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STUDY OF SOME VIRULENCE FACTORS AND ANTIMICROBIAL RESISTANCE IN COAGULASE-NEGATIVE STAPHYLOCOCCI ISOLATED FROM NEWBORNS IN MANSOURA HOSPITALS

[3]Eman M Rabie Shehab El-Din, *,[1]Mohamed M Adel El-Sokkary, [1]Ramadan Hassan and [2]Mohamed Reda Bassiouny

[1]Department of Microbiology and Immunology, Faculty of Pharmacy, Mansoura University, Egypt
[2]Department of Pediatrics, Faculty of Medicine, Mansoura University, Egypt
[3]Unit of Drug Analysis, Mansoura University, Mansoura, Egypt

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ABSTRACT

A prospective study was performed at NICUs of three Mansoura hospitals in Egypt over a period of 18 months from March 2011 to August 2012. The three NICUs are participants in Egyptian Neonatal Network (EGNN).344 newborns were diagnosed as suspected sepsis. 58.57% of 140 positive blood cultures were due to Gram positive pathogens. *Staphylococcus epidermidis* was the most common species (33 isolate), followed by *S. haemolyticus* (20). Antimicrobial resistance could have a substantial impact on healthcare by adversely affecting morbidity and mortality. 51.7% of all strains were multidrug-resistant. Oxacillin resistance was detected phenotypically in 85% (51/60) of the staphylococcal isolates, whilst genotypically, *mec A*gene was found in 88.3% (53/60) of the isolates. Characterization of SCC*mec* in MR-CoNS can generate useful information on the mobilization and evolution of this element which is epidemiologically important for infection control. Using multiplex PCR, only 37.7% of the *mec A*-positive strains could be assigned into known SCC*mec*. Among them, 12 had a single type, including type II (n = 4), V (n = 4), III (n = 3) and IV (n = 1), while 7 isolates had two types, II+IV (n= 2), II+V (n= 2), III+V (n= 2) and I+III (n= 1), and one isolate had three types including I+III+V. SCC*mec* types V, II and III (alone and combined) were the most prevalent among MR-CoNS isolated from neonatal sepsis cases. SCC*mec* types II and III were the most prevalent among *S. epidermidis*, while among *S. haemolyticus* isolates, was type V. Vancomycin is still the most effective antimicrobial agent against staphylococci isolates in NICUs, in addition to amikacin and imipenem and ciprofloxacin. Biofilm production and phenol soluble modulins (PSMs) are considered the main factors contributing to their virulence. Biofilm production was positive in 44 isolates (73.3%) with a different adherence strengths. *Psm* β was found in 60% of all isolates, 86.1% (31/36) of them were *S. epidermidis*. Hemolysin production was mainly observed in *S. haemolyticus* isolates.

INTRODUCTION

Staphylococcus genus is one of the disease associated pathogens, usually identified in neonatal sepsis. They are the most important cause of life-threatening bloodstream infections in the United States and in some European countries (Mehta *et al.*, 2009). Staphylococci, especially CoNS strains, have a great impact in the environment of neonatal intensive care units (NICUs) (Cheung and Otto, 2010). Many factors including the immature immune system of the neonates, the use of invasive procedures, aggressive antibiotic therapy protocols, and longer hospital stay enhance the development of CoNS infections among hospitalized neonates (Srivastava and Shetty, 2007; Walz *et al.*, 2010). In addition, colonization with CoNS is a risk factor associated with neonatal infection (D'Angio *et al.*, 1989). *S. aureus* is the most virulent species, in contrast to most staphylococci (CoNS) that are commonly benign commensals and only occasionally cause disease. Over the last decades, the isolated strains have increasingly become multi-resistant to antibiotics, (Paradisi *et al.*, 2001). Rates of methicillin resistance up to 80% have been observed among CoNS isolated from bloodstream infections in NICU

Corresponding author: Mohamed M Adel El-Sokkary
Department of Microbiology and Immunology, Faculty of Pharmacy, Mansoura University, Egypt

(Hira *et al.*, 2007). Resistance to oxacillin could have a substantial impact on healthcare by adversely affecting morbidity and mortality. MR-CoNSmay be considered as one of the factors that determines the generation of new MRS isolates (Ito *et al.*, 2004). This may serve as a large reservoir of SCC*mec* available for *S. aureus* to form MRSA (Hanssen and Ericson Sollid, 2006). Methicillin resistance in staphylococci is mainly mediated by expression of *mecA* gene, that results in altered penicillin-binding protein (PBP2a), which has reduced affinity for β-Lactams (Chambers, 1988). *MecA* gene is carried on a mobile genetic element called a staphylococcal cassette chromosome *mec* (SCC*mec*) (Ito *et al.*, 2003). SCC*mec* contains two essential components (the *mec* gene complex and the *ccr* gene complex) and the junkyard (J) regions (Ito *et al.*, 2001; Ma *et al.*, 2002; Ito *et al.*, 2004). The *mec* gene complex is composed of *mecA* and has been classified into six different classes, i.e. A, B, C1, C2, D and E. Cassette chromosome recombinase (*ccr*) gene complex is composed of the *ccr* genes (*ccrC* or the pair of *ccrA* and *ccrB*) which are responsible for SCC*mec* mobility (Hanssen and Ericson Sollid, 2006), and others of unknown functions. Different types of SCC *mec* elements result from different combinations between the *mec* complex and *ccr* complex, and are further classified into subtypes according to differences in their J region DNA (Ito *et al.*, 2001; Ma *et al.*, 2002; Ito *et al.*, 2004). Eleven types (I to XI) of SCC*mec* have been assigned for *Staphylococcus aureus*, and five types for CoNS ((IWGSCC), 2009). SCC*mec* elements are more diverse in MR-CoNS, with new variants of *ccr* genes continuing to be identified (Mombach Pinheiro Machado *et al.*, 2007; Descloux *et al.*, 2008; Higashide *et al.*, 2008; (IWGSCC), 2009; Pi *et al.*, 2009).

