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A study of Serum Malondialdehyde and Free Iron levels in Pregnant Women

Najat Zaid Mohammad [1], Jian Lateif Hussein[2], and Ziyad Ahmed Shareef[3].

1- Department of Chemistry, College of Science, Salahadeen University, Erbil, Kurdistan Region, Iraq.
2- Department of Chemistry, College of Science, Salahadeen University, Erbil, Kurdistan Region, Iraq.
3- Azadi Teaching Hospital – Duhok, Kurdistan Region, Iraq.

A R T I C L E I N F O

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***Corresponding Author:**
Najat Zaid Mohammad
najat.mohammad@su.edu.krd

A B S T R A C T

The aim of the current study to find relationship between oxidative stresses that illustrated by serum iron and malondialdehyde for women during the gestation period and after delivery, comparing within the subject in healthy non-pregnant women. The result obtained from the analysis of blood for 111 subjects aged (20 – 40) years, divided into two groups; the first group included 25 healthy subjects. While the second group divided to four sub groups, the first trimester which included (24 subjects), the second trimester of pregnancy included (13 subjects) and the third trimester included (38 subjects) and the last fourth subgroup for those women after delivered were included (11 women). All the chosen subjects in Erbil city. Free serum iron level showed a slight reduce at the first trimester of pregnant (449 ± 65 µg/dl) as compared with the normal control subjects (449.5 ± 65 µg/dl). While in the second trimester showed a significant increase (903.31 ± 108 µg/dl) compared to the same normal value. Also an increase of MDA concentration observed in the blood (6.527 ± 1.8 µmol/L) for the gestation women in the second trimester when compared to the normal control subjects which are (4.562 ± 0.7 µmol/L). These results improve the relationship between free serum iron and oxidative stress in pregnant women in one hand; on the other hand, free serum iron and MDA are increasing more obvious in the second trimester of pregnancy.

1. INTRODUCTION

There are many definitions of free radicals. It can be described as molecular fragments or molecules have one or more unpaired electrons. The unpaired electrons present usually considerable high degree of reactivity. In vivo which causes changing biochemical compounds, and killing cells. It means technically, free radicals are extremely unstable and very active molecules. These radicals or unpaired electron that derived from oxygen are representing the most important type of such species produced in living systems. Recently during the last two decades, there has been high interest in the function and role of oxygen-free radicals, they generally known as “reactive oxygen species” (ROS) in experimental, practical and clinical medicine (Halliwell and Gutteridge 1989), reactive oxygen species” ROS can be created (i) during X-rays irradiation or by UV light irradiation and with exposure to

gamma rays; (ii) are produced by reactions of metal-catalyzed ; (iii) are found as pollutants in the atmosphere; (iv) are generated by macrophages and neutrophils during inflammation;(v) are by-products of electron transport reactions by mitochondria-catalyzed and other mechanisms (Cadenas et al.,1989) Superoxide anion, that produces by physical irradiation increase their level either by following oxygen "activation" or with metabolic operations, Superoxide anion is represented the "primary" ROS, and can further lead to interact with other molecules to created "secondary" ROS, either directly or indirect by through metal- catalyzed processes or enzymes (Fridovich, 1986). Superoxide radical ion does not react directly with proteins, sugars, or nucleotide, and its ability to peroxidase lipids is and exciting to inquire controversially. Superoxide is undergoing to a mutation reactions (Desideri and Falconi 2003).

$$2O_2^{\cdot -} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$$

Super oxide dismutase (SOD) enzymes are increasing this reaction in biological systems more than four orders of magnitude. It was found that SOD enzymes do not work alone, but it was in conjunction with enzymes that removing H_2O_2, such as peroxidases, catalases, and glutathione (Marcel ZaÂmocky Âand Franz Koller, 1999). The redox-active metals closely linked with the participation of the generation of different free radicals (Michiels et al., 1994), it has been suggested and expected that iron regulation level ensures the absence and nonexistence of free intracellular iron; So in vivo, under stress conditions, an excess of free radical and superoxide are generated "free iron" from molecules that containing iron. The release of iron by superoxide has been illustrated with enzymes of the dehydratase, lyase family for [4Fe–4S] cluster-containing enzymes (Liochev and Fridovich, 2002). The Fenton reaction can participate with the released Fe (II), producing highly reactive hydroxyl radical (Fe (II) + $H_2O_2 \rightarrow$Fe (III) + $OH+OH^-$). The end product of the lipid peroxidation process being malondialdehyde (MDA), as well as to the MDA there are 4-hydroxy-2-nonenal (HNE) (Marnett et al., 1999).The malondialdehyde (MDA) is one of the high reactive three carbonal aldehyde produced from lipid hydroperoxidation. It can be also derived from the hydrolysis of deoxyribose, pentose, hexoses, from some amino acids and DNA. The MDA has most been measured by the thiobarbituric acid reaction (TBA test) (Pryor, 2000).

2. MATERIALS AND METHODS

1. Subjects:

The study comprised 111 healthy pregnant women subjects, which were divided into two groups, the first group included (25) healthy normal control, while the second group divided into four subgroup: the first trimester included (24) of pregnant women ,then the second trimester included (13) and the third trimester included (38) and the last subgroup included (11) subject after delivery, all the subjects were in the age range of (20-40) years, and were attending for antenatal examine at Erbil city.

2. Statistical Analysis

Statistical analysis was done by using GraphPad prism program to find ANOVA *and t*-test study. All the data were

presented as mean ± standard deviation. *P* values < 0.05 were considered significant.

3. Materials:

Used Chromogen, ascorbic acid, Buffer is Acetate buffer, thiobarbituric acid (TBA) and trichloroacetic aced (TCA) made from BDH laboratory reagent Product England.

4. Instruments:

The U.V visible spectrophotometry, Cecil CE 3021, 3000 series used, Centrifuge, Hermle labortechnic Z 200A, Made in Germany and thermostatic, Con .tem. Plate, England.

5. Method:

The collection of blood sample: - For serum collection tubes were used without anticoagulants. All samples were centrifuged at 3000 rpm for around 15 min and then serum was collected from each sample separately.

- Determination of serum iron: - A spectrophotometric method was used for serum iron determination. It is based on comparing the color that develops when the (ferrous) iron in serum is treated by a chromogen reagent, with that which develops from a standard solution .The kit was purchased from (Randox–United Kingdom) (Cerriotti and Ceriott 1980).

- Determination of serum malondialdehyde (MDA):- The MDA serum levels were assessed by spectrophotometric methods based on measuring the concentration of pink chromogenic color product, which produce when MDA reacted with thiobarbituric acid. The lipid peroxidation was expressed as µ moles MDA per dl serum. All chemicals were used in high purity commercially available. One molecule of MDA reacts stoichiometrically with two molecules of 2-thiobarbituric acid, the reaction occurs at a pH of 2 - 3 but excess acid (pH < 2) inhibits the color development (Sinnhurber, R.O. and Yu 1958) and (Yu et al., 1986).

The Method of determination of serum MDA:- The level of serum MDA was determined spectrophotometrically with a TBA solution. In brief, to 150 µL serum samples added the followings chemicals:.

3. RESULTS AND DISCUSSION

Normally, pregnant women need more food in normal cases to fill the shortfall of food during pregnancy that associated high metabolic byproducts (Arimond, et al., 2017). This leads to reduced macro and micronutrients in normal pregnancy, provoking oxidative stress, lipid peroxidation process is one of a mechanism of cell can be used as the parameter from of oxidative stress reaction in vivo and vitro study. Lipid peroxide is the product of an oxidation reaction of polyunsaturated fatty acids, that are very reactive and unstable, that typically lead to decompose and produce a various series of compounds. These include reactive carbonyl compounds, which is the considerable malondialdehyde. Therefore measurement of malondialdehyde is very widely used to providing specific indicator about lipid peroxidation. The MDA measured by the thiobarbituric acid reaction (TBA test) was used, but unfortunately, this method is not highly specific and accurate (Esterbauer et al., 1991). Concerning Free Iron concentration levels, changes were detected in iron level during different trimesters of pregnancy. As shown in figure (1) there is a slight decrease in iron

during the first trimester (449.5± 65.55 µg/ dl) compared with normal women level (460.1 ±45.9 µg/ dl). The results may be due to the time of embryonic development which requires Iron for hemoglobin synthesis and body cell growth (Marnett et al., 1999).

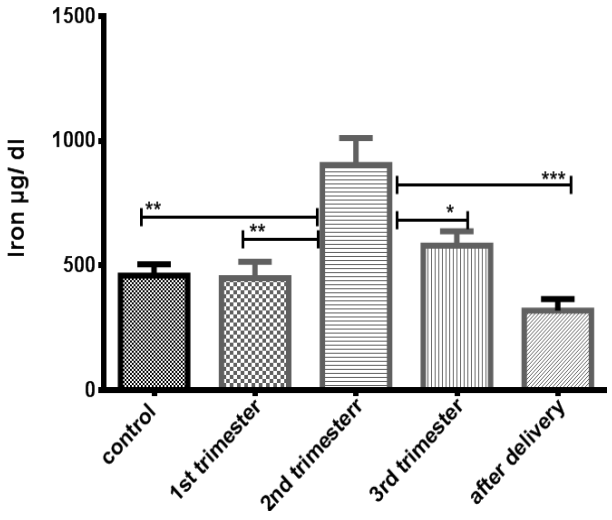

Figure 1:- The Serum iron concentration during three trimesters for pregnancy , after delivery and normal control level.

The serum iron level was increased during the second trimester (903 ± 108 µg/ dl). Ferrous Sulphate intake by the pregnant women during this period may have caused these results. Other factors may also contribute to the increase in iron levels at this stage. However, further future studies are required in order to hypothesize about. Serum iron declined after the second trimester reached (579.9 ± 57 µg/ dl) as well as after delivery table (1). The results may be explained by the decrease in intake of iron supplements during this trimester and subjects not taking proper care of their health or failing to follow their doctor's directions. And the main reason is bleeding also contribute to the reduction of serum iron after delivery. As shown statically the second trimester is high significant with control subject p value =0.0004, and the second trimester period also has significant relationship with other periods trimesters and after delivery as shown in figure (1).

Table -1- The Bio statistic ANOVA calculation for serum iron (µg/dl) level for pregnant women.

Groups	Control	First trimester	second trimester	Third trimester	After delivery
No.	25	21	10	45	10
Mean(µg/dl)	460.1	449.5	903.3	579.9	319.0
Std. Error of Mean	45.91	65.55	108.3	57.53	46.96
P vale to control	NS		P value = 0.0004	NS	NS

NS .non-significant

The malondialdehyde concentration was physiologically raised but statically non-significant as shown in table 2. The results shows an increase in MDA level in second trimester (6.5 ± 1.79 (µmol/L) if compared with other groups figure (2), this attributed to an increase in serum iron levels which is obtained in the present results as shown in

Figure (1), because Fe^{+2} participate in reduction of H_2O_2 to form $^{\cdot}OH$, these enhance lipid peroxidation increased by OH free radical (hydroxyl radical) according to the Fenton reaction (Bhasin et al., 2002).

Table -2- The Bio statistical ANOVA calculation for serum MDA level for pregnant women.

Groups	Control	First trimester	second trimester	third trimester	After delivery
No.	25	24	13	38	11
Mean (µmol/L)	4.562	3.611	6.527	5.421	5.048
Std. Error of Mean	0.7015	0.5453	1.798	0.6665	0.8020
P value	NS		NS	NS	NS

NS .non-significant

According to these and other investigators results, recommend pregnant to take care and not intake excess amount of iron (ferrous ion Fe (II) drug) due to high level of iron lead to oxidative stress which have great role in the many diseases (Abraham, et al.,1999) and (Gratacos et al., 2000).

Also there are many other factors that affect oxidative stress. where our society (especially the pregnant woman) does not take it seriously and consider on these factors. The most important factor is food, one the important factors antioxidant intake, omega-3- fatty acid and vitamins antioxidants as reported by other investigators (Taghizadeh et al., 2016).Other research Published in 2016 for woman with and without iron supplementation, when comparing with the group of pregnant women with iron supplementation shows decreased the level of oxidative stress (De Lucca et al., 2016), these improve that the high iron drug supplementation cause oxidative stress while the normal iron level has not effect.

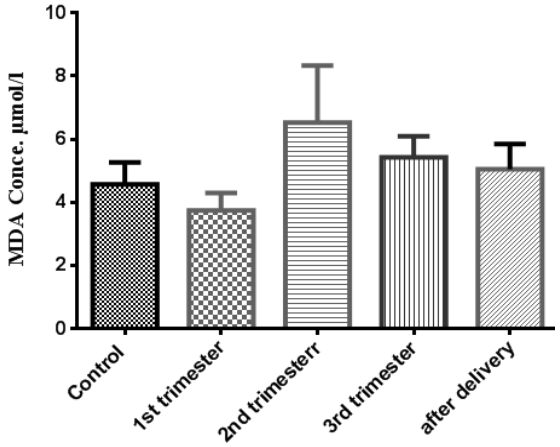

Figure 2:- The relationships and comparing of MDA concentration during pregnancy semesters, after delivery and control (not pregnant woman).

4. CONCLUSIONS

the Conclusion of this research is shows in the second trimester of pregnant woman appears high Iron concentration level and high MDA level, these results attributed to high Iron intake more than her requested and unhealthy diets which are necessary and important for pregnant woman's health.

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Dispersion Compensating Radio over Fiber (RoF) for 5G Radio Access Network

Jalal J. Hamad Ameen[1]

1- Department of Electrical Engineering, College of Engineering, Salahaddin University, Erbil, Kurdistan Region, Iraq.

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***Corresponding Author:**
Jalal J. Hamad Ameen
Jalal.hamadameen@su.edu.krd

ABSTRACT

The new developments and generations of mobile systems needs new radio access networks, this is because of higher frequency spectrum and lower coverage of base stations and then higher number of base stations, causes higher impairments and the signal quality will be decreased, this is because of the increase of interference. In this paper a new proposed radio access network has been presented for the Fifth Generation (5 G) mobile system with the use of radio over fiber (RoF) system instead of microwave link and a mesh topology. Because of the dispersion effect in optical fibers, data rate transmission will be decreased, in this paper using radio over fiber means minimization of this problem has been studied. Moreover, a new technique to compensate the dispersion effect presented, the results shows that the proposed work will decrease the effect of dispersion and then higher data rate and better signal quality between the base stations in the mobile system as shown in the results.

1. INTRODUCTION

Next generation mobile system is the future mobile system that might integrates all other mobile generations specially Fourth Generation (4 G). Today's conversation is centered on predicting what the mobile industry will look like in 2020 and beyond. Some countries have indicated that they would like to see 5G deployed even before 2020, The consensus emerging is that the industry must give enough time for technology breakthroughs that deserve the moniker 5 G. In addition, there is a consensus that because Long-Term Evolution (LTE) and LTE-Advanced are presently being deployed, there is considerable life left in 4G. In fact, the LTE family of

technologies should remain viable through at least 2020 because they will continue to evolve and advance in terms of higher speeds and greater capacity. Carrier Aggregation (CA), small cell enhancements and device-to-device signaling are just some of the examples of how LTE is advancing (4G Americas, white paper, 2015).

Many researches has been introduced each with a certain part of the system, for example, some of them about the radio access network,

others about the channel assignment, cell planning, …etc. the questions are; when will 5G be deployed? What will characterize networks in 2020 and beyond? What are the likely solutions and technologies that will come in to play? These are some questions currently being discussed among operators, the supplier community, research institutions, standards bodies, trade organizations and governments (4G Americas, white paper, 2015).

Examination of 5G requirements and solutions is basically an exercise in planning a network evolution plan that spans six to seven years. While past generations have been identified by a major new technology step, such as the definition of a new air interface, the expectation is that 5G will be approached from an end-to-end system perspective and include major technology steps both in the radio access network and core network. These steps can be evolutionary or revolutionary by introducing a completely new concept (4G Americas, white paper, 2015).

In this paper, the radio access network part has been designed with a new proposed topology using Radio over Fiber (RoF) system, challenges of RoF like dispersion has been studied that how can be minimized and then compensated for the proposed radio access network, finally, some test and examined results presented with conclusions.

2. 5G MOBILE SYSTEM

Mobile telecommunication system grown very fast motivating the companies to plan continuously and work from first generation until fourth generation, many companies in this field planned and started their scenarios toward fifth generation (5G) mobile, this is because of the need of higher data rate transmission and wireless system radio network (Wei Xiang , et. Al, 2017). The fifth-generation (5G) mobile communications system will emerge to meet new and unprecedented demands beyond the capability of previous generations of systems, 5G will support a large variety of use cases which are emerging now or will emerge in the future (Wei Xiang , et. Al, 2017). The Radio Access Network (RAN) design for the next generation, Radio Access Technologies (RAT) shall be designed to fulfill many requirements, for example the RAN architecture shall support tight interworking between the new RAT and LTE, considering high performing inter-RAT mobility and aggregation of data flows via at least dual connectivity between LTE and new RAT. This shall be supported for both collocated and non-collocated site deployments, the RAN architecture shall support connectivity through multiple transmission points, either collocated or non-collocated, the RAN architecture shall enable the separation of control plane signaling and user plane data from different sites, RAN-CN interfaces and RAN internal interfaces (both between new RAT logical nodes/functions and between new RAT and LTE logical nodes/functions) shall be open for multi-vendor interoperability, and the RAN architecture shall support operator-controlled side link (device-to device) operation, both in coverage and out of coverage. 5G networks are expected to hugely increase network densification, The large number of small cells, together with the growing coexistence of multiple radio access technologies (RATs) like global system for mobile communications (GSM), Universal Mobile Telecommunications System (UMTS), Long Term Evolution (LTE), etc., leads to an increasing complexity in the Operations, Administration, and Management (OAM) of cellular networks (Sergio Fortes, et. al, 2016).

3. RADIO OVER FIBER (RoF) SYSTEM

Radio over Fiber (RoF) is a promising technology for short range transmission applications within multimode optical fiber, radio over fiber (RoF) system is an analog link transmitting modulated Radio Frequency (RF) signals. It serves to transmit the RF signals down and uplink, i.e. to and from Central Stations (CS) to Base Stations (BS) called also radio ports. RF modulation in most cases is digital in any usual form, such as Phase Shift Keying (PSK), Quadrature Amplitude Modulation (QAM), Trellis Coded Modulation (TCM). Moreover, RoF also called Fiber to the air, which is the one emerging technology applicable in high capacity, broadband millimeter-wave access systems.

In this system, the costs of BSs will be decreased because most of signal processing including coding, multiplexing, RF generation and modulation, etc. is made in CSs rather than in the BSs. The signal to and from these is transmitted in the optical band, via a fiber optic network. The design of BSs really be simple, in the simplest case a BS doesn't comprise else than optical-to-electrical (O/E) and electrical-to-optical (E/O) converters, an antenna and some microwave circuitry (two amplifiers and a diplexer).

In the last decade or so significant research work was done in this field with significant results, the number of publications is abundant, some problems to be solved and there will be some benefits in the new architecture using RoF, but there are still special problems like resource management, channel allocation, interference, data rate improvement. RoF technology allows a micro cellular network system to be implemented by using a fiber-fed distribution antenna network.

Simplified RoF system block diagram is given in Figure 1. The RF part is between the input signal and Electrical to Optical conversion (E/O), and the received signal with that in Optical to Electrical Conversion (O/E).

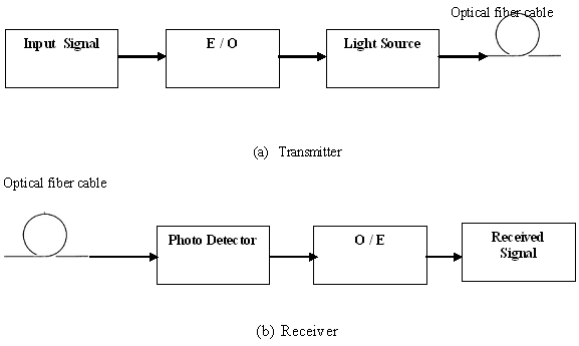

Figure (1): Simplified RoF system block diagram (a) Transmitter (b) Receiver

There are three main RoF system architectures proposed for commercial in-building wireless deployments: Radio Frequency (RF) transmission over fiber, Intermediate Frequency (IF) transmission over fiber and digitized IF transmission over fiber(John M. Senior, 2009).

Optical fiber system like other communication channels has many impairments for example, attenuation, linear and non linear scattering losses, fiber bend loss, and dispersion, this work focused on dispersion effect because it has a great effect on the data rate as given in equation (1) (John M. Senior, 2009) :

$$\text{Data rate} = 0.2 / \sigma_T \quad (1)$$

Where, σ_T is the total dispersion given in equation (2) with chromatic and modal dispersion :

$$\sigma_T=(\sigma_c{}^2 + \sigma_n{}^2)^{1/2} \quad (2)$$

Where,

σ_c is the chromatic dispersion, σ_n is the modal dispersion

And the chromatic dispersion is given in equation (3) while the modal dispersion depends on the fiber type step index or graded index, equation (4) is the modal dispersion for the step index and (5) for the graded index fiber :

$$\sigma_m \cong (\sigma_\lambda L/c)\lambda \frac{d^2 n_1}{d\lambda^2} \quad (3)$$

Where, σ_m is the material dispersion, σ_λ is the spectral width of the laser, L is the fiber length, λ is the wavelength, and n_1 is the core refractive index.

$$\sigma_s=(L\ n_1\ \Delta) / (3.4\ c) = (L\ (NA)^2)) / (6.8\ c \quad (4)$$

Where, σ_s is the modal dispersion for step index fiber, Δ is relative refractive index difference, NA is the numerical aperture and c is the velocity of light = 3 x 10 ^ 8

σ_g =(L$n_1\Delta^2$)/34c (5)

Where, σ_g is modal dispersion in graded fiber

4. DESIGN AND METHODOLOGY

The proposed topology for the 5G Radio Access Network (RAN) in this paper is that the connections between Base Transceiver Stations (BTS) and Central Base Station (CBS) will be using RoF instead of microwave (MA Zheng1 et. al, 2015). This is because of many disadvantages of microwave link like maintenance, interference, atmosphere effects, etc. therefore, RoF will be the alternative for the microwave link because these disadvantages will be solved by RoF, the topology is mesh topology because in this topology an interrupt in the link will not affect the data transmission because there will be other path links even as a disadvantage of mesh topology the cost of the fiber used. All the Base Transceiver Stations (BTS) will be connected via RoF to Columbia Broadcasting System (CBS) instead of many Base Station Controller (BSC) like that used in 3G or GSM or even 4G mobile systems, this will facilitate the cell planning procedure also and channel assignment.

After the design of RAN is proposed, the dispersion effect has been studied, first, using equations (1-5), the optimized values and relations between dispersion and other fiber parameters has been obtained and presented using MATLAB package, and then the data rate versus fiber length this is because of the connection between each BTs and CBS depends on the fiber length.

Figure 2 shows the relation between data rate and the fiber length using multimode graded index fiber optics because dispersion affecting on them more than single mode with different relative refractive index differences (Delta). It is shown that fiber length is until about 6km, but for 4 G the coverage radius is about 2km and for 5 G about 1 km because of higher frequency bandwidth. Thus, relative refractive index difference must be less than 0.04 for a higher data rate.

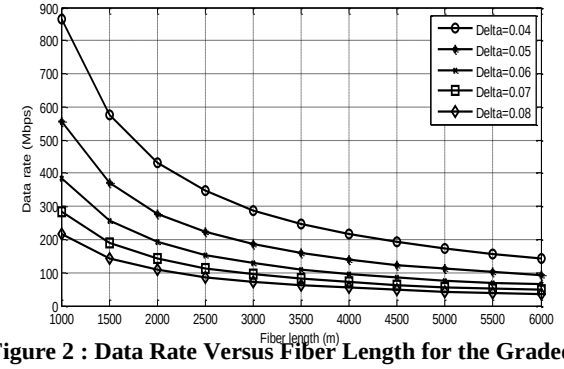

Figure 2 : Data Rate Versus Fiber Length for the Graded Index Fiber.

Figure 3 shows the relationship between fiber length and data rate with different numerical apertures (Na), using multimode step index fiber optics, it is found that for higher data rate and fiber length about 2km for 4 G and about 5 G, numerical aperture must be less than 0.028.

