ORIGINAL ARTICLE

Investigation of biofilm formation on contact eye lenses caused by methicillin resistant *Staphylococcus aureus*

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Abstract

Objective: The objective was to investigate the biofilm-forming capacity of methicillin resistant *Staphylococcus aureus* (MRSA) isolated from eye lenses of infected patients.

Materials and Methods: A total of 32 MRSA isolated from contact lenses of patients with ocular infections were screened for their biofilm-forming capacity using tube method (TM), Congo red agar (CRA), and microtiter plate (MtP) methods. The effect of some stress factor on the biofilm formation was studied. The biofilm-forming related genes, *icaA*, *icaD* and 10 microbial surface components that recognize adhesive matrix molecule (MSCRAMM), of the selected MRSA were also detected using polymerase chain reaction.

Results: Of 32 MRSA isolates, 34.37%, 59.37%, and 81.25% showed positive results using CRA, TM or MtP, respectively. Biofilm production was found to be reduced in the presence of ethanol or ethylenediaminetetraacetic acid and at extreme pH values. On the other hand, glucose or heparin leads to a concentration dependent increase of biofilm production by the isolates. The selected biofilm producing MRSA isolate was found to harbor the *icaA*, *icaD* and up to nine of 10 tested MSCRAMM genes, whereas the selected non biofilm producing MRSA isolate did not carry any of the tested genes.

Conclusions: The MtP method was found to be the most effective phenotypic screening method for detection of biofilm formation by MRSA. Furthermore, the molecular approach should be taken into consideration for the rapid and correct diagnosis of virulent bacteria associated with contact eye lenses.

Key words: Biofilm formation, eye lenses, methicillin resistant *Staphylococcus aureus*, microtiter plate, polymerase chain reaction

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Introduction

Differentiation of staphylococci respecting to its biofilm might help to elucidate the impact of staphylococci in diagnosis of infections related to contact eye lenses.[1-9] Phenotypic methods used to identify biofilm-producing strains are the microtiter plate (MtP), Congo red agar (CRA) and tube method (TM).[10-12] Recently, polymerase chain reaction (PCR) was used for detection of microbial surface components that recognize adhesive matrix molecules (MSCRAMMs) and *ica* genes, providing the genetic basis of biofilm production complementary to the phenotypic tests.[13-16]

The ability of methicillin resistant *Staphylococcus aureus* (MRSA) isolates obtained from contact lenses of patients to form biofilm was determined. Additionally, the genetic basis for biofilm formation of two selected strains was determined by PCR.

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Materials and Methods

Bacterial isolates

The contact lenses of patients suffering from an eye infection associated with their use and visiting Ophthalmology Department, Tanta University Hospital, Egypt (during 2010–2011) were aseptically removed and immediately immersed individually in a test tube containing 2 ml nutrient broth containing 7.5% NaCl and transferred to microbiology department where it was incubated aerobically at 37°C for 24 h. Subculture from each eye lens culture was made on mannitol salt agar plates. Yellow colonies of Gram-positive cocci growing on the later high salt medium were preliminary identified as *S. aureus*. Conformation of identification of these isolates was performed using standard biochemical test including Gram-stain, catalase, coagulase and DNase tests.

The biofilm-producer *Staphylococcus epidermidis* American Type Culture Collection (ATCC) 35984; the nonbiofilm producer *S. epidermidis* ATCC 12228, and *S. aureus* ATCC 29213 kindly provided from culture collection of Faculty of Pharmacy, Tanta University, were used as reference strains.

Antimicrobial susceptibility testing

All the recovered *S. aureus* isolates were tested for methicillin resistance[17] on Muller Hinton agar using oxacillin (OX) discs (1 μg). The isolates were considered methicillin resistant if the zone of inhibition was 10 mm or less. Further, the antimicrobial susceptibility pattern of MRSA isolates was determined using ampicillin; amoxicllin-clavulanic acid; cephalothin; cefepime; imipenim (IPM); tobramycin; amikacin; gentamicin; streptomycin; ciprofloxacin (CIP); chloramphenicol; tetracycline; erythromycin and vancomycin by modified Kirby-Bauer single-disk diffusion technique. The results of the tests were interpreted according to the criteria established by the Clinical and Laboratory Standards Institute.[17] *S. aureus* ATCC 29213 was used as reference strain.