The SCC*mec* types I, II, and III are predominantly found in hospital acquired methicillin resistant staphylococci (HA-MRS) isolates. The SCC*mec* types II and III are responsible for the multiple non β-lactam antimicrobial resistance. The SCC*mec* types IV and V are typically found in community acquired methicillin resistant staphylococci (CA MRS) strains and lack other multidrug resistance genes (Ünal, 2006). These are small, mobile genetic elements, more easily transferred to other strains of *S. aureus* than larger SCC*mec* (types I, II, and III) elements (Vandenesch *et al.*, 2003; O'Brien *et al.*, 2004). A thorough understanding of the molecular epidemiology and evolution of MRS is required to help detect, track, control and prevent human disease associated with this organism. Furthermore, SCC*mec* typing is an important molecular tool and is considered as epidemiologically important for infection control (Oliveira and de Lencastre, 2002). Compared to *Staphylococcus aureus*, CoNS produce a very limited number of virulence factors. The biofilm forming capacities of CoNS are thought to be the main virulence determinant.

Biofilm development is a classic 3-step process, which includes initial attachment, accumulation and detachment, with intricate regulatory pathways involved in each step (Otto, 2013). Biofilms are sticky, surface-attached agglomerations of bacteria embedded in self-produced extracellular polymeric substances (Costerton *et al.*, 1999). The significance of the biofilm mode of growth has multiple features affecting its virulence activity where the extracellular matrix provides a barrier protection against many antibiotics and human immune attack (Mah and O'Toole, 2001). In addition, bacteria in biofilm show the down-regulation of metabolic activities (Yao *et al.*, 2005), thus reducing the efficiency of antibiotics that target fast-growing bacteria, such as β-lactam antibiotics and amino glycosides. Moreover, under certain conditions, bacteria may detach from biofilm to reach a secondary infection site, resulting in the dissemination of infections (Otto, 2013). The virulence potential of a pathogen is to a large extent defined by its capacity to produce toxins. In contrast to its close relative *S. aureus*, the production of toxins in *S. epidermidis* is mostly limited to Phenol-soluble modulins (PSMs). PSMs are short staphylococcal peptides with α-helical structure, strictly regulated by the quorum-sensing system *agr* (Peschel and Otto, 2013). They can be divided into α-type peptides and β-type peptides (Otto, 2014). α-type PSMs can lyse many types of cells including neutrophils and thus cause tissue damages (Cheung *et al.*, 2010). The production pattern of PSMs in *S. epidermidis* is shifted in favor of non-cytolytic β-type peptides over α-type peptides (Cheung *et al.*, 2010). However, concerns have been expressed that under nosocomial conditions, the production of aggressive PSMs may be up-regulated in *S. epidermidis* (Cheung *et al.*, 2014). This possibly leads to life-threatening morbidities. Biofilm development and the dissemination of cellular clusters from biofilm (biofilm detachment) are considered to be mediated by β-type of phenol-soluble modulins (PSMs) due to their detergent-like properties (Peschel and Otto, 2013). Other virulence factors including extracellular polymers, teichoic acids (TA), exoenzymes and other antigens, may also contribute to the pathogenicity of *S. epidermidis* (Stevens *et al.*, 2009; Cheung *et al.*, 2010; Holland *et al.*, 2011; Peschel and Otto, 2013).

The aim of this study was:(1) to determine the incidence of staphylococci sepsis at the NICUs of three Mansoura hospitals, Egypt from March 2011 to August 2012; (2) to identify the CoNS isolated from blood cultures of septic newborns to the species level, (3)to assess antibiotic susceptibility of each strain, (4) to detect and characterize the oxacillin-resistant CoNS strains through SCC*mec* typing, (5) to identify some of the virulence factors that contribute to the pathogenesis of infection including biofilm forming properties of strains, hemolys in production and the prevalence of toxin producing genes (*psmβ* genes).

MATERIALS AND METHODS

Study design and study population

This study was prospectively conducted over a period of 18 months from March 2011 to August 2012, at three NICUs in Mansoura City, Egypt namely; Mansoura University Children Hospital (MUCH), Health Insurance Hospital (HIH) and Mansoura General Hospital (MGH). During the study period, all admitted neonates (0-28 days) with clinical signs and symptoms of sepsis at the time of admission or who developed sepsis during their hospital stay were assessed using Egyptian neonatal network (EGNN) sepsis screening tool and included in the study. Using a standard structured data collection form designed according to EGNN guidelines, social demographic, clinical and laboratory data were recorded by qualified medical staff. All neonates were subjected to full clinical examination stressing on gestational age, birth weight, mode of delivery, and risk factors for sepsis: premature rupture of membranes (PROM), maternal fever, insertion of an umbilical catheter, etc….

Sepsis was evaluated clinically: poor reflexes, lethargy, respiratory distress, bradycardia, apnea, convulsions, abdominal distension and bleeding, and by laboratory investigations: C-reactive protein, CBC and blood culture.

Sepsis is defined as presence of at least 3 out of the following 4 criteria

Presence of risk factors of sepsis (e.g. prematurity, chorioamnionitis), presence of two or more clinical signs of sepsis, abnormal hemogram and/or positive CRP and/or positive culture, and finally patient receives antibiotics and/or antifungal for at least five days (or < 5 days if the neonate is transferred or died before completion of these 5 days)(Egyptian Neonatal Network (EGNN), 2010). According to the infant age at the onset of symptoms, neonates were classified into two groups: EoNS (≤72 hours of life) and LoNS (>72 hours of life) (Bizzarro *et al.*, 2008). Nosocomial infection was defined by Standard Centre for Disease Control and Prevention (Garner *et al.*, 1988).