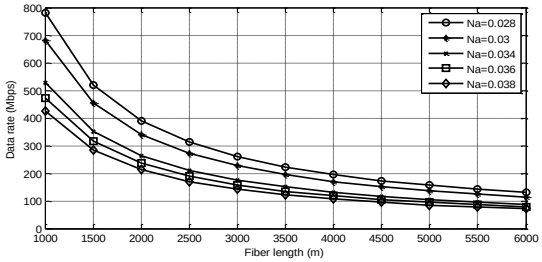

Figure 3 : Data Rate Versus Fiber Length for Step Index Fiber.

Figure 4 shows the relationship between data rate and relative refractive index difference (Delta) for different cluster sizes (N) using multimode step index fiber optics. It is shown that without clustering (N=1), the data rate is higher, but this causes higher Co-channel interference (CCI) and Adjacent Channel Interference (ACI), and hence Signal to Interference Ratio (SIR) will be decreased. Therefore, N=3 and N=7 clustering would be more optimal because these cluster sizes has less CCI and ACI specially where N = 7 during cell planning procedure. Using the cluster size N=4, the distribution for the base stations will not be uniform, and using N=7, base station numbers will be increased, but the number of carriers will be decreased. On the other hand, using N=3, the number of base stations is decreased, but the carrier frequencies used will be increased. Therefore, this work proposed that for the RAN, the two cluster sizes N =3 and N=7 are to be used. In addition, for step index multimode fiber, the relative refractive index difference must be less than 0.0004 for higher data rate towards 1Gbps outdoor as shown in Figure 4.

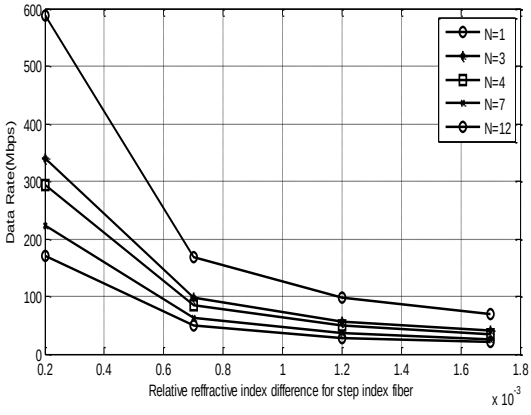

Figure 4 : Data Rate Versus Relative Refractive Index Difference for Step Index Fiber.

Figure 5 shows the relation between data rate and relative refractive index difference (Delta) for different cluster sizes (N) with graded index multimode fiber optics. It is demonstrated that without clustering (N=1), the data rate is increased, but this causes higher CCI and ACI, and hence SIR will be decreased. Therefore, again, N=3 and N=7 were deemed better. Using the cluster size N=4, the distribution for the base stations will not be uniform, and using N=7, the base station numbers will be increased, but carriers will be decreased. Again, using N=3, the number of base stations decreased, but the carrier frequencies used increased. Thus the two cluster sizes N =3 and N=7 are used. For graded index multimode fiber, the relative refractive index difference must be less than 0.04 for a higher data rate, as shown in Figure 5:

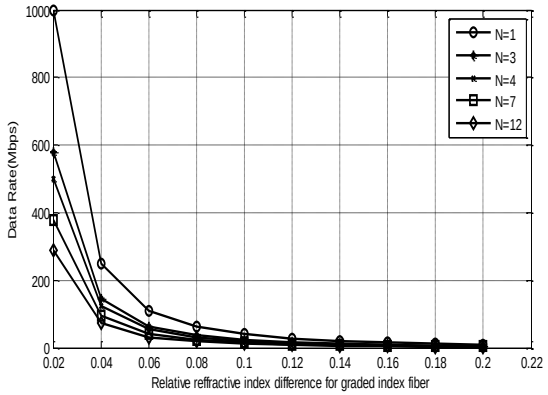

Figure 5 : Data Rate Versus Relative Refractive Index Difference for Graded Index Fiber.

Next step after the optimization, using Opti System software the special software for the optical design, the design of the RAN with compensating dispersion using Fiber Bragg Grating (FBG), and the Dispersion Compensating Fiber (DCF) to minimize the dispersion and then higher data rate, In addition the optical amplifier Erbium Doped Fiber Amplifier (EDFA) to attenuation compensation by amplifying the signal and increasing the signal quality by minimizing the dispersion has been used as shown in Figures 6-8. Figure 6 is the basic dispersion compensation using FBG for each BTs. Considered by user defined bit sequence and O/E by RoF.

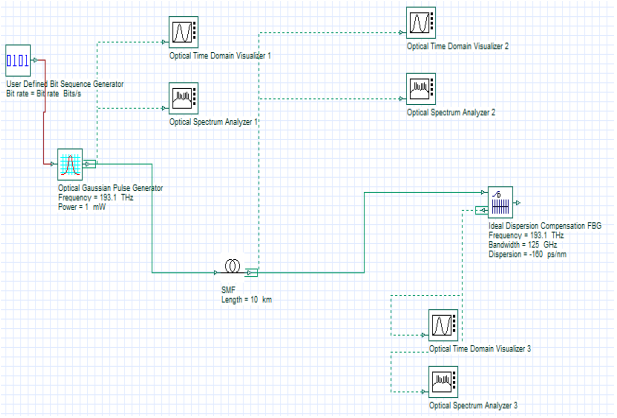

Figure 6 : Dispersion compensation with FBG using Opti System

Figure 7 is the dispersion compensating fiber (DCF) to compensate the loss caused by dispersion with the use of opt Grating linear chirp and anodization for each BTs.

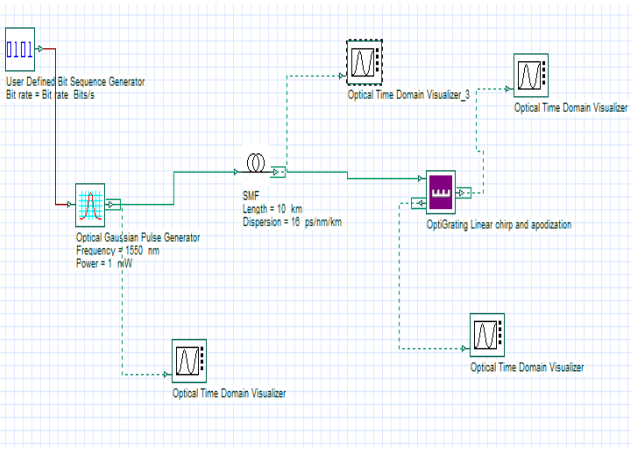

Figure 7 : The link between each BTs using RoF with FBG

Figure 8 is the overall proposed RAN with eight BTSs using the WDM/WDM DEMUX. Figure 9 a is the subsystem for the transmitting side and Figure 9 b for the receiving side.

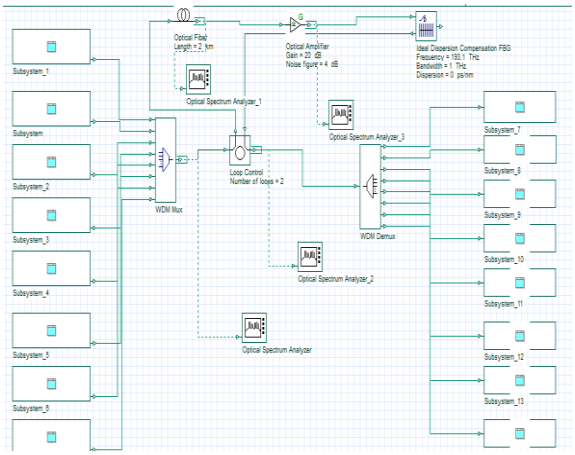

Figure 8 : The link between BTs using DCF

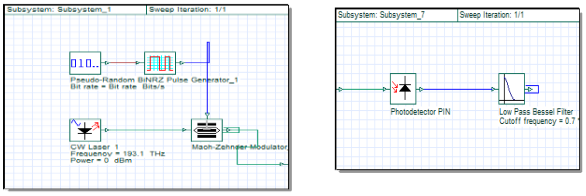

Figure 9 Subsystem of the project

a. (Subsystem Tx side) b. (Receiving side)

5. RESULTS

Many RAN designs and topologies has been presented by many researchers, in this paper after the design of a new RAN topology has been presented using RoF, the main impairments in optical system which is the dispersion is studied and the optimum level of dispersion and then the maximum data rate for the RAN obtained. Figure 10 shows the power spectrum of the transmitted signal after WDM from eight BTSs, the power is about -7dBm and the frequency is 193.1 THz – 193.8 THz spacing 0.1 THz. Figure 11 shows the power spectrum of the received signal by the BTSs after 2 km length of the fiber which is the distance between each two neighbor BTSs, each with 1 km radius. Figure 12 is the received signal after the use of loop control (2 loops) and finally, Figure 13 is the received signal after the FBG and the EDFA which will be demultiplexed by WDM/DEMUX then to each BTs, thus the received signal by each BTs

will be dispersion compensated as well as attenuation compensated with higher signal quality.

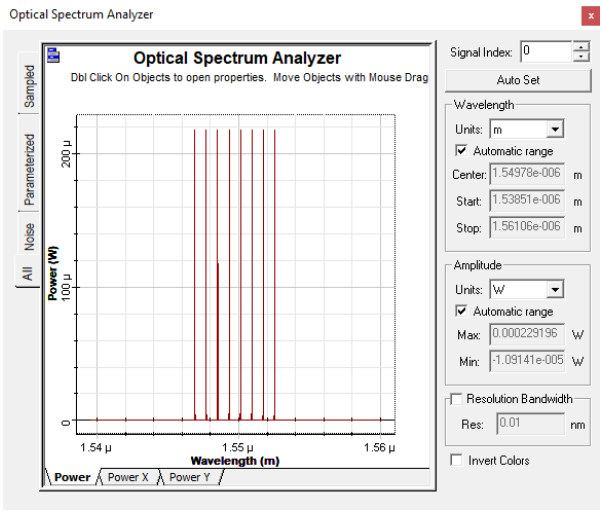

Figure 10 : Power spectrum of the transmitting signal with eight WDM BTSs

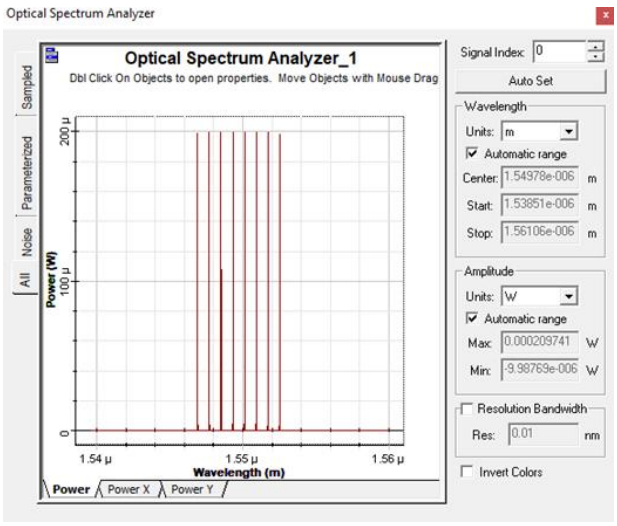

Figure 11 : Power spectrum of the Received signal by eight BTSs

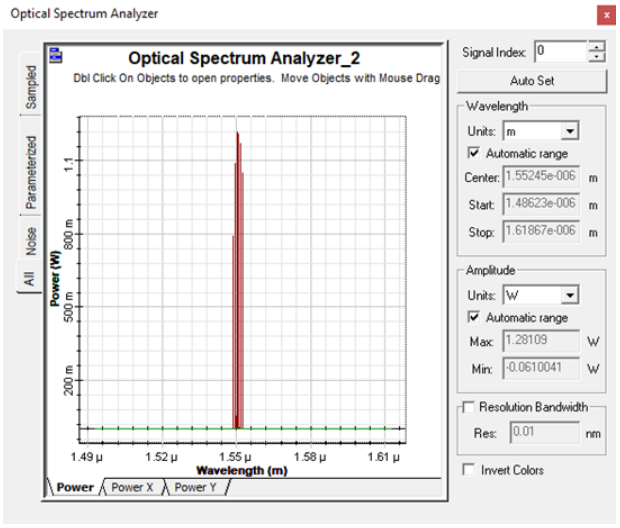

Figure 12 : Power spectrum of the received signal from the loop control

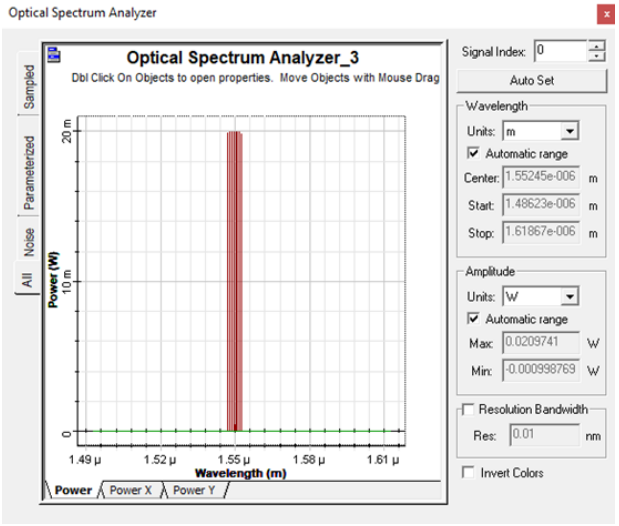

Figure 13: Power spectrum of the received signal from the FBG and the EDFA and input to WDM/DEMUX

Figure 14 is power spectrum of the input signal to the proposed RAN from each BTs, it is shown that the duration of the signal is small.

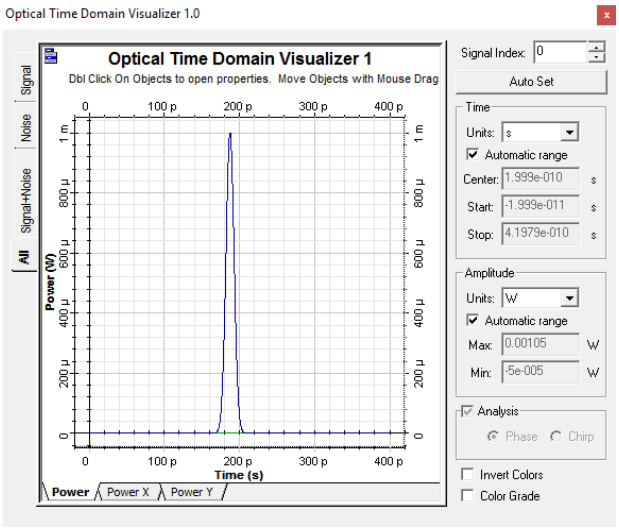

Figure 14 : Power spectrum of the input signal to the proposed RAN from each BTs

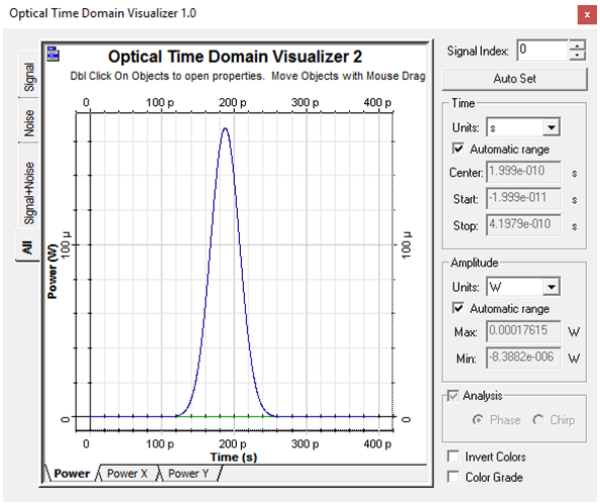

Figure 15 : The received signal by each BTs before dispersion compensating (without dispersion compensation)

Figure 15 is the power spectrum of the received signal by each BTs before dispersion compensating (without dispersion compensation). It is shown that the duration of each signal is broadened caused by dispersion which affects the data rate and causes Inter Symbol Interference (ISI), when all the signals received together by the BTSs, with the use of the proposed work in this paper, this pulse broadening will be minimized and then the dispersion effect will be minimized, ISI will be decreased, then the data rate will be increased and the signal quality will be increased as shown in Figure 16, finally, Figure 17 shows the power spectrum of the three signals together for the purpose of comparison of the transmitting, receiving signal with and without dispersion compensation.

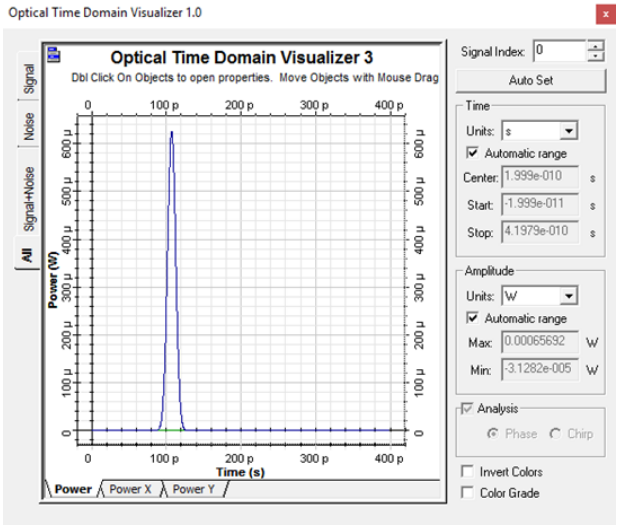

Figure 16 : The received signal by each BTs before dispersion compensating i.e. without using proposed dispersion compensation

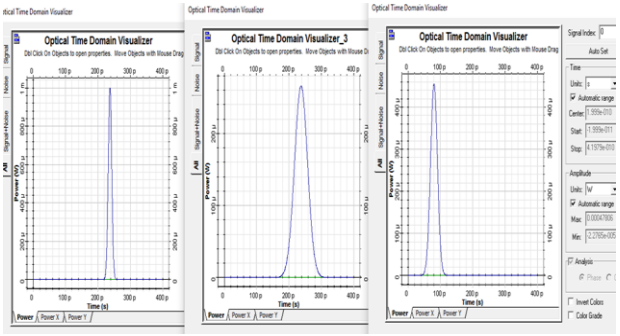

Figure 17 l The transmitting, receiving signal by each BTs before dispersion compensating (without dispersion compensation) and after proposed dispersion compensation

4. CONCLUSIONS

In this paper a new proposed radio access network for 5G mobile system has been presented, radio over fiber system proposed to be the link between base stations and a central station with a mesh topology. The main impairment of fiber system which is dispersion has been studied with the main methods of this effect compensation. A new model proposed radio access network with minimum dispersion has been presented, the new model is a combination of more than one method of dispersion compensation like dispersion compensation fiber, fiber brag grating and opt Grating linear chirp and anodization, it shown in results that with the use of the proposed model the pulse broadening and then the dispersion will with minimum level which leads to higher data rate and signal quality, it is a contribution in the field of 5G mobile system architecture and radio access network.

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Susceptibility Pattern and Detection of Some Antibiotic Resistance Genes in *Staphylococcus aureus*

Rastee Hassan Saeed[1], Zirak Faqe A. Abdulrahman[2], and Safa A. Naje[3]

[1,2-] Department of Biology, College of Education, Salahaddin University – Hawler, Erbil

[3-] Department of Biology, College of Science, Tikrit University, Tikrit

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***Corresponding Author:**
Zirak Faqe A. Abdulrahman
zirak.ahmed@su.edu.krd

ABSTRACT

In this study a total of fifty samples were collected from patients who were admitted to West Erbil Emergency, Emergency, and Rizgary teaching Hospitals during the period from 1 October 2015 to 20 February 2016. Samples collected from different clinical sources: 20 from burns, 10 from surgical wounds, 10 from dental carries, and 10 from urine samples. Isolates were identified using cultural, morphological, biochemical tests, and confirmed by VITEK2 compact system. Twenty isolates were identified as *Staphylococcus aureus.* Antibiotic Sensitivity test by disk diffusion method was done for all *S. aureus* isolates against 20 commonly used antibiotics and the resistance percentage was as the following: 100% for AMC, AP, AX, and PG, 90% for ME, 65% for CAZ, 65% for TM, 60% for T, 60% for KF ,50% for CRO, 45% for CTX, and S, 45% for L, 40% for RA, 40% for DA, 35% for CIP, 35% for C%15, for KF and 15% for GM, and 10% for E, while all isolates were sensitive to vancomycin. PCR technique was used for the detection of *tetk*, *blaZ*, and *Fem(A)*, gene in *S. aureus* isolates and the results showed that 7 (35%),18(90%), and 11(55%) of isolates were *tetk*, *blaZ*, and *Fem(A)* positive respectively.

1. INTRODUCTION

Staphylococcus aureus is a major cause of nosocomial infections and remains a versatile and dangerous pathogen in human. The frequency of both community acquired and hospital acquired staphylococcal infections have increased steadily (Lowy, 1996). *S. aureus* is coagulase-positive, often hemolysis blood and produces a variety of extracellular enzymes and toxins (Brooks *et al*., 2001). In human, infection with *S. aureus* may cause suppuration, abscess formation, a variety of pyogenic infections and even fatal septicemia. It can also cause food intoxication due to elaboration of heat-stable enterotoxin (Colle *et al*., 1996; Kloose and Bannerman 1999). Antimicrobial resistance is an increasing threat afflicting hospital worldwide (Graffunder and Venezia, 2002). Antimicrobial drug resistance in hospitals is driven by failures of hospital hygiene, selective pressures created by overuse of antibiotics, and mobile genetic elements that can encode bacterial resistance mechanisms (Wienstein, 2001). Methicillin-resistant *S. aureus* (MRSA) isolates were once confined largely to hospitals, other health care environments, and patients frequenting these facilities (David and Daum, 2010). A large number of tetracycline resistance genes have

been identified. The main mechanisms conferring resistance to tetracycline to bacteria are active efflux proteins, ribosomal protection proteins and enzymatic inactivation (David and Daum, 2010). Methicillin-resistance in staphylococci is expressed by the mecA gene that produces a "Penicillin Binding Protein 2a" (PBP2a). The Staphylococcal Chromosome Cassette mec (*SCCmec*), a mobile genetic element, is composed of the mec gene complex which includes mecA. However, mecA alone does not solely confer the methicillin resistance. Studies have shown that fem the auxiliary genes like fem A/B/X in addition to mecA are also important in the expression of methicillin resistance. The femABX operon encodes factors which are responsible for the formation of pentaglycine bridges in the cell wall of Staphylococci (Chikkala *et al*.,2012) Another gene involved in penicillin resistance in staphylococci is *blaZ* which encodes β-lactamase2. PCR, have become more important in the diagnosis of MRSA and have greatly improved the speed, sensitivity, and specificity of diagnostic tests, which in turn facilitates early detection of MRSA patients and helps in infection control and prevention (Al-Talib,2014). Therefore, this paper concerned with isolation and identification of *S. aureus* from different clinical specimen and study both antibiotic resistance pattern and detection of *tetk*, *fem (A)*, and *blaZ* genes in *S. aureus* isolates using PCR technique.

2. MATERIALS AND METHODS

2.1 Samples collection

In this study a total of fifty samples were collected from patients who were admitted to West Erbil Emergency, Emergency, and Rizgary teaching Hospitals during the period from 1 October 2015 to 20 February 2016. Samples collected from different clinical sources: 20 from burns, 10 from surgical wounds, 10 from dental carries, and 10 from urine samples. The age of patients ranged from one to 45 years. Samples were taken from different sites: burns, surgical wounds, dental carries and urine. The samples were obtained by rubbing the inflamed or discharged wound, burn, or decayed teeth by a sterile disposable swab with normal saline to keep samples fresh while transporting it to the laboratory for further processing. Urine samples were collected by taking a loop full from the midstream of urine sample and streaked it directly on the culture media.

2.2 Identification of the isolates

Identification of these isolates was carried out using microscopical, morphological, biochemical tests and VITEK2 compact system. Microscopical test like gram stain, also morphological tests like culture characteristics of isolates on media Mannitol salt agar and Blood Agar (oxoid), while biochemical test include coagulase test, catalase test, DNase test (Brooks *et al*., 2001).

