Infection and Drug Resistance

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ORIGINAL RESEARCH

Association between biofilm formation, structure and antibiotic resistance in *Staphylococcus epidermidis* isolated from neonatal septicemia in southwest Iran

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Background: *Staphylococcus epidermidis* has emerged as the pathogen from neonatal septicemia. Antibiotic resistance and the capability of biofilm formation make these infections much harder to treat. Hence, the aim of this study was to investigate the association between biofilm formation, structure and antibiotic resistance in *S. epidermidis* isolated from neonatal septicemia.

Methods: Overall, 65 *S. epidermidis* isolates were recovered from blood cultures of neonatal septicemia. Antibiotic resistance pattern and the biofilm production were determined using phenotypic methods. The presence of *ica* operon, the *bhp*, the *aap* genes and SCC*mec* types were screened using PCR.

Results: Most *S.epidermidis* isolates were resistant to erythromycin, while all isolates were sensitive to linezolid and vancomycin. Fifty-three percent of *S.epidermidis* isolates were resistant to methicillin. SCC*mec* types II was found commonly among methicillin-resistant *S. epidermidis* (MRSE) strains. The biofilm formation was observed in 65% of S.epidermidis isolates and the majority have polysaccharide matrix. *icaA* and *icaD* genes were found in 40% and 19% of isolates. Twenty-three isolates (62%) produced dissolvable polysaccharide intercellular adhesion (PIA)-dependent biofilms in SM after growth in TSB with NaCl and 14 (37%) isolates produced dissolvable protein-dependent biofilms in PK after growth in TSB with glucose. Three isolates (62%) produced dissolvable polysaccharide intercellular adhesion.

Conclusion: Our data indicate the high rates of antibiotic resistance and the capability of biofilm formation among *S. epidermidis* isolates. Hence, the transmission of these strains can cause an increased risk of serious nosocomial infections.

Keywords: *S. epidermidis*, antibiotic resistance, biofilm formation

Introduction

Septicemia is one of the leading causes of neonatal mortality and morbidity worldwide. According to the World Health Organization report (WHO), more than 3 million newborns suffer from septicemia globally.[1] According to a previous study in Iran, the rate of neonatal septicemia has been reported between 12% and 16.7%.[2] In the last two decades, coagulase-negative staphylococci (CoNS) group especially *Staphylococcus epidermidis*, a normal flora of the skin, has emerged as a common cause of septicemia in the neonatal intensive care units (NICUs) especially in late-onset sepsis (LOS).[3] Neonatal sepsis can be considered either as

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early onset sepsis (EOS) occurring in the first 72 hours of age or LOS which follows after 72 hours of age.[4] LOS has been well recognized to be associated with prematurity, invasive interventions like intravascular catheterization, failure in early enteral feeding, prolonged antibiotic treatment, andhospitalization.[5] The presence of *S. epidermidis* on human skin may allow *S. epidermidis* to form biofilm over medical implants and easily invade the bloodstream through indwelling catheters.[6] The microorganisms attach to surfaces and produce extracellular polysaccharides, resulting in the formation of a biofilm.[7] Biofilms are a serious problem for public health because of the increased resistance of biofilm-associated organisms to antimicrobial agents by slow diffusion of conventional antibiotics through the extracellular polymeric substance and the potential for these organisms to cause infections in patients with indwelling medical devices.[7,8] The biofilm formation and the antibiotic resistance of the *S. epidermidis* isolates can be one of the important reasons for prolonging the period of treatment of infants.[9] In *S. epidermidis*, the expression of several genes is associated with the biofilm formation, including *icaABCD* locus that encodes the polysaccharide intercellular adhesion (PIA), the *bhp* gene that encodes a cell wall surface anchor protein and the *aap* gene that encodes an accumulation-associated protein.[10] Otto et al indicated that in strains that lack the ica locus, biofilm formation is due to the presence of *aap* gene, which enables bacteria to bind to various matrix proteins.[11] Recently, increasing resistance of *S. epidermidis* strains to glycopeptide agents and methicillin has spurred high interests in understanding molecular mechanisms of antibiotic resistance.[6,11] Similar to *Staphylococcus aureus*, the mechanism of methicillin resistance is mediated by the *mecA* gene which encodes penicillin binding protein 2a (PBP 2a) with reduced affinity for beta-lactam antibiotics.[12] It is believed that CoNS acts as an important reservoir of resistance-associated mobile genetic elements, which can be transferred between staphylococcal species. The *mecA* gene is located on a mobile genetic element called the staphylococcal cassette chromosome *mec* (SCC*mec*). Moreover, SCC*mec* compromises two main components: the *ccr* gene complex*ccr*and the *mec* gene complex. According to the combination of *ccr* allotypes with the *mec* gene complex, 11 types (I–XI) SCC*mec* have already been reported.[13] In Iran, there are few published literature on the association between biofilm formation, structure, antibiotic resistance as well as SCCmec typing in *S. epidermidis* isolated. Hence, the aim of this study was to investigate the association between biofilm formation, structure and antibiotic resistance in *S. epidermidis* isolated from neonatal septicemia.

Methods and materials

Ethics

This research was conducted according to the Helsinki Declaration. This study was approved by the Research Ethics Committee of the Ahvaz Jundishapur University of Medical Sciences (No: IR.AJUMS.REC.1395. 332), Ahvaz, Iran, after submission of the preliminary proposal, and necessary permission for sample collection was granted. The study was accepted by the Imam Khomeini hospital data protection authority. After having read the information letter concerning the study, all respondents were asked for oral and written consent to participate. We emphasized that participation was voluntary and that parents could withdraw from the research at any time.