Collection of specimens and identification of Bacterial isolates

Blood Culture was made for all neonates with suspected clinical sepsis within the first 28 day of age. Blood was collected from peripheral vein using all regulations concerning the collection of specimens for a proper sampling technique. The study included only one clinical isolate per patient. Blood cultures were processed using the BacT/Alert automated system (bioMe´rieux) and sub cultured on Blood agar containing 5% sheep blood (oxoid). Colony morphology, Gram stain reaction, the catalase test and the tube coagulase test were used to screen for staphylococci isolates. All staphylococcal isolates were stored at -80°C in glycerol stock (25%), and retrieved when necessary by culturing in double nutrient broth.

Antimicrobial susceptibility testing

Antimicrobial susceptibility of staphylococcal isolates was determined by the Kerby-Bauer disc diffusion method on Mueller– Hinton agar (oxoid) according to the recommendations of the CLSI (2010), with the following antibiotics: ampicillin(10 µg), oxacillin (1µg), amoxacillin-clavulanic (30 µg), cefoxitin (30 µg), cefotaxime (30µg), ceftriaxone (30 µg), ceftazidime (30µg), imipenem (10 µg), vancomycin (30 µg), gentamicin (10 µg), amikacin(30µg), erythromycin (15 µg), azithromycin (15µg), ciprofloxacin (5 µg) and norflxacin (10µg) (Oxoid UK). MDR-bacteria were defined by resistance to three or more antimicrobial classes (Magiorakos *et al.*, 2012).

Identification of *Staphylococci* Species

Molecular identification of *Staphylococcus* species was performed by PCR-Restriction Fragment Length Polymorphism of *gap* gene, using *Alu* I as restriction enzyme, resulting in a distinctive RFLP pattern for each species analyzed (Yugueros *et al.*, 2000).
For further confirmation, amplified gene fragments of samples, representing each RFLP pattern obtained, were identified by sequencing.

Detection of *mecA* gene and characterization of SCC*mec* type

Primers used in this study with the corresponding annealing temperatures were listed in Table 1. All PCR assays were performed directly from bacterial suspensions obtained after the rapid DNA extraction method as previously described (Zhang *et al.*, 2004). Amplification of the *mecA* gene was performedin 25 µl reactions with appropriate reaction buffer, 0.5 µM of each primer, 1.5 mM $MgCl_2$, 0.2 mMdNTPs (iNtRON BIOTECHNOLOGY), 1.25 U Taq polymerase (Promega; GoTaq® Flexi DNA Polymerase), and 5 µl of template DNA. PCR reactions were carried out using Techne progene thermocycler with the following cycling parameters: initial denaturation at 95ºC for 5 min; followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 52 °C for 30 s, and extension at 72 °C for 1 min; with a final extension at 72 °C for 10 min. PCR products were visualized by electrophoresis on 2% agarose gel and ethidium bromide staining.

Table 1. Specific amplification primers used in this study for amplifying different genes among *Staphylococcus* spp. Isolates

Primer name	Oligonucleotide sequence (5`-3`)	Amplicon size (bp)	Specificity (Gene/SCC type/region)	Annealing temp.	Ref.
GF-1	ATG GTT TTG GTA GAA TTG GTC GTT TA	933	*gap*	59°C	(Yugueros *et al.*, 2000;
GR-2	GAC ATT TCG TTA TCA TAC CAA GCT G				Yugueros *et al.*, 2001)
Fw-20	AAA ATC GAT GGT AAA GGT TGG C	532	*mecA*	52°C	(Kozitskaya *et al.*,
Rev-20	AGT TCT GCA GTA CCG GAT TTG C				2005)
Type I-F	GCT TTA AAG AGT GTC GTT ACA GG	613	*SCCmec*I	Multiplex	(Zhang *et al.*, 2005)
Type I-R	GTT CTC TCA TAG TAT GAC GTC C			at 50°C	
Type II-F	CGT TGA AGA TGA TGA AGC G	398	*SCCmec*II		(Zhang *et al.*, 2005)
Type II-R	CGA AAT CAA TGG TTA ATG GAC C				
Type III-F	CCA TAT TGT GTA CGA TGC G	280	*SCCmec*III		(Zhang *et al.*, 2005)
Type III-R	CCT TAG TTG TCG TAA CAG ATC G				
Type IV-F	ACC AAC GTT TGT AGC GGG TT	800	*SCCmec*IV		(Soge *et al.*, 2009)
Type IV-R	AAG CGT CCA CGT CAT CTT CA				
Type V-F	GAA CAT TGT TAC TTA AAT GAG CG	325	*SCCmec*V		(Zhang *et al.*, 2005)
Type V-R	TGA AAG TTG TAC CCT TGA CAC C				
PSMβ test for	TAT TTG ACG CAA TTA GAA GTG TAG	280	*psm*β	56	(Wang *et al.*, 2011)
PSMβ test rev	GCT AAC GCC ACT TTC TAC GAT GTC		(Amplifying parts of psmβ2 and psmβ3)		
PSMβ test for	TAT TTG ACG CAA TTA GAA GTG TAG	130	*psm*β	45	(Wang *et al.*, 2011)
PSMβ2rev	AAT AAT TTA GAA ATA ATA CTA ACA		(Amplifying parts of psmβ3)		

The SCC*mec* typing of MRS isolates was performed by multiplex PCR, including 5 pairs of primers specific for SCC*mec* types I, II, III, IV, and V. Multiplex PCR was performed in 25 µl reactions with 0.5µM of each of the five primer pairs, 1.5 mM $MgCl_2$, 0.2 mM dNTPs (iNtRON BIOTECHNOLOGY), 1.25U Taq polymerase (Promega; GoTaq® Flexi DNA Polymerase), and 5 µl of template DNA. Multiplex PCR reactions were performed by initial denaturation at 95ºC for 5 min; followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 50 °C for 30 s, and extension at 72 °C for 1 min; ending with a final extension step at 72 °C for 10 min.

