Frequency of *icaA* and *icaD* determinants and biofilm formation among coagulase-negative staphylococci associated with nasal carriage in neonatal intensive care units

Doaa Mabrouk Ahmed[1,*], *Mona Abdel Wahab Abel Messih*[2], *Nermin Hassan Ibrahim*[3], *Mohamed Hussein Meabed*[4], *Soha Mahmoud Abdel-Salam*[5]

Abstract

Introduction **Nasal colonization with coagulase-negative staphylococci (CoNS) may be a preliminary risk factor for systemic infection. The aim of this study was to assess the frequency of *ica A* and *D* genes and biofilm formation among hospital-acquired nasal colonizing CoNS strains isolated from neonates in the neonatal intensive care units (NICUs). Antibiotic sensitivity patterns and some relevant risk factors were estimated.**

Methods **This study assessed nasal colonization with CoNS among neonates at days one and three of admission to NICUs of Beni-Suef University Hospital and Beni-Suef General Hospital from November 2015 to May 2016. The isolates were screened and identified; susceptibility testing was performed. Biofilm formation was examined using the Congo red agar method. Isolates identified as CoNS were tested by polymerase chain reaction (PCR) for the presence of *mecA* and *icaA* and *icaD* genes.**

Results **A total of 340 nasal swabs were collected from 170 neonates. The incidence of nasal colonization with CoNS was 50%. The species most frequently isolated were *S. haemolyticus* and *S. epidermidis*. Multidrug resistance (MDR) was detected in 86% of isolates. It was found that there was a strong association between the presence of *mecA* gene and phenotypic resistance to methicillin and also the presence of the *icaA* gene and biofilm formation.**

Conclusions **Neonates admitted to NICUs can become reservoirs for CoNS strains, leading to potential dissemination of MDR strains into the community.**

Keywords **Coagulase-negative *Staphylococcus*, *icaA*, *icaD*, *mecA*, neonatal intensive care units**

Introduction

Coagulase-negative staphylococci (CoNS) have been associated with late onset sepsis in the neonatal intensive care unit (NICU), particularly in very low birth weight neonates.[1] In a clinical setting, the most common sources of CoNS are the patient's own bacterial microbiota and the hands of healthcare workers.[2]

Neonates are prone to developing infections, due to factors such as: an immature immune system, using invasive procedures, and aggressive antibiotic treatment. Consequently, CoNS strains disperse in NICUs environments. Invasive procedures favor the migration of CoNS from the skin to the catheter insertion point, leading to infection.[3]

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[1]MD, Lecturer, Medical Microbiology and Immunology Department, Faculty of Medicine, Beni-Suef University, Mohamed Hassan Street, Beni-Suef, 6251 Egypt; [2]MD, Professor, Medical Microbiology and Immunology Department, Faculty of Medicine, Cairo University, 31 Khatem El Morsalin Street, Giza, Cairo, Egypt; [3]MD, Assistant Professor, Medical Microbiology and Immunology Department, Faculty of Medicine, Beni-Suef University, Mohamed Hassan Street, Beni-Suef, 6251 Egypt; [4]MD, Professor, Pediatric Medicine, Faculty of Medicine, Beni-Suef University, 138 El Raoda st. Beni-Suef, Beni-Suef, Egypt; [5]MSc, Assistant Lecturer, Medical Microbiology and Immunology Department, Faculty of Medicine, Beni-Suef University, Mohamed Hassan Street, Beni-Suef, 6251 Egypt.

*Corresponding author: Doaa Mabrouk Ahmed, doaamicro50@gmail.com

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Moreover, the ability of CoNS strains to form biofilm on polymeric surfaces make them more pathogenic.[4] Biofilm consists of multilayered clusters of cells embedded in an extracellular polysaccharide (slime) matrix that facilitates adhesion and protects against the host immune system and antimicrobials.[5] The intercellular adhesion (*ica*) operon is responsible for the production of slime, encoded by the genes of *icaADBC*; the gene *icaA* encodes N-acetylglucosaminyl transferase, the enzyme involved in the synthesis of polysaccharide intercellular adhesion (PIA). *icaD* plays an important role in maximum expression of N-acetyl glucosaminyl transferase, resulting in the capsular polysaccharide's full phenotype expression. It has been reported that *icaA* and *icaD* play a significant role among *ica* genes in the formation of biofilms.[6,7]

