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STUDY OF SOME IMMUNOLOGICAL ASPECTS IN PATIENTS INFECTED BY *S.EPIDERMIDIS* FORM BIOFILM

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ABSTRACT

Background: *Staphylococcus.epidermidis* is one of coagulase negative staphylococci (CONS) are now well established as major nosocomial pathogens associated with infections of indwelling medical devices, In recent years many studies focused on *S.epidermidis* because of its ability of to form biofilm , which are essential step in the pathogenesits of this bacteria therefore this study aimed Isolated bacteria from different source identificated, so study effect biofilm of this bacteria on non- specific immune response through estimate levels of IL 6 and IL 8 and C- reactive protein, on specific immune response through determine Immunoglobulins (IgG, IgM, IgA and Complement concentration (C3and C4). **Results:** A total of 50 isolate of *staph. Epidermidis* isolated from different sources include blood, catheter urine specimen, wound as well as burn swabs and swabs of skin and nasal hospital staff, these sample had been cultured and identified by using VITEK-2 system within 6 h. Blood samples occupied the first place in isolation *S.epidermidis* 60%, followed by catheter urine in the second place 22%. Most *Staphylococcus epidermidis* was isolated from male patients (58%) compare to female (42%), prevalence of *Staphylococcus epidermidis* with age were peaked in the third group (46 - 65) years, as well as male preponderance in all years age group, excepted in third group, the proportion of females (65%) appeared higher than it is in males (35%). so 60% bacterial isolate were positive biofilm produced by microtiter plate. Normal level both immunoglobulin M and A (90.23±44.23, 124.4±41.20 and 220.8±90.209, 200.10±79.004) mg / dl respectively($p>0.001$), but IgG increased level is highly elevated (1535±812.333, 1521.3±475.440) mg / dl respectively($p< 0.001$) in sera of patients with produced and non-produced biofilm, and normal levels of (C3) (120±36.5, 120±36.49) mg/dl respectively ($P> 0.001$), in sera of patients with produced biofilm compare to non-produced biofilm whilst elevated levels of (C4) (41.9±14.09 42±14.28) mg/dl respectively ($p>0.001$)to each two groups. (produced and non-produced biofilm), so level of cytokines (IL-6, IL-8) appear variations between patients and controls, levels of IL-6 (24.8 ±18.20 , 24.5 ±19.09) pg/ml respectively in sera of infected patients by *S.epidermidis* (produced and non- produced biofilm) respectively compare to the control (15.88±6.8) pg/ml($P< 0.001$), while serum level of IL-8 (38±9.52,37±7.13) pg/ml respectively in sera of infected patients by *S.epidermidis* (produced and non – produced biofilm) respectively compare to the control(20.3±7.07) pg/ml ($P< 0.001$). **Conclusions:** I can conclude from our study that level of immunoglobulin and complement in study group were within the normal range excepted (IgG and C4) so *S.epidermidis induced higher levels of IL-8, as well as IL-8 correlated positively with CRP and IL-6 in patients.*

KEYWORDS: Staphylococcus.epidermidis, interleukin: IL, C- reactive protein: CRP, Immunoglobulin: I.g,

INTRODUCTION

S.epidermidis is a versatile agent, a commensal and a nosocomial pathogen usually with an opportunistic role association with implanted foreign body materials, it's recognized as important cause of disease in the world and generally hospital-acquired, through ability to form potent biofilms on adherent surfaces [Prag *et al.*, 2014].

S.epidermidis has emerged as an important nosocomial pathogen, especially associated with individuals of compromised immune system. Biofilms play a key role in bacterial resistance against antibacterial agents an issue that causes multiple problems in medical fields, the immune response is highly specific for a particular pathogen, humoral immunity as antibodies and complement, so cytokines are produced mainly by immune cells and cytokines are involved in almost every aspect of immunity and inflammation. Among proinflammatory cytokines, IL-6 is a pro-inflammatory cytokine which has an important role in immunity also

Interleukin-8 is a pro- inflammatory chemokine, It is secreted from a range of cell types.

S.epidermidis is the main bacteria among the CoNS. It's a member of the microbiota of the human skin and wet mucosa, but may act as a pathogen causing infections wh ich may have a significant incidence especially in the immune compromised patients [Kools and Bannerman 1994].