Detection of *psm*β genes

Presence of *psm*β genes were determined by PCR using the previously indicated reaction conditions with initial denaturation at 95ºC for 5 min; followed by 40 cycles of denaturation at 95 °C for 30 s, annealing for 30 s at 56 °C or 45°C according to primers used (Table 1), and extension at 72 °C for 1 min; with a final extension at 72 °C for 10 min.

Biofilm Production

Quantitative determination of biofilm formation by *Staphylococci* spp. was performed as previously described (Stepanovic *et al.*, 2000; Stepanovic *et al.*, 2007). Briefly, 200 µl of each bacterial suspension (adjusted to 1McFarland) was inoculated in triplicate into 96-well flat bottomed sterile polystyrene micro titer plate. Six wells containing broth only (TSB supplemented with 0.25% glucose as a medium) were used as negative control. The plates were incubated at 37°C for 24 hrs under static conditions. The content of each well was aspirated and each well was washed two times with 300 µl of PBS (pH 7.4) with vigorous shaking to ensure removal of all non-adherent bacteria. The remaining attached bacteria were fixed with absolute methanol (160 µl per well) and after 20 minutes the plates were emptied and left to dry. Then, plates were stained for 15 minutes with 1% crystal violet (160 µl per well). Excess stain was rinsed off by placing the plate under running tap water. The dye bound to the adherent cells was resolubilized with 160 µl of 33% (V/V) of glacial acetic acid per well. The optical density (OD) of each well was measured at 492 nm using an ELISA plate reader. For each strain, the mean OD of the three wells was calculated (OD_T). The cut-off OD (ODc) was defined as three standard deviations above the mean OD of the negative control wells.

Determination of hemolysin production

The production of hemolysins was determined on plates containing blood agar base consisting of 5% rabbit blood and 5% sheep blood incubated at 37°C for 24 h. A positive result was indicated by the formation of hemolysis zones around the isolated colonies.

Determination of DNAse

DNAse production was determined by the DNAse agar diffusion technique as previously reported (Lachica *et al.*, 1971).

RESULTS

Studied Population

During the study period, a total of 344 neonates (0 to 28 days of age) with suspected cases of sepsis were enrolled. Among 140 positive blood, Sixty isolates of staphylococci were included and assessed in our study. 38.3% and 61.7 % were the causative pathogens in EOS and LOS, respectively. Among them, 58 isolates were classified as CoNS (96.7%) and 2 isolates were identified as *S. aureus* (3.3%). Among staphylococci septic cases, 21.7% of cases were with LBW (birth weight <1500g), and 51.7% were preterm (<37 weeks). CRP level was measured in 57 cases, and 71.9% of them were positive. CBC was determined in 54 cases, showing abnormalities in 27 cases; 55.6% showed thrombocytopenia (platelets <140,000/mm^3), 14.8% leucopenia (WBC <5,000/ mm^3), 33.3% leucocytosis (WBC >20,000/ mm^3), 33.3% neutropenia.

Identification of *Staphylococci* species

According to the genotypic method used, of the isolated staphylococci; 55% were identified as *S. epidermidis*, 33.33% as *S.haemolyticus*, 5% as *S. hominis*, 1.67% as *S. saprophyticus*, 1.67% as *S. pasteuri*, and 3.33% as *S. aureus*. The distribution of different CoNS species in respect to type of sepsis was illustrated in Table 2.

Table 2. Distribution of *Staphylococci* species in respect to the type of sepsis

	EOS (n=23) No. (%)	LOS (n=37)No. (%)	Total (n=60)
S. epidermidis	14 (42.42)	19 (57.58)	33
S.haemolyticus	6 (30.00)	14 (70.00)	20
S. hominis	2 (66.67)	1 (33.33)	3
S. pasteuri	1	-	1
S. saprophyticus	-	1	1
S. aureus	-	2	2

Antibiotic susceptibility pattern of different Staphylococci

Sensitivity patterns of different *Staphylococci* spp. to different antimicrobial agents, commonly used in management of neonatal sepsis, were illustrated in Table 3. Generally, staphylococci isolates showed high resistance to ampicillin (95%) and intermediate effect (50%) to Amoxicillin-Clavulanic acid. In contrast to gentamicin, amikacin was highly effective on staphylococci isolates. Best sensitivity was also observed to imipenem and ciprofloxacin. All isolates were sensitive to vancomycin. According to the oxacillin (1 µg) and cefoxitin (30 µg) disks were used for phenotypic detection of MRS, 86.7% of Staphylococci were methicillin resistant staphylococci.

Multi-Drug Resistance (MDR) of different Staphylococci

MDR was observed in 31 (51.67%) staphylococci isolates. Among *S. epidermidis* isolates, 30.3% were multi-drug resistant, while 95% were detected among *S.haemolyticus*.