Sample collection

A total of 130 nonduplicate CoNS isolates were consecutively collected from April 2016 to February 2017 from blood cultures of 521 neonates with suspected septicemia hospitalized in the NICU, Imam Khomeini hospital. At least 1 mL of blood was collected from the peripheral vein of a neonate with suspected sepsis before starting antibiotics, either by a needle or a new cannula, using aseptic methods. The inclusion criteria of septicemia caused by CoNS were as follows: clinical signs of sepsis in a neonate older than 3 days of age, positive monomicrobial blood culture and elevated CRP >10 mg/L within 2 days of blood culture. Alternatively, CoNS blood cultures growing more than one organism were considered as contaminants.

Bacterial isolation

First, the isolates were subcultured on blood agar (EMD Millipore, Billerica, MA, USA) and then, the single colony was inoculated on Mannitol salt agar at 37°C for 24 hours (EMD Millipore) to identify *S. epidermidis* colonies. Then, suspicious colonies were subjected to biochemical tests, including gram staining, catalase, tube-coagulase, DNase and novobiocin susceptibility test (MAST Diagnostics, Merseyside, UK).[14] The isolates were confirmed as *S. epidermidis* using the amplification of the *sesC* gene.[15] *S. epidermidis* RP62A strain was used as positive control and distilled water as a negative control in all PCR reactions.

Investigation of susceptibility to antimicrobial agents

Antibiotic susceptibility testing was performed for 17 drugs covering all the nine antimicrobial categories comprising aminoglycosides, ansamycins, fluoroquinolones, folate pathway inhibitors, tetracyclines, glycopeptides, oxazolidinones, macrolides and incosamidesl were determined using the disc diffusion susceptibility test according to clinical and laboratory standards institute (CLSI) guidelines. Commercial antibiotic discs of rifampin (5 μg), linezolid (30 μg), ciprofloxacin (5 μg), levofloxacin (5 μg), minocyclin (30 μg), doxycycline (30 μg), tetracycline(10 μg), gentamycin (10 μg), tobramycin (10 μg), amikacin(1 μg), erythromycin(15 μg), azithromycin (15 μg), clarithromycin (15 μg),clindamycin (2 μg) trimethoprim/sulfamethoxazole (1.25/23.75 μg),(MAST Diagnostics, Merseyside UK) were used in disc diffusion test. Then, MDR/XDR/PDR phenotype of these isolates was established according to the results obtained from the disc diffusion test. Briefly, multidrug-resistance (MDR) was defined as resistance to at least three or more different classes, extensive drug-resistance (XDR) was defined as resistance to at least one agent in all but two or fewer antimicrobial categories and pan drug-resistance (PDR) was defined as resistance to all agents in all antimicrobial categories.16,17

Screening for vancomycin resistance

Resistance to vancomycin (MAST Diagnostics) was prepared by Mueller–Hinton agar (Merck, Germany) containing vancomycin 6 μg/mL and 4% NaCl. The plates were incubated at 37°C for 24 hours according to CLSI guidelines. Any visible growth after 24 hours was considered vancomycin resistant.

Screening for methicillin resistance

All of the *S. epidermidis* strains were tested for susceptibility to methicillin using a cefoxitin (30 μg) disc. Results were interpreted according to the criteria established by the CLSI. The presence of the *mec*A gene was evaluated using PCR amplification, as previously described by Shrestha et al.[17] *S. aureus* ATCC 29213 strains were used as a positive control and distilled water as negative control.

Screening for of SCC*mec* elements

The presence of SCC*mec* genes in *S. epidermidis* strains were checked using PCR amplification, as previously explained by Moosavian et al.[18] Five MRSA strains, NCTC10442 (SCC*mec* I), NCTC N315 (SCC*mec* II), NCTC 85/2082 (SCC*mec* III), NCTC CA05 (SCC*mec* IVa), and JCSC3624 (SCC*mec* V) were used as a positive control and distilled water as negative control.

Biofilm formation in 96-well microtiter plate

The biofilm formation capacity of isolates was evaluated using the crystal violet staining method. First, these isolates were inoculated in Mueller–Hinton agar at 37°C overnight. Then, these isolates were adjusted to 0.5 McFarland (~1.5×10^8 CFU/mL) with normal saline (0.85% NaCl). A 10-μL aliquot of each suspension was then diluted 1:200 in 190 μL of tryptic soy broth (TSB) containing 1% glucose in 96-well polystyrene microtiter plates. Following incubation at 37°C overnight, the plates were washed three times with PBS, fixed by adding 200 μL of methanol into each well, and stained with 200 μL of 0.1% crystal violet (CV) for 20 minutes. The plates were again washed three times to remove excess stain, and the remaining CV was solubilized by incubating with 200 μL of 95% ethanol for 10 minutes. The optical density at 570 nm (OD570) of each well was measured by the ELISA plate reader (μQuant; BioTek Instruments, Winooski, VT, USA), to evaluate the biofilm formation capacity. *S. epidermidis* ATCC 35984 and TSB broth were used as positive and negative controls (ODc) for the biofilm formation, respectively. The results were interpreted according to the criteria suggested by Zhang et al. Briefly, the isolates were classified into the several groups about the biofilm formation capacity: $OD570\leq ODc$=no biofilm producer; $ODc<OD570\leq 2\times ODc$=weak biofilm producer; $2\times ODc<OD_{570}\leq 4\times ODc$=moderate biofilm producer; and $4\times ODc<OD_{570}$=strong biofilm producer.[19] All experiments were performed in triplicate.

