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Evaluation the role of IL-2, IL-10 and IFN-γ in some syphilis patients in Baghdad governorate

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KEYWORDS

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A B S T R A C T

Syphilis is a sexually transmitted disease (STD) caused by the *Treponema pallidum* and classified as acquired or congenital. This study was conducted in National Blood Transfusion Center in Baghdad as across section study including patients attending in the period from 1 August to 31 December 2014. The study included 70 patients and 10 apparently healthy individuals from both sex with age range from 24 to 57 years old as control group. The samples of patients were collected in virology unit by virologists who diagnosis the cases. The demographic information includes age, sex and address. The results of present study showed that mean of age ± SD of patients was 41 ± 7.14 years (from 24-57 years) , also the results showed (2.9%) of patients were from (21-30 years), (40%) of patients were from (31-40 years), (44.3%) of patients were from (41-50 years), (12.8%) of patients were from (51-60 years), also the results showed that the level of IL-2 higher in patients group than control group, also the mean ± SD was 64.99 ± 41.92 pg/ml and 52.94 ± 39.69 pg/ml respectively without noticing any significant statistical difference was between both groups . Additionally the level of IL10 was higher in patients group than control group, the mean ± SD were 559.10 ± 1804.20 pg/ml while in control were 377.47 ± 252.36 pg/ml. Whereas, the level of IFN-γ was lower in patients, the mean ± SD were 201.38 ± 239.35 pg/ml than in control Which were 291.49 ± 248.51 pg/ml. Finally, some change levels of interlukins (IL-2, IL10 and IFN-γ) was noticed among syphilis patients (i.e. impaired cytokine response in syphilis patients).

Introduction

Syphilis is a chronic, multistage disease caused by infection with *Treponema pallidum* subsp. *pallidum* (Tp), Syphilis is usually transmitted through contact with active lesions of a sexual partner or from an infected pregnant woman to her fetus. Although syphilis has remained endemic in sub-Saharan Africa and South-East Asia, it

has recently re-emerged in several developed countries in the form of small, sporadic outbreaks and large, widespread epidemics (Stamm and Mudrak, 2013).

The infection is transmitted from person to person through contact with a syphilis ulcer (during vaginal, anal, or oral sex). An infected mother can infect her fetus via the placenta.

Furthermore, intravenous drug addicts or other infected person can transmit syphilis through infected blood products i.e. through blood transfusion or use of infected needles for example (Workowski and Berman, 2006).

The clinical course of syphilis is divided into 4 stages primary, secondary, and tertiary stages in which characteristic manifestations occur and a latent stage in which the patient is asymptomatic but seropositive (So *et al.*, 2006). The *Treponema pallidum* particle agglutination (TPPA) and haem-agglutination (TPHA) assays are used to detect Treponema-specific antibodies. Once positive, these tests usually remain positive for life. The venereal disease research laboratory (VDRL) and rapid plasma reagin (RPR) tests are non-specific tests detecting antibodies to cardiolipin (Perine *et al.*, 1984).

IL-2, discovered more than 30 years ago in supernatants of activated T cells, is mainly produced by CD4 and CD8 T cells, and to a lesser extent by activated DCs and NK and NK Tcells (NKT) (Morgan *et al.*, 1976).

IL-10 was first described in 1989 as cytokine synthesis inhibitory factor, a TH2-derived factor inhibiting the production of IFN-γ and other cytokines in murine TH1 cells However, in the human system, IL-10 production is not a typical feature of TH2 cells, because both TH1 and TH2 cells are capable of producing IL-10, whereas the main source of T-cell–derived IL-10 is Target cells (Fiorentino *et al.*, 1989). The present study was aimed to evaluate some cytokines (IL-2, IL10 and IFN-γ) among syphilis patients by ELISA.

Materials and Methods

Collection of samples

This study was conducted in National Blood Transfusion Center in Baghdad as across section study including all patients attending in the period from 1 August to 31 December 2014.The study included 70 patients and 10 apparently healthy individuals from both sex with age range from 24 to 57 years old.

The collection of patients sample was done in virology unit by virologists who diagnosis the cases. The demographic information includes age, sex and address.

Five milliliters of venous blood were withdrawn from each patient by vein-puncture under aseptic technique by disposable syringes, likewise control individuals. The blood was collected separately in plane tube with no anticoagulant, left to clot at room temperature then centrifuged and serum was collected in two separated tubes and stored at (-20 C) until used for investigation.

TPHA Microplate hemagglutination

Principle of the assay

The TPHA (*Treponema pallidum* Hemagglutination) is an indirect hemagglutination test for the qualitative and semi-qualitative detection of specific anti-*T. pallidum* antibodies in human serum.

