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THE PRESENCE OF ICAAD GENES IN COMMENSALS OF COAGULASE-NEGATIVE STAPHYLOCOCCUS AND EVALUATION OF PHENOTYPIC BIOFILM FORMATION BY CRA AND CRAVC METHODS

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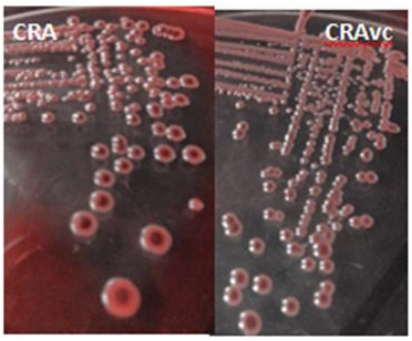

Abstract

A total of 28 commensals of coagulase negative Staphylococcus or CoNS strains were successfully isolated from healthy volunteers. Amplification of the icaAD genes showed that 21% of these isolates harbour these genes which involve in biofilm formation. The ability of these strains to form biofilm was further evaluated phenotypically using both Congo Red Agar (CRA) and a modified Congo Red Agar with vancomycin or CRAvc. Only 32% of the isolates were biofilm positive on CRA as compared to inoculation on CRAvc which displayed a higher number of biofilm producer at 92.8%. However, analysis on the correlation between icaAD genes and the biofilm formation revealed that on CRA, 14.3% of the strains were biofilm tve-ica tve and 17.9% were biofilm tve-ica–ve while on CRAvc, 21.4% were biofilm tve-ica tve and a higher number of strains at 71.4% were biofilm tve-ica–ve. This indicates that growth on CRA gives a more accurate insight on the ability of the strains to produce biofilm although the possibility of the strains forming biofilm by a different mechanism than the icaAD genes cannot be ruled out. Two strong biofilm forming isolates namely C6 and C15 were further subjected for identification via the sodA gene and were found to be S. haemolyticus and S. epidermidis respectively.

Keywords: Fibrinolytic enzyme, endophytic fungi, *Lignosus rhinoceros*

1.0 INTRODUCTION

Coagulase-negative *Staphylococcus* or CoNS were once considered relatively avirulent and have long been dismissed as culture contaminants since they are normal inhabitants of human skin and mucous membranes. While the ability of *S. aureus* to cause infections in human has long been established, CoNS were regarded as harmless skin inhabitants. These organisms are found ubiquitously residing on human skin and mucus membranes and the number varies from 10 to 10^5 colony-forming units (CFU/cm^2) on healthy adults in the community [1]. They rarely cause human disease and are often dismissed by clinicians as culture contaminants, hence given little attention.

The first report on the potential pathogenicity of CoNS in human was documented in 1958 through a study of nine cases of patients with septicemia caused by *S. albus* (now known as *S. epidermidis*) between 1935 to 1957. However, only in the 1970s that these organisms have become increasingly recognized clinically especially in hospital-acquired infections [2]. Since then, CoNS as a group has been

identified as etiologic agents in a wide variety of infections and has emerged among the most frequently isolated bacteria in clinical microbiology. In USA, out of the 24,179 cases of nosocomial bloodstream infections or BSI in 49 hospitals over a seven-year period from March 1995 through September 2002 revealed that the most-common organisms isolated were CoNS with 31% of the isolates followed by *S. aureus* with 20% isolates [3]. In England and Wales, 28% and 10% of the bacterimia cases in children involved CoNS and *S. aureus* respectively [4]. A similar trend was also observed in local study. An investigation on the etiology of blood culture isolates among patients in a multidisciplinary teaching hospital in Kuala Lumpur showed that CoNS were the most common organisms isolated, accounting for 33.0% of the total isolates, followed by *S. aureus* at 10.4% [5].