2.3 Antimicrobial susceptibility test (Disk diffusion method)

This test was performed according to (Schwalbe *et al*., 2007). Antibiotic impregnated discs include AMC, AP, AX, C, CAZ, CIP, CRO, CTX, DA, E, GM, KF, L, ME, PG, RA, S, T, TM, VA (Bioanalyse) with required concentration were dispensed on the surface of Mueller Hinton agar medium (Oxoid) that has been spread with a pure bacterial suspension of 10^5 CFU/ml. After incubation, inhibition zones were measured and translated into predetermined categories as susceptible, intermediate, or resistant.

2.4 Genomic DNA extraction

A Presto™ Mini gDNA Bacteria Kit was used for genomic DNA extraction from *S. aureus* isolates. A loop full of bacteria were incubated overnight in a tube containing LB

broth. The kit's instructions were followed carefully to obtain a good DNA extracts.

2.5 Detection of *tetk, femA,* and *blaZ* genes in *S. aureus* clinical isolates

The standard PCR assay was performed using the DNA amplification instrument Mastercycler gradient (Eppendorf, Germany) to detect *tetk* gene. The *tetk* - specific primer pairs used for amplification of 360 base pair (bps) fragment are: Forward, 5'GTAGCGACAATAGGTAATAGT-3'and Reverse, 5' GTAGTGACAATAAACCTCCTA- 3' (Bühlmann *et al*., 2008).A volume of 20µl deionized distilled water (ddH_2O), 1.3 µl Reverse primer, 1.3µl Forward primer and 2.5 µl of extracted DNA (template) was added to the ready to use PCR reagent tube (Bioneer, South Korea) which contains the following (for the 20µl reaction): 1U *Top* DNA polymerase, 250 µM of each: dNTP (dATP, dCTP, dGTP, dTTP), 10 mM Tris-HCl (pH 9.0), 30 mM KCl,1.5 mM $MgCl_2$, Stabilizer and tracking dye. The thermal cycling protocol for PCR was comprised as described by (Duran *et al*., 2012): 1. Initial denaturation at 95°C for 3 minutes. 2. Thirty-three cycles of: Denaturation at 95°C for 30sec, annealing at 54°C for 30 secs, elongation at 72°C for 30 sec and final extension at 72°C for 4 minutes. The *femA* specific primer pairs used for amplification of 360 base pair (bps) fragment are: Forward, 5'-AAAAAAGCACATAACAAGCG -3' and Reverse, 5'-GATAAAGAAGAAACCAGCAG-3'. A volume of 20 µl deionized distilled water (ddH_2O), 1.3 µl Reverse primer, 1.3 µl Forward primer and 2.5 µl of extracted DNA (template) was added to the ready to use PCR reagent tube (Bioneer, South Korea) which contains the following (for the 20 µl reaction): 1U *Top* DNA polymerase, 250 µM of each: dNTP (dATP, dCTP, dGTP, dTTP), 10 mM Tris-HCl (pH 9.0), 30 mM KCl, 1.5 mM $MgCl_2$, Stabilizer and tracking dye. The thermal cycling protocol for PCR was comprised as described by (Duran *et al*., 2012): 1. Initial denaturation at 94°C for 5 minutes. 2. Thirty-three cycles of: Denaturation at 94°C for 45 secs, annealing at 55°C for 1minute, elongation at 72°C for 1minute and final extension at 72°C for 5 minutes. The *blaZ* specific primer pairs used for amplification of 360 base pair (bps) fragment are: Forward, 5'-ACTTCAACACCTGCTGCTTTC-3' and reverse 5'-TGACCACTTTTATCAGCAACC-3'. A volume of 20 µl deionized distilled water (ddH_2O), 1.3 µl Reverse primer, 1.3 µl Forward primer and 2.5 µl of extracted DNA (template) was added to the ready to use PCR reagent tube (Bioneer, South Korea) which contains the following (for the 20 µl reaction): 1U *Top* DNA polymerase , 250 µM of each: dNTP (dATP, dCTP, dGTP, dTTP),10 mMTris-HCl (pH 9.0),30 mMKCl,1.5 mM $MgCl_2$, Stabilizer and tracking dye. The thermal cycling protocol for PCR was comprised as described by (Duran *et al*.,2012): 1. Initial denaturation at 94°C for 5 minutes. 2. Thirty-three cycles of: Denaturation at 94°C for 45sec, annealing at 55°C for 1minute, elongation at 72°C for 1minute and final extension at 72°C for 5 minutes.

2.6 Detection of PCR product

After PCR amplification, 5 µl was removed and subjected to agarose gel electrophoresis (1.5% agarose, 1× Tris-borate-EDTA, 100 V, 40 min) to estimate the sizes of the amplification products by comparison with a 100 bps O' GeneRuler™ 100 bps molecular size standard DNA Ladder, ready-to-use designed by Fermentas Life Sciences The gel was stained with ethidium bromide, and the amplicons were visualized using an ultra violet (UV) light box (Duran *et al*., 2012).

3. RESULTS AND DISCUSSION

3.1 Isolation and Identification of *S. aureus*

Twenty isolates were identified among 50 different clinical specimens including: 20 burn swabs, 10 wound swabs, 10 urines and 10 from dental carries. The identification of the isolates was carried out using conventional method based on cultural characteristics, cell morphology, Gram stain reaction, biochemical properties and VITEK2 compact system. All isolates were able to grow on mannitol salt agar (selective media for Staphylococcus). On blood agar *S. aureus* has the ability to change the color of the media with various degrees of hemolysis (mostly beta hemolysis). Prepared smears of *S. aureus* isolates appeared as purple single, diplo, and grape like Gram positive cocci under light microscope. All isolates were positive for catalase, coagulase, and DNase (Yang *et al*., 2015). All isolates were identified as *S. aureus* by VITEK2 compact system with over 95% probability percentage.

3.2 Antimicrobial resistance pattern of *S. aureus* isolates

Antimicrobial Sensitivity test by disk diffusion method for 50 isolates of *S. aureus* was done against 20 commonly used antimicrobial agents (AMC, AP, AX, C, CAZ, CIP, CRO, CTX, DA, E, GM, KF, L, ME, PG, RA, S, T, TM, VA). The resistance percentage of *S. aureus* isolates varied for different antimicrobial agents used in this study as shown in table (1). The results revealed that the resistance was 100% for AMC, AP, AX, and PG and 90% for ME. Resistance percentage for other antibiotics were 65% for CAZ, 65% for TM, 60% for T, 60% for KF, 50% for CRO, 45% for CTX, and S, 45% for L, 40% for RA, 40% for DA, 35% for CIP, 35% for C,15% for KF, and 15 % for GM and 10% for E while all isolates were sensitive for vancomycin. Our results are in agreement with that of Al-Jebouri and Madish (2003) who found that *S. aureus* isolates from patients with urinary tract infections were highly resistant to ampicillin and amoxicillin. The results of AL-Ugaili *et al*.,(2014) showed that (77%) of isolates were oxacillin-resistant *S. aureus* and exhibited multiple resistances to other tested antibiotics which is close to our results. Al-Marjan *et al*.,(2015) reported that the resistance patterns of *S. aureus* for levofloxacin (20 %), for norfloxacin (16 %), for ofloxacin (18 %), for ciprofloxacin (16 %), for lomofloxacin (14%) and for nalidixic acid (50 %), while the results of Al-Azzwai and Flayyih (2014) revealed that (8.10%) of *S. aureus* isolates were amikacin resistant, (100%) of isolates were amoxicillin resistant, (86.48 %) of isolates were ampicillin resistant, (54.05%) were resistant to (Cefotaxime, erythromycin, tetracycline), (21.62%) of isolates were methicillin resistant (MRSA), and (10.81%) were vancomycin resistant. Nurjadi *et al*., (2014) found that *S. aureus* isolates from Africa were: 54% resistant for trimethoprim, 21% for sulfamethoxazole and 19% for trimethoprim/sulfamethoxazole, while Juayang *et al*., (2014) reported that a total of 94 cases from 2010 to 2012 were diagnosed to have *S. aureus* infection using conventional bacteriologic methods. From these cases, 38 (40.6%) were identified as MRSA and 37 (39.4%) were inducible clindamycin resistant. In present study we found that vancomycin 0 (0%) the most effective antibiotics against MRSA. This finding is in agreement with study conducted by AL-Ugaili *et al*., (2014) who found that vancomycin is the most powerful antibiotic against MRSA. The antimicrobial agents are losing their efficacy because of the spread of resistant organisms due to indiscriminate use of antibiotics, lack of awareness, patient noncompliance and unhygienic condition. It is the need of the time that antibiotic policies should be formulated and implemented to resist and overcome this emerging problem. Every

effort should be made to prevent spread of resistant organisms.

Table 1- Resistance percentage of S. aureus to antimicrobial agents.

Antimicrobial agent	NO. of isolates	% of resistance	No. of resistant isolates
Amoxicillin	20	100	20
Amoxicillin+ clavulanic acid	20	100	20
Ampicillin	20	100	20
Cefotaxime	20	35	7
Ceftazidime	20	65	13
Ceftriaxone	20	35	7
Cephalothin	20	50	10
Chloramphenicol	20	45	9
Ciprofloxacin	20	40	8
Clindamycin	20	60	12
Erythromycin	20	10	2
Gentamicin	20	15	3
Lincomycin	20	45	9
Methicillin	20	90	18
Penicillin G	20	100	20
Rifampin	20	40	8
Streptomycin	20	45	9
Tetracycline	20	60	12
Trimethoprim	20	65	13
Vancomycin	20	0	0

Amoxicillin: AMC, Amoxicillin+ clavulanic acid: AX, Ampicillin: AM, Cefotaxime: C, Ceftazidime: CAZ, Ceftriaxone: CIP, Cephalothin: CRO, Chloramphenicol: CTX, Ciprofloxacin: DA, Clindamycin: KF, Erythromycin: E, Gentamicin: E, Lincomycin: L, Methicillin: M, Penicillin G: PG, Rifampin: RA, Streptomycin: S, Tetracycline: T, Trimethoprim: TM, Vancomycin: VA

3.3 Detection of *tetk* in *S. aureus* isolates

All isolates were analyzed by PCR to detect the presence of *tetk* gene using forward and reverse primers described by Duran *et al*.,(2012) and *Bioneer* master mix. The PCR products were analyzed by gel electrophoresis on 1.5% agarose gel and staining with ethedium bromide. The results showed that 7 isolates (35%) were harboring the *tetk* gene (360 bps), (figure 1). These results shows contrast with the results of disc diffusion method for tetracycline, 65% of isolates were resistant to tetracycline while only 35% of it were harboring *tetk* gene. This may be due to that the resistance may conferred by other class of tetracycline gene like *tetK, tetM, tetO* and *tetL* that not detected in our study. Our results agree with that obtained by Duran *et al*.,(2012) who reported 36% of MRSA were positive for *tet(k)* gene. In contrast to our results Adwan *et al*.,(2014) who reported that 76% isolate of methicillin resistant *S. aureus* were *tet(k)* positive. Ulah *et al*., (2012) found that only 58 (44.61%) from total 130 *S. aureus* isolates showed *tet(k)* gene positive.

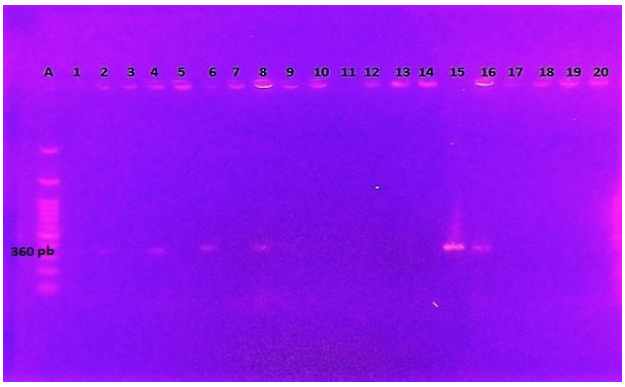

Figure 1: PCR gene product of tetK gene, A: ladder.

A large number of tetracycline resistance genes have been identified. There are 38 acquired tetracycline resistance genes that are known and all use one of three strategies to render the bacteria resistant. These include (1) efflux proteins, (2) ribosomal protection proteins and (3) enzymatic inactivation of tetracycline. The majority of these genes (60 %) code for energy-dependent efflux pumps, and different bacterial genera tend to have the same efflux or ribosomal protection genes (Speer *et al*., 1992). This indicates that tetracycline resistance genes can be transferred amongst the bacterial population. In fact, resistance to tetracycline in most bacteria is due to the acquisition of new genes; these genes tend to be associated with mobile elements such as transposons and plasmids (Chopra and Roberts,2001).

Tetracycline resistance genes: *tetK, tetM, tetO* and *tetL* are four major genes associated with tetracycline resistance amongst Gram positive bacteria. The *tetK* and *tetL* genes code

for efflux proteins; these are energy dependent membrane-associated proteins which prevent tetracycline from accumulating within the cell (Speer *et al*., 1992). The other two genes, *tetM* and *tetO*, code for ribosomal protection proteins, which reduce the affinity of tetracycline to the ribosome (Bismuth *et al*., 1990).

3.4 Detection of *femA* in *S. aureus* isolates

Figure (2) shows the PCR product of *fem A* gene. The results showed that 11 isolates (55%) were harboring the *femA* gene (132 bp), while 9 isolate (45%) were lacking the *femA* gene. Our results disagree with that obtained by Kareem (2013) who reported 100% of *S. aureus* were positive for *femA* gene. In close to our results Chikkala *et al*., (2012) reported that 29.6% isolate of *S. aureus* were *femA* positive. Yang *et al*., (2015) found that only 177 (89.4%) from total 198 *S. aureus* isolates showed *femA* gene positive.

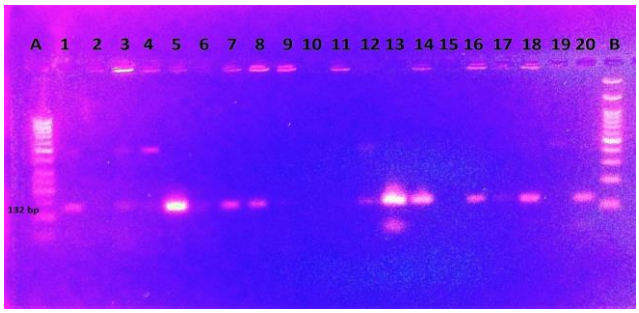

Figure 2: PCR gene product of *femA* gene, A, B: ladder.

femA was generally accepted as a species-specific marker. This gene encodes a factor which was essential for methicillin resistance and was universally present in all MRSA isolates, its product, a 48-kDa protein, has been implicated in cell wall metabolism and was found in large amounts in actively growing cultures. Analysis of the *femA* product indicated methicillin resistance without affecting Penicillin Binding Protein-2 (PBP-2`) production, the significance of the *femA* genes in the mechanism of methicillin resistance was supported by the demonstration that *S. aureus* strain with *femA* gene inactivated; lost the methicillin resistance trait, but with the transduction of *femA* genes restored the resistance. Another biochemical analysis suggested that *femA* product may be involved in the metabolism of cell wall synthesis (Al-Khafaji and Fayyhi ,2005).

3.5 Detection of *blaZ* in *S. aureus* isolates

Figure (3) referred to PCR product of *blaZ* gene which is 173 bps. The results showed that 18 isolates (90%) were harboring the *blaZ* gene, Similar results obtained by Yang *et al*., (2015) who found that 35(94%) of *S. aureus* were *blaZ* carrier. Also, Zmantar *et al*., (2013) mentioned that 59(100%) of *S. aureus* isolated from nasal cavity in pediatric service were *blaZ* positive. The activated *blaZ* could encode β-lactamase enzyme (penicillinase), which inactivates the antibiotic through hydrolysis of the peptide bond in the β-lactam ring. In this study, not all the penicillin-resistant *S. aureus* isolates exhibited genotypic resistance to penicillin (2 isolates). This is in agreement with data from Gao *et al*., (2012) showing that no resistance genes could be determined in some phenotypically resistant *S. aureus* isolates. In some isolates, phenotypic resistance may have been caused by point mutations rather than gene acquisition. Additionally, except for the general resistance mechanisms other pathways such as biofilm formation may play a major role in the resistance mechanisms (Croes *et al*., 2009).

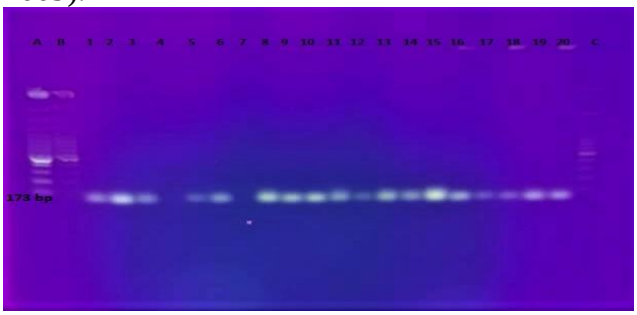

Figure 3: PCR gene product of *blaZ* gene

Our study confirms the usefulness of PCR assay for the detection of antibiotic resistance genes associated with *S. aureus* infections. The

PCR assay offers a rapid, simple, and accurate identification of antibiotic resistance profiles and could be used in clinical diagnosis as well as for the surveillance of the spread of antibiotic resistance determinants in epidemiological studies. Classical methods and molecular approaches especially PCR based techniques were more effective when used together and could provide more accurate and reliable information. Laboratory methods used to detect multidrug resistant bacteria such as MRSA should have high sensitivity and specificity.

CONCLUSION

In conclusion our study confirms the usefulness of PCR assay for the detection of antibiotic resistance genes associated with *S. aureus* infections.

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On the Stability and Convergence of linear Volterra-Fredholm Integral Equations of the Second Kind

Nejmaddin A. Sulaiman[1], Talaat I. Hasan[2], Shwan H. Hussein[3] and Shaharuddin Salleh[4]

1- Department of Mathematics, College of Education, Salahaddin University, Erbil, Kurdistan Region, Iraq.

2- Department of Mathematics, College of Basic Education, Salahaddin University, Erbil, Kurdistan Region, Iraq.

3- Physics Department, Faculty of Education and Natural Sciences, Charmo University, Sulaimani, Kurdistan Region, Iraq.

4-Department of Mathematical Sciences, College of science, Universiti Technologi Malaysia, Johor Bahru, *Malaysia.*

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***Corresponding Author:**
Talaat I. Hasan[2]
talhat.hassan@su.edu.krd

A B S T R A C T

In this paper, we consider linear Volterra-Fredholm integral equations of the second kind (LVFIE-2), we propose stability and convergence of it, also, some theorems are introduced for convergence of the iterative technique, and drive the sufficient condition for convergence of the iteration approach. It is supported by numerical examples for illustrating the usefulness of the iterative.

1. INTRODUCTION

The mathematical description of integral equation is a significant subject in mathematics and different area of science because many problems of mathematical physics, theory of elasticity and mixed problems of mechanics of continuous media reduce to an integral equation (Chen 2013, Zarebnia 2014), integral equation has a main role in mathematics because it appear naturally in many field of applied mathematics (Ibrahim 2016, Sahu 213).

The treatment schemes for solving integral equations have been arisen in the last resent years. Several techniques are introduced to achieve this goal. The numerical methods of integral equation are usefully for solving many problems in mathematic physics (Maleknejad 2011, Porshokouhi 2011). Integral equations have many advantages witnessed by the

increasing frequency of the integral equations in the literature and in many areas because some class problems have their mathematical representation appear directly in forms of it (Mathura 2011, Mustafa 2014].

Many methods with enough accuracy and efficiency have been used before by many researches to estimate the solution of integral equation (Dobritoiu 2015, Mustafa 2014, Porshokouhi 2011, Zarebnia 2014). Saadati in 2009 studied approach Variational iteration method for integral equation. Porshokouhi in 2011 used Variational iteration method for solving system of Volterra integral equations of the first kind. Ghanbari in 2015 solved Volterra and Fredholm integral equations of the second kind by this method. Hasan in 2017 studied numerical solution of VFIE-2.

To extend this work further we study Stability and convergence of linear Volterra-Fredholm integral equations of the second kind.

2. DEFINITIONS AND THEOREMS

In this section, we discuss some definitions and theorems for the main subject.

Definition 1 (Hasan 2017, Mustafa 2014).

The integral equation

$$y(s)=f(s)+\lambda\int_a^s k(s,t)y(t)\,dt+\lambda^*\int_a^b g(s,t)y(t)\,dt \quad (1)$$

is called linear Volterra-Fredholm integral equation of the second kind where λ and λ^* *are* arbitrary constants, functions $f(s)$, $k(s,t)$ and $g(s,t)$ are known functions on $W=\{(s,t), a\le t<s\le b\}$ and $y(s)$ is unknown function.

Definition 2 (Ibrahim 2016**,** Salleh 2017)

The iteration form

$$y_0(s)= f(s), \text{ for } r\ge 0$$

$$y_{r+1}(s)=T(y_r(s))=f(s)+\lambda\int_a^s k(s,t)y_r(t)\,dt+\lambda\int_a^b g(s,t)y_r(t)\,dt, \quad (2)$$

is called iterative approach for Volterra-Fredholm integral equation of the second kind which generated the sequence $\{y_r(s)\}_{r=0}^{\infty}$.

Definition 3 (Jerri 1985).

Let (X,d) be a metric space and A be a subset of X with the mapping $T: A \to X$, and $T(u)=v$, an element $p\in A$ is a fixed point of mapping T if $T(p)=p$.

Definition 4 (Saadati 2009**)**

Let $(X, \|\cdot\|)$ be a Banach space, T is a map defined by $T: X \to X$ and $y_{r+1}(s) = T(f, y_r(s))$ be the iteration form, the error defined by $e_r = \|y_{r+1} - T(f, y_r)\|$ if $\lim_{r\to\infty} e_r = 0$ implies that $y_{r+1}(s)$ converges to a fixed point p, then the iteration form $y_{r+1}(s) = T(f, y_r(s))$ is called T – stable.

Definition 5 (Jerri 1985).

The mapping T is called contractive if there is a nonnegative real number $0<\alpha<1$, such that for each $x, y\in A$ we have

$$d(T(x),T(y))\le \alpha d(x,y)$$

Theorem 1. (Density theorem of real number) **(**Lafferriere 2015)

Between any two real number their exist another real number.

Theorem 2. If $(X, \|.\|)$ be a Banach space and $T: X \to X$ satisfying

$$\|T(p)-T(q)\| \leq L\|p-T(p)\|+\beta\|p-q\|, \quad (3)$$

for all $p, q \in X$ where $L \geq 0$, $0 < \beta < 1$ then T has a fixed point. Further that it is T - stable.

Proof: We want to prove that the mapping T has a fixed point, for each $m, n \in N$, we have

$$|T(p_m(s))-T(p_n(s))| = |p_{m+1}(s)-p_{n+1}(s)|$$

$$= \Big| f(s)+\lambda\int_a^s k(s,t)p_m(t)dt + \lambda\int_a^b g(s,t)p_m(t)dt - [f(s)+\lambda\int_a^s k(s,t)p_n(t)dt + \lambda\int_a^b g(s,t)p_n(t)dt]\Big|$$

$$= \Big|\lambda\int_a^s k(s,t)p_m(t)dt - \lambda\int_a^s g(s,t)p_n(t)dt + \lambda\int_a^b k(s,t)p_m(t)dt - \lambda\int_a^b g(s,t)p_n(t)dt\Big|$$

$$= \Big|\lambda\int_a^s k(s,t)p_m(t)dt + \lambda\int_a^b g(s,t)p_m(t)dt - \lambda\int_a^s k(s,t)p_n(t)dt - \lambda\int_a^b g(s,t)p_n(t)dt\Big|$$

$$= \Big|\int_a^s \lambda k(s,t)[p_m(t)-p_n(t)]dt + \lambda\int_a^b g(s,t)[p_m(t)-p_n(t)]dt\Big|$$

$$\leq \Big|\lambda\Big|\int_a^s\int_a^s k^2(s,t)dtds\Big| \, |p_m(s)-p_n(s)| + |\lambda|\Big|\int_a^b\int_a^b g^2(s,t)dtds\Big| \, |p_m(s)-p_n(s)|$$

$$\leq \Big|\lambda\Big|\int_a^s\int_a^s k^2(s,t)dtds\Big|^{\frac{1}{2}} |p_m(s)-p_n(s)| + \Big|\lambda\Big|\int_a^b\int_a^b g^2(s,t)dtds\Big|^{\frac{1}{2}} |p_m(s)-p_n(s)|$$

Therefore, if

$$|\lambda| < [[\int_a^s\int_a^s k^2(s,t)dtds]^{\frac{1}{2}} + [\int_a^b\int_a^b g^2(s,t)dtds]^{\frac{1}{2}}]^{-1} \quad (4)$$

Since $a \leq t \leq s \leq b$.