Congo red agar test

Congo red agar (CRA) medium was composed of brain heart infusion broth (BHI, Oxoid; Thermo Fisher Scientific, Waltham, MA, USA) supplemented with NaCl 1.5%, sucrose 5% and 0.08% Congo red.[20] The inoculated CRA plates were incubated at 37°C overnight. Then, isolates were interpreted according to their colony phenotypes. Black colonies were indicative of biofilm production, while red colonies were considered as the nonbiofilm producers. *S. epidermidis* ATCC 35984 and *S. epidermidis* ATCC 12228 strains were used as biofilm-positive and biofilm-negative controls, respectively.

Biochemical characterization of the biofilm matrix

To determine the biochemical characterization of the biofilm matrix, first the biofilm formation was induced as described above. Then, all wells were washed with PBS and treated for 1 hour at37°C either with a solution of 10 mM sodium metaperiodate in 50 mM sodium acetate buffer (pH 4.5) to disrupt the extracellular polysaccharides, or with 100 µg/ml of proteinase K (SinaClon, Tehran, Iran) in 20 mM Tris (pH 7.5) and 100 mM NaCl to disrupt the biofilm extracellular protein. Subsequently, the biofilms were washed, fixed and stained with CV, and the optical absorbance (570 nm) measured as described above.[21]

Detection of genes involved in biofilm formation

All *S. epidermidis* isolates were screened using PCR for the presence of the *ica* operon (*icaA, icaB, icaC, icaD*), the *bhp* and the *aap* genes using primers and conditions as previously described. *S. epidermidis* RP62A was positive control for ica operon and *S. epidermidis* 1457 Δica was positive control for *aap* and *bhp* genes.[22]

Results

In this study, 215 out of 512 neonates with suspected septicemia had positive blood cultures. Of these 215 cases, 135(62.79%) CoNS strains were isolated from blood cultures. Moreover, of the 135 CoNS isolates, 65 (48.14%) isolates were confirmed as *S. epidermidis* using biochemical tests and PCR. Demographic and clinical features at the time of presentation of neonatal had septicemia confirmed by S. *epidermidis* are shown in Table 1. The resistance to methicillin in *S. epidermidis* was recognized by cefoxitin disk in 39 (60%) isolates and the amplification of *mecA* gene in 35(53%) isolates. The distribution of SCC*mec* types in the 35

Methicillin-resistant *S. epidermidis* (MRSE) isolates showed that SCC*mec* types II and III dominated among the tested isolates (Table 2). Of the 35 MRSE isolates, 16 (45%) carried type II SCC*mec*, 11 (31%) carried type III SCC*mec* and 4(11%) carried type I and IV SCC*mec* respectively. None of the tested isolates had type IV and two isolates were untypeable by the routine PCR assays used (Table 2). According to antibiogram results, the majority of S. *epidermidis* were resistance to the antibiotics used (Figure 1). The maximum resistance was found to erythromycin (81%), clindamycin and amikacin (52%), gentamicin (46%), ciprofloxacin (44%), tobramycin (33%), tetracycline (24%), clarithromycin (16%), rifampin and minocycline (15% each), azithromycin and trimethoprim-sulfamethoxazole (13%), doxycycline (12%), levofloxacin (10%). Also, all isolates were sensitive to linezolid and vancomycin.

Twenty-one (32.30%) of the *S. epidermidis* isolates were MDR and 37 (56%) were XDR. None of our isolates were PDR. The resistance rates to ciprofloxacin, Erythromycin, gentamicin, and clindamycin were found to be extremely high among the MRSE isolates compared to those that were methicillin-susceptible *S. epidermidis* (MSSE).

In the CRA assay, 32(49%) isolates were considered as producing biofilm and produced black colonies whereas 33 (50%) isolates produced red colonies. The biofilm production analysis by MTP method differentiated isolates into strong, moderate, weak, and nonbiofilm-forming according to the OD values at 570 nm. Again, 37of the isolates 17 (45%) were categorized as strong biofilm-formers; 12 isolates (32%) were moderate; 8(21%) were weak biofilm-formers (48%); and 28 (43%) could not form any detectable biofilm. The OD570 values for the reference strain (ATCC 35,984) and negative control were 0.416±0.048 and 0.074 ±0.010, respectively. The composition of the biofilm matrix of 23(62%) *S. epidermidis* isolates were polysaccharide and 14 (37%) isolates were protein (Table 2). In the 17 strong biofilm-formers, 13(76%) isolates were XDR and 4(23%) isolates were MDR. Among the 12 moderate biofilm-formers, 6(50%) isolates were XDR and 5(41%) isolates were MDR. Also, of the eight weak biofilm-formers, 4

Table 1 Demographic and clinical features at the time of presentation of neonatal septicemia

Characteristic		Number of neonates (%)
Gender	Male,Female	28 (43.4),37 (56.5)
Weight	>2500 g,1500–2500 g,<1500 g	0 (0.0),30 (46),35 (53)
Gestational age	Term (>37 weeks),Preterm (<37 weeks)	15 (23),50 (76.0)
Route of delivery	Normal vaginal delivery,cesarean section	11 (60.6),54 (83)

Table 2 Biofilm formation structure antibiotic resistance and biofilm related genes in all *S. epidermidis* isolat