Stabilized avian erythrocytes sensitized with an antigenic *T. pallidum* solution, agglutinates in the presence of anti-*T. pallidum* antibodies to give a characteristic patterns (VirojWiwanitkit, 2009).

Reagents

1. **R1:Test Cells (TC)** Stabilized avian erythrocytes sensitised with *T. pallidum* (Nichols) antigens, Preservative, pH 7.2.

2. **R2:Control Cells (CC)** Stabilized suspension of avian erythrocytes, Preservative, pH7.2.

3. **R3:Diluent (DIL)** Phosphate buffered saline, pH 7.2, ***T. pallidum*** (Reiter) extract, Preservative.

4. **Control +** Immune human serum prediluted 1:20 Preservative.

5. Control - Animal serum, Preservative.

Procedure

Qualitative method

1. Allow the reagents and sample to reach room temperature.
2. Dilute serum 1:20 with Diluent (10 µl serum+190 µl Diluent)
3. Pipette into adjacent wells of a microtitration plate

Sample 1:20 or Controls(µl)	25	25
Control Cells(µl)	75	..
Test Cells(µl)	..	75

4. Mix thoroughly the microplate till the complete homogenization of the mixing reaction.
5. Cover the microplate and incubate at room temperature for 45-60 min.
6. Examine macroscopically the agglutination patterns of the cells.

Human Interferon γ (IFN-γ) ELISA

Principle of the assay

The microtiter plate provided in this kit has been pre-coated with an antibody specific to IFN-γ. Standards or samples are then added to the appropriate microtiter plate wells with a biotin-conjugated antibody preparation specific for IFN-γ and Avidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated. Then a TMB (3,3'5, 5' tetramethyl-benzidine)substrate solution is added to each well.

Only those wells that contain IFN-γ, biotin-conjugated antibody and enzyme-conjugated Avidin will exhibit a change in color. The enzyme-substrate reaction is terminated by the addition of a sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450 nm ± 2 nm. The concentration of IFN-γ in the samples is then determined by comparing the O.D. of the samples to the standard curve (Mori, 2004).

Assay procedure

Bring all reagents and samples to room temperature before use. It is recommended that all samples, standards, and controls be assayed in duplicate. All the reagents should be added directly to the liquid level in the well. The pipette should avoid contacting the inner wall of the well.

1. One hundred microliter of Standard, Blank or Sample was added per well. Cover with the adhesive strip. Incubate for 2 hours at 37°C.
2. The liquid of each well was removed, don't wash.
3. One hundred microliter of Biotin-antibody working solution was added to

each well. Incubate for 1 hour at 37°C. Biotin-antibody working solution may appear cloudy. Warm up to room temperature and mix gently until solution appears uniform.

4. The liquid from each well was aspirated each well and wash, repeating the process three times for a total of three washes. Wash: Fill each well with Wash Buffer (200µl) and let it stand for 2 minutes, then remove the liquid by flicking the plate over a sink. The remaining drops are removed by patting the plate on a paper towel. Complete removal of liquid at each step is essential to good performance.
5. One hundred microliter of HRP-avidin working solution was added to each well. Cover the microtiter plate with a new adhesive strip. Incubate for 1 hour at 37°C.
6. The aspiration and wash repeated five times as step 4.
7. Ninety microliter of TMB Substrate was added to each well. Incubated for 15–30 minutes at37°C and kept the plate away from drafts and other temperature fluctuations in the dark.
8. Fifty microliter of Stop Solution was added to each well when the first four wells containing the highest concentration of standards develop obvious blue color. If color change does not appear uniform, gently tap the plate to ensure thorough mixing.
9. The optical density of each well determined within 30 minutes, using a microplate reader set to 450 nm.

Human Interleukin 2 (IL-2) ELISA

Principle of the assay

This assay employs the quantitative sandwich enzyme immunoassay technique. Antibody specific for IL-2 has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any IL-2 present is bound by the immobilized antibody. After removing any unbound substances, a biotin-conjugated antibody specific for IL-2 is added to the wells. After washing, avidin conjugated Horseradish Peroxidase (HRP) is added to the wells. Following a wash to remove any unbound avidin-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of IL-2 bound in the initial step. The color development is stopped and the intensity of the color is measured (Bodas *et al.,* 2006).

Assay procedure

Bring all reagents and samples to room temperature before use. Centrifuge the sample again after thawing before the assay. It is recommended that all samples and standards be assayed in duplicate.