Most infections in CoNS are related to implanted medical devices such as the urinary catheters, artificial heart valves or prosthetic joints [6]. As such, their potential to cause infections depends on their ability to adhere and colonize these artificial surfaces and form slime layers known as biofilms. A biofilm is defined as a community of microorganisms living together and embedded in a self-synthesized extracellular matrix that consists mainly of polysaccharides. In staphylococci, the major component of this biofilm is a specific exopolysaccharide termed PIA or polysaccharide intercellular adhesion in *S. epidermidis* or also known as PNAG or poly-*N*-acetyl-glucosamine in *S. aureus*. In 1996, an operon of genes responsible for the synthesis of PIA was identified in *S. epidermidis* by transposon insertional mutagenesis [7]. This operon was named *ica* for intercellular adhesion and is composed of the *icaABCD* genes and the regulatory gene *icaR*. PIA was also identified as the main constituent of the extracellular matrix substance or 'slime' in *S. epidermidis* and majority of clinical *S. epidermidis* isolates was found to synthesize this compound [8]

Both IcaA and IcaD are membrane proteins. IcaA is a glucosaminyl transferase which plays a role in transferring glucosamine residues from UDP-glucosamine to a growing chain of pre-PIA during the synthesis of PIA. To fully function, IcaA requires IcaD although the exact role of IcaD is still unclear. IcaC is also a membrane protein, speculated to be involved in transportation of the pre-PIA synthesized by IcaAD out of the cell [9].

The presence of these genes has also been shown in other CoNS species which include *S. hominis, S. lugdenensis and S. haemolyticus*. Hence, the identification of the *icaABCD* genes serves as a tool to determine the biofilm formation ability of *Staphylococcus* sp. This could be useful as a rapid diagnosis in providing an insight for necessary therapy management on the bacteria. In addition, the ability to form biofilm phenotypically can also be determine from a number of methods which include microtitre plate method [10], and growth on CRA [11] and a modification of the CRA method called the CRAvc method [12].

In this study, the presence of the *icaAD genes* in some commensal and clinical CoNS was detected genotypically via multiplex PCR. The ability of these commensals CoNS to produce biofilm phenotypically was also investigated using both CRA and CRAvc methods. Following that, the correlation between the ability of to form biofilm in both commensals and the clinical CoNS isolates was evaluated to further understand the role of biofilm formation as a determinant in CoNS infections. Subsequently, selected isolates were subjected for identification via the nucleotide-sequencing of the *sodA* gene.

2.0 EXPERIMENTAL

Two control strains were included in all experiments; *S. epidermidis* ATCC 12228 a non-biofilm former and *S. epidermidis* ATCC 35984 which is a strong biofilm producer.

2.1 Isolation of Commensals CoNS

The commensals strains were collected from healthy individual skin and nasal from volunteers by using sterile swabs and immediately streaked on MSA (Difco, USA) for preliminary identification of *Staphylococcus* sp. Yellow colonies were ruled out, indicating the ability to ferment mannitol which is a distinctive character of *S. aureus*. Other colonies were isolated and purified and subjected to Gram staining followed by catalase test using 3% H_2O_2 to rule out *Streptococcus.* The inability of the CoNS isolates to coagulate blood plasma was tested using the Pro-Lab Prolex™ Blue-Staph Latex Test Kit. CoNS isolates were confirmed with no visible agglutination of the latex particles.

2.2 Preparation of Genomic DNA

Genomic DNA was extracted using Bacterial Genomic DNA Isolation Kit (Norgen, Canada) from culture grown overnight at 37°C, 150 rpm, in BHI broth. The extracted DNA was maintained at -20°C.

2.3 Amplification of the *icaA* and *icaD* Genes

A multiplex PCR assay was performed to amplify the *icaA* and *icaD* genes of the isolates. For *icaA*, the primers used were icaA-F 5'-GACCTCGAAGTCAATAGAGGT-3' and icaA-R 5'-CCCAGTATAACGTTGGATACC-3' while for *icaD*, the primers were icaD-F 5'-AGGCAATATCCAACGGTAA-3' and icaD-R 5'-GTCACGACCTTTCTTATATT-3' [13]. The size of the amplified genes was 814bp and 281bp respectively.

The multiplex PCR reaction was prepared using Gotaq Flexi kit (Promega) in a total volume of 50 ul;

10 µl of 5X Go-Taq Colourless Flexi Buffer amplification buffer, 2 µl of a 200 µM concentration of mix deoxynucleoside triphosphate, 2 µl of 25mM $MgCl_2$ solution 2 µl of 10 µM of each primer, 0.5 µl of 1 U of Go-Taq DNA polymerase (Promega), 2 µl of 150 ng of DNA as the template, and 25.5 ml of deionized water. The PCR was performed in Piko Thermal Cycler (Thermo Scientific). The cycling conditions began with initial heat activation of 95°C for 15 mins followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, 8 mins extension at 65°C and a final extension step for 16 mins at 65°C. Successful amplification of the *icaAD* genes were confirmed using 1.2% agarose gel added with ethidium bromide to a final concentration of 0.5 µg/ml. The DNA bands were then visualized on a gel imaging and documentation system, AlphaImager HP (Alpha Innotech, Canada). The sizes of these bands were estimated from the DNA ladder.