In addition, the presence of *mecA* gene on the mobile genetic element staphylococcal cassette chromosome (SCC*mec*), decreased its affinity to methicillin.[8] The resistance of *mecA*-positive isolates to wide-spectrum β-lactams is spread globally in staphylococci from hospitals, farms as well as community environments, making it ineffective to treat staphylococcal infections.[9]

The aims of the present study were to evaluate the rate of hospital acquired nasal colonization by CoNS during hospitalization following the neonate's admission to NICUs, the potential risk factors for colonization, antibiotic susceptibility testing of the isolates to determine multidrug-resistant (MDR) organisms and the genetic relatedness with their phenotypic resistance to antibiotics. Moreover, the ability of the isolates to form biofilms was evaluated, as well as their relationships with certain genes (*icaA* and *icaD*).

Methods

The current study is a prospective cross-sectional one that was carried out in Beni-Suef University hospital and Beni-Suef General Hospital from November 2015 to May 2016. One hundred and seventy neonates were enrolled in the study. All neonates were eligible for nasal swab (Sterile swab ST, CK) collection at admittance and three days after. Informed written consents from guardian were obtained at enrollment. Blood cultures were done for suspected cases with sepsis.

Full history was taken, including different risk factors for CoNS colonization including: age, sex, weight, route of delivery, sepsis occurrence, prematurity, use of antimicrobial drugs during the period of hospitalization, period of hospitalization (in days), presence of external devices such as cannula, nasal cannula and use of continuous positive airway pressure (CPAP), and existence of comorbidities such as chronic diseases, genetic diseases, infectious diseases, or congenital malformations.

All samples were transported immediately in Stuart's medium to the Microbiology Laboratory of Beni-Suef University hospital for processing.

Identification of bacterial isolates

All nasal swabs and blood cultures were inoculated onto blood agar, MacConkey's agar and mannitol salt agar (Oxoid, Basingstoke, UK). After incubation, the suggestive colony was identified by the conventional microbiological standard tests. Further identification of CoNS isolates to the species level was performed by API Staph. (BioMérieux, Marcy-l'Étoile, France).[10]

Antibiogram, biological characteristics and biochemical tests were conducted for all isolates collected; we excluded the cases found on the first day and which continued to appear on the 3rd day, as they were not considered as hospital-acquired.

Susceptibility tests

CoNS isolates were submitted to a disc diffusion susceptibility test using the following antibiotics: penicillin (10 µg), oxacillin (1 µg), cefoxitin (30 µg), erythromycin (15 µg), clindamycin (2 µg), rifampicin (15 µg), gentamicin (15 µg), ciprofloxacin (5 µg), tetracycline (30 µg), vancomycin (30 µg), linezolid (30 µg), ceftazidime (30 µg), sulfamethoxazole-trimethoprim (23.75/1.25 µg) and novobiocin (Oxoid). Inhibition zones were interpreted according to Clinical Laboratory and Standards Institute (CLSI) guidelines.[11] The D zone test for inducible resistance to clindamycin

was performed. Resistance to at least three or more classes of antibiotics was considered as MDR.[12]

Detection of biofilm production

A phenotypic method was used, according to Freeman et al.,[13] by the Congo red agar (CRA) medium. A positive result was indicated by black colonies, whereas nonproducing strains produced red colonies. Darkening of the colonies in the absence of a dry crystalline morphology indicated a moderate biofilm formation.[14]

Detection of *mecA* gene phenotypically

This was done by detecting the susceptibility of the isolates to cefoxitin by disc diffusion method as a preliminary screening test for the existence of *mecA* gene. Resistant strains were tested for the existence of *mecA* gene by PCR.

Detection of *mecA, icaA* and *icaD* genes by PCR

The bacterial DNA was extracted using QIAGEN DNA extraction kit (Qiagen, Manchester, UK) and the procedure was followed according to the manufacturer's instructions.