Table 3.Comparative resistances of *Staphylococci* spp. to different antimicrobial agents

Etiologic agents		Beta-lactams								Glyco-peptides	Macrolides		Amino-glycosides		Quinolones	
		Penicillins			Cephalosporins											
		AMP	OX	AMC	FOX	CTX	CRO	CAZ	IPM	VA	E	AZM	CN	AK	CIP	NOR
S. epidermidis	n=33	96.97	81.82	24.24	81.82	33.33	33.33	90.91	15.15	0	51.52	54.55	51.52	30.30	21.21	21.21
S. haemolyticus	n=20	95	90	85	90	90	90	100	25	0	95	95	90	0	55	55
S. hominis	n=3	100	100	33.33	100	0	0	100	0	0	66.67	66.67	33.33	0	0	0
S. pasteuri	n=1	100	100	100	100	100	100	100	0	0	0	0	0	0	0	0
S. saprophyticus	n=1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
S. aureus	n= 2	100	100	100	100	100	100	100	0	0	50	50	50	0	50	50

Table 4. Distribution of SCC*mec*elements among methicillin resistant staphylococci isolated from blood cultures of neonates with N. Sepsis

Staphylococci species	SCC*mec* type										Total no. of typable isolates	Non-typable
	I	II	III	IV	V	I III	II IV	II V	III V	I III V		
S. epidermidis (n=29)	-	4	3	1	-	1	2	1	2	1	15 (51.72)	14(48.28)
S. haemolyticus (n=18)	-	-	-	-	4	-	-	1	-	-	5 (27.78)	13 (72.22)
S.hominis(n=3),	-	-	-	-	-	-	-	-	-	-	-	3
S. pasteuri (n=1),												1
S. aureus (n=2)												2

Table 5. Distribution of *Staphylococci* species according to their biofilm formation

	Total	Category			
		Non adherent isolates	No. (%)	Biofilm forming Isolates	
				No. (%)	Type of adherence
S. epidermidis	n= 33	4 (12.12)		29 (87.88)	9 (Strongly adherent) 11 (Moderately adherent) 9 (Weakly adherent)
S. haemolyticus	n= 20	10 (50.00)		10 (50.00)	1 (Strongly adherent) 9 (Weakly adherent)
S. hominis	n= 3	---		3 (100.00)	3 (Weakly adherent)
S. saprophyticus	n= 1	---		1	1 (Weakly adherent)
S. aureus	n= 2	1 (50.00)		1 (50.00)	1(Weakly adherent)

Table 6. Presence of *psm*β genes and Biofilm formation

Category	Biofilm Formation		
	Non adherent isolates	No. (%)	Adherent isolates No. (%)
*psm*β positive (n=36)	6 (16.67)		30 (83.33) (9 Strongly adherent, 11 Moderately adherent, 10 Weakly adherent)
*psm*β negative (n=24)	10 (41.67)		14 (58.33) (1 Strongly adherent, 13 Weakly adherent)

Detection of *mecA* gene in different Staphylococci

The presence of the *mecA* gene was tested in all staphylococci isolates by PCR. The *mecA* was detected in 53 isolates where it was found in all MRS isolates, and one MSS isolate.

SCC*mec* types identified in different Staphylococci

SCC*mec* typing was performed on all isolates carrying the *mecA* gene using multiplex PCR. Among the 53 *mecA*-positive strains, only 20 (37.7%) could be assigned to known SCC*mec* types. SCC*mec* type I was identified in 2/20 (10%), type II in 8/20 (40%), type III in 7/20 (35%), type IV in 3/20 (15%) and as type V in 9/20 (45%) of the strains. More than one type could be identified in one strain. Among these 20 isolates, 12 had a single SCC*mec* type including type II (n= 4), III (n =3), IV (n= 1) and V (n= 4), while 7 had two types including II+IV (n= 2), II+V (n= 2), III+V (n= 2) and I+III (n= 1), and 1 had three types including I+III+V.

Among the *S. epidermidis* strains with typable SCC*mec*, 4/15 (26.67%) were characterized as SCC*mec* type II, 3/15 (20%) as type III, 1/15 (6.67%) as type IV, while 7/15 (46.67%)were characterized as different combined types of either type II with IV or V, or type III with I or V or both, whereas among the *S. haemolyticus* strains, 4/5 (80%) were characterized as SCC*mec* type V and 1/5 (20%) as II+V. (Table 4).

Slime production

Among the 60 Staphylococci isolates, 44 (73.33%) isolates were positive for slime production, including 29/33 (87.88%) of *S. epidermidis* strains, 10/20 (50%) of *S.haemolyticus* strains, three *S. hominis* isolates, one *S. aureus* and one *S.saprophyticus* (as shown in Table 5 and Figure1)

Detection of *psm*β genes in different Staphylococci

The presence of *psm*β genes was tested in all staphylococci isolates. The *psm*β genes were detected in 36 (60%) isolates, including 31/33 (93.94%) of *S. epidermidis* strains, 4/20 (20%) of *S. haemolyticus* isolates, and one *S. saprophyticus* isolate, as shown in Figure 2. Presence of *psm*β genes and Biofilm formation were illustrated in Table 6.

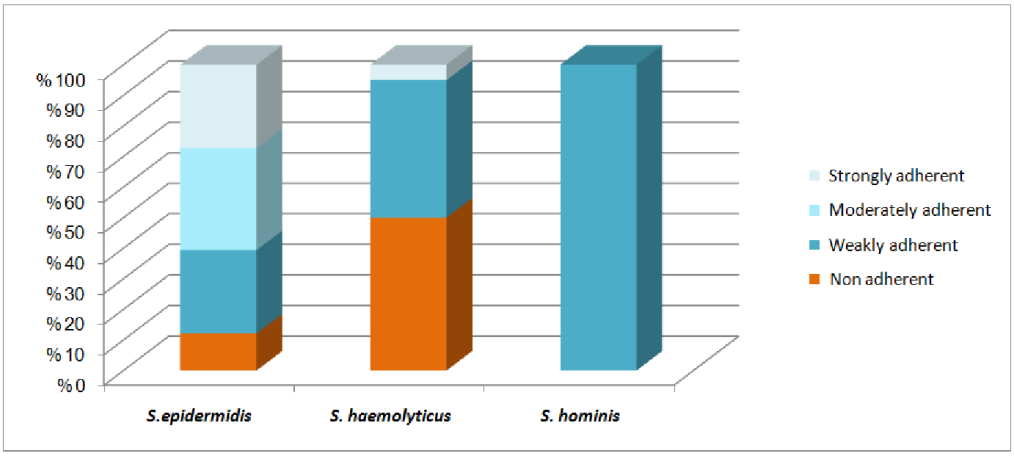

Fig. 1. Formation of Biofilm by different Staphylococci isolated from blood cultures of neonates with N. Sepsis