Sample ID	CRA	MTP	*MecA*	SCC typing	*bhp*	*aap*	*ica*	MDR	XDR	Antimicrobial resistance pattern	SM	PK
S1	P	Moderate	+	IV	–	+	*ica*A, *ica*B, *ica*D		+	MN, CD, E, T, FOX	D	Nd
S2	P	Weak	–		–	+	*ica*A, *ica*B, *ica*D	+		E, RP, FOX	D	Nd
S3	N	N	–	IV	+	–				E, TN, AM	Nd	Nd
S4	P	Strong	–		–	+	*ica*A, *ica*B, *ica*D		+	CIP, MN, CD, E, RP, GN, T	D	Nd
S5	P	Strong	+	IV	–	+	*ica*A, *ica*B, *ica*D, *ica*C	+		E, ATH, FOX	D	Nd
S6	N	N	–		+	–	*ica*A, *ica*D			CD	Nd	Nd
S7	P	Weak	–		–	+	*ica*A, *ica*D			E	D	Nd
S8	P	Moderate	–		–	+	*ica*A, *ica*D	+		CIP, MN, CD	D	Nd
S9	P	Moderate	–		–	+	*ica*A, *ica*D			CD	D	Nd
S10	N	N	–	II	+	+		+		CIP, TN, ATH, CLA	Nd	Nd
S11	N	N	–		–	–	*ica*A, *ica*D	+		E, GN, ATH, FOX	Nd	Nd
S12	P	Strong	–		–	+	*ica*A, *ica*B, *ica*D, *ica*C	+		CIP, E, GN, ATH, T	D	Nd
S13	N	N	–	I	–	-			+	CIP, MN, CD, RP, GN, AM	Nd	Nd
S14	P	Moderate	+	II	–	+	*ica*A, *ica*B, *ica*D, *ica*C		+	E, RP, TN, AM, ATH, FOX	D	Nd
S15	N	N	–		–	-	*ica*A, *ica*D	+		CIP, E, AM, ATH	Nd	Nd
S16	P	Weak	–		–	+	*ica*A, *ica*D	+		E, GN, TN, T	D	Nd
S17	N	N	–		–	-	*ica*A, *ica*D			CIP, TN, AM	Nd	Nd
S18	P	Strong	+		–	+	*ica*A, *ica*B, *ica*D, *ica*C		+	CIP, TN, AM, ATH, T, FOX	D	Nd
S19	N	N	+	II	–	-			+	E, GN, FOX	Nd	Nd
S20	N	N	–		–	-	*ica*A, *ica*D	+		CD, E, GN, AM, ATH	Nd	Nd
S21	P	Weak	+	III	–	+	*ica*A, *ica*C		+	E, GN, TN, AM, FOX	Nd	D
S22	P	Strong	+	II	–	+	*ica*A, *ica*B, *ica*D, *ica*C		+	CIP, MN, CD, RP, GN, TN, ATH, FOX	D	Nd
S23	N	N	–		–	–		+		CD, AM, ATH	Nd	Nd
S24	N	N	–		+	–			+	CIP, CD, E, T, FOX	Nd	Nd
S25	P	Moderate	+	I	–	+	*ica*A, *ica*B, *ica*D, *ica*C		+	E, GN, ATH, FOX	D	Nd
S26	P	Moderate	–		–	+		+		CIP, CD, E, TN, TS	Nd	D
S27	N	N	–		–	+		+		E, AM, ATH	Nd	Nd
S28	P	Moderate	–		–	+	*ica*A, *ica*C	+		CD, E, TN, TS	Nd	D
S29	P	Strong	+	II	–	+	*ica*A, *ica*B, *ica*D, *ica*C		+	CIP, MN, CD, E, RP, GN, TN, AM, Lev, FOX	Nd	D
S30	N	N	–		–	–		+		E, GN, Lev, ATH, CLA	Nd	Nd
S31	P	Strong	+	I	–	+			+	CIP, CD, E, RP, AM, Lev, FOX	D	Nd
S32	N	N	+	III	–	+			+	E, GN, Lev, CLA, DXT, RP, FOX	Nd	Nd
S33	P	Strong	+	II	–	+	*ica*A, *ica*B, *ica*D, *ica*C		+	CIP, CD, E, RP, GN, AM, FOX	Nd	D
S34	N	Moderate	–		–	–	*ica*A, *ica*D	+		E, GN, TN, Lev, TS	D	Nd
S35	P	Strong	+	III	–	–	*ica*A, *ica*B, *ica*D, *ica*C		+	E, GN, TN, AM, Lev, FOX	D	Nd
S36	P	Moderate	+	II	–	+			+	CIP, CD, E ,GN, TN, AM, Lev, FOX	Nd	D

(*Continued*)

Table 2 (Continued).