PCR amplification of the *mecA* gene was performed using a primer set (salt-free 0.2 µmol Germany 49 bp). Two pairs of primers SA-1 (5' CGG TAA CAT TGA TCG CAA CGT TCA 3') were used as the forward primer and SA-2 (5' CTT TGG AAC GAT GCC TAA TCT CAT 3') was used as the reverse primer to amplify a 214-bp fragment of the *mecA* gene. The set included an internal control template. For amplification of *mecA* gene the protocol described in Rigatti et al.[15] was used.

The amplified products were separated by electrophoresis on 2% agarose gel prepared in 0.5X TBE buffer and stained with SYBR Safe. The gels were photographed under UV transillumination.

PCR for *icaA* and *icaD* genes was done using the method described by Arciola et al.[6] For detection of *icaA* gene 5-TCTCTTGCAGGAGCAATCAA-3 was used as the forward primer (corresponding to nucleotides 4796-4815) and 5-TCAGGCACTAACATCCAGCA-3 was used as the reverse primer (corresponding to nucleotides 4964-4983). For *icaD*, 5-ATGGTCAAGCCCAGACAGAG-3 was used as the forward primer (corresponding to nucleotides 5422–5441), and 5-CGTGTTTTCAACATTTAATGCAA-3 was used as the reverse primer (corresponding to nucleotides 5616–5597). The amplified product sizes were estimated by comparison with 100 bp DNA ladder.[16]

Two *S. epidermidis* reference strains were used, the slime-producing strain ATCC 35984 (RP62A), as positive control for *icaA* and *icaD,* and the non-slime-producing strain ATCC 12228 as a negative control for *icaA* and *icaD*.[6] *S. aureus* strain ATCC 43300 was used as a positive control for the *mecA* gene.[15]

Statistical analysis

The collected data were coded then entered and analyzed using Statistical Package for Social Science SPSS v22.0 (IBM Corp, Armonk, NY, USA). Descriptive statistics were done for categorical variables by frequency and percentage, and for numerical variation in the form of mean and standard deviation (mean±SD). Suitable statistical tests of significance were used: Chi-square for categorical data and independent sample t-test for numerical data. P-values equal to or less than 0.05 were considered statistically significant

Results

During the period of the study, 340 nasal swabs were collected from 170 neonates (at days one and three of admission) of NICUs in Beni-Suef University Hospital and Beni-Suef General Hospital.

Using antibiograms, biological characteristics and biochemical tests, comparing the isolates found in the 1st and 3rd days, 20 of these isolates were discarded, as they were not considered as hospital-acquired colonization. Isolates that appeared only on the third day were included in the study as nosocomial colonization; they were

100 isolates, while 50 neonates revealed no growth finding.

The incidence of nosocomial colonization in the present study was (100/170) 58.8%. It was found that the most common isolates were Gram-positive organisms, reported in 86/100 isolates. CoNS were the most prevalent (50/100), followed by *S. aureus* (36/100) while Gram negative bacilli were reported in 14/100 cases. *Klebsiella pneumoniae* was the most common (8/100) followed by *E. coli* (4/100) and *Pseudomonas aeruginosa* (2/100).

In the current study, males predominated (64.2%). The age range was 1-38 days with a mean of 7.2±8.9 days. Furthermore, the most common cause of admission was respiratory distress (47.6%).

In the present study, an incidence of 50% of CoNS colonizers among isolates was recorded. Further identification of CoNS isolates was done using API Staph (BioMérieux) kit; it revealed that *S. haemolyticus* (24/50, 48%) and *S. epidermidis* (18/50, 36%) strains were the most prevalent yields, followed by *S. xylosus* (4/50, 8%), *S. hominis* (2/50, 4%) and *S. chromogenes* (2/50, 4%).

Based on the data obtained from the descriptive analysis of the important risk factors included, comparing CoNS colonized cases with no growth cases, there was a significant association with the use of intravenous cannula, patients with signs of sepsis and administration of empirical antimicrobials in cases colonized with CoNS ($p<0.001$) (Table 1). Furthermore, the age of the neonates showed statistical significance as a risk factor (Table 2).

In addition, a strong relationship was established between colonization with CoNS in neonates with cases suspected of having sepsis that revealed blood cultures positive for CoNS ($p<0.001$) (Table 3).