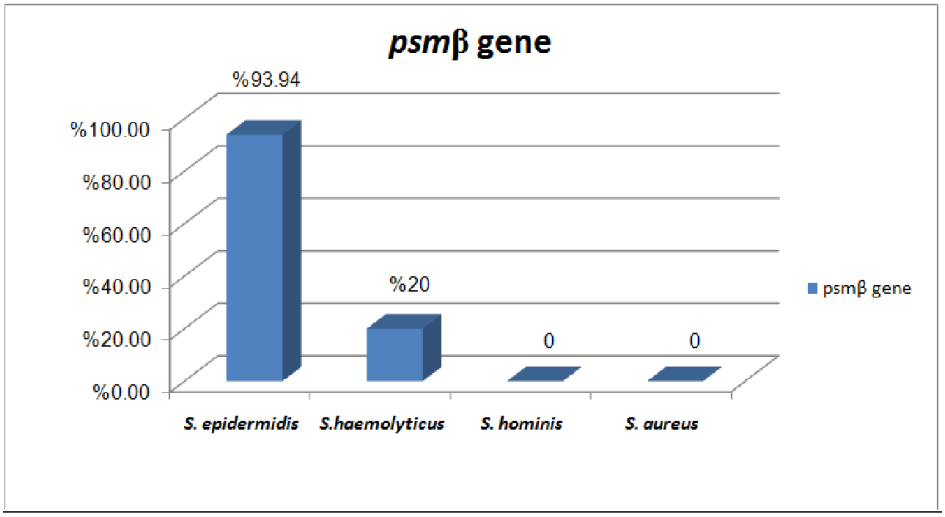

Fig. 2. Prevalence of *psm*β gene among Staphylococci. isolated from blood cultures of neonates with N. Sepsis

Hemolysin production

Thirty-one isolates produced hemolysins; including 19*S. haemolyticus*, 8*S. epidermidis*, 1 *S.pasteuri*, 1*S. saprophyticus*, and 2 *S.aureus* isolates.

DNAse production

The two *S. aureus* isolates were DNAse producers, while only one isolate among CoNS was weak producer of DNAse.

DISCUSSION

Staphylococcus is a genus of great importance, including more than 60 species and subspecies, and of concern in many researches around the world due to their implication in many human and animal infections. In spite of being commensal on host skin, they become pathogens when gaining the entry into the host tissue or in cases in which the microbial community is disturbed or in immune compromised individuals (Kloos and George, 1991; Kloos and Bannerman, 1994). CoNS have proved their clinical significance, besides *Staphylococcus aureus* (Kloos and Bannerman, 1994,1995). In our study, CoNS were at the forefront of causative agents of neonatal sepsis in both EOS and LOS. For this reason, identification of the isolated offending species was of crucial importance to permit a more precise determination of the host-pathogen relationships of staphylococci (Kleeman *et al.*, 1993; Gribaldo *et al.*, 1997), and to initiate appropriate antibiotic therapy so that a good clinical outcome can be guaranteed.

Different methods have been described for identification of *Staphylococcus* spp., but molecular methods are considered much more superior for such identification. In our study, a combined procedure of *gap* PCR and RFLP with Alu I was implemented as previously reported (Yugueros *et al.*, 2000; Yugueros *et al.*, 2001). The *gap* gene commonly has been considered a constitutive housekeeping gene (Modun *et al.*, 1998; Figge and Cerff, 2001). It encodes a 42-kDa transferrin-binding protein (Tpn), one of multifunctional cell wall-associated glyceraldehyde-3-phosphate dehydrogenases that is located within the cell wall of *S. aureus* and number of CoNS species (Modun, B. *et al.*, 1994). Among CoNS isolates in our study, *S. epidermidis*, *S. haemolyticus*, and *S. hominis* were the most frequently recovered CoNS isolates in blood cultures. They were present at 55%, 33.3% and 5% in the blood cultures, respectively. This is in consistence with other previous studies (Gatermann *et al.*, 2007; Mombach Pinheiro Machado *et al.*, 2007; Tang *et al.*, 2007; Piette and Verschraegen, 2009; Pereira and Cunha Mde, 2013).

All CoNS isolates showed high resistance to ampicillin, oxacillin, cefoxitin and ceftazidime, while *S. haemolyticus* isolates were more resistant (≥85%) than *S. epidermidis* and *S. hominis* to the other antimicrobials; amoxicillin-clavulanic acid, cefotaxime, ceftriaxone, erythromycin, azithromycin, and gentamicin. Amikacin was effective against all *S. haemolyticus* and *S. hominis* isolates, whereas it was only active against 69.7% of *S. epidermidis* isolates. Similar results were identified in other previous studies (Froggatt *et al.*, 1989; Chiew *et al.*, 2007), *S. haemolyticus* had the highest level of antimicrobial resistance among CoNS. In contrast, other authors reported *S. epidermidis* as the most resistant species (Pereira and Cunha Mde, 2013). Sensitivity of different CoNS spp. to amikacin, imipenem and quinolones were variable. *S. epidermidis* were highly sensitive to imipenem, followed by quinolones, then amikacin, whereas *S. haemolyticus* were 100% sensitive to amikacin, followed by imipenem. Quinolones had a lower activity on *S. haemolyticus* isolates. On the other hand, all *S. hominis* isolates were sensitive. Moreover, all staphylococcal isolates in our study were sensitive to vancomycin; similar observation was previously reported (Macharashvili *et al.*, 2009; Bhat *et al.*, 2011; Shah *et al.*, 2012). However, the over-prescription of this drug may result in the development of vancomycin resistant strains such as enterococci. Multi-drug resistance was detected in our study in 51.67% of staphylococci isolates with high prevalence among *S. haemolyticus* isolates (95%). This high rate of multi-resistance found in blood isolates is likely because of the extensive use of these antibiotics in our NICU. This was in consistence with other previous studies (Monsen *et al.*, 2005; Hira *et al.*, 2007; Yu *et al.*, 2010). Morever, in the study of Hira et al (2007), 75 to 80% of the strains were multi-resistant, including all *S. haemolyticus* isolates.