Testing biofilm production by the methicillin resistant *Staphylococcus aureus* isolates

Biofilm production ability of the isolates was tested using three different techniques, TM, CRA and MtP, as follows:

Tube method

Biofilm production by MRSA isolates was estimated qualitatively as described previously by Christensen.[12] After incubations at 37°C for 18–20 h, culture was decanted. Tubes were stained with crystal violet and presence of a visible stained film lined the wall and bottom of the tube was considered positive for slime production. The results were visually scored as weak/nonproducers (WPs/NPs), moderate producers (MPs) or high producers (HPs) based on the intensity of resultant film. A tube containing uninoculated Tryptic Soy Broth (TSB) was simultaneously tested and used as a negative control.

Congo red agar method

Freeman *et al*.[11] had described an alternative method of screening biofilm formation by *Staphylococcus* isolates using Brain Heart Infusion Broth (BHI) supplemented with 5% sucrose, agar no. 1 (10 g/L) and Congo red stain (0.8 g/L). Plates were inoculated with the tested isolates and incubated aerobically for 24 h at 37°C. Positive (HP) result was indicated by black colonies with a dry crystalline consistency. A darkening of the colonies with the absence of a dry crystalline colonial morphology indicated a moderate result (MP). Nonslime producers usually remained pink, though occasional darkening at the centers of colonies was observed and this gave a bull's eye appearance (WP).

Microtiter plate method

Biofilm-forming ability was measured by determination of adhesion to polystyrene MtPs as described by Christensen *et al*.[10,18] with slight modification. Briefly, 96-well flat-bottomed MtPs (Greiner Bio-One, Frickenhausen, Germany) were filled with 100 μl TSB, with/without supplements. A diluted overnight bacterial culture (1:100 in TSB, 100 μl TSB) was added to each well. Negative control wells contained TSB only. The plates were incubated at 37°C for 18 h followed by several washing with phosphate buffered saline (pH 7.3). Sodium acetate (2%) was added as fixative, decanted and wells were stained with crystal violet (0.1% w/v). Finally, the plates were rinsed under running tap water, air-dried, and read at 570 nm by a Sunrise absorbance reader (Tecan Austria GmbH, Austeria). In accordance with the original method, isolates with optical density (OD) <0.120 were considered as WP/NP, those with OD values 0.120–0.240 were regarded as MP. An OD >0.240 indicates as HP. To compensate for background absorbance, OD readings from sterile medium, fixative and dye were averaged and subtracted from all test values. The mean OD value obtained from media control well was deducted from all the test OD values. The statistical analysis was conducted using SPSS version 16.0 (SPSS Inc., Chicago, IL). A $P < 0.05$ was considered to be statistically significant.

Testing the effect of some factors on biofilm production

The modified MtP mentioned above selected for studding the biofilm forming capability of the tested isolates under different factors. To test the influence of medium composition on biofilm production diluted overnight bacterial culture with tested medium, TSB, TSB 1% glucose ($TSB_{1\%glu}$) or BHI broth with 2% sucrose ($BHI_{2\%Suc}$), was used. Presence of different concentrations of glucose (0, 0.75, 1.25, 2.5 and 5%), ethanol (0, 2, 5 and 10%), ethylenediaminetetraacetic acid (EDTA) (5, 10, 15 and 20 μM) and heparin (0, 0.01, 0.1, 1, 10 and 100 U/mL) in TSB (or TSB without dextrose medium, sigma, in case of

testing glucose) were similarly examined for their effects on biofilm production. The effect of pH level (pH 5, 7 or 9) on biofilm production by the tested isolates was also performed.

In all cases the two reference strains *S. epidermides* ATCC 35984 and *S. epidermides* ATCC 12228 were simultaneously used as control for positive and negative biofilm production, respectively. Experiments were performed in triplicate.

Genotypic characterization of biofilm formation

Genomic DNA of the two selected MRSA isolates was extracted using a DNA purification kit (Promega, USA). Three different PCR reactions were set up to detect biofilm related genes. PCR 1[19,20] was designed to detect all MSCRAMM-related genes except bap, which was detected by PCR 2.[19,20] In addition, PCR 3[21] was designed for detection of *icaA* and *icaD* genes. The name of target genes and nucleotide sequence of primers are shown in Table 1. The same PCR programs described by the corresponding author of each PCR reaction were followed.

Results

A total number of 100 *S. aureus* was isolated from 100 contact eye lenses. The results of OX susceptibility of these isolates confirmed the presence of 32 (32%) MRSA.

