



Molecular characterization of coagulase negative staphylococci isolates from clinical and carries specimens

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Abstract:

Sixty coagulase-negative staphylococci (CONS) isolates collected from patients and carries (forty of clinical origin and twenty of carries origin) during the period from October 2012 to March 2013. Isolates diagnosed depending on the morphological criteria cultural and biochemical test and Vitek-2 system test. The most common isolates species among was *S.epidermidis* which was accounted for 18 isolates (30%), *S.saprophyticus* was the second with 13 isolates (21.7%), followed by *S.hemolyticus* was 11 isolates (18.3%), *S.homins* was 7 isolates (11.7%), *S.lentus* was 6 isolates (10%), *S.capitis* was 4 isolates (6.7%) and one isolated (1.6%) of *S.auricularis*. multiple PCR was used to detection the *mecA&icaA* genes . the result indicated 30 isolates (75%) from all 40 clinical isolates produce *mecA* gene while 7 isolates (35%) produce this gene in carries isolates. the *icaA* gene produce in 12 (30%) clinical isolates while two isolates (10%) have ability this gene in carries isolates. Ten clinical isolates (25%) produce *mecA&icaA* genes while only one carries isolate (5%) produce two genes.

Introduction:

Staphylococci are important pathogenic bacteria responsible for a range of disease in human. (Adjuwon *et al.*, 2010). Coagulase-negative staphylococci (CONS) are ubiquitous microorganism and predominate in normal skin flora (Mermel *et al.*, 2009). They are commonly isolated in clinical specimens and several species are recongnized as important agents of nosocomial infection , especially in immunocompromise patients, premature newborns, prosthetic valve endocarditis, central nervous system shunt infections, intravascular catheter – related infections, urinary tract infections, arthritis and prosthetic joint infection (Heikens *et al.*, 2005). More than 30 species of CONS are recognized but only a few are commonly incriminated in human infections (Ruth *et al.*, 2006). The most frequently encountered CONS species associated with human infections are *Staphylococcus epidermidis*, in particular in associated with nosocomial bacteremia (Mermel *et al.*, 2009; Singh *et al.* 2008). Several other CONS species have been implicated at low incidence in a variety of infections. The CONS species *Staphylococcus Saprophyticus* was often regarded as a more important opportunistic pathogen than *Staphylococcus epidermidis* in human urinary tract infection (UTI), especially in young sexually active females (Garcia *et al.*, 2004).

The pathogenesis of CONS infection depends on their ability to first adhere to the substrate and then to form a mucoid biofilm referred to as slime (Otto, 2009). The biofilm produced by CONS is an important virulence factor and mainly consists of polysaccharide intercellular adhesion (PIA), which is encoded by the *icaADBC* operon (O'Gara *et al.*, 2001). Biofilm are a major clinical problem, mainly due to high levels of resistance to host immune response and antimicrobial therapy (Jefferson, 2004).

Over the last decades, there has been an enormous increases and emergence of CONS strains resistant to antibiotic methicillin, particularly in nosocomial setting (Singh *et al.*, 2008). Most of



these strains present the PBP -2 encoding *mecA*, apenicillin – binding protein which has decreased affinity for β - lactams antibiotics. Detection of resistance to methicillin in staphylococci is important to guide the therapy and prevent the patient from being unnecessarily treated with vancomycin, which is an antimicrobial agent that presents therapeutic complications, high costs, and may lead to the selection of resistant mutants (Rogers *et al.*, 2009).

The purposes of this study were:

- 1- To identify to species level the different CONS strains isolates from clinical and carries samples.
- 2- Detection and compare the present of *mecA* & *icaA* virulence genes in clinical and carries CONS strains.

Materials and Methods

Specimens collection

Staphylococcal isolates obtained during the period from October 2012 to March 2013 from clinical and carries specimens in Diwaniyah Teaching Hospital, Hakim Teaching Hospital and Zahra Hospital Maternity and Children in AL-Najaf province, were tested. Forty of clinical isolates, were obtained from culture of several specimens (16 isolates from urine, 11 from blood, 4 from vaginal and 4 also from wound, 3 from ear and 2 from pus). Twenty of carries isolates were obtained from healthy skin (12) and nasal swab (8).

Identification of an Organism

Samples were cultured (BHI broth/blood agar) and after 24-h incubation the plates were examined for colony characteristics. Isolates were identified by colony characteristics, Gram stain, catalase test and oxidase. Bacitracin (0.04 U) and Novobiocin (5 μ g) susceptibilities were determined to exclude *Micrococcus*, *Planococcus*, and *Stomatococcus* spp. Coagulase test and mannitol fermentation were done to exclude *Staphylococcus aureus* and other coagulase-positive species. These tests were performed on all samples of *staphylococcus* as per standard procedures. The isolates were identified to species level by using Vitek-2 system (bioMerieux) according to the manufacturer's instructions.