Globally, rapid increase in isolation rates of MRCoNS was previously reported. They have become an important cause of nosocomial infections since the introduction of methicillin. In our study, the rate of oxacillin resistance among the isolated staphylococci strains was 85% (51/60). Similar high rates of oxacillin resistance among neonatal CoNS isolates have been previously reported as 87% of strains were MRCoNS (Hira *et al.*, 2007). In addition, in a study conducted at the Gulhane Military Medical Academy Hospital, Turkey, the overall methicillin resistance was identified as 83.3% in 313 CoNS isolates(MERT *et al.*, 2011).In other studies in Turkey, these rates were found to vary from 67.5% to 85% (Celebi *et al.*, 2007; Koksal *et al.*, 2009; Oguz *et al.*, 2009).Intermediate rates were reported in Brazil (Pereira and Cunha Mde, 2013), in which the rate of oxacillin resistance among the 100 CoNS strains isolated from neonatal blood cultures was 69%. Another study (Piette and Verschraegen, 2009) reported that MRCoNS rates in clinical samples varies between 55% and 77%. These rates have even been reported as high as 86% in intensive care units. Lower rates of MRCoNS (47.37%) isolated from the neonatal ward were reported in a one study in Algeria (Barreto and Picoli, 2008).

Among the identified *Staphylococci* species in our study, oxacillin resistance was detected in 81.8% of *S. epidermidis* and 90% of *S. haemolyticus* isolates. Similar findings were previously reported (Pereira and Cunha Mde, 2013) in which 73.2% and 85.7% of the *S. epidermidis* and *S. haemolyticus* isolates were resistant to oxacillin, respectively. However, in another study (Ferreira *et al.*, 2003), 96% of the isolated *S. haemolyticus* strains were resistant to oxacillin. Higher resistance rates among *S. epidermidis* isolates (97%) from a NICU have been previously reported (Krediet *et al.*, 2004). Different methods can be implicated in identification of resistance to oxacillin. This includes two main methods, phenotypic and genotypic. Phenotypic method is achieved by the disc diffusion test with either cefoxitin or oxacillin. Genotypic characterization of methicillin resistance is examined by detection of *mecA* gene, which is responsible for methicillin resistance by the production of an altered penicillin binding protein (PBP2a) (Ito *et al.*, 1999). PBP2a is an enzyme involved in cell wall peptidoglycan synthesis. Unlike PBPs of Staphylococci, PBP2a does not bind to β-lactam antibiotics with high affinity (Rohrer *et al.*, 2003; (IWGSCC), 2009). According to phenotypic method, 85% of the isolates in our study were oxacillin resistant, whereas *mecA* was genotypically identified in 88.33% of isolates.

The disc diffusion test with a cefoxitin substrate is preferred over the test with oxacillin to estimate resistance to oxacillin mediated by the *mecA* gene for both *S. aureus* and CoNS(Swenson and Tenover, 2005). This was also observed by other authors (Mombach Pinheiro Machado *et al.*, 2007). However, in our study, the phenotypic test had a good correlation with the genotypic characterization of methicillin resistance. There was agreement of 96.7% in disc diffusion testing either with cefoxitin or oxacillin with the presence of the mec*A* gene. Among the 53 *mecA*-positive strains, only 20 (37.74%) could be assigned to known SCC*mec* types by multiplex PCR. This was also observed in another previous study(Barros *et al.*, 2012), as only 32 (57%) among the 56 *mecA*-positive *S. haemolyticus* strains could be assigned to known SCC*mec* types (Barros *et al.*, 2012), whereas other authors reported that 75% (63/84) of MR-CoNS could be assigned by the multiplex PCR (Zong *et al.*, 2011). The presence of non-typable strains by the method employed can be explained by the presence of novel structures or rearrangements and recombination of the SCC*mec* (Chung *et al.*, 2004; Zhang *et al.*, 2005), exhibiting substantial genetic diversity with new types continuously being identified.

In our study, type V was the most common type being present in 9 of 20 MR-CoNS isolates (45%) either alone or combined with other types, followed by type II (8/20) (40%) and III (7/ 20) (35%). On the contrary, other types could be traced as the predominant in other studies such as type III either alone or combined with other types, followed by type V and IV (Zong *et al.*, 2011), or type IV(MERT *et al.*, 2011). In these two previous studies, SCC*mec* type II was also identified but in few isolates. Difference in the methods used in SCC*mec* typing affects the interpretation of the data reported and renders comparison difficult among the different studies (Zhang *et al.*, 2005). In other studies (Mombach Pinheiro Machado *et al.*, 2007; Pereira and Cunha Mde, 2013), SCC*mec* type III was the most prevalent followed by type I. However, their results cannot be compared with ours due to difference in the method and primers used that may result in misidentification of type V in their study as type III. The co-existence of two or more SCC*mec* elements appears to be common in MR-CoNS (Barbier *et al.*, 2011). It is likely that the two SCC*mec* elements actually constitute a composite rather than two independent units. One of the limitations of the multiplex PCR-based schemes is the inability to differ composites from separate elements.