Biofilm formation is a key factor in the establishment and persistence of Staphylococcal infections, Mohammad *et al.*,(2013) define biofilm as organized communities of microorganisms embedded in a self-produced EPM, highly specific of immune response to particular pathogen humoral immunity involves molecules in solution of biological fluids [Ravindar *etal.*,2015], humoral immunity (also called the antibody-mediated system) is the aspect of immunity that is mediated by macromolecules found in extracellular fluids such as secreted antibodies, complement proteins and certain antimicrobial peptides [Janeway, 2001]), immunoglobulin bind and inactivate infectious agents, So the Cellular immunity involves the development of immune cells that are able to recognize, bind, and kill other cells that have previously been infected by foreign infectious agents [Thao Doan *et al.*,2013].

Complement test may be help diagnose the cause of recurrent microbial infections and acute or chronic autoimmune disease, increased total complement values are associated with most inflammatory responses [Mary *et al.*,2009]

Interleukins have many important functions by regulating cell growth, differentiation, cell survival and apoptosis in several diseases [Brumatti *et al*.,2010], interleukin-6 (IL-6) is very important because among proinflammatory cytokines, IL-6 is reported to have a central role in the pathophysiological process of adverse effect of inflammation in patients with renal failure [Abass,2006], as well as Interleukin 8 **(**or CXCL8) is a pro-inflammatory chemokine produced by macrophages and other cell types, it has been implicated in a number of inflammatory diseases, including rheumatoid arthritis and inflammatory bowel disease [Campbell *et al*., 2013]. Ability of biofilms to protect bacteria against host innate immune system is important in *S.epidermidis* pathogenesis, PIA protects *S.epidermidis* from effective phagocytosis by reducing opsonization of C3b and IgG binding on the bacterial surface, also activation of the complement cascade mediated by PIA biofilm [Kristian *etal.,* 2008] and *S.epidermidis* induce cytokine production by human mononuclear cells in vitro [Härtel *etal.,* 2008], so mature biofilm it can release antigens and stimulate production of antibodies, but bacteria which stay within the biofilm are resistant to these defense mechanisms also PNAG protects planktonic *S.epidermidis* bacteria against antibody independent phagocytosis, PNAG is enhanced by opsonization, that involves antibody and complement mediated phagocytosis [Kropec *etal.*,2005].

MATERIALS AND METHODS

Collection of samples

A total of 50 isolate of *staph.epidermidis* isolated from different sources include: blood; catheter urine specimen; wound and burn swabs as well as swabs of skin and nasal hospital staff, was carried out in (AL-Kindy, Imam Ali, Al- Wasete, Baghdad, Ibn Al-Balade) Hospitals and teaching laboratories of Medical City Hospitals in Baghdad from February -2014 to September – 2014.

Culturing of the samples.

Under aseptic conditions using standard bacteriological disposable plastic loop, 10µl of uncetrifuged urine and Swabs from skin and nasal from hospital staff and from burn or wound infection were streaked on MacConkey and Blood agar plates and incubated at 37ºC for 24 hours, if no growth was detected, plates were re-incubated for another 24 hours before reported negative cultures As well as 2ml of blood samples was injected into blood culture bottles and incubated at 37°C in an automated blood culture system (Bactec 9120 system).

Identification of Isolated Bacteria

In this study all isolated of S. *epidermidis* isolates were identified as follows:

Macroscopic observations (Culture Characteristics) and gram stain

Colonial morphology of grown bacteria on culture media, Colony size, color, elevation, edges, hemolysis on blood agar, and staining the isolated bacteria According to Fischbach (2001). So all *S. epidermidis* isolates were characterized using vitek 2 compact.

Quantitation of biofilm by microtiter plate (M.t.p)

In the present study, we screened the fifty clinical isolates of *S.epidermidis* for their ability to form biofilm by microtiter plate method according to the works of Christensen *et al.* (1985) with some modification. *Staph. epidermidis* isolated from fresh agar plates were inoculated in 3 ml of brain heart infusion (BHI) with 1% glucose (Mathur *et al.,* 2006), broths incubated at 37ºC for 24 h.

1. Individual wells of sterile 96 well-flat bottom polystyrene tissue culture treated plates were filled with 200µL of the diluted cultures and 200µl aliquots of only BHI + 1% glucose were dispensed into each of eight wells of the column 12 of microtiter plate to serve as a control (to check non - specific binding and sterility of media).
2. After incubation (24 h at 37°C), the microtiter plates content of each well was removed by tapping water or washed four times with 200µL of phosphate buffer saline (1 × PBS pH 7.2) to remove planktonic bacteria.
3. The plates were then inverted blotted on paper towels

and allowed to air dry for 15 min.

