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PCR detection of Biofilm formation and slime producing genes of coagulase-negative staphylococci isolated from patients with catheter related infections

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Abstract

One hundred of bacterial isolates, were identified as a CoNS represented by 53% *S. haemolyticus*, 26% *S. epidermidis* and 21% *S. hominis*. the results showed that only 93% of isolates were phenotypically biofilm formation in Christensen tube and TS-Congo red agar methods, PCR technique revealed that 93% of CoNS isolates carried *aap* gene and 98% of isolates were expressed *icaA* and *icaD* genes.

Keywords: coagulase-negative staphylococci, catheter-related infections, *icaA* gene, *icaD* gene, *aap* gene.

Introduction

Few years ago the interest in coagulase-negative staphylococci (CoNS) species has significantly increased due to their impact on human health and disease. CoNS are common bacterial colonizers normal human microflora and usually have a benign relationship with the host [1]. These microorganisms are characterized by their ability to adhere and grow on the smooth surface of catheters and other medical devices [1]. Therefore, CoNS has become the leading cause of foreign-body infections due to its biofilm formation on all kinds of medical-device surfaces. The biofilm development includes two steps: the initial attachment phase and the accumulative phase. In the accumulative phase, the polysaccharide intercellular adhesin (PIA), encoded by the *icaADBC* locus, is the major component mediating intercellular adhesion [2] . The *icaADBC* operon (intercellular cluster adhesin) and the accumulation-associated protein (AAP) as important genetic determinants of slime production [3].

The aims of present study was to determine what species were present in a collection of clinically isolated, coagulase-negative; to characterize some coagulase-negative staphylococci; to detect the *aap, icaA, icaD* genes; and to compared between phenotype and genotype biofilm production among coagulase-negative staphylococci.

Materials and Methods

Strains

From January to July 2013, three hundred isolates were collected from different clinical specimens obtained from patients undergoing catheter related infections (100 from urine, 60 from blood, 55 from high vaginal swabs (H.V.S), 35 from seminal fluid and 50 from wound swabs).

Identification of coagulase-negative staphylococci

Isolates obtained from the different clinical specimens were seeded onto mannitol salt agar and stained by the Gram method for the determination of purity, morphology, and specific staining. After confirmation of these characteristics, the isolates were identified according to Kloos and Schleifer (1975), every suspicious colony was tested for catalase and

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coagulase. If the catalase and coagulase test were positive and negative, respectively, further identification included oxidase test, Voges – Proskauer test, bacitracin and novobiocin diagnostic test, and finally used of Vitek 2 system according to Guido and Pascale (2005).

Phenotypic detection of slime layer and biofilm formation

Two methods used for detection of slime layer and biofilm formation, **first:** Christensen tube method according to Christensen *et al.* (1982) proposed qualitative method for biofilm detection. A loopful of tested organisms was inoculated in 10 mL of trypticase soy broth with 1% glucose in test tubes. The tubes were incubated at 37ºC for 24 h. After incubation, tubes were decanted and washed with phosphate buffer saline (pH 7.3) and dried. Tubes were then stained with trypan blue solution (0.4%). **Second:** TS-Congo red agar method according to Ishiguro et al. (1985) proposed this medium for detection of strains possess the cell surface protein array known as the A-layer (A+) involved in virulence formed deep red colonies on trypticase soya agar containing Congo red stain. These were readily distinguished from colorless or light orange colonies of avirulent mutants lacking A-layer (A-).

Genotype analysis

DNA extraction: Genomic DNA was isolated in method that described by Arciola *et al.* (2001). Fresh trypticase soya broth was inoculated bacterial colony and allowed to growth at 37˚C overnight in orbital shaker. The tubes were then vortexed. 100µl of the cultures were taken into eppendorf tubes. They were centrifuged at 10000 rpm for five minutes and the soup was removed. The pellets were resuspended in 45µl of distilled water and 15µl lysostaphin (100µg/µl). They were incubated at 37˚C for an hour in the water bath. 15µl of Proteinase K (100µg/µl) and 150µl TrisHCl were added and incubated at 37˚C for an hour in the water bath. The samples were then boiled at 100˚C for five minutes. Genomic DNAs were ready and stored at -20˚C.

PCR analysis: The extracted DNA, primers (CUSABIO/China) and PCR premix (SolGent/Korea), were thawed at 4˚C, vortex and centrifuged briefly to bring the contents to the bottom of the tubes. PCR mixture was set up in a total volume of 30 µL included 15µL of PCR premix, 2µL of each primer and 5µL of template DNA have been used. The rest volume was completed with sterile deionized distilled water, then vortexed. Negative control contained all material except template DNA, so instead that distilled water was added.

