Immune Response in Thalassemic Patients Associated with Bacterial Infections

A Thesis

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الأستجابة ألمناعية لمرضى الثلاسيميا المصاحبة للألتهابات البكتيرية

رسالة

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كجزء من متطلبات نيل شهادة الماجستير في الأحياء المجهرية الطبية

تقدمت بها

هبة علي هادي الخفاجي

(بكالوريوس تقنيات طبية/ بغداد 2004)

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3.1 Age distribution of thalassemic patients :

As shown in Table(3-1) a total of 150 patients with thalassemia were included in this study whose age ranged (2-28) years. The most predominant age group was 10 -20 years 90:150 (60%). While the patients below 10 years 50:150(33.3%) and more than 20 years 10:150 (6.7%) . These age groups were the most predominant to infectious agents due to multiple blood transfusions.

Table(3-1): Age distribution of thalassemic patients

Age range (years)	Number of cases	Percentage%
Less than 10 years	50	33.3%
10-20 years	90	60%
More than 20years	10	6.7%
Total	150	100%

The result in the Figure (3-1) reveals that the most predominant age group is less than 20 years of thalassemic patients 140:150 (93.3%).This means that thalassemic patients older than 20 years10:150(6.7%) were died in 2nd trimester of life (Changsri *et al*.,2006).

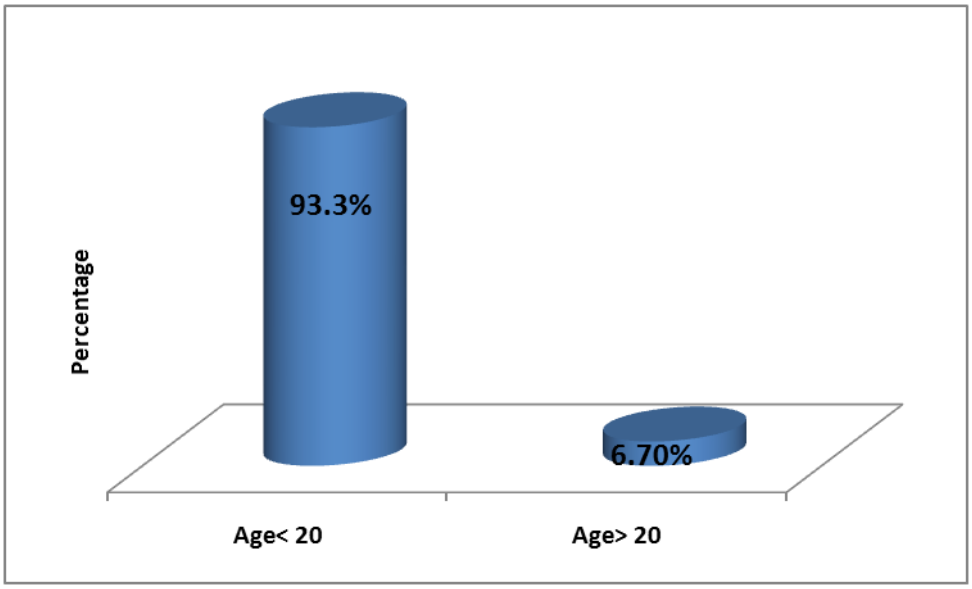

Figure (3-1): The most predominant age<20 years (140:150) 93.3%in thalassemic patients .

3. 2 Bacteriological study

3. 2.1 Bacterial isolation

A total of 92 sample from thalassemic patients with signs and symptoms of bacterial infection were presented in Table (3.2).Ear , throat swabs, blood and urine were subjected to aerobic culturing on different types of culture media. The results of sample for bacterial culture in thalassemic patients with signs and symptoms of infection revealed that 82% positive cultures included 38.6% of urine samples, 30.4% of blood and 6.5% of both ear and throat swabs which meant that thalassemic patients were highly

susceptible to Urinary Tract Infections UTI (Nowrouzian *et al* ., 2007) and high number of Bacterial isolates were found in urine which meant that urine of thalassemic patients were more favorable for bacterial growth.(Kenneth.,2002).

Table (3.2) The distribution of bacterial isolates according to the clinical site

Samples	**No .of positive bacterial isolates**	**Percentage of positive Bacterial isolates**
Urine	**37**	**38.6%**
Blood	**26**	**30.4%**
Throat swabs	**6**	**6.5%**
Ear swabs	**6**	**6.5%**
Total	**75**	**82%**

The results of bacterial culture of thalassemic patients with sign and symptom of infection revealed that (75:92) 82% were positive culture and(17:92)18% negative culture shown in Figure (3-2)

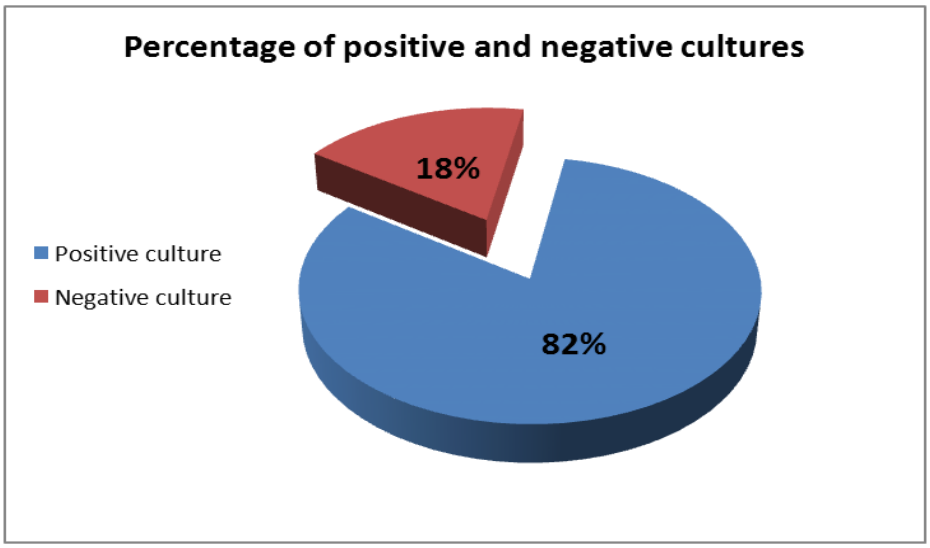

Figure (3-2): Percentage of positive and negative culture

3.2.2 Gram staining:

Figure (3-3) shows the distribution of bacterial isolates according to Gram staining. Gram-negative bacterial isolates appeared to be the predominant(58:75) (70%) when compared to Gram-positive isolates which were isolated from only(17:75) (30%) of infected thalassemic patients. These results were agreed with (Ofek *et al*.,2002; Moutschen,2005) .

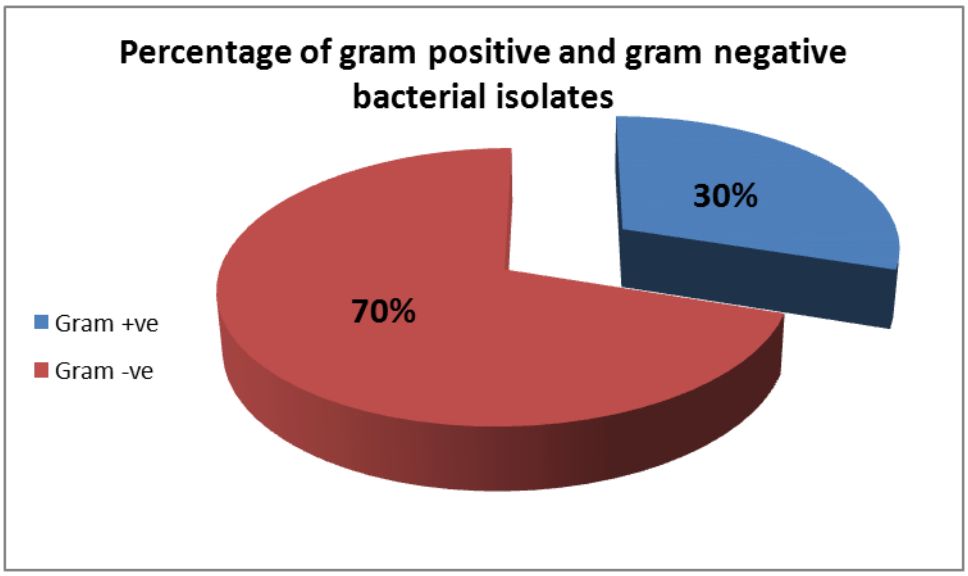

Figure (3-3) Types of bacterial isolates according to Gram staining of infected thalassemic patients.

3-2-2-1 Gram-negative bacteria:

The gram negative isolates shown in (Table 3-3) revealed that *E. coli*, *was* the predominant type among other Gram-negative bacteria (23:58) (39.65%),while the other bacterial types *Pseudomonas aeruginosa* (13:58) (22.41%) , *Enterobacter* (12:58) (20.68%), *Proteus vulgaris* (9:58) (15.51%) and *Klebseilla pneumoniae* was isolated in low percentages. These findings agreed with the results of (Kenneth.,2002). who reported that (75%)*E. coli* were the most commonly organisms associated with Urinary Tract Infections (UTI) . This may be due to the antibiotic

resistance of *E. coli* which was considered the main cause of bacteriuria.

Table (3-3) Gram-negative bacterial isolated from infected thalassemic patients

Bacterial isolates	Total number of isolates	Percentage
Escherichia coli	**23**	**39.65%**
Pseudomonas aeruginosa	**13**	**22.41%**
Enterobacter spp.	**12**	**20.68%**
Proteus vulgaris	**9**	**15.51%**
Klebseilla pneumonia	**1**	**1.72%**
Total	**58**	**100%**

The distribution of Gram-negative bacterial isolates from Infected thalassemic patients specimens as shown in Table (3-4) revealed that the large number of bacterial isolates were found in urine and *Escherichia coli.*was predominant type 23:58 (39.65%) (Nowrozian *et al* . (2007).

Table(3-4): Distribution of gram-negative bacteria isolates from Infected thalassemic patients speciemens

Samples	**NO.**	**Bacterial isolates**
Urine	37	***(22) Escherichia coli.*** ***(8) Proteus vulgaris.*** ***(1) Klebsiella pneumoniae*** ***(4) Enterobacter.*** ***(2) Pseudomonas aeruginosa***
Blood	16	***(8) Enterobacter.*** ***(1) Proteus vulgaris.*** ***(6) Pseudomonas aeruginosa*** ***(1) Escherichia coli.***
Throat swabs	2	***(2)Pseudomonas aeruginosa***
Ear swabs	3	***(3)Pseudomonas aeruginosa***

3.2.2.2 Gram-positive bacteria :

The gram posative isolates shown in (Table 3-5) revealed that *Streptococcus pyogens* was the major Gram-positive isolate (7:17) (41.17%), *Staphylococcus aureus* (5:17) (29.4%), *Streptococcus pneumonia* were (2:17) (11.76%) and *Listeria monocytogens* were (2:17) (11.76%) while *Staphylococcus epidermidi*s (1:17) (5.8%) was less commonly isolated Gram-positive species in thalassemic bacterial infections. These results agreed with (VonEiff *et al.*,2001;Ofek *et al.*,2002) who reported that *Streptococcus pyogens* was the predominant agent of infections in immuno-compromized patients.

Table (3-5) Gram-positive bacteria isolated from infected thalassemic patients

Bacterial isolates	**Total number**	**%**
Streptococcus pyogens	**7**	**41. 17%**
Staphylococcus aureus	**5**	**29. 4%**
Listeria monocytogens	**2**	**11.76%**
Streptococcus pneumonia	**2**	**11.76%**
Staphylococcus epidermidis	**1**	**5.8%**
Total of isolates	**17**	**100%**

Distribution of Gram positive bacterial isolates from Infected thalassemic patients speceimens mentiond in Table (3-6) indicates that the large number of gram positive bacterial isolates in blood and ***Streptococcus pyogens (*** 7:17)(41.17%) is causative agent of septicemia in Infected thalassemic patients (Humphreys.,2004*).*

Table (3-6) : Distribution of gram positive bacterial Isolates from Infected thalassemic patients speceimens.

Samples	NO.	Bacterial isolates
Blood	**10**	***(2) Streptococcus pyogens.*** ***(5)Staphylococcus aureus.*** ***(1)Staphylococcus epidermidis.*** ***(2) Listeria monocytogenes.***
Throat swabs	**4**	***(2)Streptococcus pneumonie*** ***(2) Streptococcus pyogens***
Ear swabs	**3**	***(3) Streptococcus pyogens***

3.3 Virulence factors of bacterial isolates

3.3.1 Coagulase production

Staphylococcus aureus is able to produce coagulase (Table 3-7) which is considered as a virulent factor for pathogenicity of these bacteria by clumping the fibrin around the bacteria (Hall, 1991; Kenneth, 2002). Possibly coagulase could provide an antigenic disguis if it was clotted fibrin on the cell surface or could make the bacterial cells resistant to phagocytes or tissue bacterial target (Humphreys., 2004).

3.3.2 Hemolysin production*:*

Hemolysin production by some bacteria isolated *Streptococcus pyogen* show narrow zone β-hemolysis(table 3-7) This result approximately fits with (Holt *et al*., 1994; Brook *et al*., 2007) who have reported that *Streptococcus pyogen* .displays β-hemolysis when cultured on a blood agar plate, and produces zones of hemolysis that are only slightly larger than the colonies themselves. Production of beta hemolysin by *E. coli* and *Listeria monocytogenes* is considered the important virulence factor which are cytotoxic due to the formation of trans membranous pores in host cell membrane (Humphreys., 2004). (table 3-7) while *Staphylococcus epidermidis, Klebsiellia sp., Pseudomonas*

aeruginosa were γ –hemolysis (non hemolysis) pattern (table 3-7) , with no color change around the bacterial colonies.

3.3.3 CAMP test

The isolates of *Streptococcus ,E. coli, Listeria monocytogenes* showed positive results for CAMP test(Table 3-7) when streaked adjacent to colonies of *Staphylococcus aureus* cultivated on sheep blood agar plates.CAMP test used for the identification of *Listeria monocytogenes* were CAMP will be positive when cultivated with *Staphylococcus aureus* (Holt *et al.*, 1994).