Antimicrobial susceptibility of the tested methicillin resistant *Staphylococcus aureus*

Among the tested drugs impenim and CIP were the most active drugs where 18.8% and 31.3%, respectively were resistant (data not shown). In general, all isolates showed a high frequency of multiple [3-15] drug resistance, and up to 50% of the isolates showed multiple resistance to >10 antimicrobial drugs [Table 2].

Phenotypic characterization of biofilm formation by methicillin resistant *Staphylococcus aureus* isolates

Production of biofilm by all tested isolates under study was assessed by three methods (CRA, TM and MtP). For ease of comparison the results of each test were classified as WP/NP, MP or HP as aforementioned. Different results were obtained with each method as shown in Table 3. As observed in Table 3 the MtP method was the most efficient one hence it detected up to 26 (81.3%) of biofilm producers (16 HP and 10 MP) compared to 19 and 11 producer isolates detected by TM and CRA, respectively.

Factors affecting biofilm formation

As shown in Figure 1a, 16 (50%) of the tested isolates displayed high biofilm producers in TSB medium, whereas with the addition of 1% glucose in TSB ($TSB_{1\%glu}$), number of highly biofilm producing isolates increased to 20 (62.5%). Also using $BHI_{2\%}$suc medium, 18 (56.25%) strongly biofilm producing isolates were detected. In the absence of glucose the percentage of high biofilm producers were 18.75% and weak or non-producing isolates were 56.25%. However, at 5% glucose, the high biofilm producers were 62.5% and weak or nonproducing biofilm isolates were only 3.125% [Figure 1b]. Similarly, enhancement of biofilm production by the tested isolates was observed in the presence of heparin and this effect was concentration dependent as shown in Figure 1c.

Generally, addition of ethanol inhibited biofilm production by the tested staphylococci and this inhibition was directly proportional to ethanol concentrations [Figure 1d]. Furthermore, addition of EDTA to the culture medium significantly reduced the biofilm production by the tested bacteria and the reduction was concentration dependent. As shown in Figure 1e, the highest reduction value was recorded at 20 μM EDTA. On the other hand, the number of WPs or nonbiofilm producers increased from 10 in the absence of EDTA to 15 at 20 μM concentration.

Table 1: Primers for detecting of MSCRAMMs, *icaA* and *icaD* genes

Genes	Nucleotide sequence of primers	Amplicon size (bp)
cna (collagen binding protein)	3'--GTCAAGCAGTTATTAACACCAGAC-5' 5'-AATCAGTAATTGCACTTTGTCCACTG-3'	423
eno (laminin binding protein)	3'-ACGTGCAGCAGCTGACT-5' 5'-CAACAGCATYCTTCAGTACCTTC-3'	302
ebpS (elastin binding protein)	3'-CATCCAGAACCAATCGAAGAC-5' 5'-CTTAACAGTTACATCATCATGTTTATCTTTG-3'	186
fnbA (fibronectin binding protein A)	3'-GTGAAGTTTTAGAAGGTGGAAAGATTAG-5' 5'-GCTCTTGTAAGACCATTTTTCTTCAC-3'	643
fnbB (fibronectin binding protein B)	30-GTAACAGCTAATGGTCGAATTGATACT-5' 50-CAAGTTCGATAGGAGTACTATGTTC-3'	524
fib (fibrinogen binding protein)	3'-CTACAACTACAATTGCCGTCAACAG-5' 5'-GCTCTTGTAAGACCATTTTCTTCAC-3'	404
clfA (clumping factor A)	3'-ATTGGCGTGGCTTCAGTGCT-5' 5'-CGTTTCTTCCGTAGTTGCATTTG-3'	292
clfB (clumping factor B)	3'-ACATCAGTAATAGTAGGGGGCAAC-5' 5'-TTCGCACTGTTTGTGTTTGCAC-3'	205
bbp (bone sialoprotein binding protein)	3'-AACTACATCTAGTACTCAACAACAG-5' 5'-ATGTGCTTGAATAACACCATCATCT-3'	575
bap (biofilm associated protein)	3'-CCCTATATCGAAGGTGTAGAATT-5' 5'-GCTGTTGAAGTTAATACTGTACCTGC-3'	971
icaA	F: 5'-TCTCTTGCAGGAGCAATCAA-3' R: 5'-TCAGGCACTAACATCCAGCA-3'	188
icaD	F: 5'-ATGGTCAAGCCCAGACAGAG-3' R: 5'-CGTGTTTTCAACATTTAATGCAA-3'	198