Genomic DNA Extraction

The bacterial DNA extracted from coagulase – negative staphylococci strains by used Genomic DNA mini kit. Single pure organisms colony that was growth on a blood agar plate was inoculated at in 5 ml of Brain Heart Infusion broth and incubated at 37°C for 24 hrs. One milliliter of the culture was pelleted in a 1.5 ml Eppendorf tube by centrifugation at 15000 \times g for 1 minute. 200 μ l of lysozyme was added to the cell suspension and re-suspend the cell pellet by shaking vigorously and incubated at room temperature for 10 minutes. Following incubation, 200 μ l of GB Buffer (proteinase) was added to cell suspension and mix by shaking vigorously. The suspension was incubated at 60°C for at least 10 minutes to ensure the sample lysate is clear. After incubation, 200 μ l of absolute ethanol was added to the clear lysate and mix by shaking vigorously for 10 seconds. At this time, GD column place in a 2ml collection tube. Transfer all mixture (including any precipitate) to the GD column by centrifugation at 15000 \times g for 5 minute. Discard the 2ml collection tube containing the flow-through and place the GD column in a new 2ml collection tube. 400 μ l of W1 Buffer added to GD column by centrifugation at 15000 \times g for 30 seconds. Discard the



flow-through and place the GD column back in the 2ml collection tube. 600µl of Wash Buffer (ethanol) added to GD column by centrifugation at 15000×g for 30 seconds. Discard the flow-through and place the GD column back in the 2ml collection tube. By centrifugation again at 15000×g for 3 minute to dry the column matrix. Transfer the dried GD column to at clean 1.5 ml Eppendorf tube. 100µl of pre-heated Elution Buffer added to the center of the column matrix. Let stand for at least 3 minutes to ensure the Elution Buffer is absorbed by the matrix. By centrifugation at 15000×g for 30 seconds to elute the purified DNA and then DNA extract was kept in deep freeze until use.

Amplification by Multiplex Polymerase Chain Reaction (PCR):

Amplification reaction mixture for Multiplex gene *mecA* & *icaA* contained: 5µl of DNA template, 1.5 µl of 10 pmole/µl of each primer (forward and reverse) and 9ul of PCR water. The total volume of mixture (20ul) was added to PCR tube master mix which content (Top DNA polymerase 1U, Each dNTP (dATP dCTP dGTP dTTP) is 250 Mm, Tris HCL pH 9.0 10mM, KCL 30Mm and MgCl₂ 1.5mM) . PCR conditions were as follows: initial denaturation at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 45 sec, ending with a final extension at 72°C for 5 min. The sequence of forward and reverse primers (Bangalore Genei, Bengaluru, Karnataka, India) used for multiplex PCR (*mecA* & *icaA* genes) were as follows:

1. *icaA* (product size 546 bp)

Forward *ica*- TTATCAATGCCGCAGTTGTC

Reverse *ica* - GTTTAACGCGAGTGCGCTAT

2. *mecA* (product size 310 bp)

Forward *mecA*- GTAGAAATGACTGAACGTCCGATAA

Reverse *mecA*- CCAATTCCACATTGTTTCGGTCTAA

Electrophoresis:

PCR products were resolved by electrophoresis in 1.5% agarose gels (0.53TBE) at 100 V stained with ethidium bromide and photographed under UV light.

Statistical analysis:

The results were analyzed statistically by Chi-square (X²) test at the level of significant when P-value ≤ 0.01 (Niazi, 2000).

Results and Discussion

A total of 60 CONS strains belonged to 7 species were identified in this study. All species were present in clinical isolation while only 5 species in carries isolation. The species included; *S. epidermidis* (18), *S. saprophyticus* (13), *S. haemolyticus* (11), *S. hominis* (7), *S. lentus* (6), *S. capitis* (4) and *S. auricularis* (1) were among them (**Table 1**). This finding was in agreement with the study from Iraq by Ali *et al.* (2009) they found 8 species in total of 60 CONS strains.