Then by theorem 1. There exist a real number t_1 such that $s \leq t_1 \leq b$,

$[\int_a^{t_1}\int_a^{t_1} k^2(s,t)dtds]^{\frac{1}{2}} \leq M_1$ and

$[\int_a^b\int_a^b g^2(s,t)dtds]^{\frac{1}{2}} \leq M_2$ where

$M_1, M_2 \in R^+$,

$\beta = |\lambda|[\int_a^s\int_a^s k^2(s,t)dtds]^{\frac{1}{2}} + \int_a^b\int_a^b g^2(s,t)dtds]^{\frac{1}{2}}]$

$\beta = |\lambda|[M_1 + M_2]$,

we obtain

$|T(p_m(s))-T(p_n(s))| \leq \beta \|p_m(s)-p_n(s)\|$ and

$L|p-T(p)| = 0 \quad (5)$

Equation (4) is the sufficient condition of convergence

Therefore putting $L = 0$ and

$\beta = |\lambda|[[\int_a^s\int_a^s k^2(s,t)dtds]^{\frac{1}{2}} + [\int_a^b\int_a^b g^2(s,t)dtds]^{\frac{1}{2}}]$

in inequality (3),

we get

$\|p-T(p)\| = 0, \Rightarrow p = T(p),$

then we obtain T has a fixed point p.

$|T(p_m(s))-T(p_n(s))| \leq \beta |p_m(s)-p_n(s)|$

Then the nonlinear mapping T is T – stable.

Note that, we can state the following results.

Theorem 3. Suppose that this iteration method holds

$$y_0(s) = f(s),$$

$$y_{r+1}(s) = T(y_r(s)) = f(s) + \lambda \int_a^s k(s,t) y_r(t)\, dt + \lambda \int_a^b g(s,t) y_r(t)\, dt, \quad (6)$$

which generate the sequence $\{y_r(s)\}_{r=0}^{\infty}$, let

$$|\lambda| \le [[\int_a^s \int_a^s k^2(s,t)\, dtds]^{\frac{1}{2}} + [\int_a^b \int_a^b g^2(s,t)\, dtds]^{\frac{1}{2}}]^{-1}$$

.If $L = 0$ and

$$\beta = |\lambda|[\int_a^{t_1} \int_a^{t_1} k^2(s,t)\, dtds]^{\frac{1}{2}} + [\int_a^b \int_a^b g^2(s,t)\, dtds]^{\frac{1}{2}},$$

then T in the norm space $L^2(a,b)$ is T - stable.

Proof: - the proof of this theorem is similar to proof of theorem 2.

Theorem 4. Let $\{y_r(s)\}_{r=0}^{\infty}$ be a sequence generate by the iteration approach

$$y_0(s) = f(s),$$

$$y_{r+1}(s) = T(y_r(s)) = f(s) + \lambda \int_a^s k(s,t) y_r(t)\, dt + \lambda^* \int_a^b g(s,t) y_r(t)\, dt,$$

for $r = 0, 1, 2, 3, \ldots,$ max,

$$\int_a^{t_1} \int_a^{t_1} k^2(s,t)\, dtds = M_1^2 < \infty,$$

and

$$\int_a^b \int_a^b g^2(s,t)\, dtds = M_2^2 < \infty,$$

$f(s) \in L^2(a,b)$,

Then the sequence $\{y_r(s)\}_{r=0}^{\infty}$ is convergent to the exact solution of LVFIE-2, in the norm $L^2(a,b)$.

Proof. We want to prove that the sequence generate by the iteration approach in equation (2) is convergence to the exact solution of LVFIE-2. Form the iterative technique we have

$$y_{m+1}(s) = f(s) + \lambda \int_a^s k(s,t) y_m(t)\, dt + \lambda \int_a^b g(s,t) y_m(t)\, dt$$

$$y_{n+1}(s) = f(s) + \lambda \int_a^s k(s,t) y_n(t)\, dt + \lambda \int_a^b g(s,t) y_n(t)\, dt$$

Then we get

$$|y_{m+1}(s) - y_{n+1}(s)| = |T(y_m(s)) - T(y_n(s)))|$$

$$= \| f(s) + \lambda \int_a^s k(s,t) y_m(t)\, dt + \lambda \int_a^b g(s,t) y_m(t)\, dt -$$

$$[f(s) + \lambda \int_a^s k(s,t) y_n(t)\, dt + \lambda \int_a^b g(s,t) y_n(t)\, dt] \|$$

$$= \| \lambda \int_a^s k(s,t) y_m(t)\, dt - \lambda \int_a^s g(s,t) y_n(t)\, dt +$$

$$\lambda \int_a^b k(s,t) y_m(t)\, dt - \lambda \int_a^b g(s,t) y_n(t)\, dt] \|$$

$$= \| \lambda \int_a^s k(s,t) y_m(t)\, dt + \lambda \int_a^b g(s,t) y_m(t)\, dt -$$

$$\lambda \int_a^s k(s,t) y_n(t)\, dt - \lambda \int_a^b g(s,t) y_n(t)\, dt] \|$$

$$= |\lambda \int_a^s k(s,t)[y_m(t) - y_n(t)]\, dt + \lambda \int_a^b g(s,t)[y_m(t) - y_n(t)]\, dt|$$

$$\le |\lambda| \| \int_a^s k(s,t)\, dt \| y_m(s) - y_n(s) | + |\lambda| \| \int_a^b g(s,t)\, dt | y_m(s) - y_n(s) \|$$

$$\le |\lambda| [\int_a^s \int_a^s k^2(s,t)\, dtds]^{\frac{1}{2}} | y_m(s) - y_n(s) | + |\lambda| [\int_a^b \int_a^b g^2(s,t)\, dtds]^{\frac{1}{2}} | y_m(s) - y_n(s) |$$

$$\le |\lambda| [\int_a^s \int_a^s k^2(s,t)\, dtds]^{\frac{1}{2}} + \int_a^b \int_a^b g^2(s,t)\, dtds]^{\frac{1}{2}}] | y_m(s) - y_n(s) |,$$

since $\int_{a}^{t_1}\int_{a}^{t_1} k^2(s,t)\,dtds = M_1^2 < \infty$ and

$\int_{a}^{b}\int_{a}^{b} g^2(s,t)\,dtds = M_2^2 < \infty$,

Then $[\int_{a}^{t_1}\int_{a}^{t_1} k^2(s,t)\,dtds]^{\frac{1}{2}} = M_1$ and

$[\int_{a}^{t_1}\int_{a}^{t_1} g^2(s,t)\,dtds]^{\frac{1}{2}} = M_2$,

We get

$$|\lambda| < [[\int_{a}^{s}\int_{a}^{s} k^2(s,t)\,dtds]^{\frac{1}{2}} + [\int_{a}^{b}\int_{a}^{b} g^2(s,t)\,dtds]^{\frac{1}{2}}]^{-1}$$

$$= [M_1 + M_2]^{-1}$$

hence we obtain

$$|T(y_m(s)) - T(y_n(s))| \le \lambda |y_m(s) - y_n(s)|$$

Then the sequence generate by the iteration technique in equation (6) is convergence to the exact solution of LVFIE-2.

Corollary. If the iteration method holds

$$y_0(s) = f(s),$$

$$y_{r+1}(s) = T(y_r(s)) = f(s) + \lambda\int_{a}^{s} k(s,t)y_r(t)\,dt + \lambda^*\int_{a}^{b} g(s,t)y_r(t)\,dt,$$

for $r = 0, 1, 2, 3, \ldots, \max$, let

$$|\lambda| \le [[\int_{a}^{s}\int_{a}^{s} k^2(s,t)\,dtds]^{\frac{1}{2}} + [\int_{a}^{b}\int_{a}^{b} g^2(s,t)\,dtds]^{\frac{1}{2}}]^{-1}.$$

$L = 0$ and

$$\beta = |\lambda|[\int_{a}^{t_1}\int_{a}^{t_1} k^2(s,t)\,dtds]^{\frac{1}{2}} + \int_{a}^{b}\int_{a}^{b} g^2(s,t)\,dtds]^{\frac{1}{2}}]$$

Then in the norm space $L^2(a,b)$ the stability of T is sufficient condition for the iteration form in equation (6) which convergent to the solution of LVFIE-2.

Proof. Is similar to theorem 4.

Theorem 5.

Let (X, d) be a complete matric space and let the mapping $T: X \to X$, be a contraction then T has exactly one fixed point.

Proof: - suppose that there are two fixed points u and v.

Then $T(u) = u$ and $T(v) = v$, since $u \neq v$,

then the distance between them is not zero, $d(u,v) \neq 0$

since u and v are fixed points of T, also we have

$$d(T(u), T(v)) = d(u,v) \neq 0 \quad (7)$$

we have, by definition of contractive mapping

$$d(T(u), T(v)) \le \alpha d(u,v), \text{ where } 0 \le \alpha < 1 \quad (8)$$

From equations (7), (8), we obtain

$$d(u,v) = d(T(u), T(v)) \le \alpha d(u,v) \neq 0$$

Then $(1-\alpha)\,d(u,v) \le 0$

By suppose we have $d(u,v) > 0$, $(1-\alpha) \le 0$

Then we obtain $1 < \alpha$, which is contradiction to the definition of contractive mapping because is strictly less than 1.

Hence the distance between u and v must be identically zero, $d(u,v) = 0$, then we get $u = v$

Which proves the uniqueness of the fixed point when it exists.

3. TEST NUMERICAL EXAMPLES

The following two examples verify and satisfy all conditions of theorem (2) then T has fixed point and it is T - stable, we present some test examples to show that the sufficient

condition for the convergence in the norm of $L^2(a,b)$ to the exact solution of LVFIE-2.

Example (1) Consider the integral equation

$$y(s) = s\cos(s) + 0.223244 + \int_0^s (t)y(t)\,dt - \int_0^1 (t^2)y(t)\,dt,$$

And $\{y_r(s)\}_{r=0}^{\infty}$ be a sequence generated by iteration form $y_{r+1}(s) = T(y_r(s))$ where T nonlinear mapping is then the sequence $\{y_r(s)\}_{r=0}^{\infty}$ convergent to the exact solution of integral equation and the iteration is T – stable. Where the exact solution is $y(s) = \sin(s)$.

Solution: - We have $\lambda = 1$, $k(s,t) = t$ and $g(s,t) = t^2$, since $0 \le t \le s$ and $0 \le s \le 1$. Then by theorem 1. There exist a real number let $t_1 = 0.8$ such that $t_1 = 0.8 < 1$,

$$\left[\int_0^{0.8}\int_0^{0.8} k^2(s,t)\,dtds\right]^{\frac{1}{2}} = \left[\int_0^{0.8}\int_0^{0.8} (t)^2\,dtds\right]^{\frac{1}{2}} = \left(\frac{(0.8)^4}{3}\right)^{\frac{1}{2}} = (0.1365)^{\frac{1}{2}} = 0.3695$$

and

$$\int_0^1\int_0^1 g^2(s,t)\,dtds = \left[\int_0^1\int_0^1 (t^2)^2\,dtds\right]^{\frac{1}{2}} = (0.2)^2 = 0.1414$$

Now

$$|\lambda|\left[\int_a^s\int_a^s k^2(s,t)\,dtds\right]^{\frac{1}{2}} + \int_a^b\int_a^b g^2(s,t)\,dtds\Big]^{\frac{1}{2}} = |\lambda|(0.3695 + 0.1414) = 0.5109 \quad |\lambda| < 1$$

$$|\lambda| < \frac{1}{0.5109} = 1.957330$$ Then the converges interval is $-1.957330 < \lambda < 1.957330$

$$\beta = |\lambda|\left[\int_a^{t_1}\int_a^{t_1} k^2(s,t)\,dtds\right]^{\frac{1}{2}} + \int_a^b\int_a^b g^2(s,t)\,dtds\Big]^{\frac{1}{2}} = 0.5109 < 1$$

Then the stable interval is $0 < \beta < 1$, then the stable interval is subset of converges interval.

The sequence $\{y_r(s)\}_{r=0}^{\infty}$ generate by the iteration form

$$y_{r+1}(s) = T(y_r(s))$$

$$= s\cos(s) + 0.223244 + \int_0^s (t)y(t)\,dt - \int_0^1 (t^2)y(t)\,dt$$

Convergent to the exact solution $y(s) = \sin(s)$, if $\beta = 0.5109$

We have $\|T(y_m(s)) - T(y_n(s))\| = |y_{m+1}(s) - y_{n+1}(s)|$

$$\le \left[\int_0^s\int_0^s (t)^2\,dtds\right]^{\frac{1}{2}} |[y_m(s) - y_n(s)]| + \left[\int_0^b\int_0^b (t^2)^2\,dtds\right]^{\frac{1}{2}} \|y_m(s) - y_n(s)\|$$

$$\le \beta \|y_m(s) - y_n(s)\|,$$

If we put $L = 0$ and $\beta = 0.5109$, then it is satisfied all conditions of theorem (2).

Hence T has a fixed point and it is T – stable.

Example (2) Consider the integral equation

$$y(s) = \frac{-1}{56}s^8 + s^6 + \frac{5}{6}s^3 - \frac{83}{24}s + \int_0^s (s-t)y(t)\,dt - \int_0^1 (st)y(t)\,dt,$$

And $\{y_r(s)\}_{r=0}^{\infty}$ be a sequence generated by iteration form $y_{r+1}(s) = T(y_r(s))$ where T is nonlinear mapping then the sequence $\{y_r(s)\}_{r=0}^{\infty}$ convergent to the exact solution of integral equation and the iteration is T – stable. Where the exact solution is $y(s) = s^6 - 5s$.

Solution: - We have $\lambda = 1$, $k(s,t) = s - t$ and $g(s,t) = st$, since $0 \le t \le s$ and $0 \le s \le 1$. Then by

theorem1, there exist a real number let $t_1 = 0.99$, such that $t_1 = 0.9 < 1$,

$$[\int_0^{0.9}\int_0^{0.9} k^2(s,t)\,dt\,ds]^{\frac{1}{2}} = [\int_0^{0.9}\int_0^{0.9}(s-t)^2\,dt\,ds]^{\frac{1}{2}} = 0.1094,$$

and

$$\int_0^1\int_0^1 g^2(s,t)\,dt\,ds = [\int_0^1\int_0^1 (st)^2\,dt\,ds]^{\frac{1}{2}} = [\int_0^1\int_0^1 (s^2t^2)\,dt\,ds]^{\frac{1}{2}} = (\frac{1}{9})^{\frac{1}{2}} = \frac{1}{3},$$

now

$$|\lambda|[\int_a^s\int_a^s k^2(s,t)\,dt\,ds]^{\frac{1}{2}} + \int_a^b\int_a^b g^2(s,t)\,dt\,ds]^{\frac{1}{2}} =$$

$$|\lambda|(0.1094 + \frac{1}{3}) = 0.4427 \quad |\lambda| < 1,$$

$|\lambda| < \frac{1}{0.4427} = 2.2589$ Then the converges interval is $-2.2589 < \lambda < 2.2589$

$$\beta = |\lambda|[\int_a^{t_1}\int_a^{t_1} k^2(s,t)\,dt\,ds]^{\frac{1}{2}} + \iint_a^b\int_a^b g^2(s,t)\,dt\,ds]^{\frac{1}{2}} = 0.4427 < 1,$$

The stable interval is $0 < \beta < 1$, then the stable interval is subset of converges interval.

The sequence $\{y_r(s)\}_{r=0}^{\infty}$ generate by the iteration form

$$y_{r+1}(s) = T(y_r(s)) =$$

$$\frac{-1}{56}s^8 + s^6 + \frac{5}{6}s^3 - \frac{83}{24}s + \int_0^s (s-t)y(t)\,dt - \int_0^1 (st)y(t)\,dt,$$

Convergent to the exact solution

$y(s) = \sin(s)$, if $\beta = 0.4427$,

We have $|T(y_m(s)) - T(y_n(s))| \le |y_{m+1}(s) - y_{n+1}(s)|$

$$\le |\lambda|[\int_0^s\int_0^s (s-t)^2\,dt\,ds]^{\frac{1}{2}}|[y_m(s) - y_n(s)]| + |\lambda^*|[\int_0^b\int_0^b (st)^2\,dt\,ds]^{\frac{1}{2}}|y_m(s) - y_n(s)|$$

$$\le \beta\|y_m(s) - y_n(s)\|,$$

If we put $L = 0$ and $\beta = 0.4427$, then it is satisfied all conditions of theorem (2).

Hence T has a fixed point and it is T – stable.

4- CONCLUSION

In this work, we studied the stability and convergent of Volterra-Fredholm integral equations of the second kind and obtained the sufficient condition of convergence. Several numerical examples were tested for supporting the iterative technique. The mentioned examples demonstrated the validity and applicability of the iterative techniques. Finally, we conclude that the stabile interval is a subset of converges interval, the nonlinear mapping T has a fixed point and it is T – stable.

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Studying of De-wetting of a PS/PMMA Polymer Bilayer

Bestoon T. Mustafa[1], Diyar A. Rasul[2]

1- Department of Physics, College of Education, Salahaddin University/ Erbil-Kurdistan Region, Iraq.

2- Department of Physics, College of Education, Salahaddin University/ Erbil-Kurdistan Region, Iraq.

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***Corresponding Author:**

Bestoon T. Mustafa

bestoon.mustafa@su.edu.krd

A B S T R A C T

The surface morphology evolution and de-wetting properties were studied of polystyrene (PS), poly(methyl methacrylate) (PMMA) thin films, and thin PS/PMMA bilayer films above silicon substrates by using Optical microscopy approach. A PS film with 18.66 nm, PMMA with 82.5 nm and the bilayer of PS/PMMA with 85.33 nm (before annealing) have been deposited, shown a fit match with the model for the both single film and a good match for the bilayer film. The heated bilayer above the glass transition temperature resulted metastable surface morphologies and growing nucleations of PS above PMMA surface, surface de-wetting were absorbed. A longer annealing time led the film producing a network above the blower layer- phase separation occurred.

1. INTRODUCTION

Long before understanding dynamics and thermodynamics behaviour, polymer thin films were used widely in many applications such as coatings to photoresists, dielectrics, adhesives, and optical elements [1] due to reduce of production requirements and low cost [2]. Up to nowadays, Polymer properties such as fracture toughness and adhesion were also improved significantly. While not long after introducing experimental as well as theoretical tools [3-7], dynamic behaviour and thermodynamic properties of polymer thin films have become increasingly recognised [2]. However, other shortcoming including surface and interface interactions and phase-separation of polymer blend bilayer thin films are still challenging researches [8, 9]. For polymer blend, the type of interface and the

nature of polymers determine the morphology of the film such as smoothness or roughness [2, 10].

In general, most of polymers are immiscible to each other [11]. The phase separation degree of blended polymer films will affect the domain morphology, which then affects the electrical and mechanical properties of the final structure of the polymer films [12]. It has been shown that de-wetting and interplay between phase separations control the surface morphology in a weak incomputable system. Yet, in strong immiscible system (i.e PS and PMMA) morphology of the surface have been widely investigated [13, 14].

Lately, to understand the de-wetting behaviour of both liquid-liquid and liquid - solid faces, several efforts were performed experimentally and theoretically. 'De-wetting is a process in which a uniform thin polymer film becomes unstable and breaks up into droplets (holes are formed) on a non-wettable surface (polymer or substrate) when it is heated above the glass transition temperature (Tg) [1, 15]. The major importance of Polymer thin films, deposited above the glass substrates, is that where they can be employed in several technological applications including optical elements and, also as dielectric materials [10]. The characteristic proceeds of de-wetting starts by producing holes somewhere at the thin film, the holes grow steadily and produce coalesce, and finally droplets are formed above the below layer [1]. The long range force (Van der Waals forces) may stabilise the film surface against de-wetting. This force for the liquid-liquid de-wetting relies on the thickness of the respective liquid layers, the liquid viscosity and involved interfacial and surface tension. There are two mechanisms proposed for de-wetting [15]: spinodal de-wetting and, nucleation and growth. In spinodal de-wetting mechanism, the fluctuation in density of the polymer cause capillary waves at the surface of the film and those waves initiate holes at the surface of the polymer film. This is due to altering amplitude of capillary wave, upon the substrate contact. In contrast, in the nucleation and growth mechanise, the impurities in the film and/or defects at the substrate initiate the holes at the polymer film surfaces.

To deposit a polymer thin film, of either polymer bilayers or a polymer blend, a variety of techniques can be used for instance dip-casting, floating or spin coating approaches [1]. For a good device performance, the thin film stability and spreading out the polymer films are highly critical. Therefore, obtaining these above rely on several factors as well as the polymer and substrate properties [15] (see figure 1). The thin film stability and spreading uniformly on the substrate are paid more attention to produce a uniform film after

annealing. Spin coating approach is one of the cheapest, easiest and cleanest approaches to use to deposit polymer thin films. However, structures which obtained due to spin coating technique will not state at thermodynamically equilibrium if the structure faces a rapid evaporation during the spin coating process [12]. As it was mentioned earlier, the final structure properties of the thin film depends mainly on the film morphology, and the initial morphology depends on polymer composition, film thickness, cast solvent, depositing and annealing temperature, chain of groups, and etc. [16-20].

Both polymers, PS and PMMA, are chosen as ideal polymers for de-wetting study. This is due to having almost the same glass transition temperature and easy to work with [21]. A blend and a bilayer of PS and PMMA polymers have been deposited above the substrate. Different substrates were used such as (Si, SiOx, Au) for PS/PMMA bilayers, with melting PS and PMMA in different solvents [12]. It was also showed that Producing domains above the film surface depend on substrates. The bilayer might be PS on PMMA or PMMA on PS. Previously, researches were conducted on PS/PMMA films as bulk and blended composite, however, less work has been done to see the de-wetting properties of these polymers as they come together in a bilayer structure. In this study, de-wetting properties will be studied of PS from PMMA, above the silicon oxide substrate, as a function of annealing temperature by using optical microscopy technique. The bilayer will be heated above Tg. This consequence separating the two faces.

2. MATERIALS AND METHODS

The substrates were rinsed with toluene for 20 min. substrates were then dried under a steam of nitrogen gas. Choosing a proper solvent is critical to well-dissolve the precursor. To prepare the polymer solutions, 0.04g of PS was dissolved in 2mL of toluene and only 0.0116g of PMMA was dissolved in 2mL of acetic acid. Films of both PS and PMMA were then deposited above SiO substrates, silicon substrates were oxidized in one side and created about 1.5nm above the Si substrates. The Oxidation helps to deposit a thin film with morphology less than 1nm. To avoid dissolving quickly, only PMMA solutions were heated at 50^0C, before depositing, to help mixing the solution well. Thin films of PS and PMMA were deposited via spin coating onto cleaned Silicon substrates.

Thin films Spin castings have been achieved at 2500 rpm for 60 seconds. After depositing the PS film on top of the silicon oxide substrate, it was quenched at 50^0C for 5 minutes, to evaporate the solvent atoms. Then, a PMMA film has been deposited on

top of the quenched PS film; this is achieved at the same deposition condition. In addition, and to compare the single film thickness of both polymers to the thickness of the polymer bilayer, two other single thin films of PS and PMMA were spin coated separately above the substrate. For measuring the thin films thickness, Ellipsometry has been employed. 'Ellipsometry is an optical technique to measure thin films characteristics such as the thickness, very accurately' [22]. The thicknesses were measured of PS, PMMA and the bilayer films (18.66nm, 82.5nm and 85.33nm) respectively. The bilayer (85.33nm thick) was then annealed from (100 to 163) ^{0}C. Optical microscopy was employed to observe the de-wetting behavior of the bilayer thin polymer films and phase separation above the T_g (~103 and 106^{0}C) for PS and PMMA respectively.