4. Adherent organisms forming biofilms in plate were fixed with sodium acetate (2%) and stained with crystal violet (0.1% w/v), and allowed to incubate at room temperature for 15 min.
5. After removing the crystal violet solution, wells were washed three times with 1 × PBS to remove unbound dye.
6. Finally, all wells were filled by 200 µl ethanol (95%) to release the dye from the cells and Optical density (OD) of stained adherent biofilm was obtained by using micro ELISA auto reader at wavelength 550 nm.

To correct background staining, the OD values of the eight control wells were averaged and subtracted from the mean OD value obtained for each strain. The experiment was repeated three times separately for each strain and the average values were calculated with standard deviation (SD), classified into the following The biofilm-forming ability of each strain was classified under one of four categories: none adherent (1), weak (2), moderate (3) and strong (4), based upon ODs of bacterial as following : OD ≤ ODc = non- adherent; ODc < OD ≤ 2 x ODc = weakly adherent ; ODc < OD ≤ 4 x ODc = moderately adherent ; 4 x ODc < OD = strongly adherent.

Immune assay

Immunoglobulin and complement determination [Fahey and Mchlevey, 1965]:

Single redial immunodiffusion (SRID) of Fahey and Mchlevey (1965) was used to determine the serum concentration of (Igs and Complement component). Endoplates were used to determine I.g.G, I.g.M, I.g.A, C3 and C4.

C-Reactive Protein

Place 50 µl of the serum on one section of the disposable slide, add a drop of reagent to the serum and mix both drops with a stirrer covering the whole surface of the slide section. Look for the presence or absence of agglutination.

RESULTS AND DISCUSSION

Isolation of Bacteria

Many of pathogens are part of endogenous flora but some may have been acquired by contamination from hospital staff or by contaminated solutions or non-sterile equipment or from other patients.

Table: 1 Prevalence of *Staph.epidermidis* in the study groups.

STUDY GROUPS	*NO. OF BACTERIAL ISOLATED*	*%*
Blood culture	30	60
Catheter urine specimen	11	22
Wound and burns swab	6	12
Swab of Skin and nasal Hospital staff	3	6
Total	50	100

S.epidermidis is the most common cause of infections associated with catheters and other indwelling medical devices, these bacteria are most prevalent bacteria of the skin and mucous membrane microflora, present unique problem in diagnosis and treatment infections involved biofilm formation.

The results in table 1 showed blood samples occupied the first place in isolation of *Staph. epidermidis* froming 60%, followed by catheter urine in the second 22%, Wound and burns swab (12%) as well as (6%) from swab of Skin and nasal hospital staff. This is almost similar to the results of Mertens and Ghebremedhin (2013) whom showed that 75% of the *S. epidermidis* isolates from blood culture, While Donlan (2001) report found *S.epidermidis* is the most common cause of infections associated with catheters and other indwelling medical devices, these bacteria are most prevalent.

Mulu *etal.*, (2012) showed in his study these organism is a commensal or normal flora on the skin, several investigations have reported these organisms as common contaminants of wounds and burns. Wound can contaminated by microorganisms that migrate from the urinary, respiratory and gastrointestinal tract [Bowler *etal.*, 2001], this indicates the idea of autoinfection that burns patients suffer from in addition to the infection acquired from the burns unit itself [Collier, 2003]. As well as Gil *etal.*, (2013) showed the biofilms produced by *S.epidermidis* strains are responsible for a number of nosocomial infections and infections on indwelling medical devices.

Table 2: Distribution of *staph.epidermidis* isolates among gender and age in relation to specimen types

AGE GROUP (YEARS)	NO. OF PATIENT (٪)	GENDER		STUDY GROUP			
		Male No. (%)	Feemale No. (%)	Catheter urine specimen No.(%)	Wound and burns swab No. (%)	Swab of Skin and nasal hospital staff No. (%)	Female No. (%)
≤ 25	5(10)	4(80)	1(20)	0(0)	1(20)	1(20)	3(60)
26-45	9(18)	5(55.5)	4(44.4)	4(44.4)	0(0)	0(0)	5(55.5)
46- 65	20(40)	7(35)	13(65)	5(25)	3(15)	2(10)	10(50)
> 65	16(32)	13(81.25)	3(18.75)	2(12.5)	2(12.5)	0(0)	12(75)
Total	50(100)	29(58)	21(42)	11(22)	6(12)	3(6)	30(60)

In table 2 Most of the *S.epidermidis* was isolated from male patients (58%) compare to female (42%), because of male always in work and more have accident from female.