Oligonucleotide	5′to3′ nucleotide sequence	Amplified product (bp)	References
icaA1	TCT CTT GCA GGA GCA ATC AA	188	Carla *et al.* (2001)
icaA2	TCA GGC ACT AAC ATC CAG CA		
icaD1	ATG GTC AAG CCC AGA CAG AG	198	Carla *et al.* (2001)
icaD2	CGT GTT TTC AAC ATT TAA TGC AA		
aap1	ATA CAA CTG GTG CAG ATG GTT G	399	Mohammad *et al.* (2011)
aap2	GTA GCC GTC CAA GTT TTA CCA G		

PCR amplification: The *icaA* and *icaD* genes amplification by PCR was performed under the conditions described by Carla *et al.* (2001): initial denaturation at 94˚C for 5min, followed by 50 cycles of denaturation at 94˚C for 30sec, annealing at 55.5˚C for 30sec, and extension at 72˚C for 30sec. after completion of the 50 cycles, the tubes were incubated at 72˚C for 1min before cooling to 4˚C.

The *aap* gene amplification by PCR was performed under the conditions described by Mohammad *et al.* (2011): initial denaturation at 97˚C for 6min, followed by 35 cycles of denaturation at 92˚C for 30sec, annealing at 55˚C for 30sec, and extension at 72˚C for 45sec.

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after completion of the 35 cycles, the tubes were incubated at 72˚C for 10min before cooling to 4˚C.

Detection of PCR products: PCR products were resolved by electrophoresis through a 0.7% agarose gel containing ethidium bromide [4]. DNA molecular weight standard 100 bp (Promega/USA) was used as the marker.

Results and Discussion

Isolation and identification

Out of the total 300 samples, only one hundred bacterial isolates were identified as coagulase-negative staphylococci and consisted of 47 isolates isolated from urine, 28 isolates isolated from blood, 12 from high vaginal swab (HVS), 13 isolates from seminal fluid, and no isolates isolated from wound infection (Table1).

Table 1: Distribution of bacterial isolates among the clinical specimens

No.	Specimens	Total No.	Total No. of bacterial isolates	Percentage
1	Urine	100	47	47%
2	Blood	60	28	46.67%
3	H.V.S	55	12	21.81%
4	Seminal fluid	35	13	37.14%
5	Wound	50	0	0%

The results showed that three species of CoNS were identified: fifty three isolates were identified as *S. haemolyticus*, twenty six isolates were identified as *S. epidermidis* and twenty one isolates were identified as *S. hominis*. The most common species isolated was *S. haemolyticus* 53(53%). The similar results were recorded in many studies of de Paulis *et al.* (2003), Sheikh and Mehdinejad (2012) they reported that majority number of isolates was *S. haemolyticus*.

CoNS species are increasingly associated with infections, *S. haemolyticus* plays an important role in hospital-acquired opportunistic infections, it is second only to *S. epidermidis* in frequency of isolation from human blood cultures and has been reported to cause peritonitis, otitis media, urinary tract infections and septicemia [5].

Study of Usha *et al.* (2013) founded among the 100 isolates, *S. epidermidis* was the most common species isolated (32%), followed by *S. haemolyticus* (18%) and *S. hominis* (10%).

The results showed that *S. epidermidis* was the second common species in 26%, similar results were recorded by Singh *et al.* (2008), and Asangi *et al.* (2011). Study by Kleeman *et al.* (1993) suggested that *S. epidermidis* was the most common isolate, *S. haemolyticus* may be important in urinary tract infections. The result of the study revealed that 35(74.47%) of *S. haemolyticus* isolates were recorded from urine specimens (Table 2) followed by *S. epidermidis* 10(21.28%) and *S. hominis* 2(4.25%).

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Table 2: Showing different species of CoNS from various clinical specimens

Species of CoNS	Specimen								Total	
	Urine		Blood		H.V.S		Seminal fluid			
	No.	%	No.	%	No.	%	No.	%	No.	%
S. haemolyticus	35	74.47	4	14.28	2	16.67	12	92.31	53	53%
S. epidermidis	10	21.28	6	21.43	10	83.33	0	0	26	26%
S. hominis	2	4.25	18	64.29	0	0	1	7.69	21	21%
Total	47	100	28	100	12	100	13	100	100	100

Other studies by Sarathbabu *et al.* (2013) founded the majority number of isolates from urine 28(14%) followed by sputum, pus and blood. Also, by Sheikh and Mehdinejad (2012) founded the majority of CoNS related to organ was obtained from urine specimens (51.5%) followed from blood 25.4%.

Phenotypic detection of biofilm production

Christensen's tube method and TS-Congo red agar were used to investigation of biofilm production, the results of two methods showed that 93% isolates were biofilm producers (Figure 1,2), represented of 53% *S. haemolyticus*, 26% *S. epidermidis* and *S. hominis* 14.