The frequent identification of co-existed SCC*mec* and the presence of ‘‘non-typable’’ elements represent great challenges for SCC*mec* typing in MR-CoNS. The distribution of different types of SCC*mec* in MR-CoNS varied depending on the host species and possibly on the geographical locations (MERT *et al.*, 2011). Different studies reported that type IV has been preferentially associated with *S. epidermidis* (Mombach Pinheiro Machado *et al.*, 2007; Ruppe *et al.*, 2009; Barbier *et al.*, 2011; Zong *et al.*, 2011; Pereira and Cunha Mde, 2013), whereas type V is appeared to be the dominant in *S. haemolyticus* (Ito *et al.*, 2004; Ruppe *et al.*, 2009; Barros *et al.*, 2012). In another study, the combination of type II and V (6/19) was the most common type among *S. haemolyticus* (n=19) (Zong *et al.*, 2011). On the contrary, other authors reported that the oxacillin-resistant *S. haemolyticus* strains carried SCC*mec* type I (83.3%) and type II (16.7%) (Pereira and Cunha Mde, 2013). In our study, distribution of SCC*mec* types among isolated CoNS illustrated that SCC*mec* types II and III were the prevalent among *S. epidermidis*, while among *S. haemolyticus*, type V was the main identified type. *S. epidermidis* generally contained SCC*mec* types II, III and IV as single or combined with types I and V.

In spite of pathogenicity of CoNS, little is known about their putative virulence determinants, especially their lack to several of the virulence factors shared with the closely related species *S. aureus* (Remington *et al.*, 2011).However, biofilm forming potentials are recognized as major virulence factor in CoNS sepsis and serves as the primary mode of immune evasion of CoNS (Foster, 2005). In our study, 73.3% of the isolates displayed the in vitro biofilm producing phenotype. This rate is higher than that detected in other studies, in which 51% or less of the strains in these studies showed adherence capability phenotype in vitro (Klingenberg *et al.*, 2005; Hira *et al.*, 2007). Slime production can vary among different species, where this characteristic is more frequent in strains of *S. capitis*, *S. epidermidis*, *S. hominis*, and *S. saprophyticus* (Christensen *et al.*, 1983), in addition to *S. Lugdunensis* as previously reported (Fleurette *et al.*, 1989).

In the same manner as previously determined (de Silva *et al.*, 2002; Klingenberg *et al.*, 2005), we found that *S. epidermidis* produced more biofilm than other CoNS species. *S. epidermidis* is clearly capable of producing strong biofilms, whereas *S. haemolyticus*is mainly a weak biofilm producer (Hira *et al.*, 2007). Phenol-soluble modulins (PSMs) are amphiphatic peptides produced by staphylococci that have multiple functions in pathogenesis; for example, they may function as cytotoxins and pro-inflammatory agents. Additionally, in a recent study of Wang *et al.* (2011), by analyzing the role of PSMs in biofilm development, they demonstrated that the β-type PSMs represent key effectors of *S. epidermidis* biofilm maturation and detachment. Furthermore, they showed that these peptides facilitate the dissemination of biofilm-associated infection, providing evidence for in vivo significance of biofilm detachment (Wang *et al.*, 2011).

In our study, *psm*β genes were found in (36/60) isolates (60%); 31 of *S.epidermidis* (93.94%), 4 of *S. haemolyticus* (20%), and *S. saprophyticus* (one isolate). All strong and moderate biofilm forming isolates were *psm*β positive, while among the weak and non adherent isolates, the presence of *psm*β was variable.
In addition to biofilm and PSMs, various metabolites, including enzymes and toxins produced by CoNS may play a role in the pathogenicity of these microorganisms (Gemmell, 1987). Production of hemolysins or cytolytic toxins as well as DNAse enzyme has been observed in CoNS. In a previous study (Cunha Mde *et al.*, 2006), hemolysins were produced by isolates of *S. epidermidis*, *S. haemolyticus*, *S. lugdunensis*, and *S. warneri*, and not by strains of *S. hominis*, *S. simulans*, and *S.xylosus*; Similar results have been previously reported (Fleurette *et al.*, 1989; Lambe *et al.*, 1990; Cunha Mde *et al.*, 2004), whilst production of DNAse enzyme was observed in strains of *S. epidermidis*, *S. lugdunensis*, *S. simulans*, and *S. haemolyticus*(Cunha Mde *et al.*, 2006).In addition, other authors verified that most *S. epidermidis*, *S. warneri*, and *S. hominis* strains included in their study produced DNAse (Lambe *et al.*, 1990). In our study, production of hemolysins

among CoNSwas observed mainly among *S. haemolyticus*, in addition to *S. epidermidis* and others, whereas DNAse was produced by *S. aureus* isolates and also only one CoNS isolate (*S. saprophyticus*).

Conclusion

CoNS were identified in our study as the most causative pathogens of neonatal sepsis. *S. epidermidis* was the most predominant among them from neonatal blood cultures, followed by *S. haemolyticus* and *S. hominis*. *S. haemolyticus* was the most multidrug-resistant species. High prevalence of methicillin resistant staphylococci isolates is still a problem in NICU. Vancomycin is still the most effective antimicrobial agent against staphylococci isolates in NICUs, in addition to amikacin and imipenem and ciprofloxacin. Among aminoglycosides antibiotics, amikacin was more effective than gentamicin. SCC*mec* types V, II and III were the most prevalent among MR-CoNS isolated from neonatal sepsis cases. SCC*mec* types II and III were the most prevalent among *S. epidermidis*, while among *S. haemolyticus* isolates was type V. More than one type can be present in the same isolate, which may constitute a composite rather than two independent units. This cannot be differentiated by the available multiplex PCR-based schemes. Biofilm production and *psm*β genes were observed mainly among *S. epidermidis* which are considered as two important virulence factors associated with sepsis in neonates.

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