Table 1: Distribution of CONS Species

Species	clinical sample		carries		Total (%)	
	NO.	%	NO.	%	NO.	%
<i>S. epidermidis</i>	10	25	8	40	18	30
<i>S.saprophyticus</i>	9	22.5	4	20	13	21.7
<i>S.haemolyticus</i>	8	20	3	15	11	18.3
<i>S.hominis</i>	3	7.5	4	20	7	11.7
<i>S.lentus</i>	5	12.5	1	5	6	10
<i>S.capitis</i>	4	10	0	0	4	6.7
<i>S.auricularis</i>	1	2.5	0	0	1	1.6
Total	40	66.7	20	33.3	60	100
Cal. $X^2 = 6.256$ tap. $X^2 = 4.3$ P-value = 0.3951						

In the present study, *S. epidermidis* was CONS species most frequently isolated from clinical samples, corresponding to 10 (40 %) isolates. The remaining species were distribution among *S.saprophyticus* (9 isolates, 22.5%), *S.haemolyticus* (8 isolates, 20%), *S.hominis* (3 isolates, 7.5%), *S.lentus* (5 isolates, 12.5%), *S.capitis* (4 isolates, 10%) and *S.auricularis* (1 isolates, 2.5%). The CONS clinical species mostly commonly isolated in the study were *S. epidermidis*, *S.saprophyticus*, *S.haemolyticus* and *S.hominis*, distribution Similar to finding by Singh *et al.* (2008), where they identified 40% of *S. epidermidis*, 14% of *S.saprophyticus*, 12% of *S.haemolyticus* and 6% of *S.hominis*. In our study, *S.lentus*, *S.capitis* and *S.auricularis* had the lower rate of clinical isolation among CONS species. The lower rates of *S.lentus* (5%) were reported by Ali *et al.* (2009), and *S.capitis* with 1.7% frequency and *S.auricularis* with 1.1% was reported by Caierao *et al.* (2004). *S.capitis* with 8.2% frequency in Iran was reported by Sheikh and Mehdinejad (2012).

In carries isolates, *S. epidermidis* was the dominating species 8 (40%) isolates followed by *S.saprophyticus* and *S.hominis* 4 (20%) isolates from each species while 3 (15%) *S.haemolyticus* and one isolates (5%) to *S.lentus*. The result indicated no a significant difference between the clinical and carries CONS isolates ($P > 0.01$).

Results of gene amplification by multiplex PCR are shown in (Table 2) (figure 1). The difference between clinical and carries isolates was statistically significant for the presence of the *mecA* & *icaA* genes ($P \leq 0.01$). A total of 30 (75%) of clinical isolates expressed the *mecA* gene with highest percentage in *S. epidermidis* and *S.haemolyticus* (Table 3) while only 7 (37%) carries isolates expressed the *mecA* gene.. This finding was very much similar to other studies done by Sharma *et al.* (2011), by Bouchami *et al.* (2011) from Tunisia and by Caierao *et al.* (2004) from Brazil in which they found 80%, 65% and 69.1% clinical CONS strains as *mecA* positive by PCR respectively.

Table 2: Comparison of *mecA* & *icaA* positivity in isolated from clinical and carries

Genes	clinical sample N=40		carries N=20		Total N=60	
	NO.	%	NO.	%	NO.	%
<i>mecA</i>	30	75	7	35	37	61.66
<i>icaA</i>	12	30	2	10	14	23.3
<i>mecA</i> & <i>icaA</i>	10	25	1	5	11	18.3
Cal. $X^2 = 3.355$		tap. $X^2 = 1.81$		P-value = 0.00265		

The *mecA* gene, which controls the synthesis of an additional penicillin-binding protein PBP2a in methicillin-resistant *Staphylococcus*. The relatively increased rate of clinical isolates in our study might be due to the fact that our specimens were taken from hospitalized patients where antibiotic policy to treat patients is lacking. Inadequate practice of antibiotic policy, indiscriminate use of antibiotics becomes common. In addition, environmental factors and hygienic conditions of the hospital were not adequate. Overcrowding of patients and attendants favour the spread of infectious agents. In such hospitals acquired infection in different wards should be quite high. As a result patients become infected during hospital staying under antibiotic therapy for prolonged period (Rahman, 2011).