Figure1: Biofilm production by tube adherence test

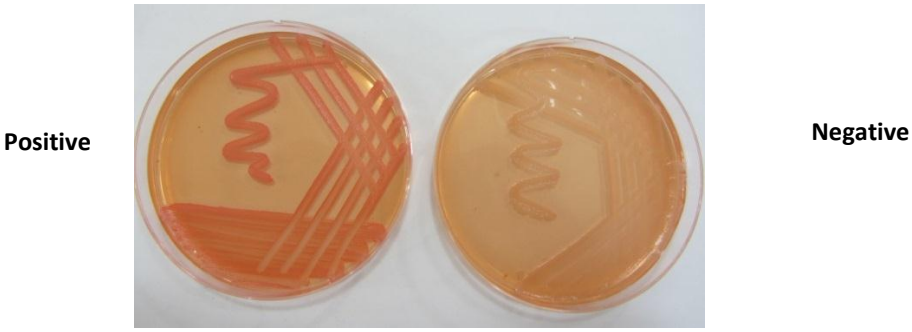

Figure2: Slime layer production by TS-Congo red agar

High level of biofilm production in our result was agreed with the study by Oliveira and Cunha (2010) they showed that 82% of the 100 isolates were biofilm producers, also higher rate of biofilm formation was demonstrated by Gad *et al.* (2009) they found that 88.6% of *S. epidermidis* isolated from urinary tract catheterized patients produced biofilm.

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However, if slime production promotes adherence to prostheses, thus acting as a virulence factor, infection control becomes more difficult since it protects CoNS cells from antimicrobial agents and the host's natural defense mechanisms [6], they reported that the mucous substance produced by CoNS can interfere with the cell-mediated immune response.

The ability of staphylococci to form biofilms helps the bacterium to avoid host immune response and is considered responsible for chronic as biofilm protects microorganisms from opsonophagocytosis and antimicrobial agents [7].

In CoNS, specifically *S. epidermidis*, biofilm formation is encoded by the icaADBC operon [8]. It is advantageous for CoNS to reside in a biofilm, these bacteria are protected from the external environment, allowing microbial communication in the form of horizontal gene transfer and have enhanced virulence due to immune evasion [9].

PCR analysis of slime layer genes

The results of PCR study revealed that 98% of CoNS isolates gave positive for detection of *aap* gene (Figure 3). Although, PCR amplification revealed 98% isolates have *aap* gene, but, phenotypically 93% have slime layer.

Figure3: PCR product of *aap* gene from CoNS species

The presence of the *aap* gene in CoNS isolates is essential for biofilm development (Latasa *et al.*, 2006). In fact, higher expression and transcription levels of aap account for a polysaccharide intercellular adhesion-independent biofilm [10].In addition to *aap* gene, the results indicated that among CoNS isolates 93% were positiveness for *icaA* and *icaD* genes (Figure4), 93% of isolates which were positive for icaA and for icaD genes most of them were related to catheter infections. The slime-producing strains was found to be positive for both genes, giving a 188-bp band for the *icaA* gene and a 198-bp band for the *icaD* gene. The non-slime-producing strains was negative for both genes.

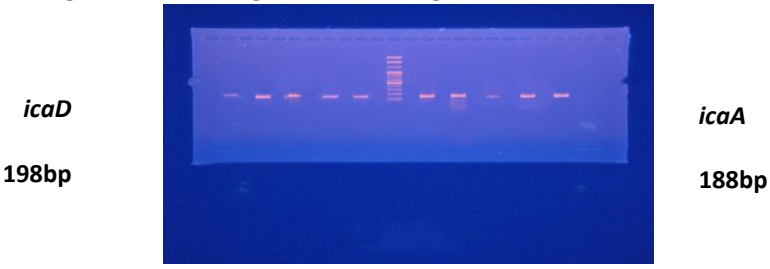

Figure4: PCR amplification of *icaA* and *icaD* genes

Results revealed that both *icaA* and *icaD* genes were either present or absent and no single strain had shown the presence of one gene. These results confirm the fact that both genes are part of one operon and so the entire operon was either present or absent. In addition, our results showed that both genes (*icaA* and *icaD*) were present in all biofilm producing strains, indicating the important role of *ica* genes as virulence markers in staphylococcal infections [11].The study of Gad *et al.* (2009) was agreement with this study, who reported that all biofilm producing strains were positive for icaA and icaD genes, and all biofilm negative strains were absent for those genes. Oliveira *et al.* (2012) demonstrated that 91.7% of the 12 children whose death was related to CoNS, were infected with strains that were positive for *icaA* and *icaD* genes, agreed with our study.

Conclusion: We conclude that the production of biofilm may be associated with the ability to cause CoNS infection. This conclusion suggests that the regulation of biofilm expression may play a central role in the disease process.

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