Detection of biofilm formation by Congo red agar (CRA)

Out of 50 CoNS isolates, 26 (52%) isolates were Congo red positive. Congo red positive strains appeared bright black or dry black

Table 1. The potential risk factors for acquisition of nosocomial colonization by CoNS in neonatal intensive care units

Risk factors	CoNS positive	No growth	P value	Chi-square value	df
Sex					
Male N=63 (63%)	32 (64%)	31 (62%)	0.500	0.043	1
Female N=37 (37%)	18 (36%)	19 (38%)			
Mode of delivery					
Caesarean section N=66 (66%)	31 (62%)	35 (70%)	0.263	0.713	1
Vaginal N=34 (34%)	19 (38%)	15 (30%)			
Prematurity					
Preterm N=68 (68%)	36 (72%)	32 (64%)	0.260	0.735	1
Full term N=32 (32%)	14 (28%)	18 (36%)			
Presence of signs of sepsis N=44 (44%)	44 (88%)	0 (0%)	**<0.001***	92.3	1
Presence of intravenous cannula N=55 (55%)	50 (100%)	5 (10%)	**<0.001***	81.8	1
Presence of other devices N=78 (78%)	42 (84%)	36 (72%)	0.114	2.98	1
Congenital anomalies N=17 (17%)	11 (22%)	6 (12%)	0.356	6.6	6
Empirical antimicrobial administration N=56 (56%)	50 (100%)	6 (12%)	**<0.001***	78.6	1
Respiratory distress on admission N=77 (77%)	39 (78%)	38 (76%)	0.500	0.056	1

Data is presented as number (percentage). **CoNS** – coagulase-negative staphylococci; **df** – degrees of freedom. *p value <0.05 is considered significant by Chi-Square test.

Table 2. Age, weight and length of hospitalization as a risk factors for acquisition of nosocomial colonization in neonatal intensive care units

		Number	Mean	SD	95% confidence interval for mean		P value
					Lower bound	Upper bound	
Age (days)	CoNS positive	50	10.5	10.2	1.7	5.8	<0.001*
	No growth	50	3.8	7.1	7.6	13.4	
Weight (Kg)	CoNS	50	2.4	782.3	2086.5	2535.1	0.716
	No growth	50	2.3	789. 4	2145.9	2590.5	

*p value <0.05 is considered significant by independent sample t-test.
CoNS – coagulase-negative staphylococci.

Table 3. Relation between blood culture positive with CoNS in neonates suspected with sepsis, neonates with nasal colonization by CoNS, and others showing no growth

		No sepsis	Clinically suspected sepsis with negative blood culture	Clinically suspected sepsis and positive blood culture	P-value	Chi-square value	df
No growth	Number (50)	49	0	1	<0.001*	85.1	1
CoNS	Number (50)	2	4	44			

*p value <0.05 is considered significant by Chi-Square test. **df** – degrees of freedom.

colonies indicating biofilm formation, while Congo red negative were 24 (48%), their color ranged from pink to red indicating non-biofilm formers (Figure 1).

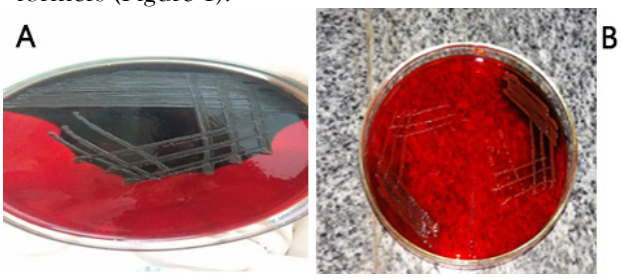


Figure 1. CoNS strains tested for biofilm formation by Congo red test
(A) Congo red positive isolates (growth appears black in color). (B) Congo red negative isolates (growth appears red in color).

Among CoNS isolates, the frequency of multidrug resistance detected was as high as (86%); the resistance was mostly demonstrated against the following antibiotics: penicillin (98%), followed by ceftriaxone (96%), oxacillin and cefoxitin (82%) (Table 4). Inducible clindamycin resistance between clindamycin and erythromycin was detected in only five cases (Figure 2).

