability to produce biofilm. Results of the resistance of these S.aureus isolates against 8 of antibiotics (Ampicillin, Chloramphenicol, Tetracycline, Vancomycin, Gentamycin, Ciprofloxacin, Cloxacillin and Trimethoprim) showed that all these isolates were susceptible (100%) to Ciprofloxacin, and S aureus which produce biofilm were more resistant to the above antibiotics compare with non-biofilm producer isolates against all 7 of antibiotics except Cloxacillin at which the non producer biofilm isolates were more resistant to antibiotics.

Keywords

Biofilm; *Staphylococcus aureus*; Antibiotics; Mastitis.

Introduction

Staphylococcus aureus is an adaptable, pathogenic, gram-positive, cocci bacterium, it form spherical clusters and it is a nonmotile, nonsporeforming facultative anaerobe (Götz *et al*., 2006). The initial attachment of staphylococcal cells on a biomaterial is followed by bacterial accumulation and formation of a mature biofilm (Götz, 2002). A microbial biofilm is a community of several species of bacteria attached to a surface through an extracellular matrix (O'Toole *et al*., 2000). This extracellular matrix is composed of polymeric proteins, polysaccharides and DNA, and allows the bacterial colony to attach itself to both biotic and abiotic surfaces (Davey and O'Toole,2000). Bacterial biofilm consists of organized slimy clusters of bacteria adhered to the inner surface area of the live organs, like mucous membranes, blood vessel and lymphatic endothelium or the surface of medical and veterinary devices. Biofilm contains slime and bacteria which surround themelves with the complex polymeric matrix they secrete, the slime

helps the bacteria embedded in it to be protected from the attack of drugs as well as phagocytosis, it inhibits chemotaxis of granulocytes and their opsonic activity, thus affecting an inflammatory response (Dorocka and Konopka, 2003; Fox *et al*., 2005; Górski and Palmer, 2007).

Numerous authors state that bacteria in a biofilm are 10 to 1000-fold more resistant to antibiotics than in their planktonic form(Górski and Palmer,2007; Melchior *et al*.,2006). This study aimed to isolate S aureus from mastitis cases (milk) and detect its ability to produce biofilm and its resistant to antibiotics.

Materials and Methods

Blood agar and mannitol salt agar were used for growth and isolation of *S. aureus* from 159 milk samples which were collected from Abu-Ghraib,Almahmodia and Khanaqeen city from cows suffering from mastitis and apparently normal cows. Morphological, Cultural and biochemical tests in addition to API staph. System were used for the diagnosis isolates, A qualitative assessment of biofilm formation was determined by tube method (Christensen *et al*., 1982).

Antibiotic susceptibility test was performed using a disc diffusion method on Mueller – Hinton Agar according to Bauer-kirby *et al*., 1966, Discs of antibiotics used were: Ampicillin, Chloramphenicol, Tetracycline, Vancomycin, Gentamycin, Ciprofloxacin, Cloxacillin, Trimethoprim.

Results and Discussion

The results of bacterial isolation showed that out of total 159 milk samples which were collected, 68 milk samples showed positive results for the presence of Staphylococci after culturing on blood agar and mannitol salt agar.

The results showed that 46 milk samples out of 61 samples of normal and mastatic cows in Abu-Ghraib zoon were grown on blood agar while just 25 samples grew on mannitol salt agar medium.

In Mahmodia 42 milk samples were collected from mastatic and apparently normal cows,36 of these samples were grown on blood agar and 27 samples gave positive results on mannitol salt agar medium.

On the other hand, 56 milk samples were collected from some villages in khanaqeen, and results showed that 38 out of these 56 samples were grown on blood agar while 16 samples only grew on mannitol salt agar medium.

After incubation at 37 □ C for 24 hours on blood agar, colonies of 1 mm in diameter appeared as yellow, round, smooth and glistening with β or α hemolytic, but after 48 hours of incubation, the isolated colonies reached to 4 mm in diameter and produced double zone of hemolysis.

The Suspected S. aureus isolates which were subcultured on mannitol salt agar medium appeared as rounded, smooth convex yellowish in color disseminated to the background of the agar indicated the ability of mannitol fermentation, these results agree with Jawetz *et al*., 2007 .