Sample ID	CRA	MTP	*MecA*	SCC typing	*bhp*	*aap*	*ica*	MDR	XDR	Antimicrobial resistance pattern	SM	PK
S37	N	N	–		+	–		+		E, GN, AM, DXT, FOX	Nd	Nd
S38	P	Strong	+	I	–	–	*ica*A, *ica*B, *ica*D, *ica*C		+	CIP, MN, CD, E, RP, GN, TN, AM, Lev, CLA, FOX	D	Nd
S39	P	Moderate	+	II	–	+	*ica*A, *ica*B, *ica*D, *ica*C		+	E, TN, AM, FOX, TS	Nd	D
S40	P	Moderate	–		–	+	*ica*A, *ica*B	+		CIP, CD, E	Nd	D
S41	N	N	+	II	–	–			+	CD, E, AM, T, DXT, FOX	Nd	Nd
S42	N	N	+	II	–	+			+	CIP, CD, E, GN, AM, FOX	Nd	Nd
S43	N	N	+	III	–	–			+	CIP, CD, E, GN, AM, FOX	Nd	Nd
S44	N	N	+	II	+	+	*ica*A, *ica*D		+	CD, E, AM, Lev, T, FOX	Nd	Nd
S45	N	N	+	III	–	+			+	CIP, E, AM, T, FOX	Nd	Nd
S46	N	N	+	IV	+	+	*ica*A, *ica*D		+	CIP, MN, GN, TN, AM, FOX	Nd	Nd
S47	P	Strong	+	II	–	–	*ica*A, *ica*B, *ica*D		+	CIP, CD, E, GN, TN, AM, CLA, FOX, TS	D	Nd
S48	N	Weak	–		–	–	*ica*A, *ica*D			E, AM	D	Nd
S49	N	N	+	II	+	–			+	CD, E, TN, FOX	Nd	Nd
S50	N	Weak	+	III	–	+	*ica*A, icaB, *ica*D, *ica*C		+	CIP, CD, E, GN, TN, AM, FOX	Nd	D
S51	P	Strong	+		–	+	*ica*A, *ica*B, *ica*D, *ica*C		+	E, GN, TN, FOX, TS	Nd	D
S52	N	N	+	III	+	+			+	CD, E, TN, T, FOX	Nd	Nd
S53	P	Strong	+	III	–	+	*ica*A, *ica*D		+	CIP, CD, AM, CLA, DXT, FOX	D	Nd
S54	N	Weak	–		–	+				CD, E	Nd	D
S55	P	Strong	+	II	–	+	*ica*A, *ica*D		+	CIP, CD, E, GN, AM, Lev, CLA, DXT, FOX	D	Nd
S56	N	N	–		–	–	*ica*A	+		E, AM, T	Nd	Nd
S57	P	Strong	–		+	–	*ica*A ,*ica*B, *ica*D	+		CD, E, AM, CLA	D	Nd
S58	N	N	+	II	–	–			+	CIP, MN, CD, E, GN, FOX, TS	Nd	Nd
S59	P	Strong	+	III	–	–	*ica*A, *ica*D		+	E, GN, AM, CLA, DXT, FOX	D	Nd
S60	N	N	–		–	+		+		CIP, CD, E, T	Nd	Nd
S61	P	Strong	–		–	+		+		E, GN, AM, T N, C LA , T, D XT, TS	Nd	D
S62	N	N	+	III	–	–			+	CD, GN, DXT, FOX	Nd	Nd
S63	P	Moderate	+	III	–	+	*ica*A, *ica*D		+	CIP, CD, E, GN, T, FOX, TS	Nd	D
S64	N	Weak	+		–	+	*ica*A, *ica*D		+	E, GN, CLA, T, FOX	Nd	D
S65	N	N	+	II	–	–			+	CIP, MN, CD, E, GN, AM, T, FOX	Nd	Nd

Note: +/positive, -/negative.

Abbreviations: *mec*A, methicillin-resistant gene; SCC, staphylococcal cassette chromosome; MDR, multidrug resistance; XDR, extensive drug resistance; *ica*, intercellular adhesion; *aap*, accumulation-associated protein; *bhp*, biofilm associated protein; AM, amikacin; GN, gentamicin; FOX, cefoxitin; CIP, ciprofloxacin; E, erythromycin; T, tetracycline; TS, sulfamethoxazole-trimethoprim; ATH, azithromycin; CLA, clarithromycin; LZD, linezolid; RP, rifampin; DXT, doxycycline; MN, minocycline; TN, tobramycin; CD, clindamycin; Lecv, levofloxacin; V, vancomycin; MTP, microtiterplate; P, positive; N, negative; CRA, Congo red agar; SM, sodium metaperiodate; PK, proteinase K; D, dissolve; Nd, Not dissolved.

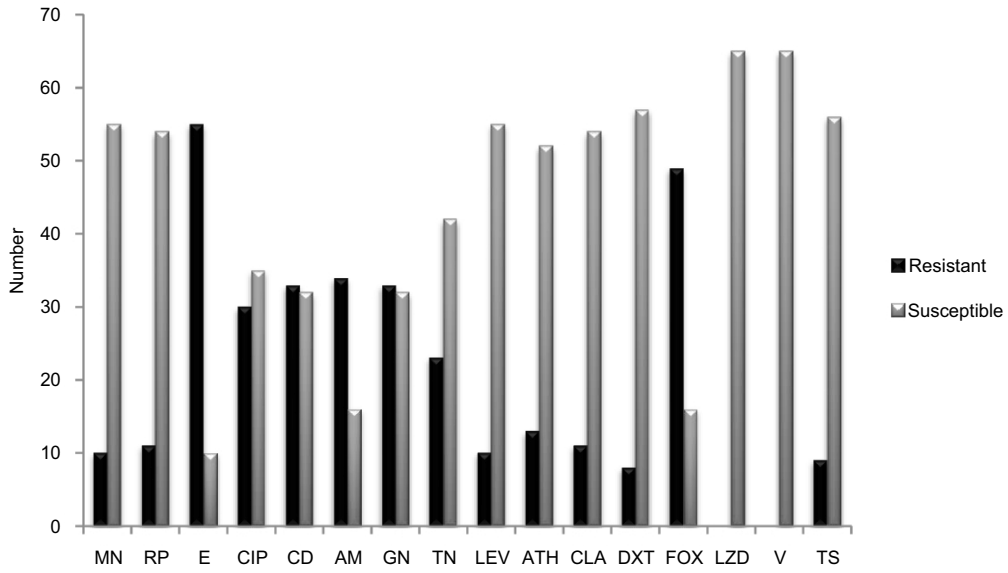

Figure 1 Antibiotic resistance rate among all 65 *S.epidermidis* isolated.
Abbreviations: AM, amikacin; GN, gentamicin; FOX, cefoxitin; CIP, ciprofloxacin; E, erythromycin; T, tetracycline; TS, sulfamethoxazole-trimethoprim; ATH, azithromycin; CLA, clarithromycin; LZD, linezolid; RP, rifampin; DXT, doxycycline; MN, minocycline; TN, tobramycin; CD, clindamycin; Lecv, levofloxacin; V, vancomycin.