2.4 Phenotypic Biofilm Formation by CRA and CRAvc Method

The CRA plates was prepared using Brain Heart Infusion (BHI, Difco USA) agar supplemented with 5% (w/v) sucrose and 0.08% (w/v) of Congo red dye (Sigma, German) [11]. The congo red dye was prepared as a concentrated solution and autoclaved separately before adding to the agar.

The CRAvc plates were prepared in a similar manner. However, the plates were also supplemented with 1.5% (w/v) NaCl and 2% (w/v) glucose. Vancomycin was prepared separately and added to the media with a final concentration of 0.5µg/mL [12].

The bacterial cultures were first grown overnight in BHI broth before inoculated into the prepared CRA and CRAvc plates and incubated aerobically at 37°C for 18 - 24 hrs. The biofilm positive strains produce colonies ranging from brown to black. Black colonies were marked as strong biofilm producer while the brown colonies indicate weak producer. Red colonies were considered as non biofilm producer.

2.5 Amplication of the *sodA* Gene

The sequence-based identification was performed using the *sodA* gene [14]. Prior to sequencing, the PCR products were purified using QIAquick PCR purification kit (Qiagen) as per the manufacturer's instructions.

DNA sequencing was performed by Eurogente AIT (Singapore) and the sequence data was used to interrogate the Genbank database using BLAST. The match with the highest percentage of similarity with a minimum of 99% similarity and 99% coverage was considered a correct identification.

3.0 RESULTS AND DISCUSSION

A total of 28 commensal CoNS were successfully isolated. The multiplex PCR designed to detect the presence of the icaA and *icaD* genes was successfully demonstrated by the amplification of the corresponding fragments with expected sizes of 814bp and 281bp respectively as shown in Figure 1. Six or 21% of the strains were positive for the *icaAD* genes

PIA is known as an important component in biofilm formation in CoNS which suggests that the expression of *icaABCD* operon is essential to promote a biofilm-positive strain. A decreased in the pathogenicity of *S. epidermidis* strain in animal models was observed with the inactivation of the *icaA* gene while a biofilm negative-*ica* negative *S. epidermidis* strain was found to be less invasive. Also an *ica*-negative strain of *S. epidermidis* was able to regain its ability to form biofilm when a plasmid with *ica* locus was transferred to the strain [16]. Thus, biofilm represents the key virulent marker of CoNS [17] indicating that the *ica* genes plays an important role in pathogenesis. The results in the current study showed that the *ica* genes were also present in commensals from healthy individuals which suggests the potential virulence of commensal isolates. In contrast, it is also possible that there is no association between the presence of the *ica* genes and virulence in CoNS which is proposed in other studies [13].

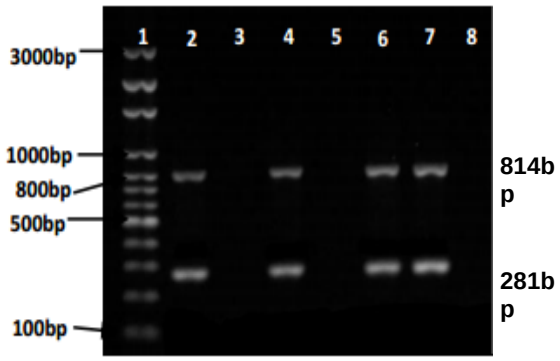

Figure 1 PCR amplifications of *icaA* and *icaD* genes. Lane 1: 100bp DNA ladder; Lane 2: S. epidermidis ATCC 35984 (positive control); Lane 3: S. epidermidis ATCC 12228 (negative control); Lane 4 – Lane 8 = isolate C_1, C_2, C_3, C_4 and C_5

On CRA, 32% of the strains were found to be positive for biofilm on CRAvc showed a stronger phenotypic biofilm formation characteristic of the commensal CoNS. Some strains which were shown to be non-biofilm producers on CRA were found to be biofilm positive instead upon inoculation on CRAvc. In total, 92.8% of the strains were positive for biofilm on CRAvc with 53.6% strong biofilm former and 39.2% were weak producers.