All CoNS strains were tested for methicillin resistance using cefoxitin and for the presence of the *mecA* gene by PCR. Forty-one isolates out of 50 (82%) proved to be *mecA* gene positive and showed resistance to cefoxitin and other β-lactam antibiotics as well, with a total of 42/50 resistance rate for cefoxitin (84%). The relationship between the presence of *mecA* and phenotypic methicillin resistance in CoNS was statistically significant (p<0.001) (Table 5). There was no evident connection between biofilm formation and methicillin resistance among the tested isolates (p=0.704) (Table 6).

The presence of the *icaA* (189 bp) and *icaD* (198 bp) genes among CoNS isolates was demonstrated by the amplification of the corresponding fragments. There were 29 (58%) isolates positive for *icaA*, while 30 (60%) strains were positive for the *icaD* gene. A clear

Table 4. Antimicrobial susceptibility of CoNS to different antibiotics

Antimicrobial	Sensitive		Intermediate		Resistant		Total
	Number	Percentage	Number	Percentage	Number	Percentage	Number
Penicillin	1	2	0	0	49	98	50
Oxacillin	9	18	0	0	41	82	50
Vancomycin	33	66	1	2	16	32	50
Erythromycin	18	36	0	0	32	64	50
Clindamycin	31	62	1	2	18	36	50
Tetracycline	16	32	0	0	34	68	50
Sulfamethoxazole-trimethoprim	23	46	1	2	26	52	50
Rifampicin	47	94	0	0	3	6	50
Cefoxitin	9	18	0	0	41	82	50
Ceftazidime	2	4	0	0	48	96	50
Linezolid	49	98	0	0	1	2	50
Ciprofloxacin	24	48	0	0	26	52	50
Gentamicin	32	64	0	0	18	36	50
Novobiocin	50	100	0	0	0	0	50

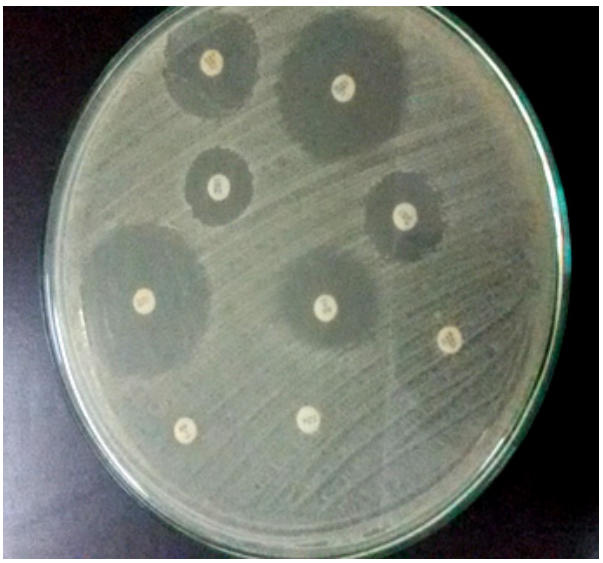


Figure 2. Inducible inhibitor D shape resistance

relationship was observed between biofilm formation and detection of *icaA* gene among the tested strains (p=0.025). On the other hand, the *icaD* gene was detected only in 43.3% of CoNS biofilm formers, statistically non-significant (p=0.133) as shown in Table 7 and Figure 3.

Discussion

The current study was conducted on 170 neonates admitted in the NICUs of Beni-Suef University Hospital and Beni-Suef General Hospital, over a period of five months. Nasal swabs (340) were taken from neonates at the time of admission and after three days. Isolates that appeared only on the third day were included in the study as nosocomial colonization.

The present findings revealed that CoNS were the most prevalent isolates (50/100), a finding that is consistent with Ternes et al.,[17] who found rates of colonization with CoNS of 55.9%.

Species identification is important for monitoring the reservoir and understanding the pathogenic potential of different CoNS species.[18] The results revealed that *S. haemolyticus* (48%) and *S. epidermidis* (36%) were the most common species.

Table 5. Relation between phenotypic expression of methicillin resistance and genotypic *mecA* gene positivity (n=50)

Presence of *mecA* gene	Methicillin phenotype, n (%)		P-value	Chi-square value	df
	Resistant	Sensitive			
Negative (n=9)	1 (11.1%)	8 (88.9%)	<0.001*	43.4	1
Positive (n=41)	41 (100%)	0 (0%)			

*p value <0.05 is considered significant by Chi-Square test. **df** – degrees of freedom.