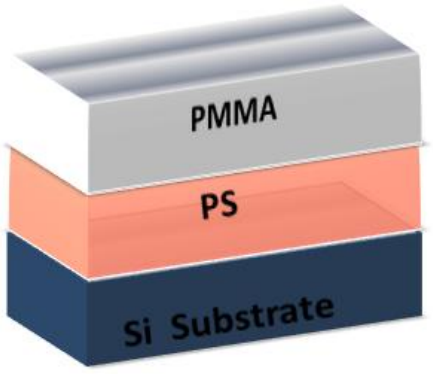

Figure (1): PS/PMMA bilayer deposited on Si substrates.

3. RESULTS AND DISCUSSION

Figure 2 shows the optical microscopy of PS/PMMA films spin coated onto silicon substrates annealed at different temperature. Figure 2a, a bilayer of PS/PMMA of 85.33nm (before annealing) has been deposited above silicon substrate (the thickness reduced to only a 2nm thick after annealing above the glass transition temperature). The bilayer appears with a good morphology unless some holes and nucleation are observed at the film surface. These holes may be due to impurities in the polymer structure or substrate defects that changes the interfacial properties between the two surfaces (PS and PMMA). The surface shows zero face separation. Figure 2(b-d) are bilayers of PS/PMMA annealed from (100-163) ^{0}C. White hole like domains appears immediately as a function of annealing the bilayer. Domain produces and surface de-wetting are absorbed with optical microscopy above T_g (after 133^{0}C) which the upper layer (PMMA thin film) only is broken up and de-wet from the underlayer (PS film) without affecting the substrate layer. This is followed by nucleation and growing holes. A further annealing increases the density of white domains and stretching the dark part of the film. The network of the de-wetting layer improves in figure (2d) and aggregated holes (dark colour) are also rising up. The rich PMMA aggregation is not inconsistent with interpalner interaction [23]. These are initiated by long-range Van der Waals forces which is stabilising the film against de-wetting.

These two polymers are immiscible [11]. That means they become either unstable or metastable when one of the polymer is deposited above the other one. That may de-wet the bilayer due to one of the de-wetting mechanisms. By deposited PMMA on top of PS, it makes the de-wetting happens due to the spinodal de-wetting mechanism. Ralf et al, have shown that stability of PS layer can be tuned by changing the oxide layer. They showed a PS layer on top of a clean Si will be stable, whereas depositing a PS layer on thick oxide layer of Si may be metastable or unstable. Nevertheless, this property depends also on the film thickness. Li et al, explained that low thickness of PMMA films reduce and collapse the hole boundaries and produce a continuous PS structure in some area. They also studied the annealing time of PMMA and showed PS rich-phase (bright part, not figured here) as the upper layer is more annealed.

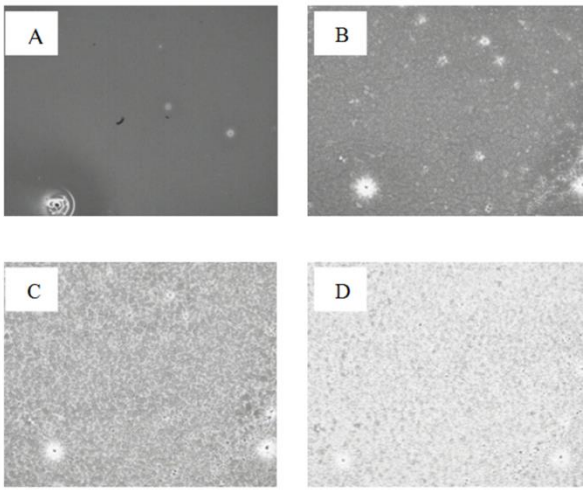

Figure (2): Optical micrographs of PS/PMMA films (2nm after annealing above T_g) as a function of annealing temperature. (a) non-annealed; (b) annealed at 100 (c) annealed above 145^0C for 27 min and (d) annealed at 165^0C for 37 min. The colour was changed steadily from a dark region that represents the PMMA layer to a white surface (PS) and a network of PMMA as a function of annealing temperature.

When the spreading coefficient (S) is negative, for a thin film above a substrate, holes grow, and this is given by young's equation (Equation 1). A part of the drawbacks, de-wetting property can be used advantageously in homo-polymer, copolymer film and a polymer bilayer to fabricate a tuneable morphology of a patterned surface [24]. The wettability of PS can be turned via the silicon oxide layer thickness.

$S = \gamma_B - (\gamma_{AB} + \gamma_A) \ldots\ldots\ldots (1)$ Young's equation [11]

Where γ_B and γ_A are surface energy of layer A and B respectively and γ_{AB} interfacial energy between the two surfaces.

Figure 3 shows the Ellipsometry parameters of psi (Ψ) and delta (Δ) fitted with the model. In Ellipsometry, the change of polarization is quantified via the ratio of amplitude which is known by ψ and the phase difference which is denoted by Δ. The value of ψ and Δ can be calculated by using Fresnel equations (Eq. 2). In addition, calculating these two parameters and matching them to the experimental data is a perfect approach to reveal the thickness parameters as well as optical constant of the samples [25].

$\rho = \psi\, e^{i\Delta}$ ….. (2)

Where ρ is the ratio of reflection coefficients. ψ and Δ are surface optical constant functions.

As it is seen in figure 3a and 3b, a PMMA was deposited above a SiO substrate and a PS layer was deposited above the SiO substrate, respectively. The experimental results of ψ (red colour) and Δ (green colour) parameters match to the model (black dashed lines). This is almost the same of depositing a bilayer of PS/PMMA and after quenching the films, the film parameters in ψ and Δ show up a good match to the model. However, a bit rescue of the ψ and Δ curve from the model line (black dash) reveals changing the optical properties of the film due to the de-wetting behaviour of the polymers. It is clear that, this happens when the film is annealed into above the glass transition temperature, which produces holes and domains above the film surface and changes the morphology of the bilayer.

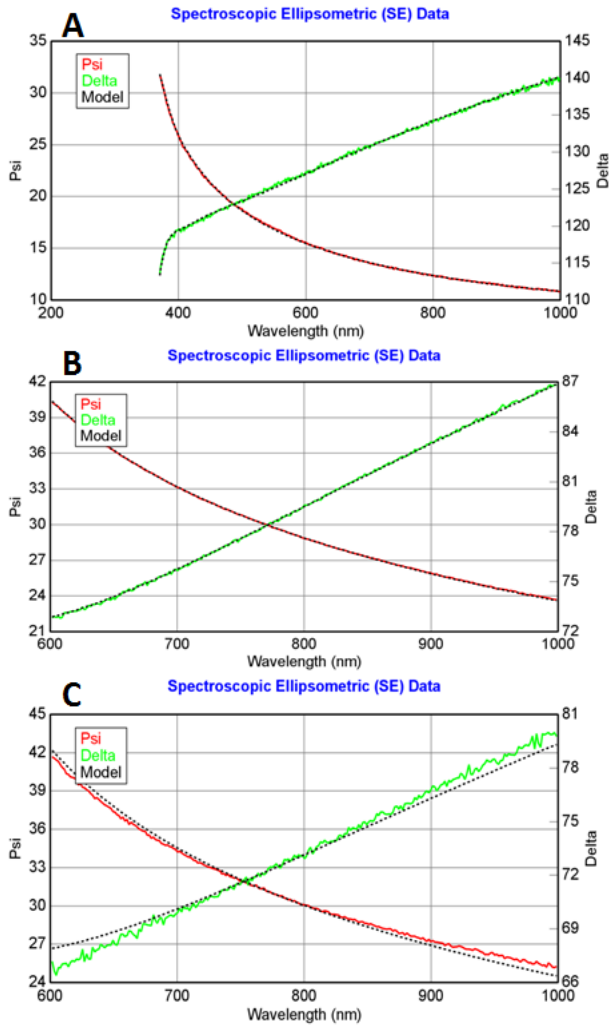

Figure (3): Ellipsometry parameters delta (green) and psi (red) and the corresponding fitted model (dashed black). Data from samples are taken 18.66nm thick of PMMA on SiO in (a), 82.50 nm thick of PS on SiO in (b) and a 2nm bilayer of PS/PMMA (after annealing) on SiO in (C).

4. CONCLUSION

In this article, the de-wetting of PS/PMMA has been studied via optical microscopy. The result showed that the PS and PMMA thin films are immiscible to each other and their phase separation appears when they are annealed above the T_g, when both polymers are deposited above each other. Two mechanisms of de-wetting were observed. The PMMA de-wetting as well as a network of PMMA (above PS film) was observed after the bilayer has been heated above the glass transition temperature. The sizes of the holes and the color were changed as temperature is raised behind the glass transition temperature. Annealing polymer surfaces at a certain temperature enhances the surface and interface properties of the bilayer films.

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New record genus and species of *Thanatophilus* Leach, 1815 (Coleoptera: Silphidae) from Iraq

Banaz S. Abdulla

Department of Biology, College of Education, Salahaddin University, Erbil, Kurdistan Region, Iraq.

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***Corresponding Author:**
Banaz S. Abdulla
banaz.abdulla@su.edu.krd

ABSTRACT

A new genus and species of carrion beetles, *Thanatophilus rugosus* (Linnaeus) is recorded for the first time in Iraq. Distinctive character of the adult male is illustrated. This species shows the posterior margin of labrum moderately emarginated, the last antennal segments (9-11gradually clubbed), Elytra with three carinas, and raised tubercles. paramere parallel, narrow at base, strongly expanded at outer margin of proximal, apical part acute. Median lobe narrows at proximal, expanding from proximal, widest at medio-proximal distinctly.

1. INTRODUCTION

Silphidae, carrion beetles consist of a small group of Coleoptera counting less than 200 species that are worldwide spread (Sikes, 2005). Silphids perform vital ecosystem functions; they promote the breakdown and recycling of organic matter into terrestrial ecosystems, most Silphids are carrion feeders (necrophagous species) but can also prey on other carrion inhabitants such as fly eggs or maggots and other small carrion beetles (necrophilous species) (Racliffe, 1996).

The genus *Thanatophilus* Leach, 1815, contains more than 24 described species distributed throughout the Holarctic and Africa (Schawaller 1981, Anderson and Peck 1985, Ratcliffe 1996). Five of them are found in North America (Anderson and Peck 1985, Peck and Miller 1993). *T. dentiger* (Semenov, 1890), *T. inutus* Kraatz, 1876 and *T. porrectus* (Semenov, 1890) are reported from India (Ruzicka, 2011). *T. dispar* (Herbs), and *T. sinuatus* (Fabricius) and *T. rugosus* (Linnaeus)

species are occurring in Italy (Angelini *et al*., 1995), the last one species also recorded in Spain (Garcia-Rojo 2004), Iran (Ghahri and Hava, 2015), Poland (Matuszewski *et al*. 2008) and Turkey (Altunsoy *et al*., 2017). There is no taxonomic study on this genus in Iraq only *Silpha arenaria* Kraatz and *Silpha laevigota* Fabricius are recorded which belong to the same family (Abdul- Rassuol, 1976).

Monophyletic Species of the *Thanatophilus* genus is well-supported in a Phylogenetic analysis of preliminary molecular data by (Dobler and Müller, 2000). Daniel *et al*., (2017) indicated that the Seven common species of the previous genus (*T. rugosus*, *T. sinuatus*, *T. mutilates*, *T. micans*,*T. lapponicus*, *T. dispar* and *T. trituberculatus*) can be distinguished by using 360 bp mtDNA sequence from the cytochrome oxidase I gene. They are found on rabbit, dog and pig carcasses during the active decay stage (Bourel *et al*., 1999; Garcia-Rojo 2004; Palanco, 2006; Matuszewski *et al*. 2008 and Dekeirsschieter *et al*.,2010).

Adults of *Thanatophilus* are characterized by black of a short row of long erect hairs behind the eyes, small eyes, labrum shallowly emarginate, and mesocoxae widely separated. They are smaller (8-15 mm long), often possessing pubescence on the dorsal surface of the pronotum. Male of most T. species have the sutural apices of the elytra rounded but not prolonged while of female prolonged and rounded, (Ratcliffe, 1996).

T. rugosus is a common species of Silphinae, distributed throughout the Palaearctic region (Schawaller 1981; Ruzicka 2002; Ruzicka and Schneider, 2002 and Lobel and Smetana, 2004). Ruzicka (2002) described *T. rugosus tuberculatus* Depoli, 1931 and *T. rubripes* Portevin, 1943 as junior subjective synonyms of *T. rugosus* (Linnaeus, 1758). Novak (1966) considered. *T. sinuatus* and *T. rugosus* are to be co-occurring species without temporally differentiated niches.The higher abundance of *T. rugosus* in autumn samples could indicate a temporal niche differentiation (Jakubec and Ruzicka, 2015). In another study of (Altunsoys *et al.*,2018) observed this species almost throughout the whole year and found in any season without habitat preference, the number of this species decreased with the reduction of insect activity in winter.

The main aim of this paper is a detailed description of the first record *Thanatophilus rugosus* (Linnaeus) from Iraq and to provide diagnoses of the species including male genital characters.

2. MATERIALS AND METHODS

Thanatophilus rugosus were collected on cat carcasses during 2017 in center of Erbil Governorate-Iraq. This paper based on 25 specimens, which collect by hand. The technique described by (Hammamurad *et al*., 2017) were used to examine the sample and to prepare the genitalia for study. A digital camera (Ucmas series microscope camera) was used to photograph the important parts the measured proportion of body parts are given in point of an eyepiece linear micrometer in binocular microscope.The species were identified with the help of available literature of (Ratcliffe, 1996; Ruzicka, 2002; Ozdemir and Sert, 2008 an Monk et al., 2016).

3. RESULTS

Thanatophilus rugosus (Linnaeus, 1758)

Synonyms

Silpharugosa Linnaeus, 1758

Thanatophilus rugosus tuberculatus Depoli, 1931

Thanatophilus rubripes Portevin, 1943

Thanatophilus ferrugatus (Solsky, 1874)

Thanatophilus sinuatus (Fabricius, 1775)

Silphaobscura orientalis Brulle, 1832

Description of the male (Fig.1).

Body length 9.6–12.5mm, black in color, oval, slightly convex, dorsal surface with densely short black setose. Antennae, legs and lateral portions of pronotum are reddish-brown.

Head. Semi globular, in dorsal view (Fig.1b) about (1.8-2.4) as wide as s long, densely, short black pubescence. Compound eyes prominent, oval, dark brown-black, diameter 0.8-1.2mm,

separated by distance of 4-5 times of eyes. Vertex and frons black, unpunctuated, high densely brown setoes, Fronto-clypeal suture absent. Labrum (Fig.2 a) brown, punctuate, sparsely pale yellow setose, posterior margin moderately emarginated, epiphrynx densely yellow setose. Mandibles (Fig. 2b) high sclerotized, semi triangular, apical bidenticates, molar area densely yellow setose, scrobe punctuate, sparsely yellow setoes. Maxillae (Fig. 2c) light brown, cardo semi globular, stipes black triangular, slightly long brown setose, galea elongate oval, densely long yellow setose, distal part of galea nearly hook like, proximal part high densely yellow setoes, lacinia basal part bare, apical part high densely fine long setoes. maxillary palps brown, 1^{st}-3^{rd}segments of maxillary palp nearly cup shaped, 2^{nd} segment 1.3 times as long as 3^{rd}; 4^{th} segment elongate oval 2.8 times as long as 1^{st} segment. Labium yellow, labial palps segments brown, 1^{st} segment glass shape, 2^{nd} segment cup shaped, 3^{rd} segment oval shape,1^{st} segment. 1.3 time as long as 2^{nd} segment, 1^{st} – 2^{nd} segment setoes, 3^{rd} seg. Paraglossa biloobed, yellow, high densely yellow fine setoes. Antennae(Fig.d) clavate, redish-brown with 11 antennomeres, widely spread and inserted on the lateral side of head, the antennomeres gradually widening into an apical club 9-11 segments,1^{st}antennomere cylindrical shaped 1.8 times as long as the 2^{nd}antennomere, 3^{rd}antennomere tubular shaped 1.5 times as long as 4^{th} - 8^{th}antennomere, 4^{th} -6^{th} antennomere globular, nearly same length, 7^{th} -8^{th} antennomeres nearly cup shaped, 8^{th}antennomeres 1.1 times as long as 7^{th}, the antennomeres sparsely short micro setose, 9^{th} -10^{th}antennomeres plate shaped , 9^{th} antennomeres1.1 times as long as 10^{th}, 11 antennomeres nearly triangular, 1.3 times as long as10^{th} .

Thorax. Pronotum (Fig.2f) Entirely black densely fine punctuate, pubescence with reddish-orange margin, semi-globrous shaped, widest toward the middle, Pubescence generally sparse, Anterior margin distinctly concave, posterior margin concave at the middle, anterior angle broadly round, posterior angle sub angulates. Scutellum triangulates, apical part acute, with some yellow pubescence.

Elytra (Fig.2 g) black, length 8.4-10.5mm, with three carina and posterior margin is straight, intercarina is irregularly wrinkled, and Interstices of elytra with raised tubercles, tubercles interspersed between costa, with large orange markings. Hind wing hyaline, stigma (S, Sc and R veins combined) elongated oval. Fore legs (Fig.2 e) dark brown, fore coxae oval, fore tibia cylindrical as long as femur, fore tarsus five segmented. 1^{st} -5^{th} segments tubular shaped, fore tarsi,1^{st} segment 2 times as long as 2^{nd} segment, 2^{nd} segmnt1.2 times as long as 3^{rd} segment, 3^{rd} as long as 4^{th} segment, 5^{th} segment 1.1time as long as 1^{st} segment. fore tibia outer margin with row (13-15) short spines, inner margin with row (6-7) short spines. Middle legs resemble to the fore legs except, the coxae semi globular, middle tibia bears moderate short white setose. Hind legs resemble to the fore legs except, the coxa bot shaped, clow simple, moderately curved.

Abdomen. Dark brown, nearly oval with six visible segments, abdominal sternites black, densely white short pubescence,1^{st} -5^{th} abdominal segment rectangular shape, posterior margin of 1^{st} -4^{th} straight, posterior margin of 5^{th} segment concave in lateral, posterior margin of 6^{th} segment rounded.

Male genitalia. Aedeagus yellow-dark yellow**,** length 2.6-3.2 mm**;** in dorsal view (Fig.2 h) strait, basal piece semi rounded, membranous outer margins slightly sclerotized, paramere parallel, narrow at base, strongly expanded at outer margin of proximal, apical part acute , the space between the apical is o.4 -0.6 mm**.** Median lobe narrow at proximal, expanding from proximal, widest at medio-proximal distinctly shorter than the paramer, strongly expanded from proximal and slightly narrowed through distal, In lateral view (Fig.2 i), slightly curved, basal piece triangular shaped. Paramere narrow at base, strongly expanded at outer margin of proximal, while slightly narrowing through distal, strongly narrowed through apical.

Figure- 1 Adult of *Thanatophilus rugosus* (Linnaeus,1758) (6X) a- Lateral view b- Dorsal view

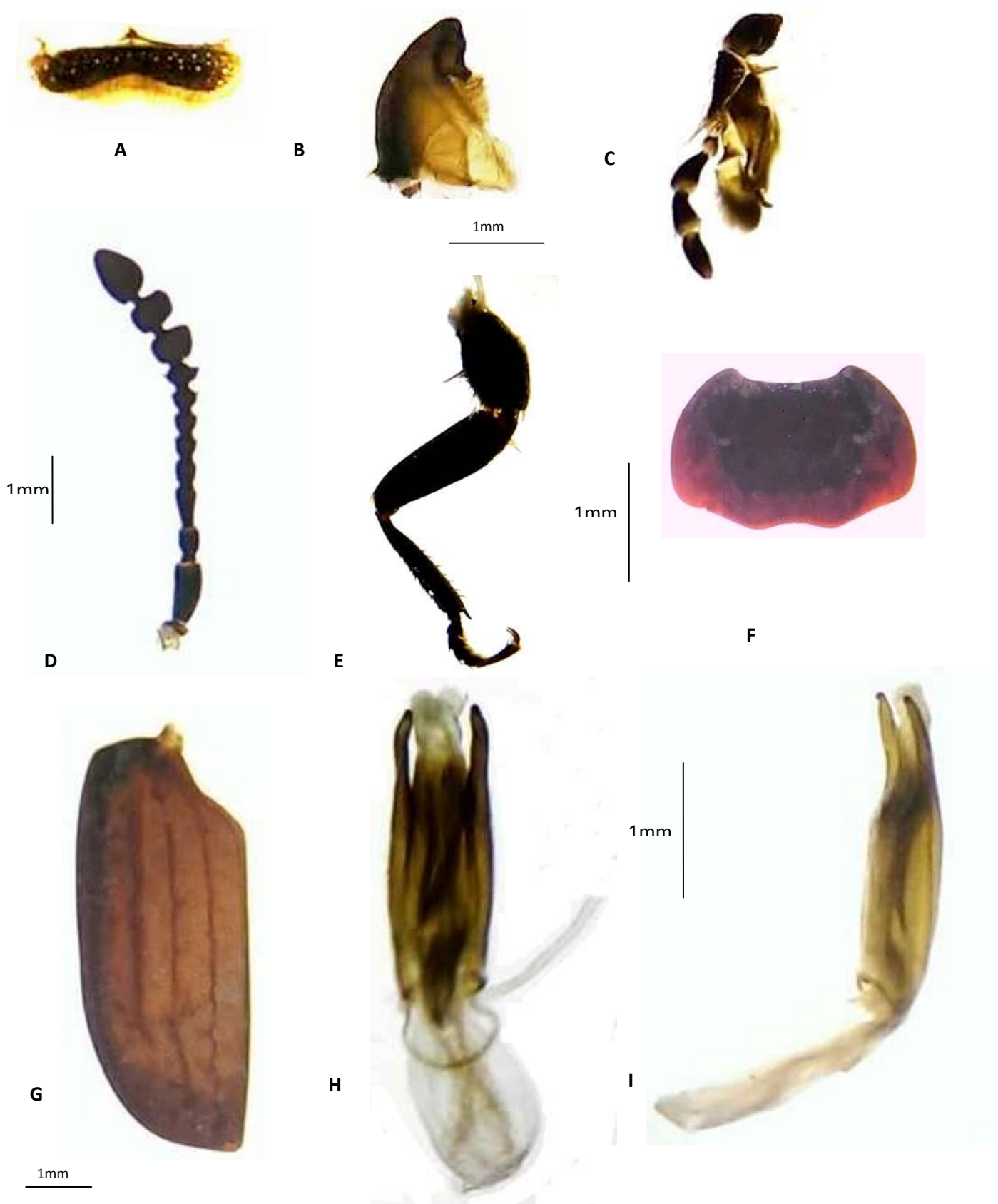

Figure (2) A: Labrum B: Mandible C; Maxilla D: Antenna E: Fore leg F: Pronotum G : Elytra H :Genitalia Dorsal view I : Genitalia Lateral view.

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Metformin ameliorates diabetes mellitus in Kurds patients by attenuation of serum cortisol and copper levels

Muslih A. Ibrahim[1]

١- Department of Pharmacology and Toxicology, College of Pharmacy, Hawler Medical University, Erbil, Kurdistan Region, Iraq

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***Corresponding Author:**
Muslih A. I.
muslih@pha.hmu.edu.krd

ABSTRACT

Metformin is one of the most commonly prescribed antihyperglycemic agent for type 2 diabetes mellitus (T2DM). However, details of exact mechanism of metformin action are not clear. The study aims to determine the roles and effects of metformin on serum cortisol, copper, and zinc levels in T2DM subjects. Venous blood samples from control and T2DM subjects were collected. The serum was separated from blood samples before concentration determination using ELISA. Quantification was done using a spectrophotometer. The present study revealed that serum levels of cortisol and copper in T2DM subjects were higher than that of control subjects whereas the serum level of zinc was lower. After 12 weeks of treatment with metformin, serum copper levels in T2DM subjects decreased significantly, while non-significant changes were recorded with respect to zinc and cortisol levels. In conclusions, the results suggested that metformin significantly changed the levels of serum cortisol in patients with T2DM, while it increased serum copper levels. On the other hands we found that there is a weak correlation between serum cortisol levels with serum copper and zinc levels.