So, the prevalence of *S. epidermidis* with age were peaked (40%) in the third group (45 - 65) years, because in this age group including the worker in differ jobs whom may have accident in worker or street or from bursting as well as many of them may have other diseases as diabetic mellitus which play important factor in all type of bacterial infections, followed (32%) in fourth age group (≥ 65).

In third age group (45- 65) the most bacterial isolated from four study groups (blood, catheter urine specimen, wound and burns swab, as well as swab of skin and nasal hospital staff) were (50, 25, 15, 10) % respectively.

Newman *etal.,* (2006) have pointed out in their some of the risk factors for bacteraemia include old age, immunosuppression, chemotherapy and invasive procedures. There was male preponderance in all age group, excepte in third group, the proportion of females (65%) appeared higher than in males (35%), Taye, (2005) showed in his study incidence of wound infection was more common in males (89.7%) than in females (81.4%), this in agree with studies done in different parts of Ethiopia [Gelaw *etal.*,2011].

BIOFILM ASSAY

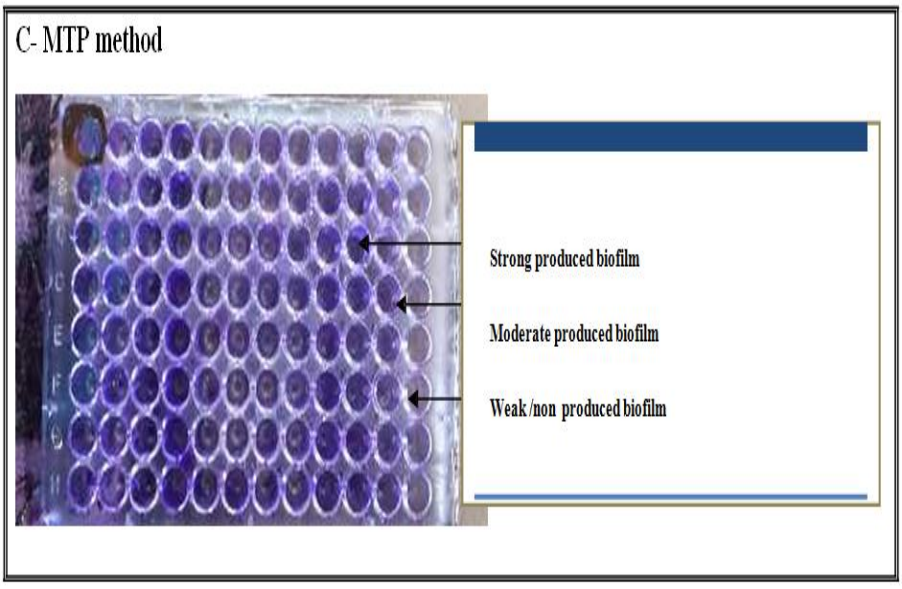

Figure 1: (a) Biofilm producer and no producer on tissue culture plate method.

Table: 3 Biofilm produced by microtiter plate method according to Source of *S.epidermidis*.

SOURCE OF *STAPH. EPIDERMIDIS*	NO.OF ISOLATE	NO.NON-BIOFILM PRODUCED (%)	NO. OF BIOFILM PRODUCED (%)
Blood	30	12(40)	18(60)
Catheter urine	11	5 (45.5)	6(54.5)
Wound and burns swab	6	1 (16.7)	5(83.3)
Swab of skin and nasal hospital staff	3	2(66.7)	1(33.3)
Total No.(%)	50(68.6)	20(40)	30(60)

Biofilm production was determined by the Microtiter plate method. Table 3 showed (60 %), of *S. epidermidis* isolates, which were gained from blood while (54.5 %) of the isolates from urine catheters, So wound and burns swab produced biofilm by 83.3% also 33.3% from swab of skin and nasal hospital staff, while (40, 45.5, 16.7)% respectively of the *S. epidermidis* isolates from blood culture , urine catheters and wound and burns swab as well as (66.7%) from swab of skin and nasal hospital staff produced no biofilm phenotypically. biofilm production is considered to be less in case of samples collected from healthy skin when compared to those collected from people associated with infections [Arciola *et al.,* 2001] .As study reported that 44.2% of the samples that were collected from patients were strong biofilm-producers compared to 0% of the samples that were isolated from healthy volunteers [Mateo *etal.*,2007].