Table 6. The relation between biofilm formation and methicillin phenotype

	Methicillin phenotype, n (%)		P value	Chi-square value	df
	Sensitive N=8	Resistant N=42			
Biofilm positive 26 (52%)	5 (62.5%)	21 (50%)	0.399	0.420	1
Biofilm negative 24 (48%)	3 (37.5%)	21 (50%)			

df – degrees of freedom.

Table 7. Comparison between Congo red agar test and PCR test results for *icaA* and *icaD* gene

	Congo red agar, n (%)		Total	P-value	Chi-square value	df
	Positive N=26	Negative N=24				
icaA						
Positive	19 (73.1%)	10 (41.7%)	29 (58.0%)	**0.025***	5.1	1
Negative	7 (26.9%)	14 (58.3%)	21 (42.0%)			
icaD						
Positive	13 (50.0%)	17 (70.8%)	20 (40.0%)	0.133	2.3	1
Negative	13 (50.0%)	7 (29.2%)	30 (60.0%)			

Similar results have been reported in other studies.[19,20]

The main risk factors for colonization in this study were the use of intravenous cannula and empirical antimicrobial therapy. These observations were supported by several studies.[21,22] These findings could be explained by the fact that the exposure to a variety of procedures such as intubation, catheters, peripheral venous lines, and parenteral nutrition allow entry of microbes.[18]

A statistically significant association was reported between the clinically featured neonates with sepsis and blood culture positive for CoNS and the clinical status of patients regarding the clinical sepsis (p<0.001). Most of the neonates with clinically suspected sepsis proved by blood culture results were nasally colonized with CoNS (44/50, 88%). This finding was comparable to the work done by Shehab El-Din et al., who reported that the rate of neonatal sepsis confirmed to have bloodstream infection with CoNS was found to be significant (52.86%).[20]

Moreover, the prevalence of multidrug resistance was evident among CoNS isolates; that could be explained by the intensive selective pressure by antibiotic therapy administered to neonates in the NICU.[23] In the current study, a high rate of resistance was detected to β-lactam antibiotics such as penicillin (98%), ceftriaxone (96%), oxacillin and cefoxitin (82%), which was confirmed by the presence of *mecA* gene in 82% of isolates. These findings paralleled the results obtained by Ternes et al.[17]

Biofilm is one of the most important virulence factors for CoNS that mediates resistance to antimicrobial therapy and guaranties chronicity of infection. CoNS isolates from the enrolled neonates (52%) were biofilm formers, using CRA medium, a finding that came in agreement with