1. Prepare all reagents, working standards, and samples was prepared as directed in the previous sections.
2. Refer to the Assay Layout Sheet to determine the number of wells to be used and put any remaining wells and the desiccant back into the pouch and seal the ziploc, store unused wells at 4°C.
3. One hundred microliter of standard and sample was added per well. Cover with the adhesive strip provided. Incubate for 2 hours at 37°C. A plate layout is provided to record standards and samples assayed.
4. The liquid of each well was removed, don't wash.
5. One hundred microliter of Biotin-antibody (1x) was added to each well. Cover with a new adhesive strip. Incubated for 1 hour at 37°C (Biotin-antibody (1x) may appear cloudy. Warm

up to room temperature and mix gently until solution appears uniform.)

6. The liquid from each well was aspirated and washing, repeating the process two times for a total of three washes. Wash by filling each well with Wash Buffer (200µl) using a squirt bottle, multi-channel pipette, manifold dispenser, or autowasher, and let it stand for 2 minutes, complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
7. One hundred microliter of HRP-avidin (1x) was added to each well. Cover the microtiter plate with a new adhesive strip. Incubate for 1 hour at 37°C.
8. The aspiration / wash process repeated for five times as in step 6.
9. Ninty microliter of TMB Substrate was added to each well. Incubate for 15–30 minutes at37°C. Protect from light.
10. Fifty microliter of Stop Solution was added to each well, gently tap the plate to ensure thorough mixing.
11. The optical density of each well determined within 5 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. Subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

Human Interleukin 10 (IL-10) ELISA

Principle of the assay

This assay employs the quantitative sandwich enzyme immunoassay technique. Antibody specific for IL-10 has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any IL-10 present is bound by the immobilized antibody. After removing any unbound substances, a biotin-conjugated antibody specific for IL-10 is added to the wells. After washing, avidin conjugated Horseradish Peroxidase (HRP) is added to the wells. Following a wash to remove any unbound avidin-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of IL-10 bound in the initial step. The color development is stopped and the intensity of the color is measured (Bodas *et al.*, 2006).

Assay procedure

Bring all reagents and samples to room temperature before use. Centrifuge the sample again after thawing before the assay. It is recommended that all samples and standards be assayed in duplicate.

1. Prepare all reagents, working standards, and samples as directed in the previous sections.
2. Refer to the Assay Layout Sheet to determine the number of wells to be used and put any remaining wells and the desiccant back into the pouch and seal the ziploc, store unused wells at 4°C.
3. One hundred microliter of standard and sample was added per well. Cover with the adhesive strip provided. Incubate for 2 hours at 37°C. A plate layout is provided to record standards and samples assayed.
4. The liquid of each well was removed, don't wash.
5. One hundred microliter of Biotin-antibody (1x) was added to each well. Cover with a new adhesive strip. Incubate for 1 hour at 37°C. (Biotin-antibody (1x) may appear cloudy. Warm

up to room temperature and mix gently until solution appears uniform.)

6. The liquid from each well was aspirated and washing, repeating the process two times for a total of three washes. Wash by filling each well with Wash Buffer (200µl) using a squirt bottle, multi-channel pipette, manifold dispenser, or autowasher, and let it stand for 2 minutes, complete removal of liquid at each step is essential to good performance.

   After the last wash, remove any remaining wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
7. One hundred microliter of HRP-avidin (1x) was added to each well. The microtiter plate was covered with a new adhesive strip and incubated for 1 hour at 37°C.
8. The aspiration/wash process repeated for five times as in step 6.
9. Ninty microliter of TMB Substrate was added to each well. Incubate for 15-30 minutes at37°C. Protect from light.
10. Fifty microliter of Stop Solution was added to each well, gently tap the plate to ensure thorough mixing.
11. The optical density of each well determined within 5 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. Subtract readings at 540 nm or 570 nm from the readings at 450 nm.

This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

Statistical analysis

Data Analysis was computer aided. Statistical analysis was done using SPSS (Statistical Package of Social Science) version 20 computer software. Frequency distribution and percentage for selected variable were done. The independent t-test was used and p-Value (less than 0.05) was considered as the level of significance (Nisi, 2004).

Results and Discussion

Syphilis is a sexually transmitted infection caused by the spirochete bacterium *Treponema pallidum* subspecies *pallidum*. The primary route of transmission is through sexual contact; it may also be transmitted from mother to fetus during pregnancy or at birth, resulting in congenital syphilis. Other human diseases caused by related *Treponema pallidum* including yaws (subspecies pertenue), pinta (subspecies cariteum) and bejel (subspecendemicum) (Gao *et al.,* 2009).