Staining the suspected isolates with gram stain showed a spherical single cocci, diplococci, quadrates, and the predominant shape was grape-like clusters of blue color under light microscope lens. The suspected isolates gave positive results for

catalase, urease, phosphatase de oxyribonuclase tests and for gelatin production, while it gave negative result for oxidase test (table 2) and to confirm the diagnosis, API Staph system was used. Finally, and according to the previous listed cultural, biochemicals and API Staph System, results confirmed that all the 56 isolates were belong to *Staphylococcus aureus*. Results showed that the rate of infection with *S. aureus* was 35.22% from the total samples collection (159), and this rate agreed with (Al-Ani, 2009) who found that (25%) milk samples were *S. aureus* positive out of total 124 milk specimens taken from Abu-Ghraib city and also agreed with Al-Marsoomy (2007) who diagnosed 173 isolates of S. aureus (28.73%) of 602 milk samples were collected from 358 cows infected with acute and subclinical mastitis of cows.

Detecting the ability of *S. aureus* isolates to produce biofilm was done by using Christensen tube method (Christensen *et al*.,1982), the wall of the tubes which contains slimy material producer isolates appear red in its color Figure (1-a) whereas large biofilm producer isolates appeared dark to red in color Figure(1-b),but tubes which not stained with saphranin appeared nearly yellow or white in its color Figure (1-c), this for tubes which their medium is spilled, tubes which their medium were not spilled a thick layer of biofilm appeared on the upper surface of the medium represented polysaccharide which form most constitution of the biofilm adhering on the wall of the test tubes Figure(1-d),while the wall of the tubes containing isolates which did not produce biofilm appeared colorless Figure(1-e).

The results showed that 46 of total 56 isolate of S.aureus in this study produced biofilm (82.14%), Table(3),this is in agreement with Vasudevan *et al*. (2003) who demonstrated that 32 of 35 S. aureus isolates were slime positive , while Zmantar *et al*., (2010) found that 26 out of 46 strains of S. aureus (56.5%) were slime producers , but Gundogan *et al*., (2006) found that 58 out of 110 S. aureus strains were slime producers. Fox *et al*. (2005) found a higher percentage (41%) of biofilm-positive isolates from milk.

Staphylococcus aureus and *Staphylococcus epidermidis* remain the two of the most commonly isolated from bovin mastitis, these bacteria are able to form biofilm, highly organized multicellular complexes that represent an important virulence factor in staphylococci. As mentioned by (Cucarella *et al*., 2001; Vasudevan *et al*., 2003; Fox *et al*., 2005; Melchior *et al*., 2006; Clutterbuck *et al*., 2007).

The result showed that isolates differed in its biofilm producing efficiency , the thickness of biofilm which measured in these isolates ranged between (0.2-1.5)mm Table(4). Al-ithawy, 2010 found that thickness of biofilm produced by P. aeruginosa ranged between (1.1- 6.5) mm Bacteria colonizing a surface produce extracellular polymeric substances(EPS) that will glue the cell to the surface and eventually form the biofilm matrix, generally extracellular polymeric substances(EPS) are composed of polysaccharides, but may also contain proteins, nucleic acids and polymeric lipophilic compounds, in terms of weight and volume extracellular polymeric substances(EPS) represent the major structural component of biofilm, being responsible for the interaction of microbes with each other as well as with interfaces (Flemming 2002; Neu *et al* ., 2001).

Table.1 Results of the isolation of *S.aureus* from milk samples

City of collected samples	Total No.	Number of acute mastitis	Number of apparently normal cows	Positive samples on	
				Blood agar	Mannitol salt agar
Abu-Ghraib	61	41	20	46	25
Mahmodia	42	34	8	36	27
Khanaqeen	56	33	23	38	16
Total No.	159	108	51	120	68

Table.2 Results of cultural, microscopical and biochemical tests

Biochemical tests	Results
Catalase	+
Oxidase	_
Gelatin hydrolysis	+
Deoxyribonuclease	+
Phosphatase	+
Urease	+
Coagulase	+
API Staph system	+
Gram stain	+
Cell morphology	Cocci with grape like clusters

Table.3 Results of biofilm production of different *S.aureus* isolates

No. of isolates	Result	No. of isolates	Result	No. of isolates	Result
1	_	20	+	39	+
2	+	21	+	40	+
3	+	22	+	41	+
4	+	23	+	42	+
5	+	24	_	43	+
6	+	25	_	44	+
7	+	26	+	45	+
8	+	27	_	46	+
9	_	28	+	47	+
10	+	29	_	48	+
11	_	30	+	49	+
12	+	31	_	50	+
13	+	32	_	51	+
14	_	33	+	52	+
15	+	34	+	53	+
16	+	35	+	54	+
17	+	36	+	55	+
18	+	37	+	56	+
19	+	38	+		