3.3.4 Capsule production:

Some isolates of *E.coli* (13).and *Klebsiela pneumonie* are capsule formation (Table 3-7) It is considered the main virulence factor that enables bacteria to avoid engulfment by phagocytic cells in a process known as Phagocytosis (Brook *et al*.,2007).

Table(3-7) Virulence factors of bacterial isolates

Bacterial isolates	**coagulase**	**Hemolysin production**	**CAMP**	**Capsule formation**
Klebsiella pneumonie(1)*	-	γ	-	+
E coli (13)	-	**B**	-	+
E coli (10)	-	**B**	+	-
Proteus (9)	-	γ	-	-
Pseudomonas (13)	-	**B**	-	-
Staphylococcus epidermidis(1)	-	γ	-	-
Staphylococcus aureus (5)	+	**B**	-	-
Streptococcus pyogens (7)	-	**α**	+	-
Streptococcus pneumonie (2)	-	**α**	+	-
Listeria monocytogens (2)	-	**B**	+	-

β-hemolysis(complete hemolysis);α-hemolysis(partial hemolysis); γ-hemolysis(Non-hemolysis)

*Number of isolates.

3-3-5 Antibiotics resistance of bacterial isolates :

A-Beta-lactam group

From the figures (3-4) (3-5) showed that bacterial isolates were showed different percent's of resistant to amoxicillin except *listeria monocytogenes*, *Staphylococcus epidermidis* and *Strep. pneumonie* which were sensitive , while most of the bacterial isolates were resistant to ampicillin (except *listeria monocytogenes* and *Strep. pneumonie*). Ampicillin was an amino penicillin when given orally or I.V (500 mg – 1g 8-hourly) was effective primarily against Gram positive organisms except for *Staphylococcus*. The resistance of to β-lactams was mediated by β-lactamase production (Hartman and Tomasz.,1981;Humphreys,2004) they expressed that *Staphylococcus epidermidis* exhibited resistance to many types of antibiotics and this resistance may be attributed to the R-plasmid acquired from pathogenic bacteria present in the site of infection. Many organisms are resistant due to β -lactamase production but the addition of B-lactum inhibitors (e.g clavulanic acid , producing co-amoxiclav) to aminopenicillin has improved clinical usefulness. Resistance to beta-lactam antibiotics in Gram-negative bacteria can be due to five mechanisms: (Kresken and Hather., 1999;Dowson *et al*.,2000; Stratton *et al*.,2000;Brooks *et al*.,2007):decreased permeability of the drug into the cell,hydrolysis of the drug by ß-lactamase.decreased

affinity of the target penicillin-binding proteins (PBPs),pump-mediated resistance (Piddock *et al*., 1997)and altered Gram –ve porin channels (Tenover *et al*.,2006).The major mechanism of resistance in bacteria causing clinically significant infection remains the expression of ß-lactamases, of which there are several classes including plasmid-encoded and chromosomally-encoded enzymes (Hall .,1997; Frere,1995 ;Livermore,1998). *Staphylococcus aureus* has the highest number of multi resistant strains and these findings are in agreement with those isolated from clinical specimens (Davies and Stone,1986). *Staphylococcus aureus* may act as a reservoir for resistance which can be transferred to *Streptococcus pyogens*. The transfer of resistance among different genera of Gram-positive and Gram-negative bacteria and between *Bacillus* species and *Staphylococci* has been reported by many researchers (Mazodier and Davies,1991; Courvalin,1994). These results are similar to the findings obtained by (Brook *et al*., 2007) who showed, that *Staphylococcus aureus* isolates were resistant to amoxicillin and less resistant to cefotaxime,the results above also agreed with (Carratala *et al*., 1995)who mentioned that bacteremia due to *streptococci pyogens* were highly resistant to penicillin's products. Among the streptococcal species, the *streptococcus pyogens* may be the most important pathogens causing bacteremia and sepsis in immunocompronized patients (Dowson and Coffey., 2000). Until the

1980s, *Streptococci* were considered to be uniformly susceptible to β-lactam antibiotics, but resistance spread rapidly in the 1990s (Dowson and Coffey., 2000).The incidence of resistance has been associated with previous use of β-lactams and varies greatly among different institutions (Carratala *et al*.,1995).

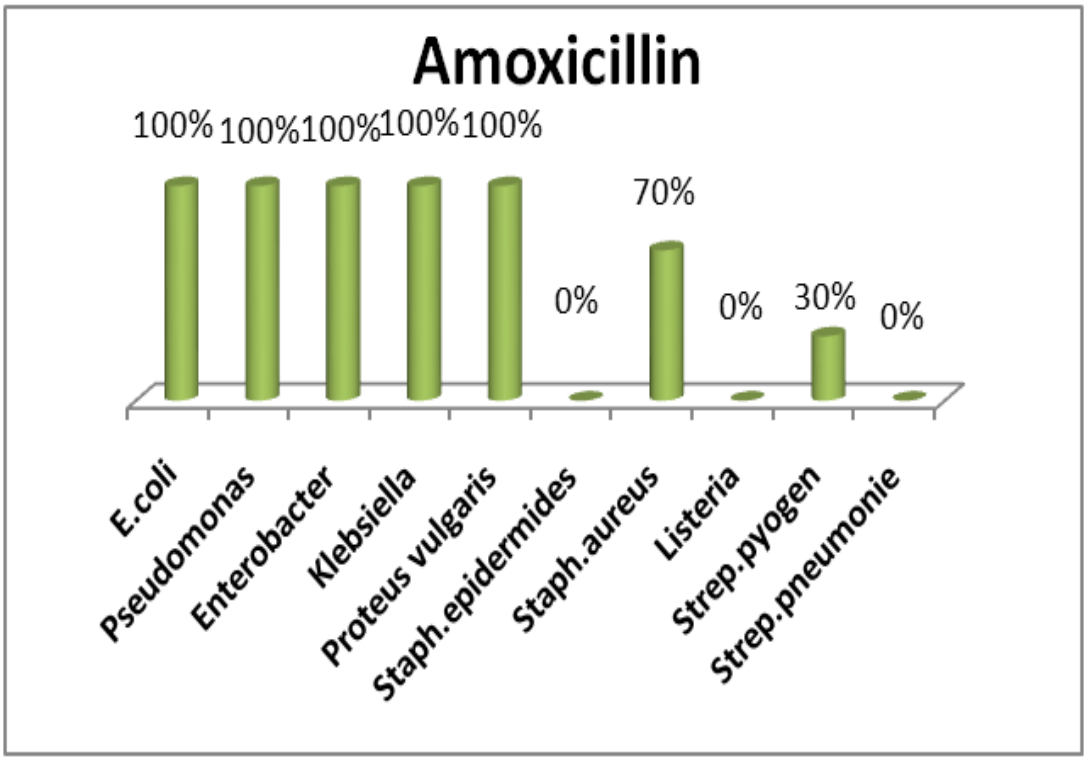

Figure (3-4) Bacterial resistance to amoxicillin

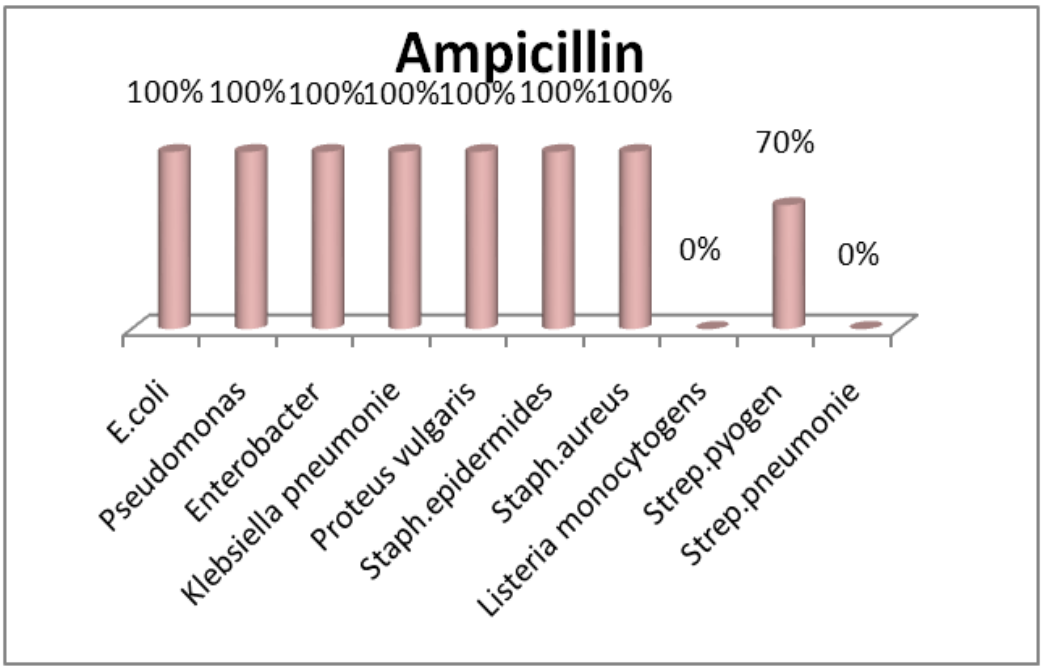

Figure (3-5) Bacterial resistance to Ampicillin

B-Aminoglycoside group

Most of the bacterial isolates show different percent's of resistant to gentamicin in Figure (3-6) except (*K.pneumonie*) was sensitive.Mechanism of action is by binding to 30 S subunits of bacterial ribosomes (require specific transport mechanism across G –ve outer/inner membranes) disrupt bacterial protein synthesis. Mechanism of resistant in this group is by membrane impermeability and enzyme inactivation of active sites (Brooks *et al*.,2007).

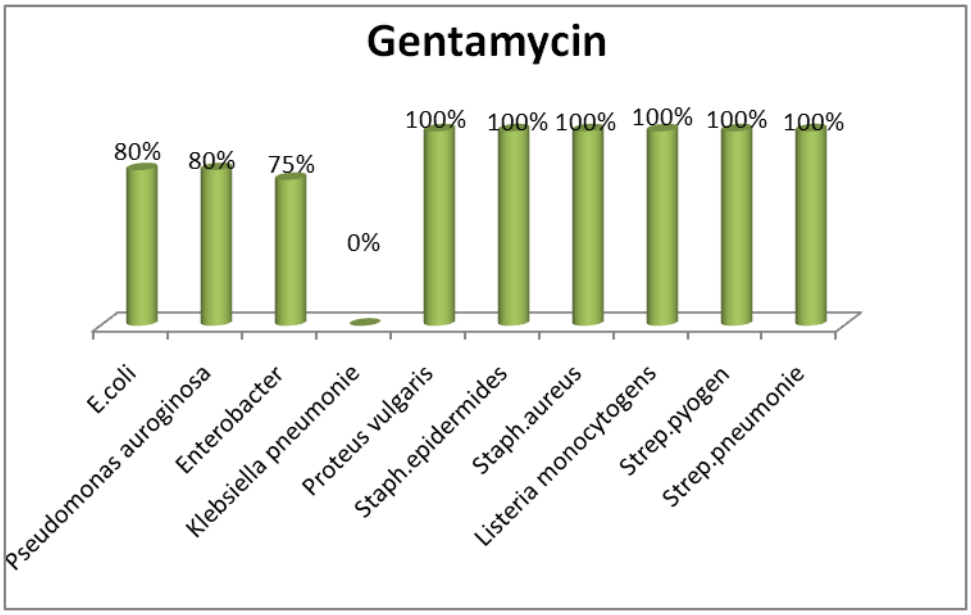

Figure (3-6) Bacterial resistance to Gentamycin

C-Cephalosporins group

Most of the bacterial isolates are sensitive to cefalexin in Figure (3-7) (except *E coli* , *Pseudomonas* and *Enterobacter*).Cephalosporin are safe and reliable antibiotics which have broad spectrum of activity and show some synergy with aminoglycosides. The most active compounds are only available in I.V form.

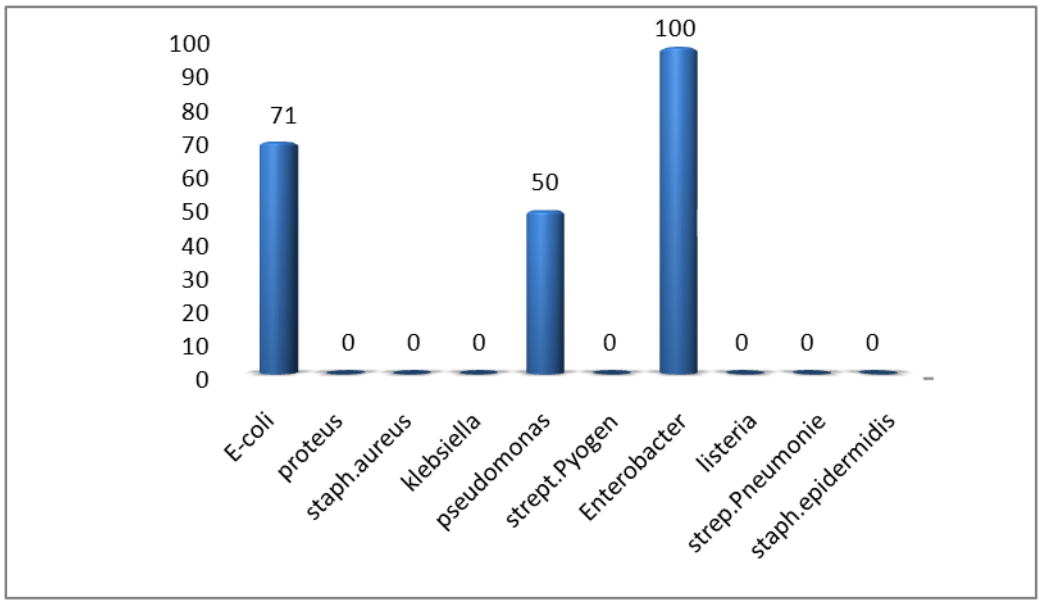

Figure (3-7) Bacterial resistance to Cefalexin

D-Quinolones Group

Most of the bacterial isolates were resistant to Ciprofloxacin in Figure (3-8). Mechanisms of resistant are by mutation of topoisomerases , porins imprmeability and active efflux(Brooks *et al*.,2007).