Ansari et al., (2015) showed (80%) isolates of *S. epidermidis* ability to produce black colonies, which are indicative of biofilm formation by CRA methods, while Gamal *etal.,* (2009), showed 88.6%of S.epidermidis were biofilm producers, as the results of Arslan and Ozkarde (2007) explained that CRA method demonstrated positive results in 38.5% of *staphylococci* isolated from clinical specimens.

Table 4: Mean values of immunoglobulin and complement Level (C3 and C4) in sera of infected patients by *S.epidermidis* (produced and non – produced biofilm).

	Sera of patients infected by *S.epidermidis* (M ± S.D)		**Normal value**	**P value**
	Produced biofilm (No.=30)	**Non-Produced biofilm (N0.=20)**		
Immunoglobulin (mg / dl)				
IgG (mg / dl)	1535±812.333	1521.3±475.440	**710-1520**	(P< 0.001) significant
IgM (mg / dl)	90.23±44.23	124.4±41.20	**40-250**	(P> 0.001) no significant
IgA (mg / dl)	220.8±90.209	200.10±79.004	**90-310**	(P> 0.001) no significant
Complements				
C3 (mg / dl)	120±36.5	120±36.49	**84-193**	(P> 0.001) no significant
C4 (mg / dl)	41.9±14.09	42±14.28	**20-40**	(P> 0.001) no significant

I.g.G: Immunoglobulin G, I.g.M:Immunoglobulin M, I.g.A :Immunoglobulin A,C3: complement3, C4 : complement 4 M= Mean;- S.D = Standard Deviation.

Table (4) showed mean values of Immunoglobulin, complement in sera of patients (infected by *S.epidermidis*) with produced and non-produced biofilm, normal level both for immunoglobulin M and A (90.23±44.23, 124.4±41.20 and 220.8±90.209, 200.10±79.004) mg / dl respectively (P> 0.001), but IgG increased level is highly elevated (1535±812.333, 1521.3±475.440) mg / dl respectively (P< 0.001) in sera of patients with produced and non-produced biofilm.

Also table showed normal levels of complements components (C3) (120±36.5, 120±36.49) mg/dl respectively (P> 0.001), in sera of patients with produced biofilm compare to non-produced biofilm whilst elevated levels of complements components (C4) (41.9±14.09, 42±14.28) mg/dl respectively (P> 0.001), to each two groups (produced and non-produced biofilm).

Increasing C3 and C4 can be explained by the IgG prevent complement attack by inhibiting C3 and C4 uptake onto target cells and tissues, therefore elevated level of IgG in sera of patients may be the source of the noticed increase in the complements, that suggest immunoglobulins can play role in active therapy in diseases accompanied by activation of classical complement pathway.

Complement may be activated by immunoglobulin bound to extracellular material in biofilms, leading to complement activation and C3 a generation but to insufficient opsonization of the bacterial surface. In addition, PIA is strongly produced in *S. epidermidis* biofilms, and may promote the lectin pathway of complement activation [Cerca *etal.*,2006]. So biofilm formation has an important role in the evasion of the host immune defense; and protects S. epidermidis from neutrophil phagocytosis, AMPs so depositions of antibodies and complement, As well as induces a significantly lower CRP response in neonatal blood compared to non-biofilm producing strains [Kristian *etal.*, 2008].

Hansen *etal.*, (2003) showed in his study third Complement component [C3] levels are normal in the

sera of all patients, however [C4] is observed elevated in sera. Results study of Mahdi *etal.*, (2009) showed significant increase in IgG, IgA, C3 and C4 in order to overcome the inflammation, IgM decreased level in IC (indeterminate colitis) patients when comparison with control, this may be due to secondary immune response intiation and IgM level return to its level because its only raised in primary immune response, presence of antigen -antibody immune complex leading to complement activations by alternative and lectin pathways and causing increased level of complement [Swidsinski *etal.*,2002]. complement activation induced by PIA biofilm are stronger than non-PIA biofilm [Granslo *etal.*,2012].

Innate inflammatory response to *S. epidermidis* infections involves the activation of the complement system (classical pathway), leukocytes and secretion of cytokines [Otto, 2009].