MSCRAMMs=Microbial surface components that recognize adhesive matrix molecules

Table 2: Resistance pattern of tested MRSA isolates

Resistance pattern	Number of resistance markers	Number of isolates exhibited the pattern
AMC, KF, OX	3	1
AMC, FEB, OX	3	1
KF, TE, E, OX	4	1
AMP, KF, FEB, OX	4	1
AMP, AMC, KF, FEB, OX	5	3
AMP, AMC, KF, FEB, CN, S, OX	7	2
AMP, AMC, KF, FEB, CN, CIP, OX	7	1
AMP, AMC, KF, FEB, TE, C, E, OX	8	3
AMP, AMC, KF, FEB, CN, TE, E, OX	8	1
AMP, AMC, KF, FEB, CN, CIP, C, OX	8	1
AMP, AMC, KF, TOB, CN, S, TE, C, E, OX	10	1
AMP, AMC, KF, FEB, CN, S, CIP, TE, C, E, OX	11	1
AMP, AMC, KF, FEB, TOB, AK, CN, TE, E, VA, OX	11	1
AMP, AMC, KF, FEB, CN, S, CIP, TE, E, VA, OX	11	1
AMP, KF, FEB, TOB, CN, S, TE, E, VA, IPM, OX	11	1
AMP, AMC, KF, FEB, CN, S, CIP, TE, C, E, VA, OX	12	1
AMP, AMC, KF, FEB, AK, CN, S, TE, C, E, VA, OX	12	1
AMP, AMC, KF, FEB, TOB, AK, CN, S, E, VA, IPM, OX	12	1
AMP, AMC, KF, FEB, TOB, AK, CN, S, TE, C, E, VA, OX	13	1
AMP, AMC, KF, FEB, TOB, AK, CN, S, TE, E, VA, IPM, OX	13	1
AMP, AMC, KF, FEB, TOB, AK, CN, CIP, TE, C, E, VA, OX	13	1
AMP, AMC, KF, FEB, TOB, CN, S, CIP, C, TE, E, VA, OX	13	2
AMP, AMC, KF, FEB, TOB, AK, CN, S, CIP, TE, C, E, VA, OX	14	1
AMP, AMC, KF, FEB, TOB, AK, CN, S, TE, C, E, VA, IPM, OX	14	1
AMP, AMC, KF, FEB, TOB, AK, CN, S, CIP, TE, C, E, VA, IPM, OX	15	2

*AMP=Ampicillin; AMC=Amoxiclin-clavulanic acid; KF=Cephalothin; FFP=Cefepime; IPM=Imipenim; TOB=Tobramycin; AK=Amikacin; CN= Gentamicin; S=Streptomycin; CIP=Ciprofloxacin; C=Chloramphenicol; TE=Tetracycline; E=Erythromycin VA=Vancomycin; MRSA=Methicillin resistant *Staphylococcus aureus*

Table 3: *Staphylococcal* biofilm formation as detected by three methods

Level of biofilm production	Number of biofilm producing isolates (%) using different methods		
	MtP	TM[a]	CRA[b]
High producer	16 (50)	12 (37.5)	6 (18.75)
Moderate producer	10 (31.3)	7 (21.9)	5 (15.6)
Weak/nonproducer	6 (18.8)	13 (40.6)	21 (65.6)
Total	32	32	32

MtP=Microtiter plate method; TM=Tube method; CRA=Congo red agar method. [a]TM showed highly significant correlation with MtP tests (r=0.557***; P=0.00); [b]CRA showed insignificant correlation (r=0.183, P=0.075) with MtP test

Additionally, the biofilm production varied according to the pH values of the medium [Figure 1f]. At highly alkaline (pH 9) levels, the percentage of HPs of biofilm were markedly decreased (25%), compared with that determined at pH 7 (50%).

Genotypic analysis of biofilm producing methicillin resistant *Staphylococcus aureus*

The PCR technique was applied to the two selected biofilm producer and nonbiofilm producer MRSA isolates. Nine of MSCRAMM related genes namely: Clumping factor A (*clfA*), clumping factor B (*clfB*), collagen binding protein, laminin binding protein (*eno*), elastin binding protein (*ebpS*), fibrinogen binding protein (*fib*), biofilm associated protein (*bap*), fibronectin binding protein A (*fnbA*) and fibronectin binding protein B (*fnbB*) could be detected from the biofilm producing isolate whilst, bone sialoprotein binding protein (*bbp*) gene was not detected in this study [Figure 2]. Moreover the *icaA* and *icaD* genes were also detected in the selected biofilm producer isolate [Figure 3]. In contrast none of such genes was detected in the nonbiofilm producer MRSA isolate.