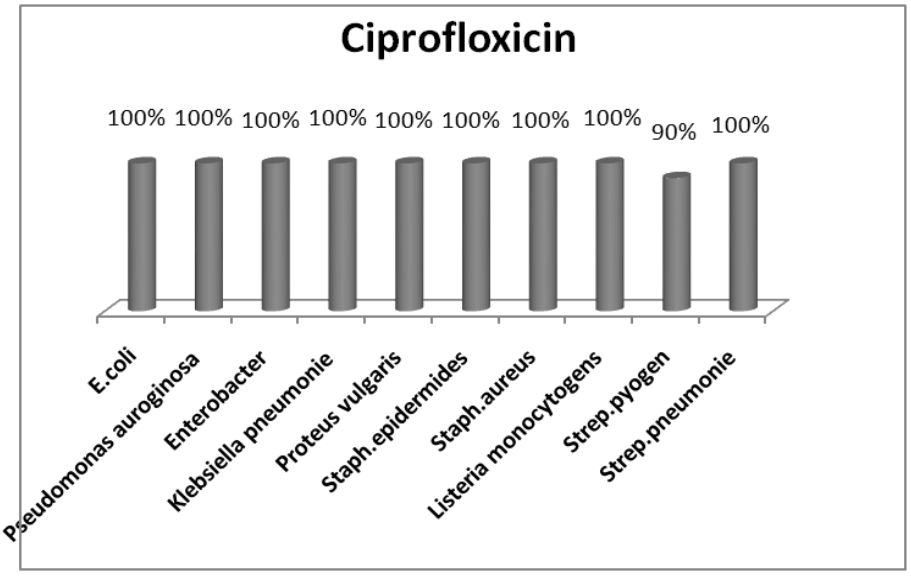

Figure (3-8) Bacterial resistance to Ciprofloxicin

E-Trimethoprim

Most of the bacterial isolates were resistant to trimethoprim in Figure (3-9) (except *K.pneumonie*) was sensitive and low sensitivity to (*Enterobacter*,*Pseudo.aeuroginosa* and *E.coli*).this drug acts by interfering with the action of bacterial dihydrofolate reductase, inhibiting synthesis of tetrahydrofolic acid. Tetrahydrofolic acid is an essential precursor in the *de novo* synthesis of the intermediate Thymidine monophoshpate (dTMP), precursor of DNA metabolite Thymidine triphosphate. Bacteria are unable to take up folic acid from the environment (i.e. the infection host) and are thus dependent on their own *de novo* synthesis. The high resistance of Gram-positive isolates for trimethoprim - sulphamethazole in this study was agreed with many researches

(Jacoby,1996;Al-Hamawandi,2005). which showed that many Gram-positive bacterial isolates showed resistant to trimethoprim-sulphamethazole (Brooks *et al*.,2004).

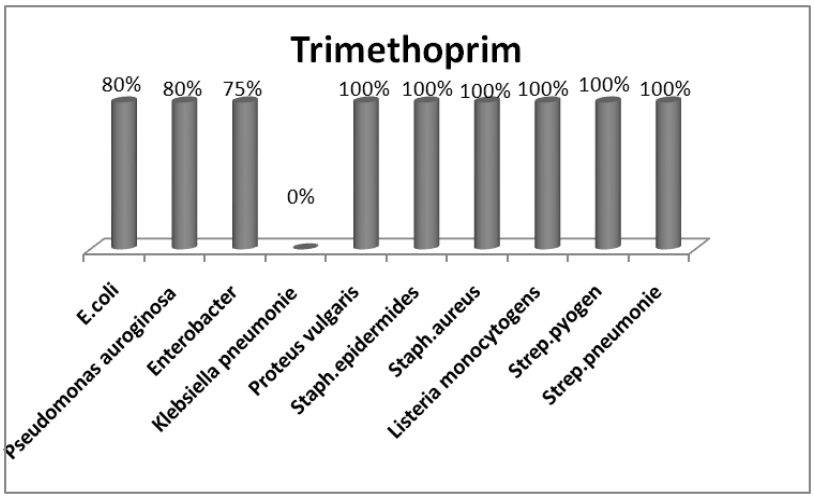

Figure (3-9) Bacterial resistance to Trimethoprim

The previously mentioned bacteria are 100% resistant to these drugs :- Chloramphenicol , and erythromycin. *P. aeruginosa* exhibits intrinsic or acquired resistance to many antibiotics. These bacteria are highly inherently resistant, and this arises from combination of unusually restricted outer membrane permeability and chromosomally encoded B-lactamase. This agrees with the results mentioned by (Hankok ,Speert .,2000; Bisiklis *et al* ., 2005). The main mechanisms for antibiotics resistance in *Pseudomonas aeruginosa* are:

a-Production of β-lactamases, cephalosparinases.

b-Alteration of outer membrane .

c-Reduction of cellular permeability to prevent entry of antibiotic into the bacterial cell (Norrby, 1996 ; James,1999).

All these antibiotics which are tested for a common type of bacteria which are resulted in our study the resistance of (*E.coli*, *Enterobacter* and *Pseudomonas aeruginosa)* to these antibiotics can be explain by un availability of all kitts of antibiotics in hospital to be tested .

3.4 Immunological parameters

3.4.1 Immunoglobulin concentration

Table (3-8) shows the levels of IgG estimated by (SRID) Figure (3-10) in infected thalassemic patients, not infected thalassemic patients and normal subjects were 17753 mg/L,14653 mg/L and 10380 mg/L respectively.Also, the levels of IgM in the infected thalassemic patients, not infected thalassemic patients and normal subjects estimated by(SRID) Figure (3-11) were 1370 mg/L , 1296 mg/L and 1275 mg/L respectively .These results indicate that the levels of IgG didn't significantly increased in both thalassemic Patients ($P>0.05$) while they significantly increased ($p<0.05$)in both thalassemic Patients as compared with controls.Also IgM levels did not significantly increase ($P>0.05$) in both thalassemic Patients and didn't significantly increase ($p>0.05$)in not infected thalassemic Patients as compared with controls.These findings of significant increasing in IgG level agreed with (Pietruska *et al*.,1989;Elting *et al*.,1997).who mentioned that the changes in

total immunoglobulines concentrations were largely affected. They may reflect exposure to infections. Because of recurrent infections to thalassemic Patients and continuous exposure to pathogens, immune system can be activated in order to defend against the infections (Abbas *et al*.,2000). Alteration in humoral immune response was less than in cellular immunity during immunosuppression process therefore the IgG and IgM levels but not at the protective levels of IgG and IgM in normal persons suffering from microbial infections without thalassemia. The high level of IgG in infected thalassemic Patients estimated in this study may indicate the presence of chronic infection, (Doan *et al*., 2005).

IgM which is the predominant type of immunoglobulines during acute infection didn't elevate in thalassemic patients with bacterial infection. This may be due to immune defect in those patients at the same time may predispose them to infections (Tovo *et al*.,2008).

Table (3-8): The Concentration of Immunoglobulin's IgM and IgG (mg/L) in studied groups.

Studied groups		IgM	IgG
A-Infected Thalassemic patients	**M*± SD****	**1370±140**	**17753±3000**
B-Not Infected Thalassemic patients	**M*± SD****	**1296±140**	**14653±3250**
	Significance between A,B	Not significant (P >0.05)	Not significant (P >0.05)
C- Normal persons (not thalassemic and not infected)	**M*± SD****	**1275±140**	**10380±2700**
	Significance between A,C	Not significant (P >0.05)	Significant (P<0.05)
	Significance between B,C	Not significant (P >0.05)	Significant (P<0.05)

* Mean

** Standard deviation

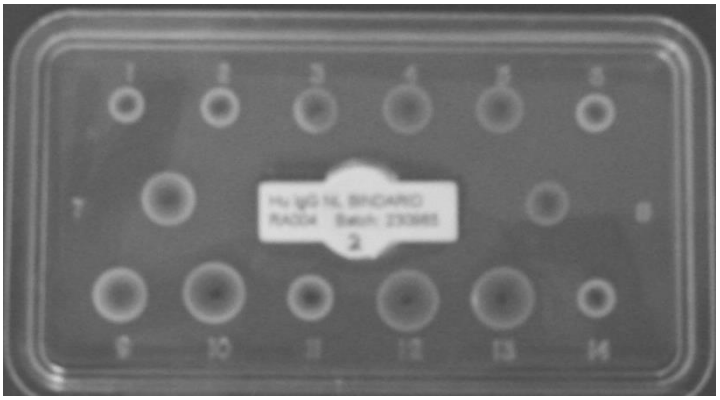

Figure (3-10):- Single radial immunodiffusion test for estimating of IgG concentration

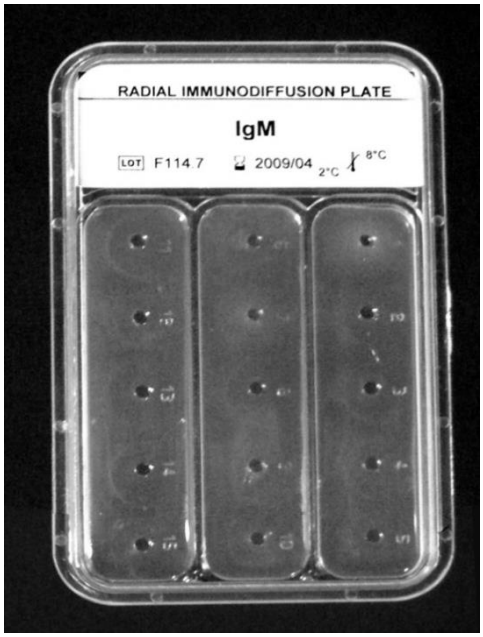

Figure (3-11):- Single radial immunodiffusion test for estimating of IgM concentration

3.4.2 Complement concentration

Table (3-9) Shows the concentration of complement component C3 estimated by(SRID) Figure (3-12) in thalassemic patients with infection was 1620 mg/L, thalassemic patients without infection was 1243 mg/L and control subjects was 1220 mg/L.The concentration of C3 shows significant differences between both thalassemic patients ($P<0.05$) and significant difference between thalassemic patients and controls ($P<0.05$).It indicates that activation of complement system so level of C3 is affected in the presence of infection in thalassemic patients and normal but shows insignificant differences between un infected thalassemic patients and controls this indicate that the level of C3 wasn't affected in absence of infection. (Doan *et al*., 2005; Moutschen. , 2005).The highest concentration of C3 was estimated in thalassemic patients with the signs and symptoms of bacterial infections when compared with uninfected thalassemic patients and controls (Tsiavon *et al* ., 2005).

The concentration of C4 was presented in table (3-9) which shows C4 level estimated by(SRID) Figure (3-13) in thalassemic patients with infection is (375 mg/L), thalassemic patients without infection is (357 mg/L) and normal is(325mg/L).

C4 reveals insignificant differences between Infected thalassemic patients as compared with not infected ones, and also insignificant differences with normal subjects (P >0.05)and this indicates that there is no activation to classical pathway of Complement components. (Doan *et al*., 2008). C4 elevated during secondary infection and is regarded as a specific immune mechanism .So the normal status in person with bacterial infection is the elevation of C4 in a manner similar to that of IgG . This is due to the fact that C4 participate in the classical pathway of complement activation.Therefore thalassemic patients with bacterial infection may have immune defect in Level of C4 these obtained results agreed with (Peng *et al*.,2005) who mentioned that deficient activity of complement system (classical or alternative pathway) in thalassemia major.

Table(3-9): The Concentration of Complement components C4 and C3 (mg/L) in Studied groups.

Studied groups		C4	C3
A-Infected Thalassemic patients	**M*± SD****	**375±128**	**1620±300**
B-Non Infected Thalassemic patients	**M*± SD****	**357±100**	**1243±200**
	Significance between A,B	**Not significant (P >0.05)**	**significant (P <0.05)**
C- Normal persons (not thalassemic and not infected)	**M*± SD****	**325±96**	**1220±204**
	Significance between A,C	**Not significant (P >0.05)**	**Significant (P<0.05)**
	Significance between B,C	**Not significant (P >0.05)**	**Not significant (P >0.05)**

* Mean

** Standard deviation

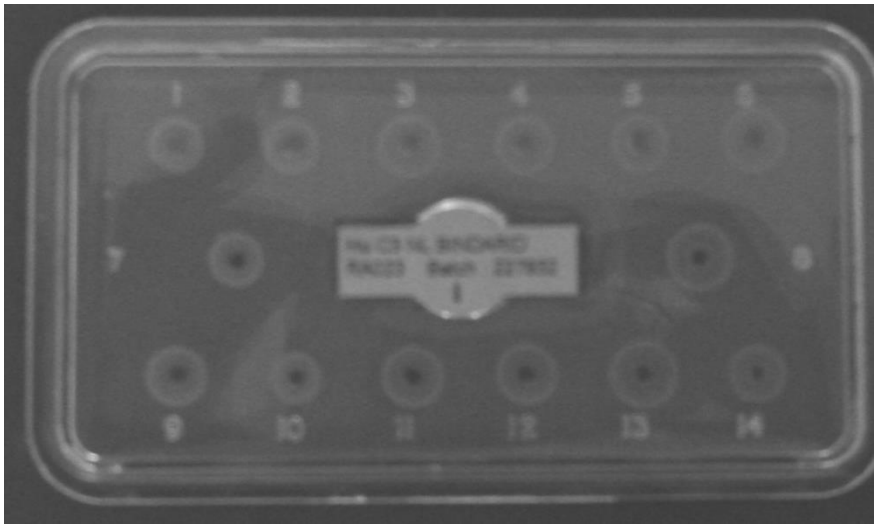

Figure (3-12):- Single radial immunodiffusion test for estimating of C3concentration

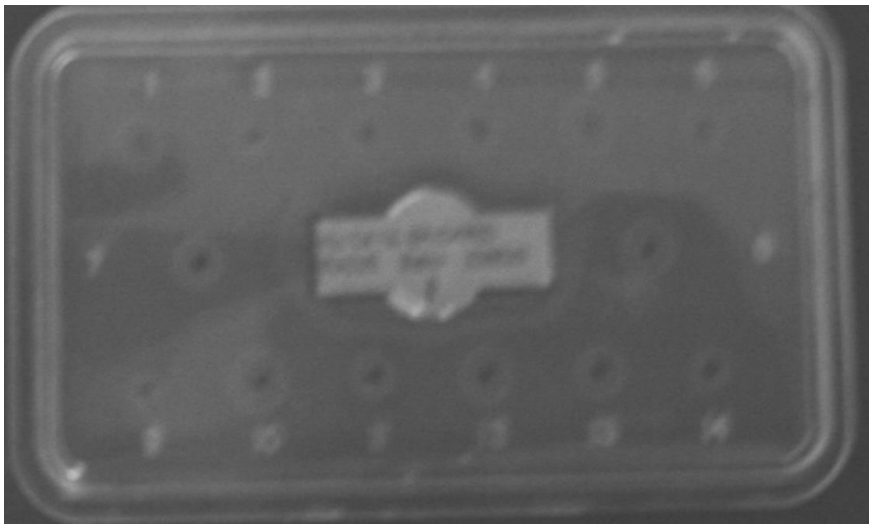

Figure (3-13):- Single radial immunodiffusion test for estimating of C4 concentration

3.4.3 Concentration of tumor necrosis factor-alpha (TNFα)

Table (3-10) shows that the Concentration of Tumor Necrosis Factor Alpha (pg/L) in thalassemic patients with infection was 67 pg/L, thalassemic patients without infection was 50.6 pg/L and normal was 15.25 pg/L.