(50%) isolates were XDR and 2(25%) isolates were MDR (Table 2). The biofilm producers were more resistant to all antibiotics used to expect linezolid and vancomycin than biofilm nonproducers. The correlation of biofilm-forming capacity and antibiotic resistance is summarized in the (Table 3) (P<0.001). The distribution of SCC*mec* types

Table 3 Correlation between the level of biofilm formation and antibiotic resistance in 65 *S. epidermidis* isolates

Antimicrobial category	Antimicrobial agent	Optical density$_{570}$		*P*-value
		Susceptible	Resistance	
Aminoglycosides	Gentamicin Tobramycin Amikacin	0.166 (0.260, 0.534) 0.270 (0.138, 0.443) 0.120 (0.097, 0.254)	0.230 (0.090, 0.119) 0.215 (0.065, 0.100) 0.312 (0.063, 0.100)	<0.001 <0.001 <0.001
Ansamycins	Rifampin Ciprofloxacin	0.150 (0.156, 0.479) 0.138 (0.123, 0.456)	0.142 (0.188, 0.113) 0.200 (0.088, 0.120)	<0.001 <0.001
Fluoroquinolones	Levofloxacin Azithromycin	– –	0.376 (0.188, 0.169) 0.200 (0.088, 0.117)	<0.001 <0.001
Macrolides	Clarithromycin Erythromycin	– 0.179 (0.100, 0.320)	0.303 (0.087, 0.119) 0.313 (0.130, 0.375)	<0.001
Incosamidesl	Clindamycin	0.237 (0.404, 0.537)	0.437 (0.260, 0.498)	<0.001
Folate pathway inhibitors	Trimethoprim/sulfamethoxazole		0.325 (0.436, 0.145)	<0.001
Oxazolidinones Tetracycline	Linezolid 0.196 (0.238, 0.468)	0.118 (0.195, 0.357) 0.125 (0.085, 0.112)	– <0.001	0.455
Tetracyclines	Doxycycline Minocycline	– 0.278 (0.010, 0.444)	0.213 (0.087, 0.118) 0.290 (0.089, 0.121)	<0.001 <0.001
Glycopeptides	Vancomycin	0.178 (0.277, 0.404)	–	<0.001

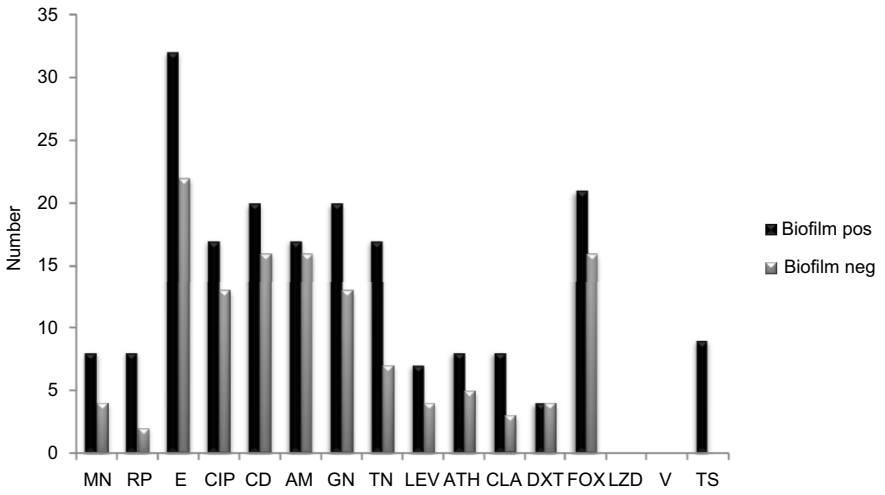

Figure 2 Comparison of antimicrobial susceptibilities between biofilm and nonbiofilm producing *S. epidermidis* isolated.
Abbreviations: AM, amikacin; GN, gentamicin; FOX, cefoxitin; CIP, ciprofloxacin; E, erythromycin; T, tetracycline; TS, sulfamethoxazole-trimethoprim; ATH, azithromycin; CLA, clarithromycin; LZD, linezolid; RP, rifampin; DXT, doxycycline; MN, minocycline; TN, tobramycin; CD, clindamycin; Lecv, levofloxacin; V, vancomycin; Biofilm pos, biofilm positive; Biofilm neg, biofilm negative.

among *S. epidermidis* isolates of biofilm producers and non-biofilm producers is shown in Figure 2. According to these results, SCC*mec* type II was commonly found among biofilm producers Figure 3. The presence of *icaA, icaB, icaC, icaD, bhp*, and *aap* genes was confirmed by PCR among 40(61%), 19(29%), 12(8%), 35(53%), 10(15%), and 36(55%) isolates, respectively (Table 2). Among 37 biofilm producing strains harbored *icaA* and *icaB, icaC*, and *icaD* genes respectively and (81%) and (24%) of strains were positive for *aap* and *bhp* genes. Furthermore, in nonbiofilm producing strains, 21%, 17%, and 30% of the isolates carried *icaA, icaB*, and *icaD* genes. *icaC* was not detected in nonbiofilm producing strains. Table 2 summarizes the antibiotic resistant, adhesions analysis of the genetic factors and biofilm production in *S. epidermidis* isolates. We assessed the relationship between biofilm formation capacity and genes involved in this process (Table 4). Out of 17 strong biofilm-formers, 88% strains were positive for the *icaA* and *icaD* genes while 60%, 52%, 60%, and 11% were positive for the *icaB, icaC, aap*, and *bhp* genes respectively. Also, among 12 moderate biofilm-formers, 10 (83%), 5 (41%), 4 (33%), 8 (66%), 1 (8%), and 11 (91%) strains were positive for the *icaA,caB, icaC, icaD, aap*, and *bhp*. Alternatively, out of eight weak biofilm-formers, 2(25%) strains were positive for the *icaB* and *icaC* genes, followed by the *icaA* 7(78%), *icaD* 6 (75%), *aap* 6(75%), and weak biofilm-formers did not harbor *bhp* gene. There is a significant association between the biofilm formation and genes encoding *ica* operon, and