Discussion

Infection by MRSA isolates is a growing concern that presents implications for both systemic and ophthalmic health. Our study enrolled 32 MRSA isolates recovered from contact lens infection cases. Herein, MRSA isolates were found to be multiply resistant to 2–15 out of the 15 antimicrobials under test. These results were comparable to the results of Ammendolia *et al.*[22] and Murugan *et al.*[23] Their high prevalence of antibiotic resistance might be due to their biofilm forming nature. Consequently, investigations to understand the pathogenesis of these infections have focused upon the process of adherence of these microorganisms on contact lenses.

Our results indicated that up to 90.62% isolates displayed a biofilm-positive and 50% of them were strong biofilm producers as determined by MtP method. Similar results were observed by Zegans *et al.*[5] and Lasa[6] also Mathur *et al.*[24] recommend the use of MtP technique due to its high specificity, sensitivity, and positive predictive value. However, Jain and Agarwal;[25] Grinholc *et al.*;[26] Hassan *et al.*[27] supported the use of CRA for biofilm detection.

Biofilm formation by tested MRSA isolates was induced by increasing glucose concentrations up to 0.5% in TSB media, which is entirely consistent with previously reported data.[28] Generally, it was noticed that the presence of various sugar supplementations is essential for biofilm formation. On the other hand, it was observed that the addition of EDTA significantly reduced the biofilm formation by the tested bacteria and such reduction was concentration dependent. Most probably EDTA was acting as antibiofilm formation by