Level of TNFα

Shows significantly differences between both thalassemic patients, ($P < 0.05$) , significantly differences between infected thalassemic patients and controls ($P < 0.05$) and significantly differences between un infected thalassemic patients and controls ($P < 0.05$) . It also indicate that TNF-α is the major mediators that may be responsible for the lowering cell-medaited immunity, and the production of this cytokine is stimulated by bacterial infection; mainly gram negative bacteria (Abbas *et al*.,2007).these results agreed with (Wanachiwanawin *et al.*,1999; Chandrasekharan *et al*.,2007) who mentioned that elevated level of TNFα in thalassemic patients.The elevated serum levels of TNF-alpha could contribute to complications of the disease, such as cachexia and thromboembolic phenomena**.** Tumor necrosis factor (TNF) is a multifunctional proinflammatory cytokine secreted predominantly by monocytes/macrophages that has effects on lipid metabolism, coagulation, insulin resistance, and endothelial function (Pennica *et al*., 1984;Shirai *et al*., 1985;Franchimont *et al*.,1999).

Table(3-10): Concentration of Tumor Necrosis Factor Alpha (pg/L) in Studied groups

Studied groups		TNFα
A-Infected Thalassemic patients	**M*± SD****	**67±15**
B-Not Infected Thalassemic patients	**M*± SD****	**50.6±20**
	Significance between A,B	**significant (P <0.05)**
C- Normal persons (not thalassemic and not infected)	**M*± SD****	**15.25±5**
	Significance between A,C	**significant (P <0.05)**
	Significance between B,C	**significant (P <0.05)**

*Mean

** Standard deviation

3.4.4 Concentration of Gamma interferon (IFN-γ)

Table (3-11) shows that Gamma interferon IFN-γ Concentration IU/ml in thalassemic patients with infection is 1.2 IU/ml, thalassemic patients without infection is 0.6 IU/ml and normal is 1.8 IU/ml.

Concentration of IFN-γ:

The study shows no significantly differences between both thalassemic patients (P >0.05) , significant differences between infected thalassemic patients as compared with controls (P < 0.05) and not infected thalassemic patients as compared with controls (P < 0.05) . The results indicates the presence of reduced cellular immunity in thalassemic patients which is agree with (Kukreja *et al*., 2002 ;Paiboonsukwong *et al* .,2003; Tsiavon *et al*., 2005 ; Puliti *et al*., 2006) who stated that (decreased serum concentration of gamma-IFN were found in thalassemic patients when compared to controls.). IFN-γ is secreted from Th1 lymphocyte, NK cells and macrophage therefore the defect in these cells leads to decreased IFN-γ production that indicates reflects impaired host resistance in thalassemic patients due to a dysregulation of cytokine network (Snapper and Paul .,2007).IFN-γ enhances the microbicidal function of macrophages by stimulating the synthesis of reactive oxygen intermediates and nitric oxide (NO). It promotes the

differentiation of naïve CD4+ T-cells to the Th1 subset and inhibits the proliferations of Th2 cells beside to activate neutrophils and stimulates the cytolytic activity of NK cells (Rajesh and Rutten,2004; Doan *et al* ., 2005).These results agreed with (Phumala et al.,2003) .Which mention that decreased serum concentration of gamma-IFN were found in thalassemic patients when compared to controls. IFN-γ was much lower in thalassemic patients than in controls due to altered lymphokine productions.

Table (3-11): Concentration of Gamma Interferon IU/ml .

Studied groups		IFN γ
A-Infected Thalassemic patients	**M*± SD****	**1.2 ± 0.4**
B-Non InfectedThalassemic patients	**M*± SD****	**0. 6 ± 0.3**
	Significance between A,B	**Not significant (P >0.05)**
C- Normal persons (not thalassemic and not infected)	**M*± SD****	**1.8± 0.5**
	Significance between A,C	**significant (P <0.05)**
	Significance between B,C	**significant (P <0.05)**

*Mean

** Standard deviation

3.4.5 Serum Ferritin concentration:

Table(3-12) shows that the serum ferritin concentration ng/ml in thalassemic patients with infection was 350 ng/ml, thalassemic patients without infection was 295 ng/ml and controls was 87 ng/ml .Serum ferritin concentration show no significantly differences between both thalassemic patients ($P > 0.05$),.Although ,there was no significantly differences between thalassemic patients with infection and thalassemic patients without infection ($P > 0.05$) but the former exhibited relatively high level of ferritin in thalassemic patients as compared with control this may be due to the large times of blood transfusion .Additionally ,high level of ferritin causes immunosuppressive effects that make thalssemic patients more susceptible to bacterial infection . Significantly differences between infected thalassemic patients ($P < 0.05$) and control and significantly differences between not infected thalassemic patients ($P < 0.05$) and control. This higher Serum Ferritin concentration in thalssemic patients revealed a high iron stores which agreed with (Underwood *et al*.,2004; Pootrakul *et al*.,2004). Iron overload is a constant and the most important complication in thalassemia. Ferritin is a globular protein complex consisting of 24 protein subunits and is the main intracellular iron storage protein in both prokaryotes and eukaryotes, keeping iron in a soluble and non-toxic form. Ferritin

which is not combined with iron is called apoferritin. This observation is compatible with that splenectomy in thalassemia is associated with increased iron deposition and increased transferrin iron saturation. The further increase in iron overload after splenectomy in thalassemia should be borne in mind in considering removal of this organ. (Phumala *et al*.,2003).Regarding pathogenesis, iron overload, a primary complication of both thalassemia itself and transfusion therapy, is thought to be the main precipitating mechanism ,due to the important immune regulatory properties of iron and its binding proteins ;iron excess may derange the immune balance in favor of the growth of infectious organisms (Cappellini *et al*.,2007).other factors include multiple transfusions, with transmission of immunosuppressive viruses ; splenectomy , resulting in increased susceptibility to infections by encapsulated bacteria and to immune system modifications; low levels of zinc, (another immune regulator); iron chelation therapy, which predisposes to serious infections by yersinia species due to appropriate environmental conditions (elevated iron level in intestine); and the circulation of abnormal native thalassemic erythrocytes, forming another permanent immune stimulus (Sunzel *et al* .,2005). Thus surveillance for infections in patients with beta-thalassemia is crucial, while further studies are warranted on

immune function abnormalities and the implicated mechanisms (Sunzel et al .,2005).

Table (3-12) Concentration of Serum Ferritin ng/ml in studied groups.

Studied groups		**Serum ferritin concentration**
A-Infected Thalassemic patients	**M*± SD****	**350 ± 20**
B-Non Infected Thalassemic patients	**M*± SD****	**295 ± 17**
	Significance between A,B	**Not significant (P >0.05)**
C- Normal persons (not thalassemic and not infected)	**M*± SD****	**87 ± 8**
	Significance between A,C	**significant (P <0.05)**
	Significance between B,C	**significant (P <0.05)**

*Mean

** Standard deviation

3.4.6 Concentration of Immunological parameters in Thalassemic patients Specimens (Blood and Urine):

In Table (3-13): shows that IgG, C4, C3 and TNFα level there was no significantly differences between bacteremia and UTI infections(P>0.05). While in IgM and IFNγ level there was significantly difference between bacteremia and UTI infections (P<0.05). (Chapel *et al* .,1999).who mentioned that decreased level of IgM and IFNγ in acute urinary tract infections.

Table (3-13): Concentration of immunological parameters in infected thalassemic patients Specimens (Blood and Urine)

Speciemens		***IgG***	***IgM***	**C3**	***C4***	**TNFα**	**IFN γ**
A-Blood	**M*± SD****	**14771 ± 3600**	**917 ± 810**	**1382±776**	**270 ± 107**	**37.67±34.3**	**1.1±0.3**
B-Urine	**M*± SD****	**16458 ± 430**	**322 ± 91**	**1794±254**	**450 ±131**	**52.2±42**	**0. 6±0.2**
	Significance between A,B	**Not (P >0.05)**	**significant (P <0.05)**	**Not (P >0.05)**	**Not (P >0.05)**	**Not (P >0.05)**	**Significant (p<0.05)**

* Mean

** Standard deviation

3.4.7 The Concentration of Immunological parameters in infected thalassemic patients Specimens (Ear and Throat swabs)

In table (3-14) : IgG and TNFα levels show not significantly differences between ear and throat swabs (P>0.05). While IgM ,C3, C4 and IFNγ levels show significantly differences between ear and throat infections (P<0.05).these results agrees with (Brooks *et al* .,2007).who mentioned that elevated level of IgM and C4 in otitis media.

Table (3-14): Concentration of immunological parameters in infected thalassemic patient's specimens (Throat and Ear swabs)

Speciemens		***IgG***	***IgM***	**C3**	***C4***	**TNFα**	**IFN γ**
C- Throat swab	**M*± SD****	**20916± 5182**	**3470±0**	**1400±822**	**430±0**	**87±50**	**0.7±0.3**
D- Ear swab	**M*±SD****	**25000 11000±**	**3700±0**	**700±0**	**650 ± 0**	**83±67**	**0.4±0.1**
	Significance between C,D	**Not significant (P >0.05)**	**significant (P <0.05)**	**significant (P <0.05)**	**significant (P <0.05)**	**Not**	**significant (P <0.05)**

* Mean ** Standard deviation

ABSTRACT

This study was aimed to isolate and identify the aerobic and/or facultative anaerobe bacteria, evaluate humoral and cellular immunological parameters against those bacterial infections and studying the effect of some antibiotics on bacterial isolates in thalassemic patients admitted to Thalassemia center in Karbalaa hospital for children from November 2008 to April 2009.
A total of 200 individuals were subjected for this study with age range (2-28 years).

Thalassemic patients with signs and symptoms of infections were 92 individuals and the rest (58) from thalassemic patients apparently not infected. it also contain 50 normal individuals as control group at the same age range.

Samples (ear swabs,throat swabs ,blood and urine) were taken aseptically from each thalassemic patients with any clinical sign of infections . The collected samples were processed for bacterial isolation and determination of virulence factors (coagulase, hemolysin , CAMP (Christie, Atkins and Munch-Peterson) test and capsule formation). Antibiotic sensitivity tests were assessed for each isolate.

Blood samples were collected from both patients and controls to estimate IgM , IgG ,C3 and C4 concentration by single radial immunodiffusion (SRID) test, tumor necrosis factor alpha (TNF-α),gamma interferon (IFN-γ) by ELISA (Enzyme linked immunosorbant assay) method and concentration of serum ferritin

The results of bacterial culture were positive in 75:92 (82%)(gram negative bacteria were more predominant 58:75) (70%) then (gram positive bacteria 17:75 (30%)) ; *E. coli* was the main predominant gram negative bacteria that constitute 23:58

(39.65%) of gram negative bacteria, while *Streptococcus pyogens* was the main predominant gram gram-positive isolate 7:17 (41.17%). followed by *Staphylococcus aureus* 5:17 (29.4%) .

The immunological parameters showing that there is a significantly increased ($p<0.05$) in the level of IgG in infected thalassemic patients (17753) compared with control and significantly increased ($p<0.05$) non-infected (14653) and normal persons (10380) mg/L, IgM insignificantly increased ($p>0.05$) in the same patient groups and normal persons (1370,1296,1275) mg/L,C3 level is a significantly increased ($p<0.05$) in the same patients group and normal persons (1620,1243,1220)mg/L, C4 level insignificantly increased ($p>0.05$) in the same patient group and normal persons (375,357,325)mg/L, TNF-α level is a significantly increased ($p<0.05$) in the same patient group and normal persons (67 ,50.6 , 15.25)Pg/L, IFN-γ level significantly reduced ($p<0.05$) in the same patient group and normal persons (1.2 , 0.6 , 1.8)IU/L and serum ferritin concentration is a significantly increased ($p<0.05$) in the same patient group and normal persons (350 , 295, 87) ng/ml.

There is only significantly increased in IgM and IFNγ levels in sera of patients with bacteremia ($P<0.05$) while the IgG ,C4, C3 and TNFα levels showing insignificant difference between them($P>0.05$). There is significantly increased in IgM, C4 levels in sera of patients with otitis media ($P<0.05$) and significantly increased in C3, IFNγ levels in sera of patients with laryngitis ($P<0.05$) .