1. INTRODUCTION

Diabetes is a group of metabolic diseases defined by hyperglycemia caused by defects in insulin secretion, insulin action, or both. Chronic hyperglycemia of diabetes is linked with abnormal function and failure of various organs, especially the kidneys, eyes, heart, and blood vessels (American Diabetes Association, 2013). Diabetes mellitus (DM) is the leading cause of morbidity and mortality worldwide, with an estimated 415 million adults being affected in year 2015. This figure is estimated to increase to over 462 million by 2040 (International Diabetes Federation, 2015; WHO Diabetes Fact Sheet, 2017). T2DM is the most common form of DM, resulting from reactions between genetic, environmental, and behavioral risk factors (Olokoba *et. al.*, 2012).

Decreased secretion of incretin glucagon-like peptide 1 (GLP-1) and increased glucagon concentration, leads to hyperglycaemia and insulin resistance in T2DM patients (Seck, 2004; Charmandari *et. al*., 2011).

Metformin is believed to suppresses glucagon signal transduction, resulting in decreased gluconeogenesis (Bridges *et. al.*, 2014; Rena *et. al.*,2017). The metformin actions suppress hepatic gluconeogenesis, enhances insulin-mediated glucose uptake by skeletal muscles and fat, interferes with the mitochondrial respiratory chain, promotes glucose utilization peripherally via anaerobic glycolysis, and delays the intestinal glucose absorption (Hawley *et. al.*, 2010; Logie *et. al.*, 2012; Miller, 2013).

Glucocorticoid hormones are produced by the adrenal cortex under the control of hypothalamic-pituitary-adrenal (HPA) axis. They exert their function in different target tissues by binding two intracellular receptors (Lansang *et. al.*, 2011). The use of glucocorticoids, which is prescribed to treat inflammation, may cause side effects, including hyperglycaemia which can worsen pre-existing diabetes or precipitate new steroid induced diabetes (SID). About 50% the side effects persist despite reduction or withdrawal of the drug (Seck *et. al.*, 2004; Simmons *et. al.*, 2012). Ethnicity, age, increased glucocorticoid dosage, duration of therapy, underlying disease, and body mass index (BMI) are risk factors of SID induction (Dube *et. al.*, 2015; Hwang *et. al.*, 2014).

Inhabitants of Iraqi Kurds origin are at remarkably high risk for T2DM (Hussein *et. al.*, 2012; Skogberg *et. al.*, 2016). This is of interest because T2DM increases the risk of comorbid conditions including cardiovascular and renal diseases, which negatively effects patient's quality of life (Muilwijk *et. al.*, 2017). There are very few data on the prevalence of T2DM in Kurdistan region/Iraq as a whole. Studies that scrutinize T2DM data trends within Kurdistan, points to the evidence of significant increase in prevalence of T2DM in both rural and urban setting, affecting both genders proportionately (Iraq Family Health Survey Report, 2006/7; Iraqi Refugee Health Profile, 2014).

Divalent cations are vital minerals to maintain biomolecules homeostasis, system interactions, structure, and function of cells. They are dynamic elements that have crucial interactions at various ion transporters. Trace elements such as magnesium (Mg), selenium (Se), manganese (Mn), and copper (Cu) play important roles in insulin activity and carbohydrate metabolism although their relationship with pathogenesis and progress of DM is uncertain (Nsonwu *et. al.*, 2006). Transition metals such as copper (Cu), zinc (Zn), and iron (Fe) are critical for numerous physiological functions. However extra copper and iron raise free radical concentrations that may encourage damage in the biological system (Singh *et. al.*, 2012). Zinc prevents Fenton's reaction by competing with copper and iron to bind to vicinal sulfhydryl groups. This prevents intramolecular disulfide formation and avoid cells from forming excess ROS species [e.g. hydroxyl (.OH) and superoxide ($O_2^{\cdot -}$)] (Santon *et. al.*,, 2006; Libardo *et. al.*, 2015). The objective of this study was to determine the serum levels of cortisol, copper, and zinc elements in the control group, pre-metformin and post-metformin Kurdistan T2DM patients. This study also identified and evaluated effects of metformin on the different serum parameters and successfully clarified the relationship between hypercortisolism and DM after 12 weeks of treatment.

2. MATERIALS AND METHODS

2.1. Blood Sample Collection

Prior to the experiment, venous blood samples from all 60 subjects were collected in the morning after an overnight fasting, into special blood collecting tubes for the measurement of cortisol, copper and zinc in the serum. The same method of collection was carried out on the subjects after 12 weeks of study period. In total, measurements were taken for all subjects twice throughout the course of the study: once before the metformin administration (500 mg twice daily) and once after 12 weeks of treatment.

2.2 Serum cortisol, copper and zinc determination

Serum cortisol was measured using double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) with the Human Cortisol ELISA Kit (Abcam, USA). Serum concentration of copper and zinc were determined through spectrophotometric method (Perkin Elmer Landa 25 double beam UV/VIS) using MyBioSource kits with serum reference.

2.3 Design of the Study

All the subjects were informed of the aim of the study and the potential effects of the procedure. The subjects have provided informed consent before research was conducted. The study was conducted pursuant to the Declaration of Helsinki and was approved by Institutional Review Board Hawler Medical University/College of Pharmacy and Institutional Review Board Layla Qassim Diabetic Centre/Hawler. All the subjects undergone metformin monotherapy at a stable dose of 500 mg, twice per day. Subjects recruited were Kurd patients with T2DM, who visited Layla Qassim Diabetic Centre/Hawler and Rizgary Teaching Hospital, between September 2015 to July 2016. The study involved participation of 35 patients afflicted with T2DM, aged 44 (29-65) years, body mass index (BMI) of 31.1 ± 0.41 kg/m2, and HbA1c between 6.1-7.5%. The HbA1c was estimated using ELISA method: Tetradecyltrimethylammonium- bromide (TTAB) as the detergent in the haemolyzing reagent to eliminate interference from leukocytes (TTAB does not lyse leukocytes) (Lenzi *et. al.*, 1987). The HbA1c determination is based on the turbidimetric inhibition immunoassay for haemolyzed whole blood (Chang *et. al.*, 1998).

Twenty-five healthy control subjects that were matched in age, BMI, gender, and ethnic background volunteered for the study. The healthy subjects were aged 43 (28-63) years, with a mean BMI of 30.4 ± 0.62 kg/m2. BMI is estimated according to the following equation:

$$BMI = \frac{weight\ (kg)}{height\ (m2)}$$

Subjects were chosen based on inclusion and exclusion criteria of this project. The inclusion criteria were propensity for cooperation and affliction with T2DM. Exclusion criteria were subjects with no other chronic diseases or medical history of disorders involving glucocorticoid, mineralocorticoid, or androgen excess, subjects without a history of type 1 diabetes or secondary forms of diabetes, subjects without previous insulin therapy, subjects without triglycerides level >500 mg/dl, and subjects have not undergone treatment with any oral, systemic, topical, inhaled glucocorticoids or medicaments other than metformin within the period of the study.

2.4 Statistical Analysis

Results were expressed as mean ± SEM and median (inter-quartile range). Differences

between means of all groups were assessed using one-way analysis of variance (ANOVA) with Tukey's test for data with normally distributed and Kruskal-Wallis and Dunn's tests for non-normally distributed data. Correlation between variables were performed using Pearson's correlation coefficient test and Spearman's correlation coefficient test for parametric and non-parametric distributed continuous data respectively. Statistical analyses were performed using the GraphPad Prism 6 (California corporation, USA). A P value of < 0.05 is considered statistically significant.

3. RESULTS

The mean serum cortisol level in T2DM subjects before metformin treatment was non-significant compared to control subjects (15.17 ± 0.556 µg/dl vs.14.15 ± 0.381 µg/dl, p<0.645). Serum cortisol concentrations after 12 weeks of treatment with metformin in T2DM subjects significantly increased compared to control subjects (16.04 ± 0.369 µg/dl vs. 14.15 ± 0.381 µg/dl, p<0.044) (Figure 1).

The mean serum copper level in T2DM subjects was significantly higher compared to the control group before treatment (177.3 ± 8.948 µg/dl vs. 126.6 ± 4.345 µg/dl, p<0.035). While, treatment with metformin for 12 weeks significantly increased the mean concentration of serum copper in T2DM subjects compared to pre-treatment mean concentration (142.9 ± 5.102 µg/dl vs. 177.3±8.948 µg/dl, p<0.004). The change in mean serum copper level between the T2DM post-metformin treated and control group was not significant (142.9 ± 5.102 µg/dl vs. 126.6 ± 4.345 µg/dl, p<0.085) (Figure 2).

Total serum zinc level was non-significantly lower in T2DM subjects compared to subjects in the control group [124.3 µg/dl (96.39 µg/dl -196.4 µg/dl) vs. 131.5 µg/dl (100.0 µg/dl -211.7 µg/dl), p< 0.086] before treatment. Concentration of serum zinc level after 12 weeks of metformin therapy showed non-significant difference compared to pre-treatment concentration [122.3 µg/dl (99.09 µg/dl -184.7 µg/dl) vs. 124.3 µg/dl (96.39 µg/dl -196.4 µg/dl) µg/dl, p< 0.068). Difference in mean serum zinc level was also not significant between post-metformin T2DM group and control group [122.3 µg/dl (99.09 µg/dl -184.7 µg/dl) vs. 131.levels 5 µg/dl (100.2 µg/dl -211.7 µg/dl), p< 0.052] (Figure 3).

According to the Pearson's correlation, there was a weak correlation (r=0.311, p=0.078) between serum cortisol concentration and copper levels. On other hand, Spearman's correlation analysis, revealed that serum cortisol levels negatively correlated with zinc concentration (r=-0.2, p=0.087.)

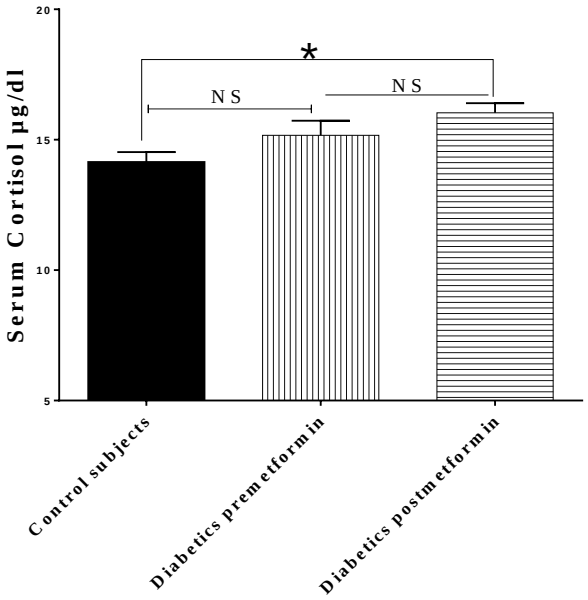

Figure 1. Effects of metformin administration (500 mg twice/day) on serum cortisol level in Kurd T2DM and control subjects.

The asterisk (*) represents significant difference between post-metformin groups and control group.

(NS) represents no significance between control and pre-metformin group, and pre-metformin and post-metformin group.

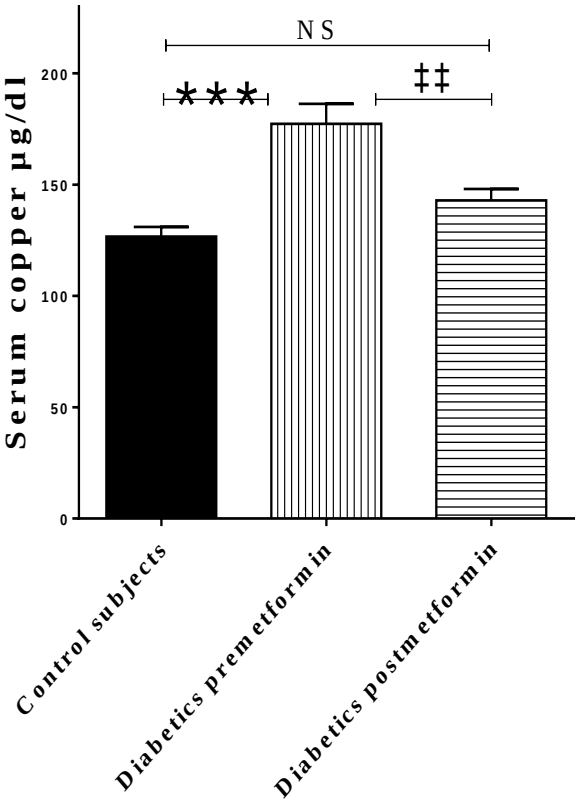

Figure 2. Effects of metformin administration (500 mg twice/day) on serum copper level in Kurd T2DM and control subjects.

The asterisk (*) represents significant comparison between pre-metformin and control groups.
The double dagger sign (‡) represents significant comparison between pre-metformin and post-metformin groups.
(NS) represents non-significant comparison control and post-metformin groups.

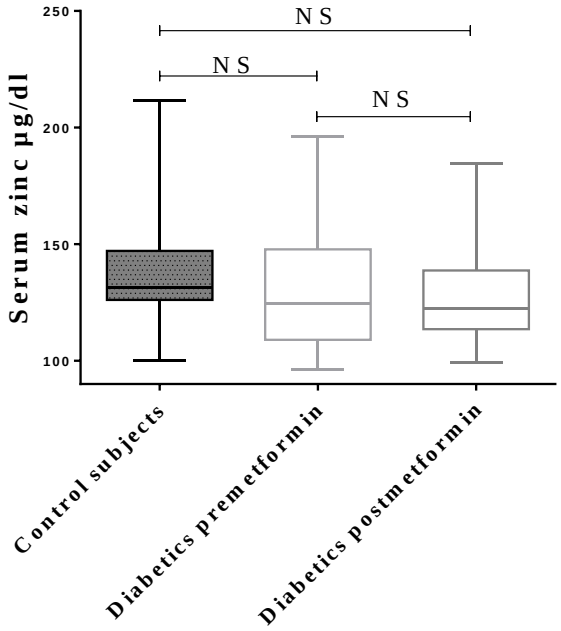

Figure 3. Box plot shows effects of metformin administration (500 mg twice/day) on serum zinc level in Kurd T2DM and control subjects.

(NS) represents non-significant comparison between the indicated groups.

4. DISCUSSION

The relationship between elevated levels of endogenous glucocorticoids and T2DM is under controversial discussion. It is well known that cortisol prepares the body for stressed conditions by supporting it with increased glucose. A decrease in cortisol secretion leads to diminished glucose secretion in the circulation (Shrayyef and Gerich, 2010). In this study we found that the level of serum cortisol was not changed in T2DM subjects before treatment (Figure 1). After 12 weeks of treatment with metformin, the serum cortisol was increased significantly in T2DM subjects compared to control subjects. These results were consistent with other studies examining the effects of biguanides on subclinical hypercortisolism in T2DM (DOŞA *et. al.*, 2013, Costa *et. al.*, 2016). In an enzyme catalysed process of steroidogenesis, electron

leakage produces cellular reactive oxygen species (ROS) (Prasad *et. al.,* 2014) through secretion of adrenaline and cortisol and speeds up over production of ROS (Kim *et. al.,* 2015; Aschbacher *et. al.,* 2013).

The results of this study (Figure2) showed a significant increase in serum level of copper in T2DM subjects compared to control group. The level of serum copper after treatment with metformin for 12 weeks showed significant difference compared to pre-metformin treated T2DM subjects ($p<0.004$) and non-significant difference with respect to control group subjects ($p<0.085$). These data were in agreement with other studies (Doşa *et. al.,* 2013). Moreover, this study found similar conclusions made by Orsedkar *et. al.,* 2011 in which T2DM subjects showed higher serum copper levels compared to subjects without T2DM. Many researchers also found that the increased quantity of copper contributes in T2DM pathogenesis (Tanaka 2009; Duan 2010). A study found that rats with deficiency in copper were subjected to higher blood sugar levels, demonstrating a possible link between DM and copper (Roughead *et. al*., 2003).

Until currently there is no previous study indicating that serum copper concentration correlated with serum cortisol level. Although our study shows weak positive relation between them. The reason behind such results needs further works to explain the exact mechanism for this relationship.

Many studies found that copper deficiency will lower superoxide dismutase enzyme (SOD) activity to lesser than normal level. A raised trend with the increased of copper intake alters the ratio of copper to zinc (Cu/Zn). The content of malonyl dialdehyde (MDA) was higher than normal in copper deficient rats. The content of MDA decreased when there was an increase in copper intake and the Cu/Zn ratio was maintained at a relatively low level (Orsedkar *et. al.,* 2011).

These findings revealed a non-significant difference between serum zinc in T2DM group versus the control group ($p<0.086$). Zinc homeostasis was disrupted in T2DM subjects (Ruz *et. al.,* 2013). Treatment with metformin for the course of 12 weeks did not significantly change the concentration of serum zinc neither with respect to pre-metformin groups ($p<0.068$), nor with control group subjects ($p<0.052$) (Figure 3). This finding was in agreement with other researchers (Nechifor *et. al.,*2014).

Zinc-induced metallothionein binds to copper and reduces its influx into the cell cytoplasm, consequently decreases free radical formation and accumulation (Marreiro *et. al.,* 2017). Current results indicated a weak negative correlation between serum zinc and serum cortisol levels, i.e., a decrease in serum zinc resulted in an increase in serum cortisol, this result is consistent with a study conducted by (Rosa *et. al.,* 2015).

Previous study found that plasma zinc deficiency occurs due to extreme renal loss of zinc and diabetes impaired gastrointestinal absorption (Doşa *et. al*., 2011). The deleterious effects of zinc deficiency may increase cases of diabetes-induced cataract among people with DM (Rahim and Iqbal, 2011). Hyperglycaemia in DM may also affect the tubular transport of zinc (Tapiero and Tew, 2003). Zinc transporter 8 (ZnT8) is responsible for the transport of zinc to insulin granules in β-cells (Tamaki *et. al.,* 2013). A genetic variation in ZnT8 gene which codes for ZnT8 transporter protein is associated with predisposed risk of T2DM (Zahari *et. al.,* 2016). This is due to the reduced activity of ZnT8 transporter protein in ZnT8 mutant gene (Sladek *et. al*., 2007; Kambe *et. al.,* 2015).

5. CONCLUSIONS

In conclusions, the results suggested that metformin significantly changed the kevels of serum cortisol in patients with DMT2, while it increased serum copper levels. On the other hands we found that there is a weak correlation between serum cortisol levels with serum copper and zinc levels.

Conflict of Interest

The author declares no conflict of interest

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SNPs Analysis in the CDKN2A /CDKN2B Genes in human assayed by DNA sequencing and ARMS™ testing

Ardalan Abdulhamid Osman Khoshnaw
Department of Pathological Analysis, College of Science, Knowledge University

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***Corresponding Author:**

A B S T R A C T

This research facilitates the detection process of single-nucleotide polymorphism (SNP) by DNA sequencing and Amplification Refractory Mutation System™ (ARMS™). Alterations of four SNPs have been investigated at the specific location in CDKN2A and CDKN2B genes. In fact, these are tumor suppressor genes. The DNA from the cultured cell were extracted and then by using specific Oligonucleotide for SNPs, the CDKN2A and CDKN2B genes were amplified by PCR later separated by gel electrophoresis. Finally, the ARMS test and sequencing test were applied for all samples to find out allele frequency and genotype frequency in this population. The result presented that the efficient selection of oligonucleotides able to discriminate a single nucleotide mismatch also shows that different people may have different SNP on the same region in the chromosome. These data will facilitate the design of custom made primer viable to gene expression profiling and sequencing analyses even it will be very interesting to compare the Kurdish People genomes to find the correlation between the SNP with several genetic backgrounds diseases. Both results ARMS and sequencing test have compared together. It was illustrated that there are different in genotype results by ARMS test and sequencing test for the same sample.

After the sequencing stage, it was concluded that ARMs test can be used for sequencing the genomes of a large number of people, additionally it is very sensitive, inexpensive and rapid to find out different SNPs of each individual and generate a single map of the human genome containing all possible SNPs (SNP maps). Depending on this map we can group the people based on the SNP profile to determine if SNP associated with a disease, also explain why some people respond to treatment and not others?

1. INTRODUCTION

The single nucleotide polymorphism (SNP**)** and refers to the predominant form of segregating variation at the molecular level. SNPs are extremely important in a wide range of biological investigation, ranging from biomedical research to ecology and evolutionary biology as well in the characterization of population structure. The human DNA sequences are 99.9% identical with small genetic differences (0.1%) between humans. it has been found that variants of the letter sequence frequently occur in the genome nucleobases can be missing, exchanged or inserted at a certain point in the genome. Many geneticists regard this genetic variation at the level of DNA structure ("DNA sequence variation") as the key to innate susceptibilities

to folk diseases, congenital responses to drugs and other differences in the phenotype of humans. By far the largest part of the variation is made by single, exchanged nucleotides, so-called (SNPs) (Hart *et al.*, 2004).

Such SNPs happen once in every 300 nucleotides on average, which implies there are roughly around 10 million SNPs in large genomes like ours, there are many polymorphic positions (Ali *et al.*, 2017). Some SNP is only present in large groups of people at all time, so that groups of people with the same haplotype can be formed. For this purpose, the members of a family clan or resident of an inaccessible region are to be counted. Numerous diseases may be linked to specific haplotypes and could be easily identified.

Generally, lots of SNP are found in the DNA between genes. They can act as biological markers, helping researchers to locate genes that are related to the disease (Helyar *et al.*, 2011). When SNPs arise within a gene, they may play a more direct role in disease by affecting the gene's activity. When this project is completed, the analysis of certain positions for broad statements is sufficient. In principle, the individuality of a person can be clearly defined by less than 100 polymorphisms and linked with all possible predispositions.

In the HapMap project, haplotypes are being studied to identify genetic similarities and differences in individuals. Using this data researcher will be able to find genes involved in disease and responses to therapeutic drugs at International HapMap Project (Jing *et al.*, 2014). Researcher work to identify all SNPs in the genome and to develop technologies (microarrays or "DNA chips") that allow a person's genome to be tested in a short time to see if certain SNPs exist (Govindarajan *et al.*, 2012).

In this study, I have been investigated alterations of two SNPs at the specific location in cyclin dependent kinase inhibitor 2A (CDKN2A) and CDKN2B genes which exist on P arm of chromosome 9 in human genome. In fact, these are tumor suppressor genes by cell cycle inhibiting. The CDKN2A gene in *Homo sapiens* locus in chromosome band 9 p21.3, complement (21,967,751.21,994,490) also stated that the CDKN2 gene consists of 3 coding exons (1β, 1α, 2 and 3) (Rahman *et al.*, 2014). CDKN2A gene makes several proteins, p16 (INK4a) and the p14 (ARF) proteins are most common. Both work as tumor suppressors, by keeping the cells from dividing too quickly.The p14(ARF) protein transcript from the first exon which protects a protein called p53 from being degraded by E3 ubiquitin-protein ligase MDM2. The p53 protein is an important tumor suppressor that is essential for regulating cell division and apoptosis as well induces G2 arrest and prevent tumor formation. Also, the p16 and p15 share a controlling role in cell cycle G1 checkpoint (McWilliams *et al.*, 2011).

The cyclin-dependent kinase inhibitor 2B (CDKN2B) gene in *Homo sapiens* locus in chromosome band 9 p21.3, also stated that the CDKN2B gene has 2 coding exons (E1, and E2). It is linked to p16INK4A and p14ARF (Fares *et al.*, 2012). CDKN2B gene encodes two separate transcript variants: p15 and p10. The most common function of p15INK4B is protein kinase inhibitors specificity towards the (CDK4) or (CDK6) also play a critical role during differentiation of the cell and cellular senescence (Takahashi *et al.*, 2007). CDKN2B is commonly mutated or deleted in wide variety diseases include Myelodysplastic syndromes, a group of clonal stem cell disorders, Acute myeloid leukemia (Brakensiek *et al.*, 2007).