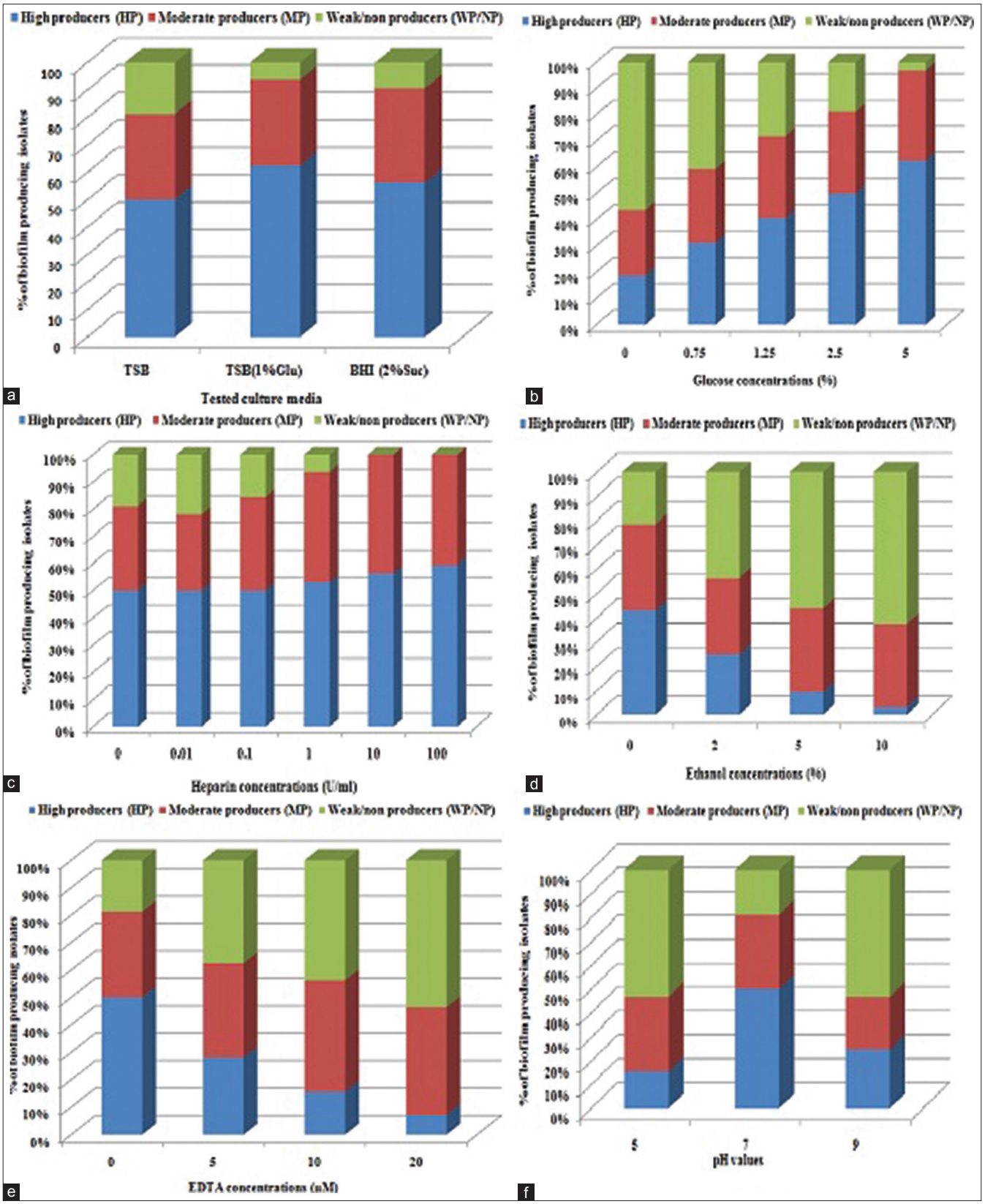

Figure 1: Biofilm production by methicillin resistant Staphylococcus aureus isolates under different conditions. Effect of different medium composition, TSB = Trypticase Soy Broth medium, TSB(1% Glu): Trypticase Soy Broth with 1% glucose, Brain Heart Infusion (BHI) (2%Suc): BHI medium with 2% sucrose, on the biofilm production (a). Biofilm Production in TSB at different pH values (b). Additions of several concentrations of ethanol, glucose, ethylenediaminetetraacetic acid or heparin in TSB to estimate biofilm produced (c-f)