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List of Abbreviations

AK	Amikacin
AMC	Amoxicillin+ clavulinic acid
APX	Ampicillin+ cloxacillin
AX	Amoxicillin
AZM	Azithromycin
CAMP	Christie , Atkins and Munch-Peterson
CAZ	Ceftazidime
CL	Cephalexin
CLR	Clarithromycin
CN	Gentamycin
CoNS	Coagulase negative staphylococci
CTX	Cefotaxim
DNA	Deoxyribonucleic acid
DO	Doxycycline
DW	Distilled water
E	Erythromycin
EASIA	Enzyme amplified sensitivity immunoassay
EMB	Eosin methylene blue
GBS	Group B *Streptococcus*
HRP	Horseradish peroxidase

Ig	Immunoglobulin
IgG and IgM	Immunoglobulin G,and M
IL-1,6,8,10	Interleukin-1,6,8,10
LPS	Lipopolysaccharide
LSD	Least significant diffrences
M	Mean
MAbs	Monoclonal antibodies
MR	Methyl red reagent
N.K cells	Natuarl killer cells
NCCLS	National committee for clinical laboratory standard
nm	Nanometer
NO	Nitric oxide
NOR	Norfloxacin
PABA	Para – Amino Benzoic Acid
PBPs	Penicillin Binding Proteins
PBS	Phosphate buffer saline
SD	Standard deviation
SRBCs	Sheep red blood corpuscles
SRID test	Single radial immunodiffusion test
Th- 2	T-cell helper 2
TNF-α	Tumor necrosis factor-alpha

TSI	Triple sugar iron
UTI	Urinary tract infection
VP	Voges-Proskauer reagent

الخلاصـــة

تهدف هذه الدراسة الى عزل وتشخيص البكتيريا الهوائية من مرضى الثلاسيميا و دراسة بعض المعايير المناعية الخلطية والخلوية ودراسة تاثير بعض المضادات الحيوية على العزلات البكتيرية عند مرضى الثلاسيميا المراجعين لمركز الثلاسيميا في مستشفى كربلاء للاطفال وللفترة من تشرين الثاني 2008 لغاية نيسان 2009

تضمنت الدراسة 200 عينة منها 150 مريض بالثلاسيميا تراوحت اعمارهم بين(2-28) عاما كان 92 منهم لديهم علامات الاصابة بالالتهابات حسب تشخيص الطبيب المسؤول والبقية(58) يبدون غير مصابين وكما تضمنت الدراسة 50 شخص (اصحاء) كمجموعة سيطرة وبنفس المدى العمري.

تضمنت العينات الماخوذة (مسحات اذن ، مسحات حنجرة ، وعينات دم وادرار) من مرضى الثلاسيميا الذين يملكون علامات الاصابة بالالتهابات.تلك العينات الماخوذة من كلا المجموعتين تم زرعها لعزل البكتيريا المسببة للالتهابات وتعيين عوامل الضراوة للعزلات البكتيرية (انتاج انزيمات التجلط ، تحلل الدم CAMP{ Christie , Atkins and Munch-Peterson} وانتاج الكبسولة) اضافة الى اجراء فحص الحساسية لعدد من المضادات الحيوية. اخذت عينات الدم من كلا المجموعتين (المرضى والاصحاء) لحساب تراكيز الكلوبينات المناعية نوع IgG ، IgM وتراكيز المتممات C3 ، C4 بطريقة الانتشار المناعي الشعاعي المفرد وقياس تركيز IFN و TNFαبطريقة ELISA وحساب تركيز Ferritin .

اظهرت النتائج ان نسبة الزرع البكتيري الموجب هي (93%) (75 : 92) وكانت البكتريا السالبة لصبغة غرام هي السائدة (70%) (17 : 75) ان *Echrichia coli* كانت المسبب الرئيسي(39.65 %) (23 : 58) من الاصابات الناتجة من البكتيريا السالبة لصبغة غرام بينما *Streptococcus pyogen* كانت المسبب الرئيسي (41.17%) (7 : 17) من الاصابات الناتجة من البكتيريا الموجبة لصبغة غرام .

كما ان الفحوصات المناعية اظهرت زيادة في تراكيز IgG بشكل معنوي ($P< 0.05$) حيث كان تركيز IgG لدى مرضى الثلاسيمياالمصابين بالإلتهابات 17753 وغير المصابين14653 مقارنة بالإصحاء 10380 (ملغم لكل لتر) وتركيز C3 اظهر زيادة معنوية ($P< 0.05$) عند نفس

المرضى مقارنة بالإصحاء حيث كانت النسب (1243,1620, 1220) (ملغم لكل لتر) على التوالي وكذلك تراكيز TNFα فقد اظهرت زيادة معنوية(P< 0.05) لدى نفس المرضى مقارنة بالإصحاء حيث كانت النسب(15.52,50.6,67) (بيكوغرام لكل لتر) و تركيز Ferritin اظهر زيادة معنوية

(P< 0.05) لدى نفس المرضى مقارنة بالإصحاء حيث كانت النسب (78,295,350) (نانوغرام لكل مللتر) بينما تركيز IgM لم يظهرزيادة معنوية (P> 0.05) لدى مرضى الثلاسيميا المصابين بالإلتهابات 1370 وغير المصابين1296 مقارنة بالإصحاء (1275 ملغم لكل لتر) وتركيز C4 لم يظهر زيادة معنوية(P> 0.05) لدى نفس المرضى مقارنة بالإصحاء حيث كانت النسب (325,357,375)(ملغم لكل لتر) على التوالي وتراكيز IFN-γ اظهر قلة بشكل معنوي (P< 0.05) لدى نفس المرضى مقارنة بالإصحاء حيث كانت النسب (1.2,0.6,1.8) (وحدة دولية لكل مليلتر) .

كذلك اظهرت النتائج ان IgGو IgM قد ازدادا وبشكل معنوي (P< 0.05) في امصال مرضى الثلاسيميا المصابين بالتهابات الاذن و تركيز,TNFα قد ازدادت وبشكل معنوي (P< 0.05) في امصال مرضى الثلاسيميا المصابين بالتهابات الحنجرة وكذلك تراكيز C3,C4 قد ازدادت وبشكل معنوي(P< 0.05) في امصال مرضى الثلاسيميا المصابين بالتهابات المجاري البولية وزيادة في تراكيز IFN-γ وبشكل معنوي(P< 0.05) في امصال مرضى الثلاسيميا المصابين بتجرثم الدم.

Examination Committee

We, the examiner committee, certify that we have read the thesis entitled ***(Immune Response in Thalassemic Patients Associated with Bacterial Infections)*** and have examined the student ***(Hiba Ali Hadi Al -Khafagii)*** in its contents, and that in our opinion it is accepted as a thesis for the degree of Master of Science in Microbiology with very good estimation.

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Hiba

Conclusions

1-Gram negative bacteria are the predominant bacteria in infected thalassemic patients, and *E. coli* is the most common one.

2- IgM level in infected thalassemic patients wasn't significantly increased but IgG level was significantly increased.

3- C3 level in infected thalassemic patients was Significantly increased but C4 level wasn't significantly increased.

4- TNF-α level in thalassemic patients was significantly increased but IFN-γ was significantly decreased.

5- Serum ferritin concentration in infected thalassemic patients was significantly increased .

Recommendations

1.Infected thalassemic patients should be screened for bacterial infection as well as other microbial infection as a prophylactic measure to prevent complications.

2.Estimation the protective level of specific antibody in infected thalassemic patients due to multiple blood transfusions.

3.Studying the pathogenicity of anaerobic bacteria, Cytomegalovirus and or other viruses and their relationship with thalassemia.

4. Needs more antibiotics kitts to be tested for these bacteria.

Supervision Certificate

We certify that this thesis was prepared under our supervision at the Department of Microbiology, College of Medicine in Babylon University as partial fulfillment of the requirements for the Degree of **Master in Medical Microbiology.**

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Dedication

To . . .

My support in life, tender hearts that surrounded me with care and courage my father

The kind heart: my mother

And all my brothers

Hiba

1.1 Introduction:

Thalassemia describes a group of inherited disorders characterized by defect in globin chain, (in beta thalassemia defect or decreased in Beta-globin chain gene .while, in alpha thalassemia result from deletion in α-globin chain gene. There are two basic groups of thalassemia disorders: alpha thalassemia and beta thalassemia. These conditions cause varying degrees of anemia, which can range from insignificant to life threatening (Bojanowski.,1999; Richard *et al*.,2000; Cooley's Anemia Foundation, 2007). Many families have thalassemia carriers, but the trait often goes undiagnosed because it produces no or few symptoms. Frequently, thalassemia is not diagnosed in a family until a baby is born with it (Rund., 2005) .The immunologic defects observed in patients with thalassemia major make them susceptible to different kinds of infections, both before and after splenectomy. The reasons for immunodeficiency are: (Blumberg *et al*.,2000; Ezer *et al*.,2002).a-The immunosuppressive effects of increased ferritin concentrations due to multiple blood transfusions .b-Cytomegalovirus (CMV) infection is responsible for increased susceptibility to infection because of the immunosuppressive properties of CMV (Germenis, Politis.,2000).c-Abnormalities in humoral immunity such as defects in alternative complement pathways and abnormal immunoglobulin levels, abnormalities in cell mediated immunity (CMI) such as decreased natural killer (NK) cell activity, defective neutrophil function, decreased T-

helper/ T-suppressor ratio and T-cell subset abnormalities. Defective neutrophil function in patients with thalassemia has been suggested in different reports (Jamsai *et al.*, 2005; Bartolomeo., 2008).Infectious complications constitute the second most common cause of mortality and a main cause of morbidity in beta-thalassemia. Besides the high risk of blood-borne infections associated with multiple transfusions, the increased susceptibility of these patients to infectious diseases has been attributed to a coexistent immune deficiency. Recent studies on immune competence in beta-thalassemia have revealed numerous quantitative and functional defects, involving T and B lymphocytes, immunoglobulin production, neutrophils and macrophages, chemotaxis, and phagocytosis, as well as the complement system. (Kutukculer *et al*.,1996;Cappellini *et al*.,2000).

Aims of the study

Thalassemia is a common disease in Iraq. There is no information about bacteriological and immunological profile about thalassemic patients in Iraq , so it is the first study which evaluates bacteriological and immunological parameters of these patients and discusses their susceptibility to infections. Thus this study aims to:

A-Isolating and identifying aerobic and or facultative anaerobic bacteria from the infected thalassemic patients.

B- Studying the aspects of humoral and cellular immunological parameters in thalassemic patients.

C- Studying the effect of some antibiotics on bacterial isolates.

1.2 Review of literatures

1.2.1 Definition of thalassemia:-

A group of genetic disorders of hemoglobin synthesis characterized by a disturbance of globin chain production (in beta thalassemia defect or decreased in Beta-globin chain gene .while, in alpha thalassemia result from deletion in α-globin chain gene. There are two basic groups of thalassemia disorders: alpha thalassemia and beta thalassemia. These conditions cause varying degrees of anemia, which can range from insignificant to life threatening (Bojanowski.,1999; Richard *et al*.,2000;Cooley's Anemia Foundation, 2007) .

1.2.2 Description of thalassemia:-

All types of thalassemias are considered quantitative diseases of hemoglobin, because the quantity of hemoglobin produced is reduced. Usual adult hemoglobin is made up of three components: alpha globin, beta globin, and heme. Thalassemias are classified according to the globin that is affected, hence the names alpha and beta thalassemia. Although both classes of thalassemia affect the same protein, the alpha and beta thalassemias are distinct diseases that affect the body in different ways (Cohen *et al*.,2004) .

1.2.3 Distribution of thalassemia:-

Beta thalassemia is seen most commonly in Mediterranean (including North African, and particularly Italian and Greek), Middle Eastern, Indian, African, Chinese, and Southeast Asian (including Vietnamese, Laotian, Thai, Singaporean, Filipino, Cambodian, Malaysian, Burmese, and Indonesian). Alpha-thalassemia is seen with increased frequency in the same ethnic groups. However, there are different types of alpha thalassemia within these populations. The populations in which alpha thalassemia diseases are most common include Southeast Asians and Chinese (particularly Southern Chinese) (Fucharoen *et al.*,2000*;* Cunningham, *et al*.,2008*)* .

1.2.4 Causes of thalassemia:-

Thalassemia is always inherited, passed on from parents to children through their genes. A child cannot develop the disease unless both parents carry a thalassemia gene. If only one parent passes a gene for thalassemia on to the child, then the child is said to have thalassemia (carrier). He will not develop into the full-blown disease, and no medical treatment is necessary. Many families have thalassemia carriers, but the trait often goes undiagnosed because it produces no or few symptoms. Frequently, thalassemia is not diagnosed in a family until a baby is born with it (Chohan *et al*.,2001 ; Rund. and Rachmilewitz,.2005; Boon *et al*., 2006) .

1.2.5 Types of thalassemia: -

1.2.5.1 Beta thalassemia

Beta thalassemia may be the most well-known type of thalassemia. It is caused by a change in the gene for the beta globin component of hemoglobin. Beta thalassemia causes variable anemia that can range from moderate to severe, depending in part on the exact genetic change underlying the disease. Beta thalassemia can be classified based on clinical symptoms and blood investigations into three categories: beta-thalassemia minor, intermedia, and major (which is called Cooley's anemia (Opartkiattikul *et al*., 1999; Fucharoen *et al*.,2000; Kiska *et al*.,2003; Chumpia *et al*. ,2006). There were other form of Beta thalassemia :

Hb S/beta thalassemia:This type results from one beta globin gene carrying a thalassemia mutation and one gene for sickle cell disease, another inherited anemia. It is most common in those of African or Mediterranean ancestry .Symptoms generally resemble those of sickle cell disease, including varying degrees of anemia, serious infections, pain and damage to vital organs (Opartkiattikul *et al*.,1997;Ong *et al*., 1998; Fucharoen *et al*.,2000;Kiska *et al*., 2003) .

1.2.5.2 Alpha thalassemia

Alpha thalassemia is the result of changes in the genes for the alpha globin component of hemoglobin .The mildest form, has one alpha globin gene missing. Affected individuals generally have no symptoms, but they can pass on the genetic

abnormality to their children it is also called Silent carrier (Doan *et al.*,2005).There are four main types of alpha thalassemia disease: alpha thalassemia trait (carrier) , alpha thalassemia , hemoglobin H disease and hydropes fetalis . These diseases are quite different from beta thalassemia as well as from one another (Bojanowski.,1999; Fucharoen *et al*.,2000).