Table.4 Thickness (mm) of biofilm produced by *S.aureus* isolates

No. of isolate	Thickness of biofilm(mm)	No. of isolate	Thickness of biofilm(mm)
2	0.6	34	0.7
3	0.5	35	0.7
4	1	36	0.2
5	1	37	0.3
6	1	38	1
7	0.7	39	1.5
8	1	40	1
10	1	41	0.9
12	0.5	42	0.9
13	0.4	43	1
15	0.2	44	0.7
16	0.5	45	0.5
17	0.5	46	0.7
18	1	47	1
19	0.3	48	1
20	1	49	1
21	0.8	50	0.9
22	1	51	0.5
23	0.5	52	0.5
26	0.3	53	0.6
28	0.3	54	0.9
30	0.3	55	1
33	0.5	56	1

Table.5 Results of susceptility test of 10 biofilm producer isolates against 8 of Antibiotics, Group (1)

Number of isolates						
Antibiotics	**Resistant**		**Intermediate**		**Susceptible**	
	Number	**%**	**Number**	**%**	**Number**	**%**
Ampicillin	**9**	**90**			**1**	**10**
Chloramphenicol	**2**	**20**	**2**	**20**	**6**	**60**
Tetracycline	**2**	**20**			**8**	**80**
Vancomycin	**4**	**40**			**6**	**60**
Gentamycin	**3**	**30**			**7**	**70**
Ciprofloxacin	**0**	**0**			**10**	**100**
Cloxacillin	**2**	**20**			**8**	**80**
Trimethoprim	**4**	**40**	**1**	**10**	**5**	**50**

Table.6 Results of susceptility test of 10 non-biofilm producer isolates against 8 of Antibiotics, Group (2)

Number of isolates						
Antibiotics	**Resistant**		**Intermediate**		**Susceptible**	
	Number	**%**	**Number**	**%**	**Number**	**%**
Ampicillin	8	88.8			1	11.1
Chloramphenicol	1	11.1	1	11.1	7	77.7
Tetracycline	1	11.1			8	88.8
Vancomycin	3	33.3	1	11.1	5	55.5
Gentamycin	1	11.1	1	11.1	7	77.7
Ciprofloxacin	0	0			9	100
Cloxacillin	2	22.2	1	11.1	6	66.6
Trimethoprim	2	22.2	2	22.2	5	55.5

Susceptibility test of (10) biofilm producer isolates against (8) of antibiotics showed that all these isolates were susceptible 100% to Ciprofloxacin, and susceptible to other antibiotics at various ratio as illustrated in Table(5) :

These results are in accordance with Ambrina *et al* .,2010 who found that 85% of MRSA isolates in their study were resistant against ampicillin, and (24%) isolates were resistant to Gentamycin, and only 8% were resistant to Ciprofloxacin.
The comparative results of biofilm producer (group 1) and non-biofilm producer (group 2) isolates against these antibiotics showed that all these isolates of both groups were susceptible (100%) to Ciprofloxacin ,and all isolates of group 1 were more resistant than group 2 against all 7 antibiotics except Cloxacillin at which group 2 was more resistant than group1 .

Studies have shown (Nickel *et al*., 1985; Mah and O'toole, 2001) that 10 - 1000 times more antibiotics are required to treat an infection caused by a biofilm-associated organism than a planktonic microbe of the same species. Biofilms also provide an ideal niche for the exchange of extra chromosomal DNA responsible for antibiotic resistance, making it a perfect milieu for emergence of drug resistant pathogens (Donlan, 2002).

Generally, much of biofilm-associated antibiotic resistance observed in this study can be attributed to:

The extra cellular polymeric substances (EPS) secreted by biofilm bacteria, acts as a physical/chemical barrier, thus preventing penetration by many antibiotics (Thien and O'toole, 2001; Jefferson *et al*., 2005).

EPS is negatively charged and functions as an ionexchange resin which is capable of binding a large number of the antibiotic

molecules that are attempting to reach the embedded biofilm cells (Prakash *et al*., 2003).

Embedded biofilm bacteria are generally not actively engaged in cell division and are smaller in size and less permeable to antibiotics (Thien and O'toole, 2001). Virtually all antimicrobial agents are more effective in killing rapidly-growing cells.

The close contact between bacteria within a biofilm and the matrix may inhibit the penetration of antibiotics through the exopolysaccharid matrix (Jefferson *et al*., 2005).

Biofilm-associated bacteria are often less susceptible to antimicrobial agents and host defenses and, as such, infections involving biofilm may be harder to treat and clear (Yarwood *et al*., 2004).

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