Figure 2 shows some examples of the different characteristic of the strains when grown on both CRA

and CRAvc. In both methods however, the phenotype of both control strains remain the same.

The CRA method is known to be a fast and reproducible method for the determination of phenotypic of biofilm formation in *Staphylococcus*. On the other hand, CRAvc was a modification of CRA with added NaCl, glucose and vancomycin believed to enhance the production of biofilm in *Staphylococcus* sp ([12]. The detail of the biofilm producing ability of each strain is shown in Figure 3.

Strains that were strong and weak biofilm producers on CRA showed similar results on CRAvc. In contrast, the majority of the non-biofilm producer strains on CRA displayed different phenotypic characteristics whereby about half of the strains were strong biofilm producer while the other half were weak producers instead when inoculated on CRAvc. Hence the number of positive biofilm producer of the commensal CoNS determined by the CRA and CRAvc method showed a big difference with 32% and 92.8% respectively. Due to that, the correlationship between the phenotypic biofilm formation on CRA and CRAvc against the genotypic method by detecting the presence of icaAD genes was further analysed as shown in Table 1.

The *icaABCD* operon has been known to be an indicator of biofilm formation in *Staphylococcus* that denotes pathogenicity. An almost similar result was observed whereby strains that harbour the *icaAD* genes also displayed biofilm positive results phenotypically when cultured in both CRA and CRAvc. Some strains that do not harbour the genes were shown to be biofilm producers suggesting a different mechanism in biofilm formation in agreement with other studies [18-19]. However, only 17.9% of the *icaAD* –ve strains were biofilm positive on CRA as compared to a high 71.4% on CRAvc. In addition, only 2 of the 28 commensal CoNS strains were biofilm negative on CRAvc while the rest were biofilm formers. Thus, the ability of CRAvc to differentiate between biofilm and non-biofilm forming strains is questionable as commensals were known to have a low ability to form biofilm.