1.2.6 Genetic Basis:-

Humans normally make several types of the oxygen-carrying protein hemoglobin. An individual's stage in development determines whether he or she makes primarily embryonic, fetal, or adult hemoglobins. All types of hemoglobin are made of three components: heme, alpha (or alpha-like) globin, and beta (or beta-like) globin. All types of Thalassemia are caused by changes in either the alpha- or beta-globin gene. These changes cause little or no globin to be produced. The thalassemias are, therefore, considered quantitative hemoglobin diseases (Weatheral *et al*., 2006). All types of thalassemia are recessively inherited, meaning that a genetic change must be inherited from both the mother and the father (Wongseree *et al*.,2007) .The severity of the disease is influenced by the exact Thalassemia mutations inherited, as well as other genetic and environmental factors(Fucharoen *et al*.,1999,2001;lshwar *et al*., 2007;Sarakul *et al*., 2008).Most individuals have two normal copies of the beta globin gene, which is located on chromosome 11 and makes the beta globin component of normal adult hemoglobin, hemoglobin A. There are

approximately 100 genetic mutations that have been described as causing beta Thalassemia, designated as either beta0 or beta+ mutations. No beta globin is produced with a beta0 mutation, and only a small fraction of the normal amount of beta globin is produced with a beta+ mutation (Furuumi *et al*., 1998; Ngiwsara *et al*.,2004).Most individuals have four normal copies of the alpha globin gene, two copies on each chromosome 16. These genes make the alpha globin component of normal adult hemoglobin, which is called hemoglobin A (Lacerra *et al*.,2000; Ma *et al*.,2007) . Alpha globin is also a component of fetal hemoglobin and the other major adult hemoglobin called hemoglobin A2. Mutations of the alpha globin genes are usually deletions of the gene, resulting in absent production of alpha globin. Since there are four genes (instead of the usual two) to consider when looking at alpha globin gene inheritance, there are several alpha globin types that are possible (Ezer *et al*.,2002) .Absence of one alpha globin gene leads to a condition known as silent alpha Thalassemia carrier. This condition causes no health problems and can be detected only by special genetic testing (Imai *et al*.,2001).

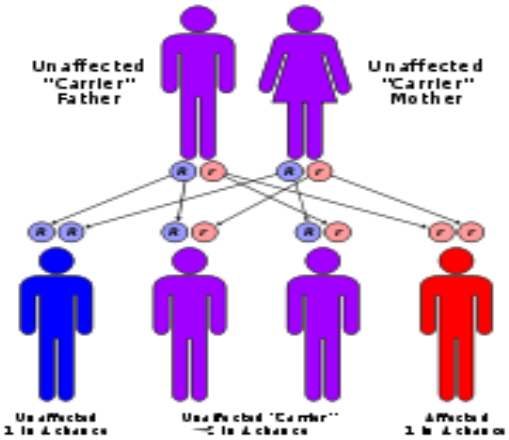

Figure (1-1)

Thalassemia has an autosomal recessive pattern of inheritance

1.2.7 Diagnosis:-

Thalassemia may be suspected if an individual shows signs that are suggestive of the disease. In all cases, however, laboratory diagnosis is essential to confirm the exact diagnosis and to allow for the provision of accurate genetic counseling about recurrence risks and testing options for parents and affected individuals. Screening test is likewise recommended to determine trait status for individuals of high-risk ethnic groups (Watanapokasin *et al*.,2000; Winichagoon *et al* 2005; Tang *et al*.,2001; Svasti et al.,2006).The following tests are used to screen for thalassemia disease:

A-Complete blood count .

B-Hemoglobin electrophoresis with quantitative hemoglobin A,A2 and hemoglobin F

C-Free erythrocyte-protoporphyrin (or ferritin or other studies of serum iron levels).

D-Prenatal diagnosis of beta-thalassaemia by reverse dot-blot hybridization (Winichagoon *et al* .,1999; Winichagoon *et al* 2005).

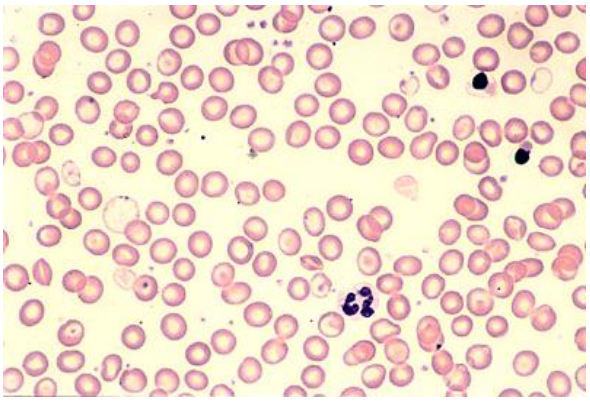

Figure (1-2) Blood cells of beta thalassemia major patient (Dacie *et al*., 2007)

1.2.8 Microbial infections associated with thalassemia:-

Infections are major complications and constitute the second most common cause of mortality and a main cause of morbidity in patients with thalassemia (Jonson *et al*.,2005). Predisposing factors for infections in thalassemic patients include severe anemia, iron overload, splenectomy, and a range of immune abnormalities (Jintaridth *et al*., 2006). Major causative organisms of bacterial infections in thalassemic patients are *Klebsiella spp* in Asia and *Yersinia enterocolitica* in western countries (Gale.,2006). Transfusion-associated viral infections (especially hepatitis C) can

lead to liver cirrhosis and hepatocellular carcinoma (Murry *et al*., 2003). A unique and challenging infection detected in Asian patients is pythiosis, caused by a fungus-like organism (Wang *et al*.,2003;Doan *et al*.,2005) .The incidence and clinical spectrum of severe bacterial infection were studied (Huber *et al*.,2005) in patients with thalassemia major included liver abscess , septicemia , soft-tissue infection , osteomyelitis , corneal ulcer , enteritis and abscesses of the lung, kidney, intra-abdominal region, retropharynx, gums, and buttocks (Cisterna *et al*.,2001). The leading causal microorganisms were gram-negative bacilli, especially *Klebsiella pneumoniae* Other responsible pathogens were *Pseudomonas aeruginosa , Vibrio vulnificus, Acinetobacter baumanii, Streptococcus spp., Listeria monocytogenes:, Yersinia enterocolitica, Staphylococcus aureus , Escherichia coli , Salmonella* species and *Proteus* spp.(Chin .,2000).

A-Streptococcus spp.

Streptococci is a genus of spherical Gram-positive bacteria, (Holt *et al*.,1994).In addition to sore throat, certain Streptococcus species are responsible for many cases of meningitis, bacterial pneumonia, endocarditis, erysipelas and necrotizing fasciitis (the 'flesh-eating' bacterial infections). However, many streptococcal species are non-pathogenic. *Streptococci* are also part of the normal commensal flora of the mouth, skin, intestine, and upper respiratory tract of humans (Chart *et al*.,2004).The incidence and clinical

spectrum of severe bacterial infection caused by Streptococcus spp in thalassemic patients was reported. (Kiska *et al*.,2003).

b-Listeria monocytogenes:-

Listeria monocytogenes are a Gram-positive rod-shaped bacterium. It is the agent of listeriosis, a serious infection caused by eating food contaminated with the bacteria .affect the adults with weakened immune systems (Kiska *et al*.,2003).Listeriosis is an infection occur in thalassemic patients (Dondorp *et al*.,1999). Contaminated food is the usual source of infection, and transmission appears to be blood-borne following gastrointestinal infection (Collee *et al*. ,1996).

c- Escherichia coli

E. coli is Gram-negative, facultative anaerobic and non-sporulating cells are typically rod-shaped**.** *E. coli* normally colonizes an infant's gastrointestinal tract within 40 hours of birth, arriving with food or water or with the individuals handling the child. In the bowel, it adheres to the mucus of the large intestine (Cassels *et al*., 1995). It is the primary facultative organism of the human gastrointestinal tract. As long as these bacteria do not acquire genetic elements encoding for virulence factors, they remain benign commensals (Chart *et al*., 2004).Virulent strains of *E. coli* can cause gastroenteritis, urinary tract infections, and neonatal meningitis. In rarer cases, virulent strains are also responsible for haemolytic-uremic syndrome (HUS), peritonitis,

mastitis, septicemia and Gram-negative pneumonia in thalassemic patients (Johnson ., 1991 ;Maria *et al*.,2007).

d-Enterobacter microorganisms:-

Enterobacter is a genus of common Gram-negative, facultatively-anaerobic, rod-shaped bacteria of the family *Enterobacteriaceae*. Several strains of the these bacteria are pathogenic and cause opportunistic infections in immunocompromised in thalassemic patients (Hancock *et al.*,2000; Ryan and Ray.,2004). The urinary and respiratory tract are the most common sites of infection. *Enterobacter* can be distinguished from other Gram-negative rods (GNR) by virtue of being a 'fast fermenter' of lactose (as are *Escherichia coli* and *Klebsiella* (Maria *et al*.,2007).

e- Staphylococcus epidermidis and *S. aureus* were also more prevalent in the blood of infected thalassemic patients (Humphreys.,2004).

1.2.9 Immunity in thalassemia:-

Adaptive immunity has been intensively studied while it has been only recently that we have gained some understanding of innate immunity (Chapel *et al*., 1999). In particular, cellular responses in innate immunity have been poorly understood compared with humoral responses. In addition, the mechanisms and roles of innate immune responses could be distinct

between the organisms that possess both innate and adaptive immunity and those possessing only innate immunity. On the other hand, invading pathogenic microbes employ various strategies to inhibit the host immune system for their survival (Chapel *et al*.,1999).The dynamics of the interactions between a pathogen and its host's immune response consists of two different equations, one is for pathogen load while the other one is for an index of specific immunity (Kiska *et al*.,2003).According to the hosts' or pathogen's parameter values, or to the initial infection size, a rich repertoire of behaviours:can be mentioned immediate clearing of the pathogen through specific immune response; or acute infection followed by clearing of the pathogen through specific immune response; or uncontrolled infections; or acute infection followed by convergence to a stable state of chronic infection mimic some features of immune response after vaccination (Kiska *et al*.,2003).The immunologic abnormalities in Beta-thalassemia major patients show significantly increased absolute lymphocyte counts compared with the control group. The increased number of activated T cells which suggest chronic stimulation of immune system (Cotton.,2004; Cohen *et al*.,2004).Neutrophils the body’s primary defense against bacterial invasion, engulf and destroy bacterial and foreign particles by a process known as phagocytosis. In patients with bacterial infections, neutrophil function tests may show that neutrophils are unable to kill target bacteria or migrate to the infection site (chemotaxis). Chemotaxis in patients with thalassemia major was found to be defected when compared with healthy

controls (Giuntoli *et al*.,1984;Matzner *et al*.,1993 ; Palacios *et al*.,1993;Kutukculer *et al*.,1996). On the contrary, T-cell proliferation and interleukin 2 (IL-2), interferon gamma (IFN-γ), and IL-4 production were suppressed in patients compared to controls according to (Kiska et al.,2003) who mentioned that (decreased serum concentration of gamma-IFN were found in thalassemic patients when compared to controls).Patients with high serum ferritin levels produced significantly less IFN-gamma and IL-2, indicating the immunosuppressive effect of iron overload in beta-thalassemia patients (Jittangprasert *et al*.,2004; Pootrakul *et al*.,2004) .The serum levels of tumor necrosis factor alpha and absolute counts and percentages of B and T cells were higher (Sternbach *et al*.,1998).T-lymphocytes express activated phenotype in polytransfused beta-thalassemia major patients, while T cell proliferation and effector function are significantly suppressed(Pattanpanyasat *et al*.,2000). Multiple blood transfusion and continuous immune stimulation could be responsible for making such a double-faced immune response (Kiska *et al*.,2003).Effects of iron overload include decreased antibody-mediated and mitogen-stimulated phagocytosis by monocytes and macrophages, alterations in T-lymphocyte subsets, and modification of lymphocyte distribution in different compartments of the immune system (Peng *et al*.,2006).The importance of iron in regulating the expression of T-lymphocyte cell surface markers, influencing the expansion of different T-cell subsets, and affecting immune cell functions can be demonstrated in vitro and in vivo (Jittangprasert

et al.,2004; Pootrakul *et al*.,2004) .The poor ability of lymphocytes to sequester excess iron in ferritin may help to explain the immune system abnormalities in iron-overloaded patients. Iron overload as seen in hereditary hemochromatosis patients enhances suppressor T-cell (CD8) numbers and activity, decreases the proliferative capacity, numbers, and activity of helper T cells (CD4) with increase in CD8/CD4 ratios, impairs the generation of cytotoxic T cells, and alters immunoglobulin secretion when compared to treated hereditary hemochromatosis patients or controls.(Ezer *et al*.,2002;Kiska *et al*.,2003) who mentioned that there was decrease in CD4+ / CD8+ ratios in the beta thalassemia major group .Iron overload, with its associated increases of serum iron levels and transferrin saturation, may cause a poor response to interferon therapy. Iron overload with hyperferremia is associated with suppressed functions of the complement system (classic or alternative types) (Peng *et al*.,2005).High plasma ferritin content in patients with chronic, diffuse diseases of the liver (cirrhosis, chronic hepatitis), beta-thalassemia major, dyserythropoiesis, and hereditary hemochromatosis may induce the development of anti-ferritin antibodies with the production of circulating immune complexes (Srichairatanakool *et al*., 2006).Increased body stores of iron in various clinical situations may tip the immunoregulatory balance unfavorably to allow increased growth rates of cancer cells and infectious organisms, and complicate the clinical management of preexisting acute and chronic diseases (Jittangprasert *et al*.,2004; Pootrakul *et al*.,2004) .Aseries of immunological

abnormalities has been described in patients with beta-thalassemia. The measurement of serum levels of selected cytokines and soluble molecules (deriving from cell membrane antigens) involved in the immune response could be useful for a better definition of such alterations (Hyde.,2000).Serum levels of interleukin-2 (IL-2), IL-6, tumor necrosis factor (TNF), soluble (s) CD4, sCD8, sCD23 and sCD25 were measured using immunoenzymatic assays in 45 transfusion-dependent patients affected by beta-thalassemia major and correlated to conventional immunological indexes, such as peripheral lymphocyte subpopulations and circulating immunoglobulins (Hyde .,2000).Patients with beta-thalassemia major showed increased TNF,, sCD23 and sCD25 and lower sCD4 values compared to normal controls. IL-2 and IL-6 were found to be undetectable or within the normal range in all patients. Splenectomized patients presented lower levels of sCD8 and sCD23 than those observed in unsplenectomized ones. A series of correlations involving TNF, sCD8, sCD23, sCD25, serum immunoglobulins and some lymphocyte subpopulations were observed. In addition, serum markers of immune activation (TNF, sCD23, sCD25) correlated directly with the annual blood transfusion requirement (Ezer *et al*.,2002).Polytransfused beta-thalassemic patients are characterized by a partial functional immunodeficiency determined by increased activity of CD8+ suppressor/cytotoxic lymphocytes and possibly reduced activity of the CD4+ helper/inducer subset (Hyde .,2000; Ezer *et al*.,2002).B-lymphocytes also appear highly activated. The allo-antigenic

stimulation of transfusions seems to play a major role in the determination of these defects; however, this functional immunological imbalance does not seem to have any clinical relevance (Kiska *et al*.,2003). Recent studies on immune competence in beta-thalassemia have revealed numerous quantitative and functional defects, involving T and B lymphocytes, immunoglobulin production, neutrophils and macrophages, chemotaxis, and phagocytosis, as well as the complement system (Corry *et al*.,1981).Regarding pathogenesis, iron overload, a primary complication of both thalassemia itself and transfusion therapy, is thought to be the main precipitating mechanism, due to the important immunoregulatory properties of iron and its binding proteins; iron excess may derange the immune balance in favor of the growth of infectious organisms (Cappellini *et al*.,2000).Other factors include multiple transfusions, associated with transmission of immunosuppressive viruses; splenectomy, resulting in increased susceptibility to infections by encapsulated bacteria and to immune system modifications (Blumberg *et al*.,2000).Low levels of zinc, (another immune regulator);(Sunzel *et al*.1995) iron chelation therapy, which predisposes to serious infections by yersinia species; and the circulation of abnormal native thalassemic erythrocytes, forming another permanent immune stimulus (Peng *et al*.,2005).Thus surveillance for infections in patients with beta-thalassemia is crucial, while further studies are warranted on immune function abnormalities and the implicated mechanisms.