2. MATERIALS AND METHODS

2.1. Experimental background

In the present study, the SNPs rs199907548 and rs1024022739 have been investigated for different DNA samples. The ability to genotype large numbers of SNPs in large numbers of individuals rapidly and cost-effectively is essential for many applications, from linkage and association mapping to mutation detection and diagnosis and there are some methods for detection of SNP genotyping as following:

1) Low-Technology Methods, (ASO)
2) Hybridization-Based Methods
3) Mini-sequencing Methods
4) Homogeneous Fluorogenic Dye-Based Methods
5) Haplotype phasing Methods
6) Amplification refractory mutation system (ARMS)
7) DNA sequencing assay

In this study, the ARMS test and DNA sequencing assay was used**.**

2.2. DNA samples

A total of 11 human fibroblast cultures, established from Lung and skin samples derived from fetal (14–20 wk gestational age) and two samples were 43 and 47 years old, all the samples were ordered from the National Institute on Aging cell repository at the Coriell Institute for Medical Research (CIMR) Camden, NJ. All the samples were medically examined and diagnosed as "healthy" as well they have a normal karyotype, *i.e.*, 46, XX or 46, XY. Cells were grown without antibiotics and supplied with 2 mM l-glutamine and 10% (vol/vol) fetal bovine serum. 100 ng of genomic DNA was taken from each sample to identify CDKN2A/B genes and amplify it by available PCR kits.

The starting material for amplification The DNA extraction was done from 11 cultured cell samples that has been mention above (Table1) as described by the manufacturer from Coriell institute and Lundby laboratory from Gothenburg University.

Table 1- show the name of samples, age, type of cell, source and karyotype.

	Sample	Taken from	Source	cell	Origin	Karyotype
1	AG01522p3	aborted fetus 20 w	Skin	Fibroblast	Caucasian	46, XY
2	AG06556	aborted fetus 20 w	Lung	Fibroblast	Caucasian	46, XY
3	AG04393p3	aborted fetus 16 w	Lung	Fibroblast	Caucasian	46, XX
4	AG04452	aborted fetus 16 w	Lung	Fibroblast	Black	46, XX
5	AG09033	aborted fetus 17 w	Skin	Fibroblast	Caucasian	46, XY
6	AG09117	49 years	Lung	Fibroblast	Caucasian	46, XY
7	AG04525	aborted fetus 17 w	Skin	Fibroblast	Caucasian	46, XY
8	AG06173	aborted fetus 5 m	Lung	Fibroblast	Asian	46, XX
9	AG04931	49 years	Lung	Fibroblast	Caucasian	46, XY

10	AG06814	aborted fetus 3 m	Lung	Fibroblast	Caucasian	46, XX
11	AG05965	aborted fetus 14 w	Lung	Fibroblast	Caucasian	46, XY

2.2 PCR Amplification and Sequencing of CDKN2A and CDKN2B genes.

Oligonucleotide primers were synthesized by cyanoethyl phosphonamidite chemistry on an Applied Biosystems model 392 DNA synthesizer (Applied Biosystems Inc., Foster City, CA) Multiplex PCR was performed in a final volume of 25 µL containing 100 ng of genomic DNA, 50 mM KCl, 10 mM Tris-HCl (pH 8.8), 2.5 mM MgCl2, 5% (vol/vol) DMSO, 200 µM deoxyribonucleotide triphosphates, 1 U of Taq polymerase and 25 pmol of each of the primers used.

Primers used to amplify p14, p15 and p16 exons of CDKN2A/B are listed in Table 2. were reported elsewhere (Fujiwara-Igarashi *et al.*, 2013).

Table 2. Primer sequences for p14, p15 and p16 analyses.

Primar		Amplify	Fragment
P14-1F	5′-GGGAGCAGCATGGAGCCG-3′	p14. exon 1	204 bp
P14-1R	5′-AGTCGCCCGCCATCCCCT-3′	pI4, exon 1	
P14-2F	5′-GGAAATTGGAAACTGGAAGC-3	p14, exon 2	498 bp
P14-2R	5′-TCTGAGCTTTG-GAAGCTCT-3′	pI4, exon 2	
P15-1F	5´-CCAGAAGCAATCCAGGCGCG-3'	p15. exon 1	400 bp
P15-1R	5´-AATGCACACCTCGCCAACG-3'	pI5, exon 1	
P15-2F	5´-CCTTAAATGGCTCCACCTGC-3'	p15, exon 2	
P15-2R	5´-CGTTGGCAGCCTTCATCG-3'	pI5, exon 2	
P16-1F	5´-GAAAGGAGAGGAGGGGCT-3'	pl6, exon 1^{α}	340 bp
P16-1R	5´- GCGCTACCTGATTCCAATTC-3'	pl6, exon 1^{α}	
P16-2F	5´-GGAAATTGGAAACTGGAAGC-3'	p16, exon 2^{α}	
P16-2R	5´-TCTGAGCTTTGGAAGCTCT-3'	p16. exon 2^{α}	
P16-3F	5´-TTTTCTTTCTGCCCTcTGCA-3'	plo, exon 3	

P16-3R	5´-TGAAGTCGACAGCTTCCG-3'	p16. exon 3

Primers used to amplify p16 exons are listed in Table 2 and named A and G according to the consequence of the Allele, also primers used to amplify p15 exons are listed in Table2 and named T and G. All PCR reactions were carried out and mixed with 100 ng genomic DNA. The PCR solutions are prepared as have been described by (Lorenz, 2012)**.** The PCR program used for all samples by using different specific primer for each sample as showing in table 3, 4 and 5 in Appendix. The PCR products were separated by gel electrophoresis on 1.5% agarose or 10% nondenaturing polyacrylamide gels Figure 5.

The Pure Link® Quick Gel Extraction Kit from thermos fisher scientific is used to purify CDKN2A and CDKN2B fragments from agarose gels in less than 30 minutes. For sequencing the CDKN2A and CDKN2B fragments the ABI PRISM 377 DNA sequencer are used and the sample prepared according to the procedure from the AB Applied Biosystems. The ABI PRISM 377 DNA Sequencer analyzes fluorescently labeled DNA fragments by gel electrophoresis. The DNA sections labeled with four diverse fluorescent colors and stacked into a gel made of polymerized acrylamide. Once the samples are loaded, voltage is applied, causing the fragments to move through the gel and separate according to size. Later the gel exposed to the laser and the fragments which attached by fluorescent dyes will produce the light at a specific wavelength. Then the results from the sequencer collected and blasted in database program.

The amplification refractory mutation system (ARMS) are used for detecting any mutation relating to SNP. ARMS method depends on the use of allele-specific PCR primers (ASP) that permit enhancement of the DNA fragment just when the objective allele is contained inside the sample. Subsequent presence of a PCR product is diagnostic for the presence of the target allele and vice versa.

A total of 11 samples of CDKN2A and CDKN2B fragments was extracted, and PCR reactions were prepared in two separate tubes for each sample. One test tube was used for the amplification of the normal ARMS primer and the second for the amplification of the mutant ARMS primer. The primers used for ARMS were kindly provided in Table 2. The procedure used according to the instructions provided by the manufacturer and Institute of Clinical Chemistry, Rudolfstiftung Hospital (Najmabadi *et al.*, 2001).

3. Results

One µl of extracted DNA from different samples were run on 1% agarose gel to be sure if the DNA is extracted. The size is approximately between 11000-12000 bp.

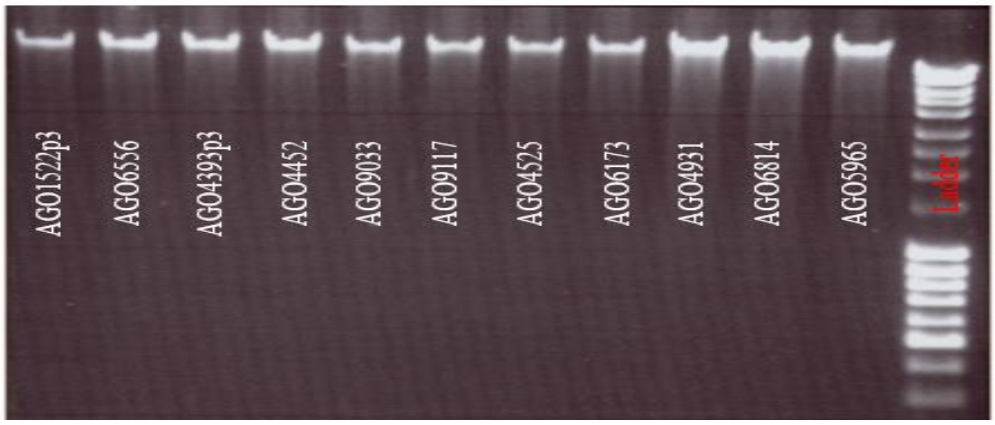

Figure 6. the extracted DNA from 11 samples

ARMS test

Five µl of each DNA sample was run on 1% agarose gel.

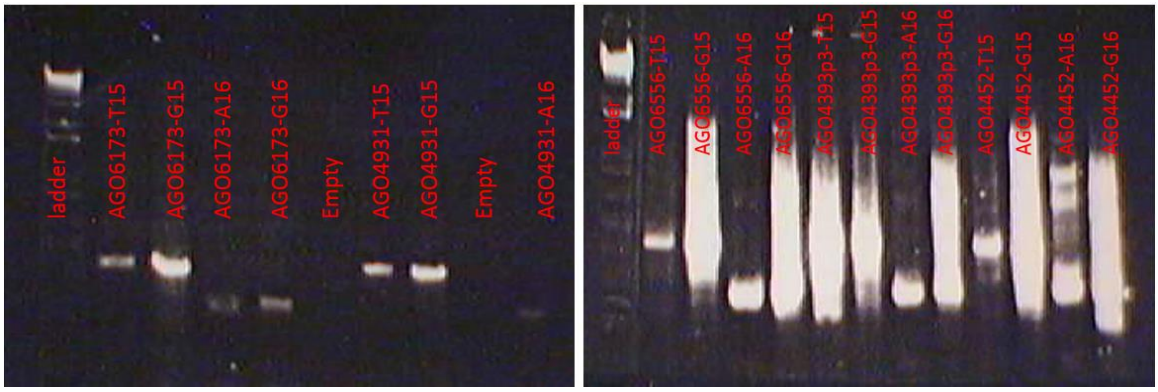

Figure 7- ARMS test for some samples with four allele specific primer.

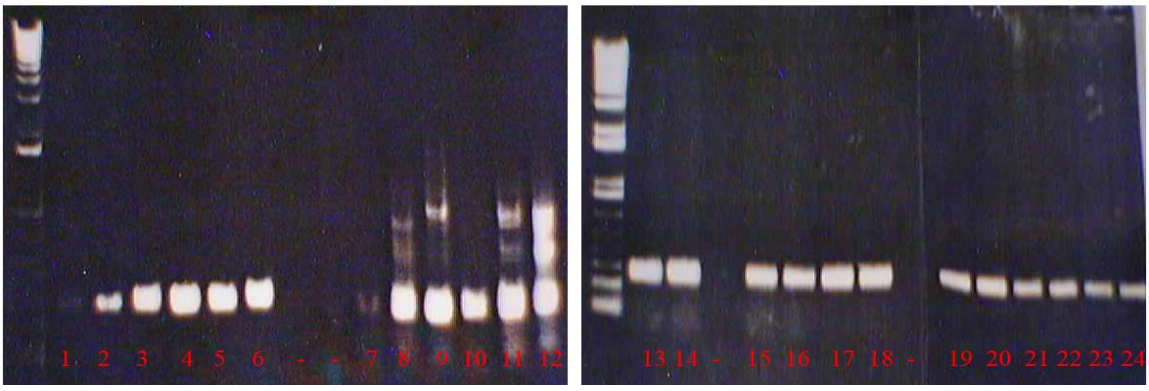

Figure 8. Represent bands for purification of P15 DNA and P16 DNA fragment.

Table 6 - Genotypes of SNPs rs199907548 Alleles: A/G in CDKN2A (P16) and SNPs rs1024022739, Alleles: T/G in CDKN2B (P15) in 11 human samples assayed by ARMS test

Samples	Amplified fragment by specific primer		Size bp	Genotype
AGO1522p3	T15-	G15	400-420 , 400-420	**T/G**
AGO1522p3	A16-	G16	280-300 , 280-300	**A/G**
AGO6556	T15-	G15	340-380 , 340-380	**T/G**
AGO6556	A16-	G16	280-300 , 280-300	**A/G**
AGO4393p3	T15-	G15	340-380 , 340-380	**T/G**
AGO4393p3	A16-	G16	280-300 , 280-300	**A/G**
AGO4452	T15-	G15	340-380 , 340-380	**T/G**
AGO4452	A16-	G16	280-300 , 280-300	**A/G**
AGO9033	T15-	G15	400-420 , 400-420	**T/G**
AGO9033	A16-	G16	280-300 , 280-300	**A/G**
AGO9117	T15-	G15	- , -	**- / -**
AGO9117	A16-	G16	- , 280-300	**G/G**
AGO4525	T15-	G15	400-420 , 400-420	**T/G**
AGO4525	A16-	G16	280-300 , 280-300	**A/G**
AGO6173	T15-	G15	400-420 , 400-420	**T/G**
AGO6173	A16-	G16	- , 280-300	**G/G**
AGO4931	T15-	G15	400-420 , 400-420	**T/G**
AGO4931	A16-	G16	- , 280- 300	**G/G**
AGO6814	T15-	G15	400-420 , 400-420	**T/G**
AGO6814	A16-	G16	- , -	**- / -**
AGO5965	T15-	G15	400-420 , 400-420	**T/G**
AGO5965	A16-	G16	- , -	**- / -**

The results mentioned above shows that different people may have different SNP on the same region. The remaining of the same samples that had been amplified for both primers (R and L), were run on agarose gel for purification and sequencing test.

Both results ARMS and sequencing test have compared together in (Table 7). It was illustrated that there are different in genotype results by ARMS test and sequencing test for the same sample especially in P15 but the result was same in P16.

Table 7- comparison between genotypes of SNPs rs199907548 Alleles: A/G in CDKN2A (P16) and SNPs rs1024022739, Alleles: G/T in CDKN2B (P15) in 11 human samples assayed by ARMS test and sequencing test.

Row	samples	ARMS test p16	Sequencing test p16	ARMS test p15	Sequencing test p15
1	AGO1522P3	A/G	A/G	T/G	T/T
2	AGO6556	A/G	A/G	T/G	T/T
3	AGO4393P3	A/G	A/G	T/G	-/-
4	AGO4452	A/G	A/G	T/G	-/-
5	AGO9033	A/G	A/G	T/G	T/G
6	AGO9117	G/G	G/G	- /-	T/T
7	AGO4525	A/G	A/G	T/G	T/T
8	AGO6173	G/G	G/G	T/G	T/T
9	AGO4931	G/G	G/G	T/G	T/T
10	AGO6814	- /-	G/G	T/G	T/T
11	AGO5965	- /-	A/G	T/G	T/G

4. Discussion

DNA amplification by methods such as primer extension pre-amplification, produce relatively low molecular weight DNA that is not representative of the entire genome. The product is often error prone and unlikely to completely preserve heterozygosity information or correct copying of short repeated elements, but the allele-specific PCR has been rationalized by using primary allele-specific PCR primers that contain a universal 5´-tail sequence that becomes part of the PCR product of amplification, this technique is the best principle of choice genotyping of SNPs, and has been improved in numerous assay formats.

Allele-specific primer extension, two primers that anneal to their target sequence adjacent to the SNP and have the nucleotide complementary to the allelic variant at their 3´-

end are used in primer extension reactions catalyzed by a DNA polymerase. Only primers with perfectly matched 3´-ends will be extended. The Allele-specific PCR depend on exact base pairing at the 3´end of the primer that can distinction between alleles that differ at just a single nucleotide. In the popular ARMS method, primers are designed whit their 3´end nucleotides designed to base-pair with the variable nucleotide which distinguishes the two alleles, and with the remaining primer sequence designed to be complementary to the sequence immediately adjacent to the variable nucleotide. Under suitable experimental conditions, amplification will not take place where the 3´end nucleotide is not perfectly base-paired thereby distinguishing the two alleles.

APS1 will bind perfectly to the complementary strand of the allele 1 sequence, permitting amplification with the conserved primer. However, the 3´ terminal C of the ASP2 primer mismatches with the T of the allele 1 sequence, making amplification impossible. Similarly, ASP2 can bind perfectly to allele 2 and initiate amplification, unlike ASP1.

There are a lot of SNPs in CDKN2A and CDKN2B genes and two of them are SNPs rs199907548, Alleles: A/G in CDKN2A (P16) that exist on Exon 3 and SNPs rs1024022739, Alleles: G/T in CDKN2B (P15) that exist on Exon 2 respectively. In this experiment, the mentioned SNPs detected by using the Allele-specific primer in 11 human DNA samples then by running DNA sequencing and ARMS™ test analysis the result collected and compared.

In contrast to results from the Table 6 that show different people may have different SNP on the same region. likewise, there was a different result in DNA sequencing and ARMS™ test for the same SNP at the same region as showing in Table 7.

This is the first study to report on resequencing of the locus 9p21.3 containing CDKN2A and CDKN2B. This region is one of the most imperative loci in modern genetic studies, other studies have reported sequencing 9p21.3 with sign that supports risk associated alleles having effects on expression of CDKN2A and CDKN2B (Pilbrow *et al.*, 2012).

After the sequencing stage, it was concluded that ARMs test can be used for sequencing the genomes of a large number of people, additionally it is very sensitive, inexpensive and rapid to find out different SNPs of each individual and generate a single map of the human genome containing all possible SNPs (SNP maps). Depending on this map we can group the people based on the SNP profile to determine if SNP associated with a disease, also explain why some people respond to treatment and not others?

According to our results it can be proposed that the genetic variance is mainly accounted by many variants and loci while there are significant differences in tissues, investigations, and isoforms targeted across these studies that may limit direct comparisons. The overall findings of the present study are consistent with several prior findings.

Additionally, detailed studies in multiple tissues and in large clinical accomplices are necessary to determine how these variations influence disease and which ones are most important for potential clinical applications.

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Molecular Characterization of Cutaneous Leishmaniasis Isolated from Human in Erbil Province- Kurdistan Region/ Iraq

Zuber I. Hassan

Department of Veterinary, Shaqlawa Technical Institute, Erbil Polytechnic University, Erbil, Kurdistan Region-Iraq.

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***Corresponding Author:**
Zuber I. Hassan
Vetro_11@yahoo.com

ABSTRACT

Cutaneous leishmaniasis (CL) is a parasitic disease of public health problem in Erbil Province, Kurdistan Region/ Iraq. The identification of the parasite species causing these diseases is a pre-requisite for understanding disease epidemiology. In the current study a higher rate of infection was found in males than females (60.34% versus 39.66%). Most lesions were found on upper limbs (41.38%), while lower rates were found in the legs (24.14%). Regarding age, the highest rate (27.59%) of infections was observed in the age group 1-9 years, while the lowest rate infections (13.79%) were observed in the age group over 40years. Regarding the residency, the overall rate of CL in the inhabitants of the rural area was higher as compared to that of urban inhabitants. The polymerase chain reaction products were sequenced and further examined by sequence analysis, 33 isolates, independent of the host, exhibited L. tropica while in 15 samples, Nucleotide substitution generate polymorphism at position 132 nucleotide (C-G), Furthermore, ten human samples have more than one nucleotide substitution (G to T) at positions 246 and 276 and G to A at position 281. The sequencing of the PCR products recognized 3 haplotypes, which are not described in Kurdistan Region and Iraq. Thus, monitoring of CL and sustained surveillance system is crucial in counteracting the disease, and if possible, to control it.

1. INTRODUCTION

Cutaneous leishmaniasis (CL) is a protozoan Parasitic disease transmitted by sand fly of the genus *Phlebotomus*, with a wide spectrum of clinical manifestations; the most typical are ulcerative skin lesions developed near or at the vector's bite site (Khatri *et al*., 2009 and Soares *et al*., 2014). This protozoan is an obligatory intracellular parasite which exists in two distinctive forms. In humans and other hosts, it occurs as none flagellated amastigotes, while in culture and in the gut of sandflies the flagellar or the promastigotes form is seen (Farahmand *et al*., 2011 and Traore *et al*., 2016). They are neither found in the peripheral blood nor in any visceral organ. The amastigotes are small, round to oval, bodies measure about 2-5µm in diameter and found only in the macrophages of infected vertebrate hosts, The flagellar or the promastigote forms are seen in the culture media and in the gut of the sandfly (Rahi, 2015).

In Iraq, Turkey, Syria, Saudi Arabia, Iran, Israel, Libya, Sudan and Morocco *Leishmania major*, *Leishmania tropica* and less

frequently *Leishmania infantum* cause cutaneous leishmaniasis (Bensoussan *et al*., 2006; Qader *et al*., 2009; Eroglu *et al*., 2011; Farahmand *et al*., 2011; Osman, 2011; Amro *et al*., 2012; Al-Nahhas and Kaldas, 2013; Ajaoud *et al*., 2013; El-Beshbishy *et al*., 2013; Rahi *et al*., 2013; Rahi, 2015 and Al-Obaidi *et al*., 2016 and El-Badry *et al*., 2016). CL is most frequently diagnosed by clinical evaluation (AL-Zaidawi, 1997 and Bensoussan *et al*., 2006) and its typical acute lesions can be confused with other dermatological problems, such as sporotrichosis (Eroglu *et al*., 2011). The traditional diagnostic methods of CL consist of microscopic observation of the parasites in tissue smears after Giemsa staining, and/or culture of promastigotes from tissue (Khalaf *et al*., 2016), additional serological, biochemical, biological, and other techniques have been used to characterize the parasites (Bensoussan *et al*., 2006 and Abass *et al*., 2013). These techniques are adequate for the diagnosis of CL but are not adequate for the discrimination of the species due to the morphological similarity of different *Leishmania* species (Rehman *et al*., 2016).

Molecular methods are increasingly employed for the diagnostic and the epidemiological purposes in order to confirm *Leishmania* infection and to characterize the parasites at the species or genotype level in hosts and vectors (Schonian *et al*., 2003; Al-Heany *et al*., 2014 and Al-Ajmi *et al*., 2015). The detection of *Leishmania* parasites by PCR methods is highly specific and sensitive, with values reaching up to 100% (Al-Nahhas and Kaldas, 2013 and Ajaoud *et al*., 2013). The targets for amplification with PCR serve either nuclear DNA, such as the SSUrRNA gene, mini-exon regions, the gp63 gene locus, microsatellite DNA, the internal transcribed spacer 1 (ITS1) region or extra chromosomal DNA, such as the repetitive kDNA minicircles (Bensoussan *et al*., 2006 and Eroglu *et al*., 2011). kDNA PCR using universal minicircle primers is considered the most sensitive diagnostic tool to date for detecting leishmaniasis. Diagnostic PCR using the internal transcribed spacer 1 (ITS1) region, located between the 18S and 5.8S rRNA genes, has been shown to be a sensitive and specific method for detecting *Leishmania* DNA in patients with CL or VL (Nasereddin *et al*., 2008). The aim of the present study was to use the PCR method for the direct identification of *Leishmania* species on clinical samples.

2- MATERIALS AND METHODS:

2.1- Sample Collection:

Specimens were collected from infected patients of cutaneous leishmaniasis visited Rizgary and Komary Teaching Hospitals and the Erbil Dermatological teaching center in Erbil Province. Suspected CL is diagnosed clinically by the appearance of diffuse infiltrative erythematous nodules or ulcerated lesions, which present on an exposed area of the body (Fig. 1). Out of 58 patients, 35 were

male and 23 were female with ages of 4-56years, the lesions were located on the: face 20, arms 24 and legs 14. Skin biopsies (skin scrapings 5-12mm in diameter) were taken under sterile condition with the help of scalpel in one direction until blood came out from the lesion and an incision was made mostly in the inflamed border of lesion. Half of the sample was kept in 70% ethanol for PCR and stored at 4 ºC (Amro *et al*., 2012) and the second half was mixed with 1% formalin and kept in a sterile tube for future processes (Eroglu *et al*., 2011).