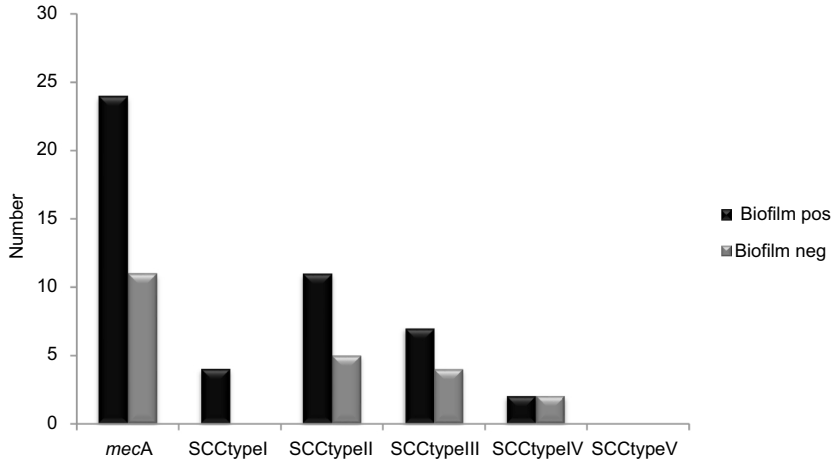

Figure 3 Distribution of SCC typing in *S. epidermidis* biofilm producer and nonbiofilm producer.
Abbreviations: *mec*A, methicillin-resistant gene; SCC, staphylococcal cassette chromosome; Biofilm pos, biofilm positive; Biofilm neg, biofilm negative.

Table 4 The pattern of the genes that make up biofilms is based on the biofilm capacity

Strong biofilm formation	*aap*	2
	icaA, icaB, *icaD*	3
	icaA, *icaB*, *icaC*, *icaD*, *aap*	5
	icaA, *icaB*, *icaC*, *icaD*, *aap*	1
	icaA, *icaB*, *icaD*, *icaC*, *aap*, *Bhp*	1
	icaA, *icaD*	1
	icaA, *icaB*, *icaD*, *icaC*	2
	icaA, *icaD*, *aap*	2
Moderate biofilm formation	*icaA*, *icaD*	1
	icaA, *icaD*, *aap*	3
	icaA, *icaC*, *aap*	1
	Aap	2
	aap, *icaA*, *icaD*	1
	icaA, *icaB*, *icaD*, *icaC*, *aap*	3
	icaA, *icaD*, *aap*	1
	icaA, *icaB*, *icaD*, *icaC*, *aap*	1
	icaA, *icaD*, *aap*	1
Weak biofilm formation	*icaA*, *icaB*, *icaD*, *icaC*, *aap*	1
	icaA, *icaD*, *aap*	1
	icaA, *icaB*, *icaD*, *aap*	1
	icaA, *icaD*, *aap*	2
	icaA, *icaC*, *aap*	1
	icaA, *icaD*	1
Nonbiofilm formation	*aap*	7
	icaA, *icaD*	4
	icaA, *icaD*	1
	icaA, *icaD*, *aap*	1
	icaA, *icaD*, *aap*	1
	icaA	1

aap gene ($P<0.001$). The presence of all genes involved in biofilm formation among biofilm positive strains was significantly higher than nonbiofilm-producing strains (Figure 4).

Discussion

Bacteria present in human skin thus have the opportunity to control cell behaviors below the surface. Examples of beneficial functions induced by specific skin bacteria include the capacity[23] Also, a prior study has described that *S.epidermidis* induces the secretion of antimicrobial peptide, which increased the capacity of cell lysates to inhibit the growth of group A *Streptococcus* and *S.aureus*. Nevertheless, *S.epidermidis* biofilm-associated infections are increasing the use of indwelling or implanted medical devices. These often can spread into the bloodstream and cause nosocomial sepsis.[23,24] In this study, the prevalence of septicemia associated with CoNS in the neonatal population during one year was 62.8%. In agreement with our study, the incidence of septicemia associated with CoNS evaluated in other studies ranged from 34% to 48%.[25,26] The incidence of LOS increases up to 50% in the immature preterm infants and very low birth weight (VLBW) infants have a high danger of septicemia associated with CoNS.[27] In our study, the clinical isolate of *S. epidermidis* was more common in neonatal have immature preterm infants and in very low birth weight (<1500 g) (Table 1).