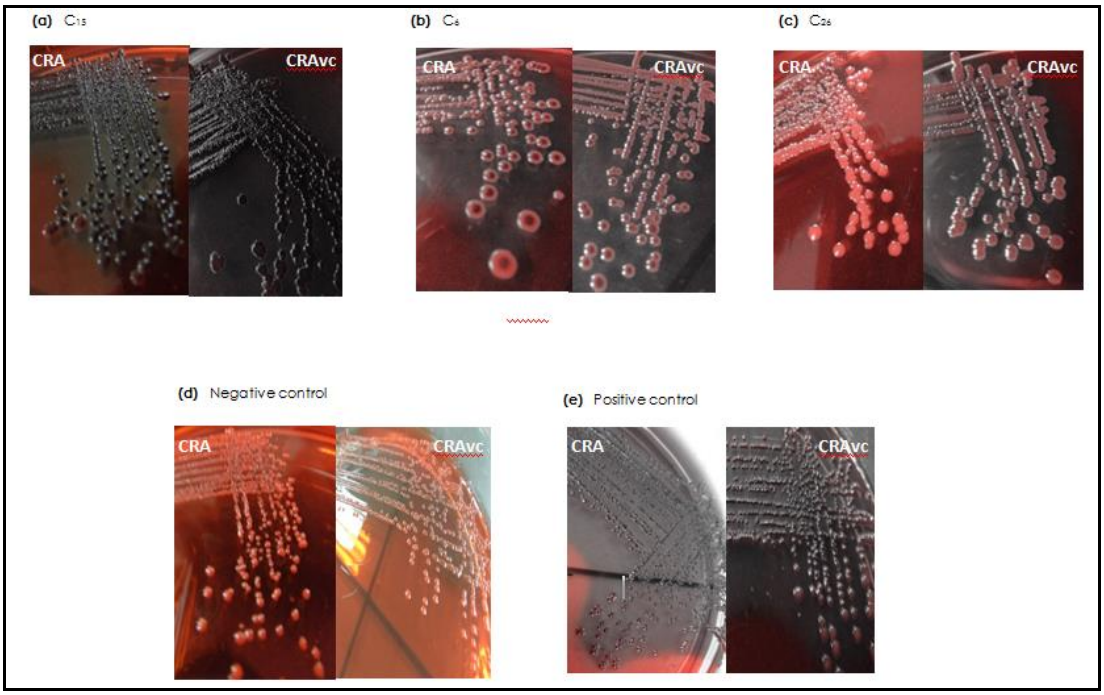

Figure 2 Characteristics of selected *Staphylococcus* strains grown on both CRA and CRA$_{VC}$. Isolate C15 (a), C6 (b) and C26 (c) on both CRA and CRAvc plates. The ability of the isolates to form biofilm was found to be enhanced upon growth on CRAvc, noticeable by the darker colonies formed. However, growth of the control strains (d&e) remains similar on both plates

		Biofilm formation				Biofilm formation	
Isolate	*icaAD*	CRA	CRAvc	Isolate	*icaAD*	CRA	CRAvc
C_1	+ve	Strong	Strong	C_{15}	+ve	Strong	Strong
C_2	-ve	Strong	Strong	C_{16}	-ve	Non	Strong
C_3	+ve	Strong	Strong	C_{17}	-ve	Non	Strong
C_4	+ve	Weak	Strong	C_{18}	-ve	Non	Strong
C_5	-ve	Non	Strong	C_{19}	-ve	Weak	Weak
C_6	-ve	Strong	Strong	C_{20}	-ve	Weak	Weak
C_7	-ve	Non	Weak	C_{21}	-ve	Weak	Weak
C_8	-ve	Non	Weak	C_{22}	-ve	Non	Strong
C_9	-ve	Non	Non	C_{23}	-ve	Non	Weak
C_{10}	-ve	Non	Weak	C_{24}	-ve	Non	Weak
C_{11}	-ve	Non	Weak	C_{25}	+ve	Non	Strong
C_{12}	-ve	Non	Weak	C_{26}	+ve	Non	Strong
C_{13}	-ve	Non	Non	C_{27}	-ve	Non	Strong
C_{14}	-ve	Non	Strong	C_{28}	-ve	Non	Weak

Figure 3 Summary of phenotypic and genotypic screening of Staphylococci isolates for their ability to form biofilm. The ability of each of the commensal isolated to form biofilm on both CRA and CRAvc plates are as shown. The presence (+) or absance (-) of icaAD genes on each of the isolate are also included

Table 1 Correlation between the *icaAD* genes and the ability to form biofilm on CRA and CRAvc

	Number of biofilm producer (%)			
	CRA		CRAvc	
	tve	-ve	tve	-ve
icaAD tve	4 (14.3%)	2 (0.1%)	6 (21.4%)	0 (0%)
icaAD -ve	5 (17.9%)	17 (60.7%)	20 (71.4%)	2 (0.1%)

The biofilm formation by both phenotypic and genotypic methods in commensal Staphylococcus

Isolates C_6 and C_{15} were identified as shown in Figure 3. Isolate C_6 and C_{15} were *S. haemolyticus* and *S. epidermidis* respectively. *S. epidermidis* is commonly known for its ability to form biofilm but not in *S. haemolyticus* thus it would be interesting to further explore this strain. The Figure 4 shows the amplification of the soda gene in commensal Staphylococcus with sizes 429bp.

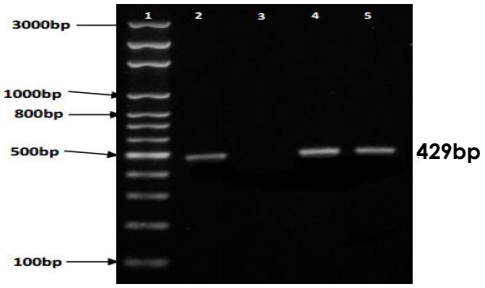

Figure 4 Amplification of the *sodA* gene in commensal *Staphylococcus*

4.0 CONCLUSION

Six or 21% of the commensals were positive for *icaAD* genes indicating the potential of virulence strains among commensals *Staphylococcus* although the possibility of lack association between the genes and virulence could not be ruled out. Biofilm determination on CRA was more reliable as compared to CRAvc.