Table 1 demonstrates the distribution of both groups according to the age that showed no significant relationship at $p>0.05$. The mean of age ± SD of patients was 41 ± 7.14 years (range from 24-57 years). The mean of age ± SD of control was 38 ±8.89 years (range from 25-52 years).

Distribution of age in both groups

In the table (2) showed (2.9%) of patients were from (21-30 years), (40%) of patients were from (31-40 years), (44.3%) of patients were from (41-50 years), (12.8%) of patients were from (51-60 years). (20%) of controls were from (21-30 years), (50%) of controls were from (31-40 years), (20%) of controls were from (41-50 years), (10%) of controls were from (51-60 years).

Age was classified into four categories (ten years for each category), (21-30) years, (31-40) years, (41-50) years, and (51-60) years.

This was due to the age capacity used in the study and the different interferences. This agrees favorably with the findings in the study by Karki *et al.* (2008).

Immunologic study

The results in table 3 and 4 shows increase the level of IL-2 in patients group than control group, there were mean ± SD was 64.99 ± 41.92 pg/ml and 52.94 ± 39.69 pg/ml respectively with no significant statistical difference was noticed between both groups .In addition to the level of IL-10 was more in patients group than control group, the mean ± SD were 559.10 ± 1804.20 pg/ml while in control were 377.47 ± 252.36 pg/ml. On other hand the level of IFN-γ was decreased in patients, the mean ± SD were 201.38 ± 239.35 pg/ml than in controls were 291.49 ± 248.51 pg/ml.

It was found that cells of syphilitic patients were able to produce IL-2, IFN-γ and IL-10 already in primary sero-negative syphilis. The growing ability to produce IL-10 was accompanied with a diminished production of IL-2, IFN-γ nearly in all stages of syphilis. The healing of syphilitic lesions was accompanied by increased ability of lymphocytes to IL-2, IFN cytokines may stimulate the cell-mediated immune response and in this way contribute to protection against *T. pallidum* infection (Podwinska *et al.,* 2000). During acute infection, high levels of interleukin (IL)–2 and interferon (IFN)–γ and low levels of IL-10 transcripts have been obtained from rabbit splenocytes incubated with treponemalsonicates or recombinant proteins (Arroll *et al.,* 1999).

From the results on table below (table 5) shows that the immunological markers was increase or decrease on different patients age groups, these due to different stages of disease in syphilis patients

The results in this study clarified that positive correlation between IFN- γ and IL-10 , this due to activation of T-helper 1 in syphilis patients.

Correlation between values of IL2, IL10 and IFN-γ in different levels

Figure 1 illustrates the values of Cytokines (IL2, IL10 and IFN-γ) for its three levels: low, normal and high. IL2 showed the highest rate, nearly 98.6% of the normal values and the low level was1.4%. IL10 presented a high percentage of the normal values. and a low portion at the low level, whereas, IFN-γ showed a high rate at the normal level and a low one at the high level. It is noticeable that the normal rate has increased and the high and low rates have converged in the three indicators.

The IL-2 and IFN-γ, cytokines of Th1 lymphocytes, are very important for the cell-mediated immune response; we have taken into account the ability of syphilitic patients' cells to produce them. Recent data have indicated that the ability of lymphocytes to produce IL-2 and IFN-γ is also distinctly suppressed at some stages in syphilis (Podwinska *et al.,* 2000). The IL-10 inhibits the ability of Th1 cells to secrete cytokines in syphilis. A strong correlation between high IL-10 levels and low IFN-γ, IL-2 levels was also observed (Mosmann and Sad, 1996). Finally, some change levels of interlukins (IL-2, IL10 and IFN-γ) was noticed among syphilis (i.e. impaired cytokine response in syphilis patients).

Table.1 Descriptive statistics of age in both groups

	No.	Minimum	Maximum	Mean	S.D.	t-test	P value
Patients	70	24	57	41	7.14	1.503	0.137
Controls	10	25	52	38	8.89		

Table.2 Distribution of age in both groups

Age group (year)	patients		controls	
	No.	%	No.	%
21-30	2	2.9%	2	20%
31-40	28	40%	5	50%
41-50	31	44.3%	2	20%
51-60	9	12.8%	1	10%
Total No.	70	100%	10	100%

Table.3 Levels of IL2, IL10 and IFN-γ in both groups

		Min.	Max.	Mean	S.D.	T test	P value	Total No.
IL2	patients	2.4	191.04	64.99	41.92	0.85	0.395	70
	controls	18.4	149.81	52.94	39.69			10
IL10	patients	7.73	15213	559.10	1804.20	0.31	0.753	70
	controls	83.2	855.21	377.47	252.36			10
IFN-γ	patients	0.49	893.73	201.38	239.35	1.10	0.271	70
	controls	2.13	627.42	291.49	248.51			10