Results study of Kristian *et al.*, (2008) explained form biofilm induced to release of C3a, but also protected *S. epidermidis* from IgG and complement opsonisation whilst *Staphylococci* are known to produce immune evasive molecules that also may inhibit the complement system [Foster, 2005; Laarman *et al.*, 2010], So PIA as a strong activator of the complement system as well as Kristian *et al.*, (2008) shown in his results the *S. epidermidis* embedded in a biofilm is protected from polymorph nuclear cells (PMN) killing. So PIA important factor for immune evasion and strains which produce PIA are persistent colonizers of indwelling medical devices and that biofilm is a major virulence factor in chronic *S. epidermidis* infections [Begun *et al.*, 2007], Whilst Granslo *etal.*, (2012) showed in his study the *S.epidermidis* biofilms induce a lower complement activation in neonates as compared with adults which induced stronger complement.

Table 5: Mean of interleukins (6 and 8) and C-reactive protein (C-RP) in sera of infected Patients by *S.epidermidis* (produced and non – produced biofilm)

TEST		SERUM OF PATENTS INFECTED BY S.EPIDERMIDIS M ± S.D		CONTROL	P- VALUE
		Produced biofilm (No.=30)	Non- Produced biofilm(No.=20)		
Interleukins (pg/ml)	IL 6	(24.8 ±18.20)	(24.5 ±19.09)	(15.88±6.8)	(P< 0.001) significant
	IL 8	(38±9.52)	(37±7.13)	(20.3±7.07)	(P< 0.001) significant
C-reactive protein (C-RP)		+	+		

- M= Mean; - S.D = Standard deviation.

The level of cytokines (IL-6, IL-8) was determined in sera of patients and control, cytokines determined, showed variations between patients and controls. Serum levels of IL-6 (24.8 ±18.20, 24.5 ±19.09) pg/ml respectively in sera of infected patients by *S.epidermidis* (produced and non- produced biofilm) respectively compare to the control (15.88±6.8) pg/ml (P< 0.001)., while serum level of IL-8 (38±9.52, 37±7.13) pg/ml respectively in sera of infected patients by *S.epidermidis* (produced and non-produced biofilm) respectively compare to the control (20.3±7.07) pg/ml (P< 0.001).

In the present results, patients shared a significant increased serum level of IL-8, IL-6 compared to controls, increased production of IL-6 has been implicated in various disease processes, including bladder cancer. In some instance, IL-6 is implicated in proliferation pathways, because it acts with other factors, such as, heparin-binding epithelial growth factor and hepatocyte growth factor [Wang *et al.*, 2002].

The innate inflammatory response to *S.epidermidis* infections involves activation of the complement system and leukocytes and Cytokines activate the innate immune system and facilitate the defense against invading pathogens[Rogier *et al.*, 2003], PIA as an important immunogenic component of the *S. epidermidis* biofilm that can regulate pro-inflammatory cytokine production (IL-1α) and (IL-8) but inhibit other cytokine secretion, so IL-6 alone or together with IL-1β and TNF-α, induce the production of acute-phase proteins as C-reactive protein (CRP), as well as Klingenberg *et al.*, (2005) of his study explained reduced IL-6 and IL-1β secretion in response to the PIA-positive strain therefore explain reduced CRP-response, in neonatal infections caused by biofilm positive coagulase-negative staphylococci.

Result of Ivarsson (2013) reported showed *S. epidermidis* induced higher levels of IL-8 (mean 38.5 ng/mL) than *S. aureus* (IL-8 mean 22.2ng/mL) and induced a higher chemo attractive response, So Dinarello (1996) showed in results study IL-8 are elevated in patients with Gram- positive bacterial sepsis and *S.epidermidis* induced significant increases in TNF-a and IL-8 (1.9 - 0.9 and 94.7-67.2 ng/ml, respectively) and high CRP levels have been observed in bacteremic patients[Honkinen *et al.*, 2000].

This study is agreement with Snijder and Meulenberg (2001) whom suggested that CRP is a good marker for systemic inflammation, as well as increased bacterial infections that mean increased in CRP Titer which is considered as a defense against any bacterial infections [Challis *et al.,* 2009].

CONCLUSIONS

We can conclude from our study that level of immunoglobulin and complement in study group were within the normal range excepted (IgG and C4). as well as C.reactive protein is a sensitive but non- specific marker for the diagnosis of acute infection, because it can increase in other causes of tissue injury and Inflammation , so *S.epidermidis* induced higher levels of IL-8, as well as IL-8 correlated positively with CRP and IL-6 in patients infected by *S.epidermidis*.

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