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2. Materials and Methods

2.1 Materials

2.1.1 Samples–Patients:

A total of 150 thalassemic patients (70 males and 80 females) whose ages ranged between (2-28) years were admitted to Thalassemia Center in Karbalaa hospital for children ,92 of them have signs and symptoms of bacterial infections(38 males and 54 females) and the others(58) apparently healthy without infections (32 males and 26 females) included in this study during the period from November/2008 to April/2009.Those patients have been diagnosed by specialist physicians depending on clinical suspicion and proved by laboratory investigations including blood film , Hb-electrophoresis, serum iron , ferritin and total iron binding capacity . The blood, urine, throat and ear swab specimens were generally collected from Thalassemic patients with fever , urinary tract infection tonsillitis, laryngitis and otitis media respectively for bacteriological isolation Serum samples were also collected from infected , not infected thalassemic patients and controls for immunological study .

2.1.2 Healthy controls:

A total of 50 apparently healthy controls (not thalassemic and not infected) (26 males and 24 females). Whose age ranged between (2-28) years ; which is similar to that of thalassemic patients age range were included in this study.

2.1.3 Laboratory Equipment's and Apparatuses used in study are illustrated in table (2-1)

Table (2-1) The laboratory Equipments and Apparatus

Equipment	Company (Origin)
Autoclave	Stermite- Japan.
Bunsen burner	Germany
Centrifuge	Hermle- Japan
Hot air oven	Memmert-Germany
Hot plate	Classico-India
Incubator	Memmert- Germany
Light microscope	Olympus-Japan
Micropipette	Oxford, USA
Millipore filter paper	Satorius Membrane Filters GmbH- W.Germany
Ocular lens	Olympus-Japan
pH meter	Hoeleze&Cheluis,KG-Germany
Refrigerator	Concord- Italy
Sensitive electricbalance	A & D-Japan
Sterile syringe 22G	Discardit-Spain
Water bath	Memmert- Germany
Water distillatory	GFL- Germany
ELISA Reader	Beckman-Germany
ELISA Mixer	Denley-U.K
ELISA Washer	Beckman-Germany
ELISA Shaker	Jean Robin-France

2.1.4 The Chemical and Biological Material used in the study were illustrated in Table (2.2)

Table (2.2)The Chemical and Biological Materials .

Items	Company (Origin)
A-Chemical Materials Na_2HPO_4, KH_2PO_4, NaCL, $MgSO_4$, $CaCL_2$, K_2HPO_4	Merk, Switzerland.
-α-naphthol, KOH, ferric ammonium citrate, HCl, isopropyl alcohol,methylred, tetramethyl-P-paraphenylene diamine dihydrochloride, Phosphate buffer saline (PBS-PH= 7.2).	B.D.H.- United kingdom
-99% ethanol, glucose, H2O2, *p*-dimethyl amino benzaldyde, 99% methanol, urea solution.	Flukachemika-Switzerland
B-Stains: -Gram's stain set -Indian ink (capsule stain)	Crescent-Saudi Arabia Crescent-Saudi Arabia

2.1.3 Ready made culture media:

These culture media were prepared according to the manufacture instructions and autoclaved at 121°C, 15 pound/ $inch^2$ for 15 minutes.

Culture media	Origin
Brain heart infusion broth	Mast diagnostic/U.K
Nutrient agar	Mast diagnostic/U.K
Blood agar base	Mast diagnostic/U.K
MacConky agar	Mast diagnosti/U.K
Müller Hinton agar	Mast diagostic/U.K
Manitol Salt agar	Mastdiagnostic/U.K
Kligler's Iron agar	Difco/U.K

2.1.3.1 Blood agar media (B.A) :

It was prepared by suspending 37 grams of blood agar base in one liter distilled water. then heated the mixture and was sterilized by autoclave at 121^{o}C, 15 pound/$inch^2$ for 15 minutes and supplemented with 10% of fresh human blood after cooling it to 45^{o}C. Finally pH was adjusted to 7.1 by adding 0.1Nof NaoH or HcL. It was used for cultivation of the fastidious bacteria and detection of hemolysine production (MacFaddin,2000) .

2.1.3.2 Brain heart infusion broth (BHIB):

This medium was prepared by adding 37 grams of brain heart infusion broth base in one liter distilled water.Then heated the mixture and was sterilized by autoclave at 121^{o}C, 15 pound/ inch^{2} 15 minutes. The BHI agar medium supplemented by adding 2% agar to the above mentioned medium.

2.1.5.3 Semi- Solid medium:

This medium was prepared by adding 0.5 gram agar A to 100ml nutrient broth, and was used for detecting the motility of bacteria (Collee *et al*.,1996).

2.1.5.4 Müller Hinton agar:

This medium was prepared by adding 38 grams of müller hinton agar base in one liter distilled water. Then heated the mixture and was sterilized by autoclave at 121^{o}C, 15 pound/ inch^{2} 15 minutes. This medium was used in antibiotic sensitivity test.

2.1.6 Immune reagents kits that are used through-out the study:

i-Interferon Gamma IFN- γ kit detected by ELISA (Biosource, Europe, S. A).

ii- Tumor Necrosis Factor alpha TNFα kit detected by ELISA (Biosource, Europe, S. A).

iii- Immunoglobulin's (IgG , IgM) and complement components (C3 , C4) endoplates of single radial immunodiffusion (SRID) test (The binding site, United kingdom).

2.1.7 Solutions

2.1.7.1 Normal saline

Normal saline was used in washing of SRBCs, and for making the bacterial suspensions. It was prepared by dissolving 8.5 grams of sodium chloride (NaCl) in one liter of distilled water(D.W.) and was sterilized by autoclaving at 121C˚ for 15 minute, and was kept at 4^{o}C (Cruickshank *et al*., 1975).

2.1.8 Biochemical reagents :

2.1.8.1 Methyl red(MR) reagent

Methyl red 0.1 gram was dissolved in 300 ml of 99% ethanol and then completed the volume was completed to 500 ml by distilled water. This reagent was used to detect ability of organisms to produce acid as an end product when fermenting dextrose (McFadden, 2000).

2.1.8.2 Voges –Proskauer (VP) reagents:

It is composed of two solutions. (Collee *et al.*,1996) .

Reagent A:

Five grams of alpha-naphthol were dissolved in 100 ml of 99% ethanol.

Reagent B :

Fourty grams of KOH were dissolved in 100 ml of distilled water (D.W.) and used to detect ability of organisms to produce acetylmethylcarbinol end products when fermenting dextrose.

2.1.8.3 Oxidase reagent

It was prepared by dissolving 0.1 gram of tetramethyl-paraphenylene diamine dihydrochloride in 10 ml of distilled water which was, then, and stored in a dark container. It was used for the detection of the ability of bacteria to produce oxidase enzyme (Brooks *et al*., 2007).

2.1.8.4 Catalase reagent

Three percent of H2O2 was used to detect the ability of bacteria to produce catalase enzyme (Brooks *et al.*,2007). It was stored in a dark container.

2.1.8.5 Kovac's reagent

This reagent was prepared by dissolving 5 grams of (*P*-dimethyl-aminobenzaldehyde) in 75 ml amyl alcohol, and then 25 ml of concentrated hydrochloric acid was added. This reagent was used for detection of indole production (MacFaddin, 2000).

2.1.9 Stains:

2.1.9.1 Gram's stain:

This stain was used to detect shape, morphology and to differentiate Gram-negative from Gram-positive bacteria according to (Collee *et al*.,1996).

2.1.9.2 Indian ink stain :

It is a negative stain used to stain capsule **(**Collee *et al*.,1996).

2.2 Methods

2.2.1 Samples Collection

2-2.1.1 Ear and Throat swabs:

Total of 22 swabs were collected (10 ear swab and 12 throat swab). Each swab was placed in a sterile tube containing 5ml brain-heart infusion broth and incubated aerobically for 24-48 hours at 37C,˚ and then swab or loop full was taken from this medium and inoculated on culture media which were Blood agar, MacConkey agar and Nutrient agar. All plates were incubated aerobically for 24-48 hours at 37C˚(Collee *et al*., 1996).

2.2.1.2 Blood Samples

Blood samples were collected from 150 patients and 50 controls (five milliliters of venous blood) were drawn by 22G

disposable syringe under aseptic technique. each blood sample was divided into two parts:

a- Three milliliters were put directly in a sterile bottle containing 30ml brain-heart infusion broth and incubated aerobically for 48 hours at 37˚C and then loop full was taken from this medium and inoculated on culture media (Blood agar, MacConkey agar and Nutrient agar) and then incubated aerobically for 24 hours at 37C˚. If no growth appeared the bottles were re incubated and checking for growth each 24 hours for 7 days (Collee *et al*., 1996). (33 blood sample from infected thalassemic patients were cultured).

b- Two milliliters were placed in a sterile plane tube and allowed to clot, then serum was separated by centrifugation at 2500 r.p.m for 15 minutes. The serum was stored at -10 C˚ . These 200 sera (150 thalassemic patients and 50 controls) were used for estimating:

a- The concentration of Immunoglobulin's (IgG, IgM).

b- The concentration of complement (C3, C4).

c- The concentration of tumor necrosis factor alpha TNFα.

d- The concentration of gamma interferon IFN γ.

2.2.1.4 Urine sample

Fourty five urine sample were collected 5 ml mid stream urine was taken in aseptic manner by using sterile tube and urine

bags were used to patients less than 5 years after sterilizing genital area by normal saline . then loop full was taken and inoculated on culture media (Blood agar, MacConkey agar and Nutrient agar) and incubated aerobically for 24 hours at 37C˚(Collee *et al*., 1996).

2.2.2 Laboratory diagnosis:

2.2 .2.1 Bacterial identification**:**

A single colony was taken from each primary positive culture on blood agar, and on MacConckey agar. The microorganism was identified on the basis of its morphology (colony shape, size, colour, borders, and texture) and then stained with Gram's stain and examined by the microscope. The biochemical tests were processed on each isolate to complete the final identification of species (Collee *et al.*, 1996; MacFaddin., 2000; Murray *et al.*, 2003, and Brooks *et al*.,2007)

2-2-2-1-1 Detection of capsule:

A negative stain was used and carried out as follows:(Collee *et al.*, 1996):-

a- A large loopful of undiluted Indian ink was placed on a clean glass slide.

b- A very small portion of bacterial colony was emulsified in the ink drop.

c- A clean grit-free cover- slip was placed on the ink drop.

d- The presence of capsule was examined with the oil immersion objective lens, and the highly refractive out-line of the bacterium was seen.

2.2.2.2 Biochemical Tests

2.2.2.2.1 Catalase Test

A colony of the organism was transferred by sterile wooden stick to the surface of a clean, dry glass slide; and one drop of 3% H_2O_2 was added to it. The formation of gas bubbles was considered indicate the positive result (Brooks *et al.,* 2007).

2.2. 2.2.2 Oxidase Test

A piece of filter paper was saturated with oxidase reagent; then required colony of organism was spread onto the filter paper. If the color turned from rose to purple, the oxidase test was considered positive (Brooks *et al.*,2007).

2.2. 2.2.3 Coagulase Test

This test is used to detect the ability of an organism to clot plasma by the action of the enzyme coagulase. Coagulase slide method is used to detect the bound coagulase that is found on the surface of cell wall as follows :

After emulsifying staphylococcal colony with a drop of sterilized normal saline on a clean slide, one drop of human plasma was added, then mixed gently. Coagulase positive

organisms became clumped after few seconds. To compare the result, control test was done by mixing saline and bacteria without plasma to ensure that the organisms did not clump spontaneously (Bennerman., 2003) .

2.2.2.2.4 Indole Test

This test is used for the determination of the organisim's ability to produce indole from deamination of tryptophan by tryptophanase. The formation of red color ring at top of broth indicates a positive reaction, while a yellow color ring indicates a negative reaction (MacFaddin., 2000).

2.2.2.2.5 Methyl Red Test

It is employed to detect the production of sufficient acid during the fermentation of glucose. The change of color to orange indicates a positive reaction (Murray *et al*., 2003).

2.2.2.2.6 Voges-Proskauer (acetoin production) Test

The VP test is used to detect acetoin (acetyl-methyl-carbinol), which is produced by certain bacteria during growth in peptone glucose broth (MR-VP broth). The positive result is indicated by changing the color of the medium to red (MacFaddin, 2000).

2.2.2.2.7 Simmon's Citrate Test

The citrate test is used to determine the ability of a bacterium to utilize citrate as it's the only source of carbon. The

positive result changes the color of media from green to blue (Forbes *et al.*,2007).

2.2.2.2.8 Triple Sugar Iron(TSI) Test

The aim of this test is to differentiate the enterobacteriaceae according to carbohydrate fermentation and hydrogen sulfide production (Murray *et al.*, 2003).