Table.4 Values of IL2, IL10 and IFN-γ in both groups

		patients		controls		P-value	P-value
		No.	%	No.	%		
IL2	Normal	69	98.6%	10	100%	0.224	0.000
	High	_	_	_	_		
	Low	1	1.4%	_	_		
IL10	Normal	59	84.3%	9	90%	0.066	0.001
	High	6	8.6%	1	10%	0.088	
	Low	5	7.1%	_	_		
IFN-γ	Normal	33	47.1%	5	50%	0.053	0.066
	High	16	22.9%	4	40%	0.001	
	Low	21	30%	1	10%	0.02	

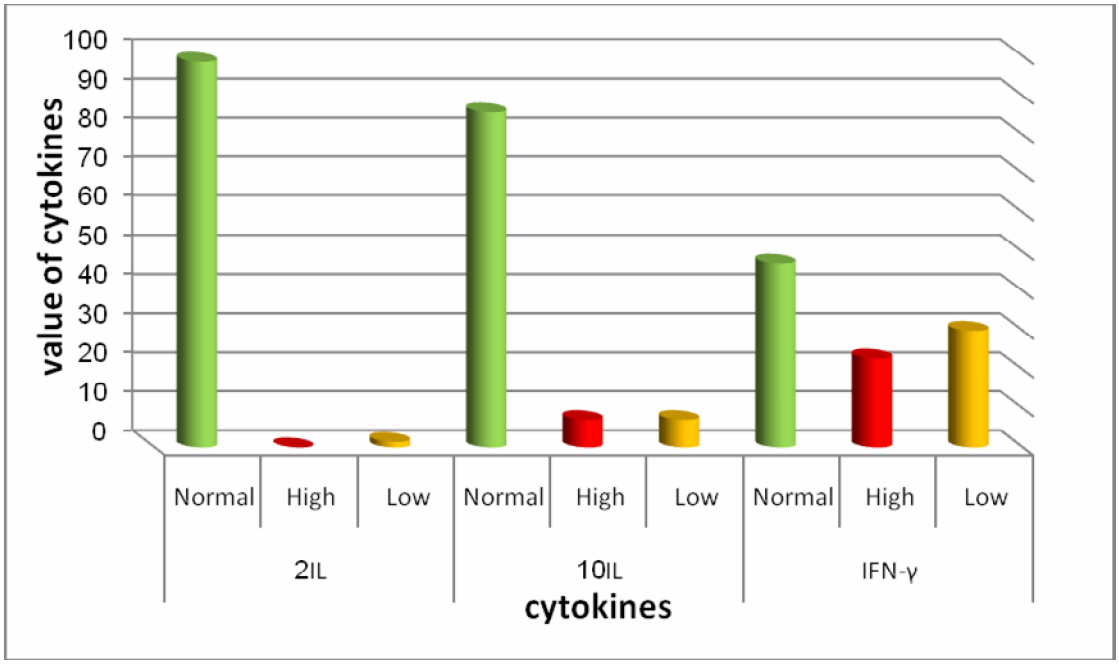
Table.5 Values of IL2, IL10 and IFN-γ regarding age of presentation in patients

		(21-30) years		(31-40) years		(41-50) years		(51-60) years		Total No.		P value	P value
		No.	%	No.	%	No.	%	No.	%	No.	%		
IL2	Normal	2	2.9%	27	39.2%	31	44.9%	9	13%	69	100%	0.044	0.0002
	Low	-		1	100%	-		-		1	100%	----	
IL10	Normal	2	3.4%	23	38.9%	30	50.9%	4	6.8%	59	100%	0.032	0.001
	High	-		3	60%	-		2	40%	5	100%	0.042	
	Low	-		2	33.3%	1	16.7%	3	50%	6	100%	0.001	
IFN-γ	Normal	2	4.5%	14	31.8%	27	61.4%	1	2.3%	44	100%	0.031	0.033
	High	-		11	55%	4	20%	5	25%	20	100%	0.044	
	Low	-		3	50%	-		3	50%	6	100%	0.774	

Table.6 Correlation between IL2, IL10 and IFN-γ in patients

	IL2	IL10	IFN-γ
IL2	1		
IL10	.146	1	
IFN-γ	.215(*)	.052	1

Figure.1 Values of IL2, IL10 and IFN-γ in different levels



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