In the present study, the high prevalence of MRSE in the NICUhighlighted the importance of a suitable choice

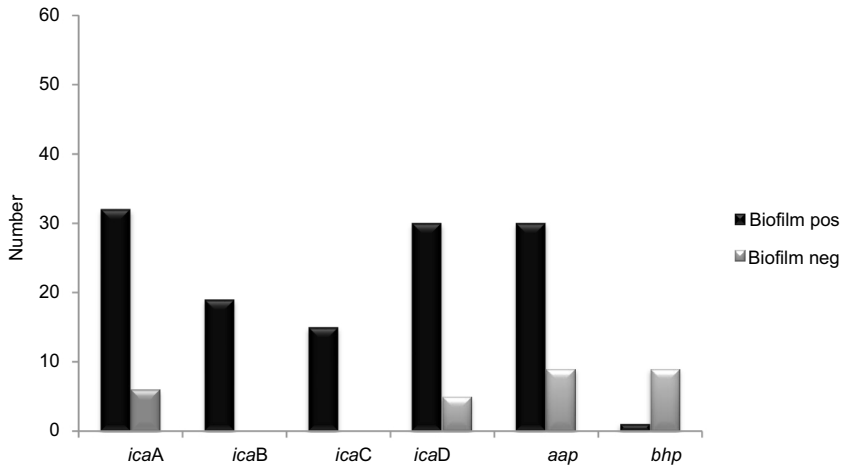

Figure 4 Distribution of biofilm formation genes among biofilm producer and nonbiofilm producer *S. epidermidis* isolated.
Abbreviations: *ica*, intercellular adhesion; *aap*, accumulation-associated protein; *bhp*, biofilm associated protein; Biofilm pos, biofilm positive; Biofilm neg, biofilm negative.

of antibiotic therapy and to develop treatments against *S. epidermidis* infections. Earlier studies have shown that the proportion of MRSE in Iran, with an incidence of 31% to 89%.[28,29] Due to the wide variation seen in the different reports in Iran it could be in part due to different populations and geographical locations, and the quality of hospital sampling carried out. Four of the MRSE isolated appeared susceptible to cefoxitin but were negative to harbor *mecA*. Such strains are perhapsvery heterogeneous in their expression of methicillin resistance. Also a previous study reported the complete absence of five major SCC*mec* types and *mecA* gene as well as the gene product of PBP2a in isolates which were phenotypically MRSA suggesting a probability of hyper production of β-lactamase as a cause of the phenomenon.[30] In agreement with this study, another study has shown most *S. epidermidis* isolates were resistant to aminoglycoside, fluoroquinolone, and macrolide agents.[31] In our study, 56.3% of *S. epidermidis* isolates were XDR (≥3 antibiotic classes). Considering the presence of XDR *S. epidermidis* in NICUcan cause infection and would be more complicated to treat. In our study, the majority of the *S. epidermidis* isolated had the ability of biofilm production but with different capacities. The mechanisms responsible for antimicrobial resistance in organisms producing biofilms may be one or more of the following, such as the poor diffusion of the antimicrobial penetration through the biofilm extracellular matrix, the different growth rate of biofilm organisms, etc. Thus, the ability to form biofilm could be an effective strategy to enhance the survival and persistence under stressed conditions like host invasion or antibiotic treatment.[32–35] Confirming these, in our study; we found a significant correlation between the capacity of biofilm formation and antibiotic resistance ($P<0.001$). In other words, the biofilm density in *S. epidermidis* resistance strains was more than susceptible strains. Inconsistent with our study, some researchers demonstrated that the resistant isolates were stronger producers of biofilm than the susceptible isolates.[16] Together, in this study, the evaluation and comparison of biofilm formation between nonMDR and MDR/XDR have shown that the majority of the MDR/XDR isolates have a significantly higher capacity to form biofilms compared to nonMDR isolated. In *S. epidermidis* isolates, the *ica* operon appears to play an essential role in biofilm formation. As found by other authors, our data indicate that the prevalence of the *ica* operon in *S. epidermidis* isolates from neonatal septicemia was 61.50%.[36,37] In our study, even though a significant difference was found among biofilm producers and nonproducers in the *bap* gene (24% vs 2.7%). This percentage is significantly lower than previously reported.[38] Biofilm formation is also associated with the presence of *ica* operon and *aap* genes which is responsible for the production of the PIA and proteinaceous structure of biofilm respectively. Previous studies have shown that the biofilm formation in staphylococci is associated with the presence of both *icaA* and *icaD* genes.The expressions of these genes are essential for the full phenotypic expression of biofilm in clinical staphylococcal isolates.[39,40]

However, in accordance with our findings which demonstrated that the presence of the ica operon was not always associated with biofilm production. Infact four biofilm-producing isolates did not carry the *ica* operon but indicated the ability of biofilm formation. In conclusion, to our knowledge, recognized risk factors for postpartum septicemia may not always be present, and signs of severe septicemia may be masked or present atypically. In this study, we report a high prevalence of virulence/antimicrobial resistance determinants in *S. epidermidis* from neonatal septicemia, although the reasons for the increases of invasive *S.epidemidis* infection are unclear because it has different reasons. Environmental factors that influence *S.epidemidis* septicemia such as hygiene, maternity setting, health-care workers, social and family contacts, etc, and the host and the pathogen factors affecting the transmission of *S.epidemidis*. However, processes of infection control including identifying patients at risk of nosocomial infections, observing hand hygiene, include identifying sources of organism, identification of organisms, isolation if required, antibiotic prophylaxis to be used selectively following standard precautions to reduce transmission and strategies to reduce infections. Fortunately, the result of antibiotic resistance in *S. epidermidis* isolates demonstrated thatlinezolid and vancomycin showgood effects in treatment. Furthermore, a high rate of prevalence of biofilm-forming strains among the tested *S. epidermidis* isolates was detected, but, *S. epidermidis* forms a prototypic biofilm, and that biofilm formation in vitro does not necessarily correlate with biofilm formation in vivo.

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Author contributions

All authors contributed to data analysis, drafting or revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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