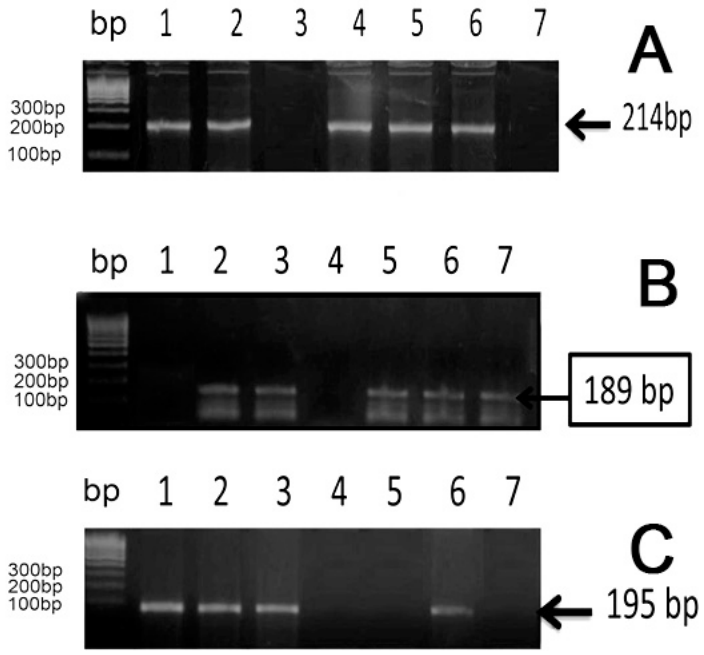


Figure 3. PCR detection **of *icaA, icaD* and *mecA*. Lane 1, 100 bp DNA molecular size marker**
(A) PCR detection of *mec*A gene locus (A, 214-bp band). Lane 1 shows the DNA marker, lanes 1, positive control for *mec*A gene S. *aureus* strain ATCC 43300. Lane 7 negative control. Lanes 2, 4, 5, 6 were positive. Lane 3 negative case.
(B) PCR detection of *ica*A gene locus (B, 189-bp band). Lane 2, positive control for *ica*A gene S. *epidermidis* reference strain (ATCC 25923). Lane 1, negative control for *ica*A gene S. *epidermidis* reference strain (ATCC 12228). Lanes 3, 5, 6, 7 were positive. Lane 4 negative case.
Figure (C) PCR detection of *icaD* gene locus (C, 195-bp band). Lanes 2, 3, 6 positive cases. Lanes 4, 5 negative cases. Lane 1, positive control for *icaD* gene S. *epidermidis* reference strain (ATCC 25923). Lanes 7 negative control for *icaD* gene S. *epidermidis* reference strain (ATCC 12228).

that of Nasr et al.,[7] where 56% of CoNS isolates were biofilm producers.

It was found that co-expression of some genes such as *icaA and icaD* are necessary for phenotypic expression of biofilm in many isolates.[24] *icaA* and *icaD* genes were detected by PCR as a reference of phenotypic method in 30 (60%) CoNS isolates in the present study. These results were consistent with Sharma et al.[23] who found that 68.2% of the clinical isolates were positive for the *ica* genes. A clear relationship was present between biofilm formation and detection of *icaA* and *icaD* genes among the tested strains in this study, which was statistically significant with the *icaA* gene (p=0.025). A good correspondence between phenotypic characterization methods and the presence of *ica* genes was reported by Nasr et al.[7]

Unexpectedly, in the current study, 26.9% and 50% of the isolates were *icaA* and *icaD* negative and produced biofilm as detected by CRA methods, this concurs with the findings reported by Nasr et al.,[7] and enlightened by the presence of genes inducing an alternative PIA-independent mechanism of biofilm formation in both *S. aureus* and *S. epidermidis*.[25] However, lack of biofilm production by certain isolates despite their positivity for the *ica* genes like our findings, was elucidated by Post et al.[26] They reported that although *ica* genes are responsible for biofilm formation, full phenotypic expression could be conditioned by additional genes (*atlE, sarA, agrA*

and *mecA*) that have direct or indirect regulatory impact.

This study had the following limitations: follow-up of the neonates for colonization at several intervals and at discharge as well as following up the outcome of each case could have added more data to the present findings. However, due to lack of funding and materials available, we couldn't extend it any further.

Conclusions

Taken together, it can be concluded that nasal screening of neonates should be considered in NICUs to preclude nasal colonization that may predispose to neonatal sepsis. Prematurity, use of devices and abuse of antibiotics are serious risk factors for colonization with CoNS. Consequently, the quality of medical services in the NICU environment should be improved with constant training and education of healthcare providers for measures and procedures of infection control, over and above, the implementation of strict antibiotic policies.

Furthermore, the biofilm forming CoNS stains showed resistance to antibiotics potentially leading to treatment failure and development of more resistance. Hence, a better tool is recommended for screening of biofilm formation that might help to modify antibiotic therapy and averting hospital-acquired infections in NICUs. Similarly, anti-adhesive strategies could be applied to inhibit bacterial surface adhesion or to disrupt an already established biofilm, targeting the biofilm matrix. It is vital and imperative to continue investigating the environmental factors, which might contribute to increasing incidence of nasal colonization of neonates with resistant strains.

Authors' contributions statement: DMA designed the research plan and organized the study. SMAS carried out all laboratory tests, coordinated the data analysis, and had a major contribution in writing of the manuscript. MHM supervised all clinical issues of patients. NHI participated in the writing of the manuscript and had a major role in following-up all steps of the study. MAWAM participated in the main role of editing and revising the manuscript. All authors reviewed and approved the final version of the manuscript.

Conflicts of interest: All authors – none to declare.

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