2.2.2.2.9 Urease Test

Urease is an enzyme that breaks the carbon-nitrogen bond of amides to form carbon dioxide, ammonia and water. The urea base agar was sterilized by autoclave, after that it was cooled to 50 Cº, and urea substrate was added to it after sterilizing by Millipore's filter. It was poured in sterile tubes; then it was inoculated by bacterial cultures, which were incubated for (24 - 48) hours at 37Cº. When urea broke down, ammonia was released and the pH of the medium increased. This pH change was detected by a pH indicator that turned pink in a basic environment. The pink medium indicated a positive test for urease. Failure of deep pink color to develop was taken to mark a negative reaction (Collee *et al.,* 1996).

2.2.2.2.10 Motility Test (Semisolid Media)

Non motile bacteria give growth that is confined to the stab-line and have sharply defined margins, leaving the surrounding medium clearly transparent. Motile bacteria typically give diffuse

hazy growth that spread throughout the medium, rendering it slightly opaque (Murray *et al*., 2003).

2.2.2.2.11 CAMP Test

CAMP reaction is named for its original descriptors: Christie, Atkins and Munch-Peterson (Christie et al .,1994). Certain organisms (including group B streptococci) produce a diffusible extra-cellular protein (CAMP factor) that acts synergistically with the beta-lysin of *staphylococcus aureas* to cause enhanced lysis of red blood cells (Brooks *et al* .,2007).

2.2.2.2.12 Haemolysin production

Haemolysin production was carried out by inoculating a blood agar medium with bacterial isolate at 37ºC for 24-48 hrs. An appearance of clear zone around the colonies refered to complete haemolysis (β-haemolysis). Greenish zone around the colonies refered to partial haemolysis (α-haemolysis); while no change refered to non-haemolysis (γ- haemol ysis) (Brooks *et al*.,2007).

2.2.2.2.13 Mannitol Salt Agar

The medium was inoculated with bacterial colonies, then incubated at 37C° for 24 hours. The color changed from pink to bright yellow when the bacteria was a mannitol fermenter and this meant a positive result, while unchanging color of the medium indicated a negative result (Collee *et al*., 1996).

2.2.2.2.14 Eosin Methylene Blue (EMB) Agar

Lactose fermenting colonies are either dark or have dark centres with transparent colorless peripheries, while organisms that do not ferment lactose remain uncoloured (Murray *et al*., 2003).

2.2.3 Detection of virulence factors:

2.2.3.1 Coagulase factor test

This test was carried out as described in (2.2.2.2.3) .

2.2.3.2 Haemolysin production :

Haemolysin production was carried out by inoculating of blood agar medium with bacterial isolate at 37ºC for 24-48 hrs. An appearance of clear zone around the colonies referred to complete haemolysis (β-haemolysis) or greenish zone around the colonies referred to partial haemolysis (α-haemolysis), while no change referred to non-haemolysis (γ- haemolysis) (Brooks *et al*.,2007).

2.2.3.3 Capsule production:

This test was carried out as described in (2.3.2). it is considered the main virulence factor that enable bacteria to avoid phagocytosis (Brooks *et al*., 2004).

2.2.4 Antibiotics Sensitivity Test

Antibiotic diffusion test (Kirby-Bauer susceptibility test) was carried out according to(MacFaddin, 2000). Antibiotic disc potency was supplied from Bioanalyse (Turkey).

Table(2-3) : Antibiotic Disc Potency

Type	Abbreviation	Disc potency(µg)
Norfloxacin	NOR	10
Amikacin	AK	30
Azithromycin	AZM	15
Doxycycline	DO	30
Clarithromycin	CLR	15
Ceftazidime	CAZ	30
Ampicillin + Cloxacillin	APX	25 + 5
Cefotaxim	CTX	30
Gentamycin	GN	10
Erythromycin	E	15
Cephalexin	CL	30
Amoxicillin	AX	25
Amoxicillin + Clavulanic aci	AMC	20 + 10

2.2.4.1 Procedure of the Antibiotics Sensitivity Test (MacFaddin, 2000).

a-It is performed by using a pure culture of previously identified bacterial organism. The inoculums to be used in this test are prepared by adding growth from 5 isolated colonies grown on blood agar plate to 5ml of broth. This culture is then incubated for 2hr. to produce bacterial suspension of moderate turbidity. A sterile swab is used to obtain inoculums from the standardized broth culture. These inoculums are then streaked on a Mueller-Hinton plate.

b-The antibiotic discs are placed on the surface of the medium at evenly spaced intervals with flamed forceps or a disc applicator.

C-Incubation is usually overnight with an optimal time being 14hr. at 37°C. Antibiotic inhibition zones are measured using a caliper.

D-Zone size is compared to standard zones of NCCLS to determine the susceptibility of the organism to each antibiotic (NCCLS, 2003a).

2.2.5. Immunological tests:-

2.2.5.1 Estimation of IgG , IgM,C3 and C4 concentrations :

A- Principle:

The Single Radial Immunodiffusion (SRID) test is immune precipitation in agarose between an antigen and its homologous antibody. It is performed by incorporating one of the two immune reactants (usually antibody) uniformly throughout a layer of agarose gel, and then introducing the other reactants (usually antigen) into wells duly punched in the gel. Antigen diffuses radially out of the well into the surrounding gel-antibody mixture, and a visible ring of precipitation forms. Ring diameters are measured by viewing device (ocular). Unknown concentration is determined from the tables supplemented with each type of plate (Dacie *et al*., 2006).

B- Procedure:

a- Endoplates and the serum of (both patients and controls)were removed from refrigerator. Reagent was equilibrated to room temperature.

b- Plate was removed from ziplock bag. After lid was removed, the wells were inspected for moisture .If moisture was present, uncovered plates were allowed to remain at room temperature until moisture evaporated. Sera of patients were thoroughly shacked by inversion pipette 5 µL of the serum into the appropriate well, and putting wet cotton in the plate center.

c- Plates were incubated at room temperature for 48 hrs. to IgG and 72 hrs. for IgM test.

d- End point of diffusion was indicated by a sharp precipitation ring.

e- Diameter of ring was measured with 0.1mm precision by ocular lense and then compared to the standard diameter to conclude the concentrations of serum immunoglobulin IgG,IgM and complement components C3,C4 (Dacie *et al*., 2006).

2.2.5.2 Estimation of Tumor Necrosis Factor Alpha TNF-α EASIA test (BIOSOURCE)

A-Principle:

TNF-α EASIA is a solid phase Enzyme Amplified Sensitivity Immunoassay (EASIA) performed on microtiter plate. The assay was based on an oligoclonal system in which a blend of monoclonal antibodies (MAbs) directed against distinct epitopes of TNF-α are used. Antibody-producing cells were immortilized using the myeloma cell fusion method of Kohler and MilsteinR. A hybridoma cell was produced which secretes specific homogeneous antibodies. The use of a number of distinct MAbs was to avoid hyper specificity and allow high sensitive assay with extended standard range and short incubation time. Standards or samples containing TNF-α react with capture monoclonal antibodies (MAbs 1) coated on the microtiter well. After incubation, the occasional excess of antigen was removed

by washing. MAbs 2, the horseradish peroxidase (HRP)-labelled-antibody, was then added. After an incubation period allowing the formation of a sandwich: coated MAbs 1- TNF-α – Mabs 2 –HRP , the microtiter plate was washed to remove unbound enzyme labelled antibodies. Bound enzyme-labelled antibodies were measured through a chromogenic reaction. Chromogenic solution (TMB+H_2O_2) was added and incubated. The reaction is stopped with the addition of stop solution (H_2SO_4) and the microtiter plate was then read at the appropriate wavelength. The amount of substrate turnover number was determined colourimetrically by measuring the absorbance which was proportional to the TNF-α concentration. A standard curve was plotted and TNF-α concentrations in a sample were determined by interpolation from the standard curve. The use of the EASIA Reader (linearity up to 3 OD units) and a sophisticated data reduction method (polychromatic data reduction) resulted in high sensitivity in the low rang and in an extended standard range.

B-Assay Procedure**:**

A- The required number of strips were Selected for the run. The unused strips were resealed in the bag with desiccant and stored at 2-8° C.

B- The strips were secured into the holding frame.

C- Fifty µl of incubation buffer was Pipetted into all wells.

D- Two hundred µl of each standard, control, or sample were Pipetted into the appropriate wells.

E-Incubated for 2 hours at room temperature on a horizontal shaker was set at 700 rpm.

F- The liquid was aspirated from each well.

G- The plate was washed three times by :

1-dispensing of 0.4 ml of BioSource wash solution into each well.

2 The content of each well was aspirated.

H- One hundred µl of standard 0 was Pipetted into all well.

I- Fifty µl of anti- TNF-α conjugate was Pipetted into all the wells.

J-Incubated for 2 hours at room temperature on horizontal shaker set at 700 rpm.

K- The liquid was aspirated from each well.

The plate was washed three times by :

1-Dispensing of 0.4 ml of BioSource wash solution into each well.

2- The content of each well was aspirated.

L- Two hundred µl of freshly prepared chromogenic solution was Pipetted into each well within 15 min. following the washing step.

M- The plate was incubated for 30 min. at room temperature on horizontal shaker set at 700 rpm, sunlight was avoided directly.

N- Fifty µl of stop solution was Pipetted into each well.

O- Absorbance was read at 450 nm and 490 nm (reference filter:630 or 650 nm) within 3 hours and the results were calculated.

2.2.5.3 Estimation of serum Gamma Interferon IFN-γ (BIOSOURCE)

A-Principle:

IFN-γ was tested by solid phase enzyme amplified sensitivity Immune assay (EASIA) performed on micro titer plate. Samples or standards containing IFN-γ react with capture monoclonal antibodies (MAbs-1) coated on the micro titer well and with a monoclonal antibody (MAb-2) labeled with horseradish peroxidase (HRP). After an incubation period the formation of a sandwich was allowed: (coated MAbs-1-IFN-γ - MAb2- HRP). The microtiter plate was washed to remove unbound enzyme labeled antibodies. Bound enzyme labeled antibodies were measured through a chromogenic reaction. Chromogenic solution (tetra methyl benzidin, TMB) was added and incubated. The reaction was stopped with the addition of

stop solution (H_2So_4, 1.8N) and the micro titer plate was then read at the appropriate wavelength. A standard curve was plotted and IFN-γ concentration in a sample is determined by interpolation from the standard curve. The procedure of IFN-γ estimation: (according to the information supplied by Biosource Co.).

B-Reagent preparation:

Standard	Concentration I.U/ml
0	0
1	0.6
2	1.3
3	3.9
4	7.9
5	22.5

a-Standards provided that were prepare by addition 0.5 ml D.W to each one were to be used to draw standard curve.

b-Controls were prepared as in standards and there were two controls (control 1: 2.2 ± 0.6 I.U/ml ; control 2: 5.1 ± 1.3 (I.U/ml), used as internal laboratory controls.

c-Wash solution was prepared by adding 2 ml of the provided solution in 400 ml of D. W.

d-Chromomenic solution was prepared by pipetting 0.2 ml of the concentrated chromogen into one vial of substrate buffer (21 ml) that is provided with the kit.

C- Assay procedure of IFN-γ estimation :

a-Required numbers of the strips were selected for the run.

b-The strips were secured into the holding frame.

c-Fifty μL of each standard or sample was pippetted into the appropriate wells.

d- Fifty μL of anti- IFN- γ conjugate was pippetted into all the wells.

e-The plate was incubated for two hours at room temperature on a horizontal shaker set at 700 r.p.m .

f-The liquid from each well was aspirated.

g-The plate was washed three times by washing solution .

h-Two hundred μL of freshly prepared chromogenic solution was pippetted into each well, within 15 min. following the washing step .

i-The plate was incubated for 15 minutes at room temperature on horizontal shaker set at 700 r.p.m .

j- Fifty μL of stop solution was pippetted into each well.

k-Absorbance was readed at 450 nm (reference filter 620 nm) within three hours.

l-The curve was drawn on linear graph paper, plotting the concentration of IFN-γ standards on horizontal axis and the absorbance on the vertical axis.

m-The IFN-γ concentration for unknown sample was read and content from the standard curve plotted in step (**a**).

2.2.6 Determination of Serum ferritin concentration

A-Principle:

The ferritin quantitative test is based on a solid phase enzyme-linked immunosorbant assay(ELISA) .the assay system utilizes on rabbit anti-ferritin antibody for solid phase (microtiter wells) immobilization and amouse monoclonal anti-ferritin antibody in the antibody-enzyme (horseradish peroxidase) congucate solution . the test sample is allowed to react simultaneously with antibodies , resulting in the ferritin molecules being sandwiched between solid phase and enzyme-linked antibodies .after a 45 minute incubation at room temperature the wells were washed with water to remove unbound –labeled antibodies .solution of TMB is added and incubated at room temperature for 20 min resulting the development of blue color the color development was stopped with the addition of stop solution and the color was changed to yellow and measured spectrophotometrically at 450 nm .ferritin conc. Was directly proportional to the color intensity of tested sample. : (according to the information supplied by BioCHECK, INC.).

B-Material and components:

a-Standards provided that ready for use each one were to be used to draw standard curve.

Ferritin Conc. ng/ml	Absorbance (450nm)
0	0.074
15	0.150
80	0.362
250	1.017
500	1.699
1000	2.728

b- Antibody coated microtiter plate with 96wells.

c- Enzyme congucate reagent.

d-TMP (Tetra Methyl Benzidin) reagent 11ml.

e- Stop solution (1N HCL) 11ml.

C- Assay procedure of Serum ferritin estimation : (according to the information supplied by BioCHECK, INC.).

a- Required numbers of coated wells in the holder were secured..

b- Twenty µl of each standard or sample was pippetted into the appropriate wells.

d- One hundred µl of enzyme conjugate was dispensed into all the wells.

e- Gently mixing for 30 min.

f- Incubate at room temperature for 45 minute.

g-The plate was washed with distilled water.

h- One hundred µl of TMB was dispensed in to each well gently mixing for 10 sec.

I-Incubate at room temperature for 20 minute in the dark.

j- One hundered µL of stop solution was pippetted into each well.

k-Gently mixing for 30 sec.

l- Absorbance was readed at 450 nm (reference filter 620 nm) within 15 min.

m-The curve was drawned on linear graph paper, plotting the concentration of ferritin (ng/ml) standards on horizontal axis and the absorbance (450nm) on the vertical axis.

n-The ferritin concentration for unknown sample was read and content from the standard curve plotted in step (**a**).

2.2.7 Statistical analysis:

Mean, standard deviation, and T-test ($p < 0.05$) were carried out according to Bowers (1997).