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References

[1] Kloos, W. E. 1980. Natural Populations of The Genus Staphylococcus. *Annu Rev Microbiol.* 34: 559-92.
[2] Piette, A. and G. Verschraegen. 2009. Role of Coagulase-Negative Staphylococci in Human Disease. *Vet Microbiol.* 134(1-2): 45-54.
[3] Wisplinghoff, H., *et al.* 2004. Nosocomial Bloodstream Infections in US Hospitals: Analysis of 24,179 Cases from a Prospective Nationwide Surveillance Study. *Clin Infect Dis.* 39(3): 309-17.
[4] Henderson, K. L., *et al*. 2010. The Changing Aetiology of Paediatric Bacteraemia in England and Wales, 1998-2007. *J Med Microbiol.* 59(Pt 2): 213-9.
[5] Karunakaran, R., *et al*. 2007. Etiology of Blood Culture Isolates Among Patients in a Multidisciplinary Teaching Hospital in Kuala Lumpur. *J Microbiol Immunol Infect*. 40(5): 432-7.
[6] Mack, D., *et al*. 2006. Biofilm Formation in Medical Device-Related Infection. *Int J Artif Organs.* 29(4):343-59.
[7] Heilmann, C., *et al*. 1996. Molecular Basis of Intercellular Adhesion in The Biofilm-Forming Staphylococcus Epidermidis. *Mol Microbiol.* 20(5): 1083-91.
[8] Rohde, H., *et al*. 2007. Polysaccharide Intercellular Adhesin or Protein Factors in Biofilm Accumulation of

Staphylococcus Epidermidis and Staphylococcus Aureus Isolated from Prosthetic Hip and Knee Joint Infections. *Biomaterials.* 28(9): 1711-1720.

[9] Gerke, C., *et al*. 1998. Characterization of Then-Acetylglucosaminyltransferase Activity Involved in the Biosynthesis of the Staphylococcus Epidermidispolysaccharide Intercellular Adhesin. *Journal of Biological Chemistry*. 273(29): 18586-18593.

[10] Stepanovic, S., *et al*. 2000. A Modified Microtiter-Plate Test for Quantification of Staphylococcal Biofilm Formation. *Journal of Microbiological Methods.* 40(2): 175-179.

[11] Jain, A. and A. Agarwal. 2009. Biofilm Production, A Marker of Pathogenic Potential of Colonizing and Commensal Staphylococci. *J Microbiol Methods.* 76(1): 88-92.

[12] Kaiser T. D. *et al*. 2013. Modification of The Congo Red Agar Method to Detect Biofilm Production by S. epidermidis. *Diagn Microbiol Infect Dis*. 75(3): 235-9.

[13] De Silva, G. D., *et al*. 2002. The Ica Operon and Biofilm Production in Coagulase-Negative Staphylococci Associated with Carriage and Disease in a Neonatal Intensive Care Unit. *J Clin Microbiol.* 40(2): 382-8.

[14] Poyart, C., *et al*. 2001. Rapid and Accurate Species-Level Identification of Coagulase-Negative Staphylococci by using the sodA Gene as A Target. *J Clin Microbiol.* 39(12): 4296-301.

[15] Zhang, Y. Q., *et al*. 2003. Genome-Based Analysis of Virulence Genes in A Non-Biofilm-Forming Staphylococcus Epidermidis Strain (ATCC 12228). *Mol Microbiol.* 49(6):1577-93.

[16] Li, H., *et al*. 2005. Conversion of Staphylococcus Epidermidis Strains from Commensal to Invasive by Expression of The Ica Locus Encoding Production of Biofilm Exopolysaccharide. *Infect Immun*. 73(5): 3188-91.

[17] Raad, I., A. Alrahwan, and K. Rolston. 1998. Staphylococcus Epidermidis: Emerging Resistance and Need For Alternative Agents. *Clin Infect Dis*. 26(5): 1182-7.

[18] Agarwal, G., *et al*. 2005. Characterization of Pseudomonas Aeruginosa Isolated from Chronically Infected Children with Cystic Fibrosis in India. *BMC Microbiol.* 5: 43.

[19] Chokr, A., *et al*. 2006. Correlation Between Biofilm Formation and Production of Polysaccharide Intercellular Adhesin in Clinical Isolates of Coagulase-Negative Staphylococci. *Int J Med Microbiol.* 296(6): 381-8.