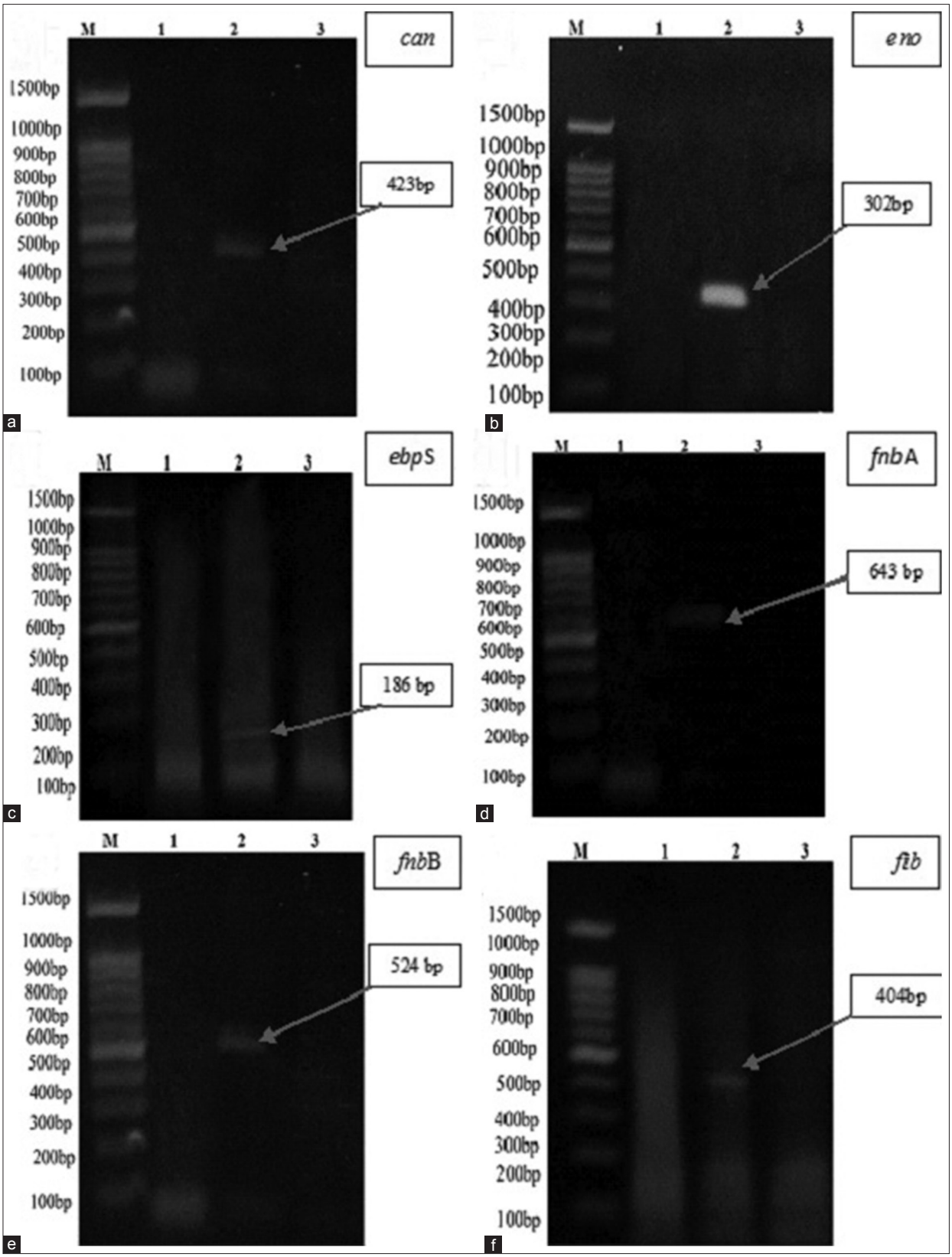

Figure 2: Polymerase chain reaction detection of microbial surface components that recognize adhesive matrix molecule gens. M: Molecular weight markers; lane 1: Negative control (DNA template absent); lane 2: Band for under studied gene obtained with DNA from a biofilm-producing clinical isolate; lane 3: Absence of bands obtained with DNA from a biofilm non-producing clinical isolate

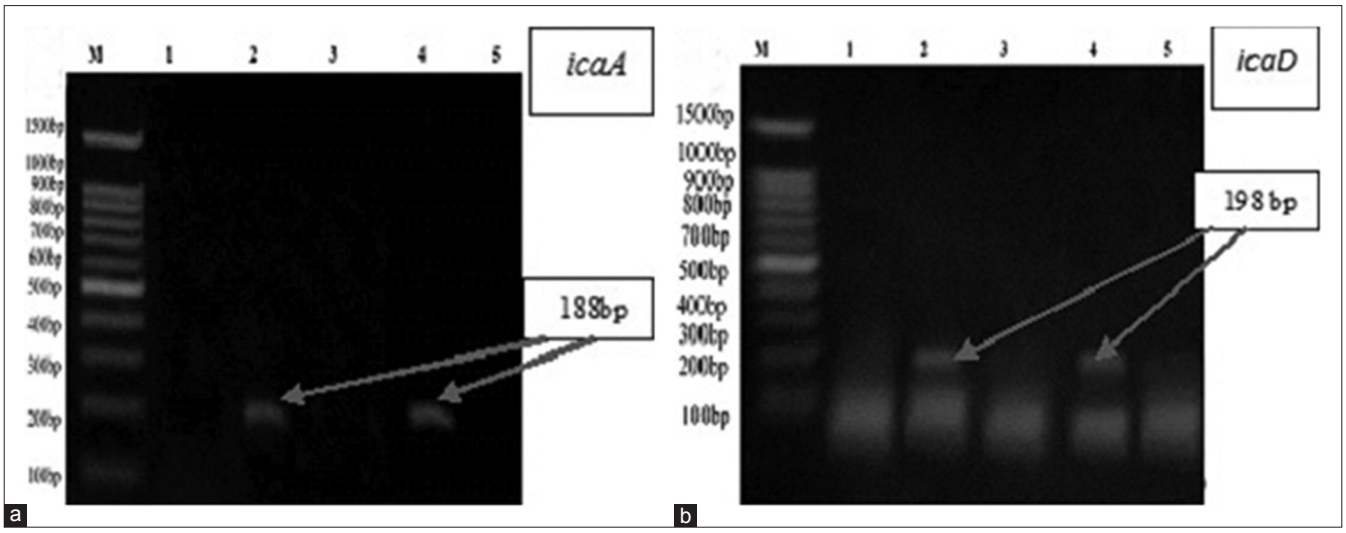

Figure 3: Polymerase chain reaction detection of icaA and icaD genes. M: Molecular weight markers; lane 1: Negative control (DNA template absent); lane 2: Band for under studied gene obtained with DNA from a biofilm-producing clinical isolate; lane 3: Absence of bands obtained with DNA from a biofilm nonproducing clinical isolate. Lane 4: 188-bp band for icaA and 198-bp band for icaD obtained with DNA from the biofilm-producer Staphylococcus epidermides American Type Culture Collection (ATCC) 35984, respectively; lane 5: Absence of bands obtained with DNA from a biofilm-non producer Staphylococcus epidermides ATCC 12228