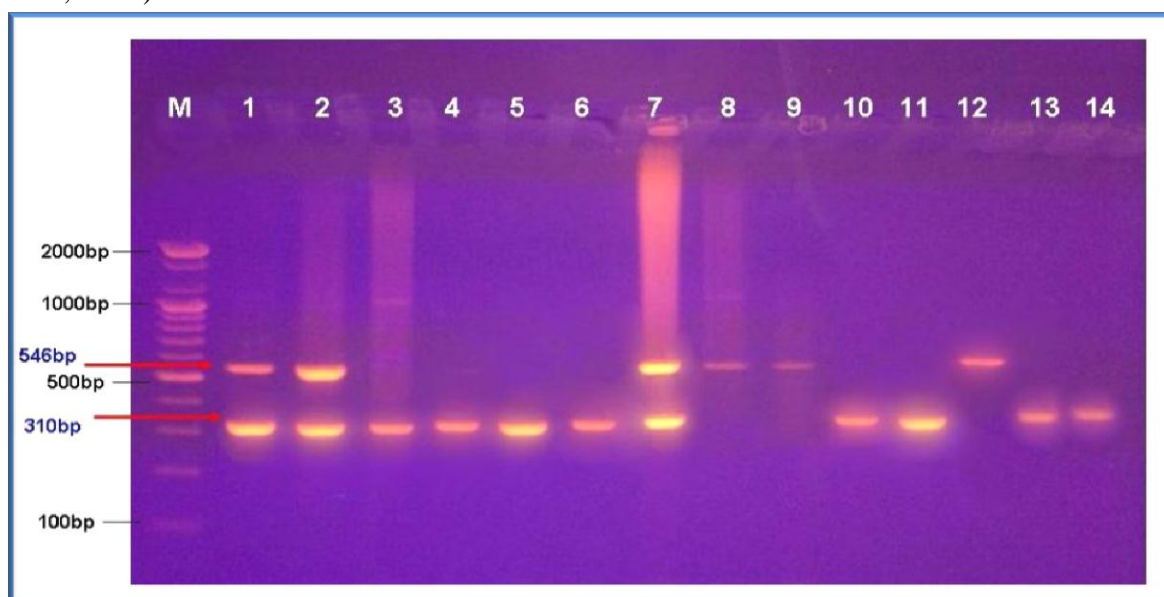


Figure 1 : (Agrose gel electrophoresis image explain the PCR product result of *mecA* & *icaA* genes at positive product in (310 bp & 546bp respectively), M: Marker (100bp).

- Lane (1,2,3,4,5,6,7,10,11,13&14) positive *mecA* gene.
- Lane (1, 2,7&12) Positive *icaA* gene.
- Lane (1, 2&7) Positive *mecA* & *icaA* genes.
- Lane (8&9) Negative *mecA* & *icaA* genes.

The *icaA* gene was expressed by 12 (30%) of clinical isolates while only 2 (10%) of carries isolates expressed the *icaA* gene.



Hussain (2011) compared the presence of *icaA* gene in clinical and non-clinical strains where found 25% of clinical CONS isolates expressed the *icaA* gene while 10% of non-clinical strains similar to our study.

Table 3: : frequency of *mecA* & *icaA* positivity genes CONS isolated from clinical and carries isolates according to species

Species	<i>mecA</i>		<i>icaA</i>		<i>mecA</i> & <i>icaA</i>	
	clinical	carries	clinical	carries	clinical	carries
<i>S. epidermidis</i>	9	3	5	1	5	1
<i>S. haemolyticus</i>	7	1	3	1	2	0
<i>S. saprophyticus</i>	6	2	2	0	1	0
<i>S. hominis</i>	2	1	1	0	1	0
<i>S. lentus</i>	3	0	1	0	1	0
<i>S. auricularis</i>	1	0	0	0	0	0
<i>S. capitis</i>	2	0	0	0	0	0
Total	30	7	12	2	10	1

Polysaccharide synthesis is mediated by the *ica* operon; it encodes an *N*-acetylglucosaminyl transferase enzyme that catalyzes the synthesis of the capsular polysaccharide (β -1, 6-glucosaminoglycan) from *N*-acetylglucosamine. The production of polysaccharide intercellular adhesin (PIA) is an important component in the process of biofilm formation, suggesting that the *ica* operon plays an important role in pathogenicity of CONS (Kozitskaya *et al.* 2005).

PIA production is not always correlated with biofilm formation, in recent studies; it has become clear that there are at least two mechanisms of biofilm development in CONS. One mechanism requires the production polysaccharide intracellular adhesin (PIA) by the intercellular adhesion (*ica*) operon. Other *ica*-independent mechanisms described biofilm production in absence of PIA as process which depend upon multiple factors such as the biofilm – associated protein (Bap) and the aggregation - associated protein (Aap) (seidl *et al.*, 2008; Lauderdale *et al.*, 2009).

The result shown presence of *icaA* along with *mecA* was observed in 10 (25%) of clinical CONS strains while one isolate (5%) of carries CONS strains and this combination is related to more resistance and treatment failures. Sharma *et al.* (2011) detected the present both genes in 52 (52%) out 100 CONS isolates from different clinical cases. The lack of the *mecA* gene in biofilm negative phase variants suggests its possible role in pathogenicity. The development of resistance to multiple drugs in CONS is important and is related to biofilm production and its growth pattern (Donlan, 2000).

Mohammad *et al.* (2011) reported the presence *icaA* operon associated with antibiotic resistance. Production of biofilm reduces penetration and access of antibacterial components such as complement, antibodies and various antibiotics to bacteria, hence decreasing the activity of these substances against bacteria (Costerton, 2005).

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