the chelation of several divalent cations that are required to stabilize the biofilm matrix.[29-31] Furthermore, increasing of heparin concentrations enhanced biofilm production by our isolates. A possible explanation might be that heparin increased the frequency of clumped cells in the planktonic phase and on polystyrene also it stimulates the formation of adhesion molecules that make *S. aureus* better able to adhere to one another in either a heparin dependent (where heparin acts as a cross-bridge) or heparin independent manner.[32]

Previous reports suggested an essential role for the MSCRAMMs gene in the initiation of staphylococcal corneal infection.[15] In this regard, genotypic characterization of the two selected isolates (biofilm producing isolate and nonbiofilm producing isolate) for the detection of the MSCRAMM genes was performed using PCR. Interestedly the selected biofilm producing clinical isolate was shown to possess genes encoding the MSCRAMMs including *clfA, clfB, cna, eno, ebpS, fib, bap, fnbA* and *fnbB* while, only *bbp* gene not detected. On the other hand, the nonbiofilm producing isolate was negative for all tested genes.

Compared with a previous study regarding MSCRAMM genes, *Cna*, *fnbA* and *fnbB* have been proved significantly contributing to tissue colonization in various pathological conditions including eye keratitis.[33] In addition, Rhem *et al.*[34] suggested that the collagen-binding adhesin (*Cna*) is involved in the pathogenesis of *S. aureus* infection of the cornea. Moreover, invasive strains of staphylococci have been reported to bind fibronectin, *fnbA* and *fnbB*, more avidly than commensal strains.[35] Typically, clumping factor A has also been shown to be important in the binding of *S aureus* in adhesion to both polyethylene and polyvinyl surfaces. Furthermore, all *bap*-positive *S. aureus* strains tested by Cucarella *et al.*[36] were highly adherent and strong biofilm producers. Therefore, it may be suspected that MSCRAMM genes in MRSA isolate are associated with their virulence. Biofilm formation consists of two-independent processes: The initial attachment of bacteria to a solid surface followed by proliferation and accumulation of bacteria cells, which results in biofilm maturation.[37] The process of bacterial attachment is characterized by a number of variables, including the species of bacteria, the surface condition of supporter, environmental factors, the growth medium, and essential gene products.[15,38,39] To monitor the biofilm formation of bacteria, it is required that these various factors be considered together. The results obtained from this study suggest that the analysis of MSCRAMM genes with phenotypic assays might be an essential step for bacterial control in the environment.

The ability of biofilm formation by *S. aureus* also depends on the production of polysaccharide intercellular adhesion molecules, encoded by the intercellular adhesion (*ica*) locus including the *icaA* gene, *icaB* gene, *icaC* gene, and *icaD* gene. PCR amplification of the *icaA* and *icaD* genes demonstrates the inherent biofilm producing nature of the isolates.[40] The data reported here indicate that biofilm producing clinical isolate was positive for both *icaA* and *icaD* genes. Conversely, no band was obtained from nonbiofilm-producing clinical isolate for both studied genes. Therefore, presence and association of *icaA* and *icaD* genes with the strain ability to produce biofilm strongly suggest a role of these genes in the pathogenetic mechanisms of infection associated with eye.[41] This finding should allow routine diagnostic identification of particularly virulent *Staphylococcus* strains.

Finally, our observations indicate that *Staphylococcal* biofilms from an eye lenses may participate in ocular infections by allowing bacteria to persist on abiotic surfaces that come in contact with the eye. Although, the MRSA isolates collected from contact lenses of patients were relatively susceptible to IPM and CIP, treatment of eye infection using antimicrobial drug may cause serious medical problems. This adds urgency to the search for new infection-fighting compounds to control microbial infections by eradicating biofilms. These substances could be chelating agents (EDTA) or other chemical compounds. Probably, a study of the presence and expression of genes for early and intracellular adhesion molecules, such as the MSCRAMM and ica genes, could be help in clarifying the relevance of the different adhesion mechanisms in the pathogenesis of infections associated with contact eye lenses. It could also be of value in the development of new preventive and therapeutic measures. Further studies to demonstrate this hypothesis are recommended.

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