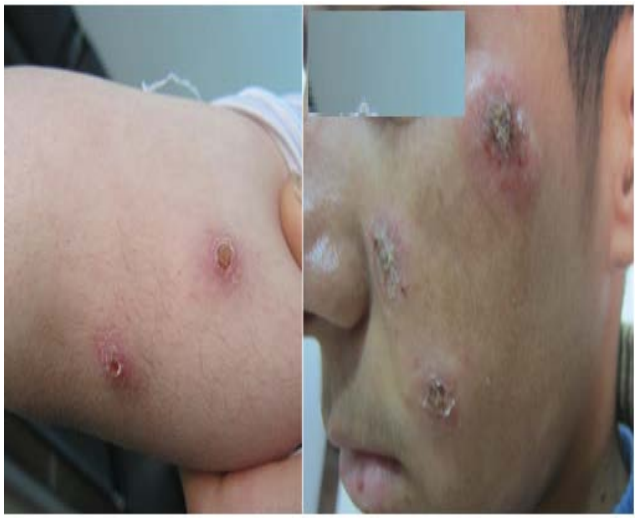

Figure 1: Shows the presence of cutaneous leishmaniasis lesions on arm and face of infected patients in Erbil Province

2.2- DNA Extraction:

Clinical samples were used for DNA extraction, from which 80-100µl was obtained, using the Qiagen DNeasy Blood and Tissue DNA Extraction Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions, PCR product was amplified using Go Taq (Promega, Madison, WI, USA). To define the species and genotyping of extracted DNA fragment; using ribosomal internal transcribed spacer 1; the Primer sequence LITSR (forward) (5'-CTGGATCATTTTCCGATG-3') and L. 5.8S (reverse) (5'-TGATACCACTTATCGCACTT-3') to amplify 320bp for the *Leishmania* species (Akhoundi *et al.*, 2013). The amplification reaction mixture (50µl) consisted of 25µl of (2X) Go-Tag master mix, 30 pmol (3µl) of each primers (forward and reverse), 3 µl of template DNA and 16µl nuclease-free water. The amplification reaction was performed using AB Applied BioSystem thermo-cycler (Veriti 96 Well Thermal Cycler- Singapore), under the following conditions: a pre-amplification step at 95^{o}C for 2 min, followed by 32 cycles with denaturing at 95^{o}C for 20s, annealing at 53^{o}C for 30s and extension at 72^{o}C for 1 minute, and for the final elongation of DNA strands, an ending-extension at 72^{o}C for 6 min. Water instead of DNA was included in each set of PCR reactions as the negative control. The PCR products were visualized by using 2% agarose gel electrophoresis (Promega, USA) at 70 Volts, run for approximately 15minutes initially to move slowly after that increased to 100volts for 45 minutes in 1x TAE buffer (2M Tris-acetate, 50mM EDTA), Prepared by Cleaver Scientific Company-U.K. All PCR products were purified (BeckMan Coulter AMP Pure Xp-USA) and submitted to sequencing using the same primers *Leishmania* used in the PCR (Macrogen EZ- Sequence-Amsterdam,

Netherlands) in both directions and nucleotide data was alined with nuclotides deposited in the NCBI database through the use of BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST/).

2.3- Data Analysis:

Statistical analysis of epidemiological study was carried out using t-test and Chi-square (X2) test to determine the probability value (p-value) using the graph pad (6.1). A total of 58 samples were sequenced to determine the intraspecific genotype. The sequencing was done using the National Center for Biotechnology Information BLAST programs and databases (Accelrys\Accelrys Gene 2.5) (http://accelrys-gene.software.informer.com/).

3- RESULTS:

Fifty-eight patients were diagnosed with cutaneous leishmaniasis. The highest rate of infection was found in males, which was 60.34% (35/58) versus 39.66% (23/58) in females. Furthermore, the highest rates of lesions/or scars were found on the upper extremities (Arms) which was 41.38% , followed by the face (34.48%) and the lowest rates was found on the lower extremities (legs) which was 24.14%,as shown in Table (1). These differences were statistically nonsignificant (P>0.05). On the other hand, the distribution of human CL cases according to age and residency was shown in Table (2). It is indicated that the highest rate of CL was found in the age group 1-9 years which was 27.59% (16/58 cases) and then the rate slightly declined at older ages, but still was high in the age group 30-39 years which was 22.41% (13/58 cases). The lowest rate of the infections was recorded among ages over 40 years, which was 13.79% (8/58 cases). Regarding the residency, the overall rate of CL in the inhabitants of the rural area was higher as compared to that of urban inhabitants (68.97% and 31.077%, respectively).

Table 1: Prevalence of CL lesions and scars among both sexes in Erbil Province

Lesion location	Sexes with Percentage					
	No. of Male (35)	% (60.34)	No. of Female (23)	% (39.66)	Total No. (58)	%
Face	11	31.43	9	39.13	20	34.48
Arms	15	42.86	9	39.13	24	41.38
Legs	9	25.71	5	21.74	14	24.14

P =0.8285

Table 2: Prevalence of CL according to age and residency in Erbil Province

Age group (years)	Residency					
	Urban (18)	% (31.07)	Rural (40)	% (68.97)	Total (58)	%
1-9	7	12.07	9	15.52	16	27.59
10-19	4	6.90	7	12.07	11	18.97
20-29	4	6.90	6	10.34	10	17.24
30-39	1	1.72	12	20.69	13	22.41
Over 40	2	3.45	6	10.34	8	13.79

All PCR products were sequenced with primer LITSR (forward) and L. 5.8S (reverse), the accuracy of the sequencing data was confirmed by sequencing in both directions. The sequence alignment was compared with the previously recorded sequences under the following accession number {*L. tropica* (GU561644, GU561643, FJ948456, FN395025, FN395026, EU683618, EU683617, FJ940894, FJ940895 and AY045763), *L. aethiopica* (GQ920674), *L. major* (HF586399), *L. infantum* (AJ000289) and *L. donovani* (GQ332356)}. The neighbor-joining method was used depending on the sequence sample alignments with CL reference for the classification. Thirty three human samples(1, 2, 6, 8, 9, 11, 13, 15, 16, 18, 19, 22, 23, 24, 25, 29, 30, 31, 34, 35, 36, 37, 39, 41, 43, 46, 47, 48, 50, 54, 55, 56 and 57) were clustered within *L. tropica* which previously described by Toz *et al*.(2009) (FJ940894) in Turkey and Mahdy *et al*.(2010) (GU561644) in Yemen, while 15 human samples (3, 4, 5, 10, 17, 20, 26, 27, 38, 40, 42, 49, 51, 52 and 58) has one nucleotide substitution (C to G) at position 132 with previous records (FJ940894 and GU561644) and they were clustered within (GU561643) Mahdy *et al*. (2010). Furthermore, ten human samples(7, 12, 14, 21, 28, 32, 33, 44, 45 and 53) have more than one nucleotide substitution (G to T) at positions 246 and 276 and (G to A) at position 281 with previous records (FJ940894 and GU561644) and were cluster within (EU683618) Nasereddin *et al*. (2008) as shown in figure (2).

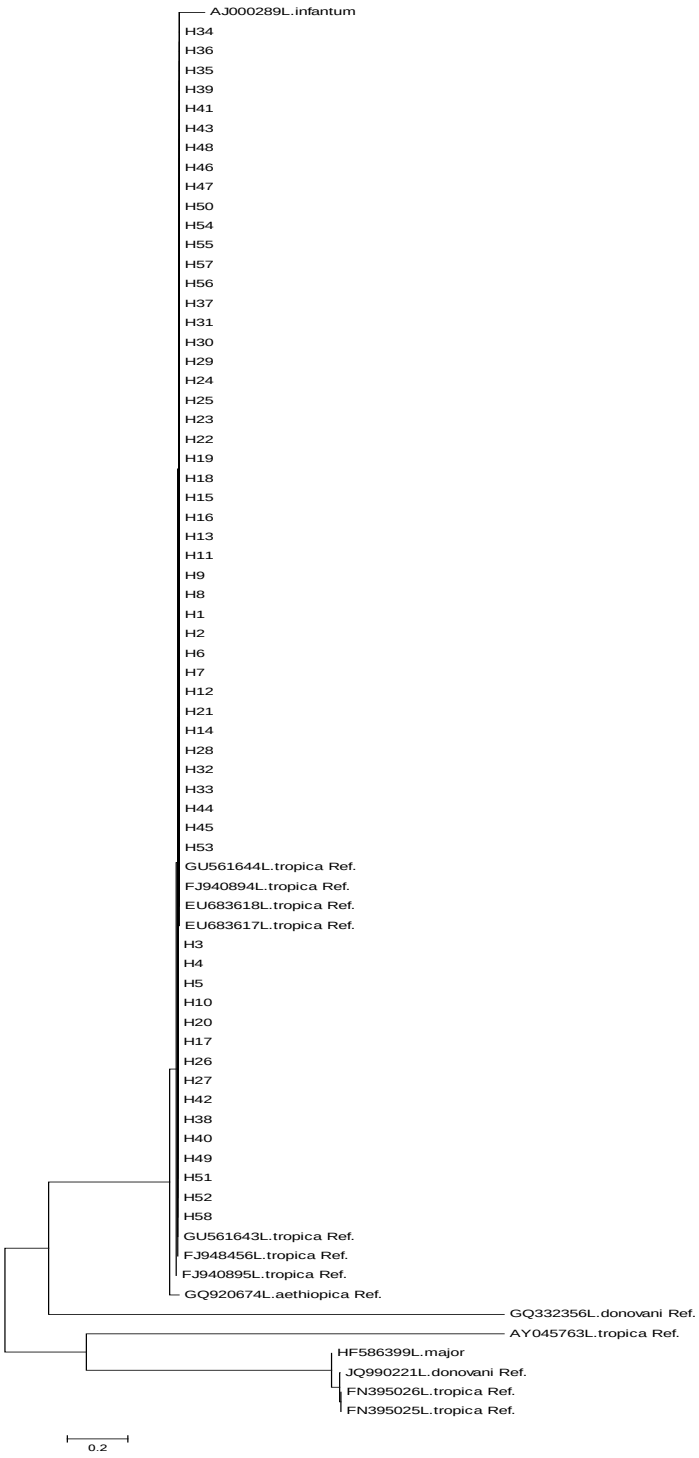

Figure (2): Phylogenetic Tree of the Representative Sequences of CL and Reference Sequences of *L. tropica*, *L. aethiopica, L. major*, *L. infantum* and *L. donovani* Deposited in GenBan

4- DISCUSSION:

Cutaneous leishmaniasis represents a major public health problem in Iraq: from 2008-2015, the incidence rate was 2.9-10.5 cases per 10,000 (Al-Samarai and Al-Obaidi, 2009 and Al-Obaidi, *et al*., 2016). Regarding gender differences, CL have been reported more frequently in males (60.34%) in comparison to females (39.66%). The reason is frequently related with migrants, seasonal and sex workers, truck drivers and employment. Furthermore, males usually work and sleep in open areas in addition to less coverage of their bodies than females, therefore, have more chances of exposure to the infected sand flies. This result is in agreement with the results of Al-Samarai and Al-Obaidi(2009); Curti *et al*. (2011); Soares *et al*. (2014); Bsrat *et al*. (2015); Al-Obaidi *et al*.(2016) ; Hijjawi *et al*. (2016) and Traore *et al*.(2016). The present result disagree with those of Kahime *et al*. (2016) in Morocco, who stated that leishmaniasis affects both genders with an equal distribution of cases between them (56% female and 44% male). This may be attributed to the fact that women suffering from the disease seek medical advice more often than men.

The highest rates of lesions/or scars were on the arms or upper extremities (41.38%) and the lowest rates (24.14%) on the legs (lower extremities) because the arms are more exposed to insect bites than the legs. The present results are in agreed with other studies performed in Iraq (Al-Samarai and Al-Obaidi, 2009 and Khalaf *et al*., 2016), who also reported that the predominant part of the body was arms (57% and 52.94%, respectively). On the other hand, the result disagree with those of Rahi *et al*. (2013) who represented that most affected part of the body was the face (64.3%) with single lesion (46.4%). Furthermore, Bsrat *et al*. (2015) also, showed that the majority of active lesions were observed on the face in which the cheeks and the nose were most affected. A similar pattern was observed regarding the distribution of the scars on different parts of the body.

All ages were affected by different forms of the disease, with a higher incidence (27.59%) in young ages (1-9 years) this may be due to several factors, like, children's spending more time outdoors for playing, sleeping outdoors during hot season, thus the chances of their contact with the insect vector will be increased, the lower rates in older ages may be due to better protection from flies or to a higher immune status (Arroub *et al*., 2012; Rahi *et al*., 2013; Bsrat *et al*., 2015; Al-Obaidi *et al*., 2016 and Kahime *et al*., 2016). While the present result disagree with those of Al-Samarai and Al-Obaidi (2009) and Khalaf *et al*. (2016) in Iraq and Talari *et al*. (2006) in Iran, who reported higher rate of CL infection (57%, 52.94% and 38%, respectively) in patients aged 10-20 years. The higher rate (68.97%) in rural inhabitants as compared to urban (31.07%) one, may be due to agricultural

activities and the long duration of work expose them to insects bites (Rahi *et al*., 2013).

The usual diagnosis of leishmaniasis in Iraq is the clinical features and the examination of stained smears prepared from lesions to look for amastigotes stage (Al-Hamash, 2012). This classical method is not able to differentiate between *Leishmania* species due to their homologous morphology, this may pose a problem particularly in the chronic phase of CL where parasite levels in skin lesions are very low (Rahi *et al*., 2013 and Khalaf *et al*., 2016).Therefore, it is necessary to apply a sensitive and specific method such as, the molecular approach which led to more precise diagnosis and differentiation between different *Leishmania* species, and improve therapy (Laskay *et al*., 1995; Mohammed, 2006; Qader *et al*., 2009 and Al-Nahhas and Kaldas, 2013).

Several PCRs against polymorphic regions, such as the spliced leader mini-exon (10 parasites/ reaction), heat shock protein 70 (30 parasites/reaction), RFLP and kDNA PCR have been developed (Toz *et al*., 2009; Fraga *et al*., 2010; Al-Nahhas and Kaldas, 2013; Auwera *et al*., 2013; El-Beshbishy *et al*., 2013; El-Badry *et al*., 2016 and Hijjawi *et al*., 2016). They can identify the infecting *Leishmania* species, the sensitivity of these assays are not comparable to sequencing techniques used for strains detection (Almeida *et al*., 2011). The results shed light on the presence and the distribution of *Leishmania* spp. for the first time, in Erbil Province. Furthermore, this study reported the existence of three isolates of *L. tropica*. The expansion of this species in Erbil could be due to the movements of immigrants from Mosul, Anbar, Tikriert, and from Baghdad and Syrian refugees. The result in agreement with Serin *et al*., 2005 and Mahdy *et al*., 2010, identify the *Leishmania* species causing CL based on sequencing.

5- CONCLUSIONS:

The PCR-based assays increased the speed and sensitivity of species-specific leishmaniasis diagnosis compared to the conventional techniques and is considered the method of choice for identifying the causative species of CL. The current sequencing results confirmed that CL is mainly caused by *L. tropica* in Erbil Province which is anthroponotic transmission. The test may be used as a new test for the detection of parasites in suspected patients with negative microscopic examination and/or culture results. Species identification is a vital target in the control strategies, therapy, in determining the epidemiology and dynamics of leishmaniasis

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Molecular Characterization of Cutaneous Leishmaniasis Isolated from Human in Erbil Province- Kurdistan Region/ Iraq

Zuber I. Hassan

Department of Veterinary, Shaqlawa Technical Institute, Erbil Polytechnic University, Erbil, Kurdistan Region-Iraq.

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***Corresponding Author:**
Zuber I. Hassan
Vetro_11@yahoo.com

ABSTRACT

Cutaneous leishmaniasis (CL) is a parasitic disease of public health problem in Erbil Province, Kurdistan Region/ Iraq. The identification of the parasite species causing these diseases is a pre-requisite for understanding disease epidemiology. In the current study a higher rate of infection was found in males than females (60.34% versus 39.66%). Most lesions were found on upper limbs (41.38%), while lower rates were found in the legs (24.14%). Regarding age, the highest rate (27.59%) of infections was observed in the age group 1-9 years, while the lowest rate infections (13.79%) were observed in the age group over 40years. Regarding the residency, the overall rate of CL in the inhabitants of the rural area was higher as compared to that of urban inhabitants. The polymerase chain reaction products were sequenced and further examined by sequence analysis, 33 isolates, independent of the host, exhibited L. tropica while in 15 samples, Nucleotide substitution generate polymorphism at position 132 nucleotide (C-G), Furthermore, ten human samples have more than one nucleotide substitution (G to T) at positions 246 and 276 and G to A at position 281. The sequencing of the PCR products recognized 3 haplotypes, which are not described in Kurdistan Region and Iraq. Thus, monitoring of CL and sustained surveillance system is crucial in counteracting the disease, and if possible, to control it.

1. INTRODUCTION

Cutaneous leishmaniasis (CL) is a protozoan Parasitic disease transmitted by sand fly of the genus *Phlebotomus*, with a wide spectrum of clinical manifestations; the most typical are ulcerative skin lesions developed near or at the vector's bite site (Khatri *et al*., 2009 and Soares *et al*., 2014). This protozoan is an obligatory intracellular parasite which exists in two distinctive forms. In humans and other hosts, it occurs as none flagellated amastigotes, while in culture and in the gut of sandflies the flagellar or the promastigotes form is seen (Farahmand *et al*., 2011 and Traore *et al*., 2016). They are neither found in the peripheral blood nor in any visceral organ. The amastigotes are small, round to oval, bodies measure about 2-5µm in diameter and found only in the macrophages of infected vertebrate hosts, The flagellar or the promastigote forms are seen in the culture media and in the gut of the sandfly (Rahi, 2015).

In Iraq, Turkey, Syria, Saudi Arabia, Iran, Israel, Libya, Sudan and Morocco *Leishmania major*, *Leishmania tropica* and less

frequently *Leishmania infantum* cause cutaneous leishmaniasis (Bensoussan *et al*., 2006; Qader *et al*., 2009; Eroglu *et al*., 2011; Farahmand *et al*., 2011; Osman, 2011; Amro *et al*., 2012; Al-Nahhas and Kaldas, 2013; Ajaoud *et al*., 2013; El-Beshbishy *et al*., 2013; Rahi *et al*., 2013; Rahi, 2015 and Al-Obaidi *et al*., 2016 and El-Badry *et al*., 2016). CL is most frequently diagnosed by clinical evaluation (AL-Zaidawi, 1997 and Bensoussan *et al*., 2006) and its typical acute lesions can be confused with other dermatological problems, such as sporotrichosis (Eroglu *et al*., 2011). The traditional diagnostic methods of CL consist of microscopic observation of the parasites in tissue smears after Giemsa staining, and/or culture of promastigotes from tissue (Khalaf *et al*., 2016), additional serological, biochemical, biological, and other techniques have been used to characterize the parasites (Bensoussan *et al*., 2006 and Abass *et al*., 2013). These techniques are adequate for the diagnosis of CL but are not adequate for the discrimination of the species due to the morphological similarity of different *Leishmania* species (Rehman *et al*., 2016).

Molecular methods are increasingly employed for the diagnostic and the epidemiological purposes in order to confirm *Leishmania* infection and to characterize the parasites at the species or genotype level in hosts and vectors (Schonian *et al*., 2003; Al-Heany *et al*., 2014 and Al-Ajmi *et al*., 2015). The detection of *Leishmania* parasites by PCR methods is highly specific and sensitive, with values reaching up to 100% (Al-Nahhas and Kaldas, 2013 and Ajaoud *et al*., 2013). The targets for amplification with PCR serve either nuclear DNA, such as the SSUrRNA gene, mini-exon regions, the gp63 gene locus, microsatellite DNA, the internal transcribed spacer 1 (ITS1) region or extra chromosomal DNA, such as the repetitive kDNA minicircles (Bensoussan *et al*., 2006 and Eroglu *et al*., 2011). kDNA PCR using universal minicircle primers is considered the most sensitive diagnostic tool to date for detecting leishmaniasis. Diagnostic PCR using the internal transcribed spacer 1 (ITS1) region, located between the 18S and 5.8S rRNA genes, has been shown to be a sensitive and specific method for detecting *Leishmania* DNA in patients with CL or VL (Nasereddin *et al*., 2008). The aim of the present study was to use the PCR method for the direct identification of *Leishmania* species on clinical samples.

2- MATERIALS AND METHODS:

2.1- Sample Collection:

Specimens were collected from infected patients of cutaneous leishmaniasis visited Rizgary and Komary Teaching Hospitals and the Erbil Dermatological teaching center in Erbil Province. Suspected CL is diagnosed clinically by the appearance of diffuse infiltrative erythematous nodules or ulcerated lesions, which present on an exposed area of the body (Fig. 1). Out of 58 patients, 35 were

male and 23 were female with ages of 4-56years, the lesions were located on the: face 20, arms 24 and legs 14. Skin biopsies (skin scrapings 5-12mm in diameter) were taken under sterile condition with the help of scalpel in one direction until blood came out from the lesion and an incision was made mostly in the inflamed border of lesion. Half of the sample was kept in 70% ethanol for PCR and stored at 4 ºC (Amro *et al*., 2012) and the second half was mixed with 1% formalin and kept in a sterile tube for future processes (Eroglu *et al*., 2011).

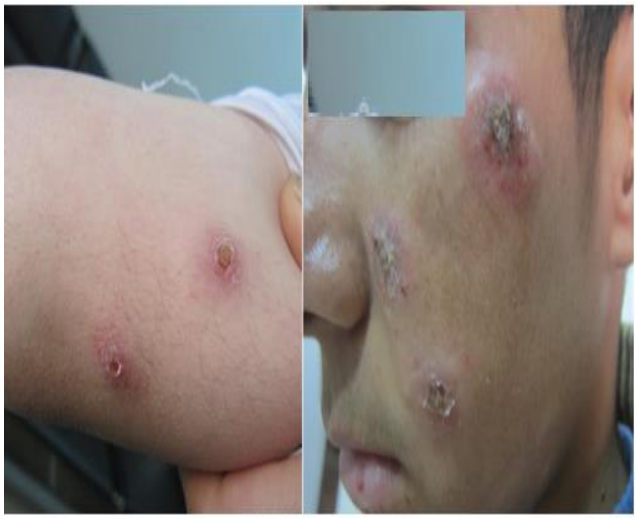

Figure 1: Shows the presence of cutaneous leishmaniasis lesions on arm and face of infected patients in Erbil Province

2.2- DNA Extraction:

Clinical samples were used for DNA extraction, from which 80-100µl was obtained, using the Qiagen DNeasy Blood and Tissue DNA Extraction Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions, PCR product was amplified using Go Taq (Promega, Madison, WI, USA). To define the species and genotyping of extracted DNA fragment; using ribosomal internal transcribed spacer 1; the Primer sequence LITSR (forward) (5'-CTGGATCATTTTCCGATG-3') and L. 5.8S (reverse) (5'-TGATACCACTTATCGCACTT-3') to amplify 320bp for the *Leishmania* species (Akhoundi *et al.*, 2013). The amplification reaction mixture (50µl) consisted of 25µl of (2X) Go-Tag master mix, 30 pmol (3µl) of each primers (forward and reverse), 3 µl of template DNA and 16µl nuclease-free water. The amplification reaction was performed using AB Applied BioSystem thermo-cycler (Veriti 96 Well Thermal Cycler- Singapore), under the following conditions: a pre-amplification step at 95^{o}C for 2 min, followed by 32 cycles with denaturing at 95^{o}C for 20s, annealing at 53^{o}C for 30s and extension at 72^{o}C for 1 minute, and for the final elongation of DNA strands, an ending-extension at 72^{o}C for 6 min. Water instead of DNA was included in each set of PCR reactions as the negative control. The PCR products were visualized by using 2% agarose gel electrophoresis (Promega, USA) at 70 Volts, run for approximately 15minutes initially to move slowly after that increased to 100volts for 45 minutes in 1x TAE buffer (2M Tris-acetate, 50mM EDTA), Prepared by Cleaver Scientific Company-U.K. All PCR products were purified (BeckMan Coulter AMP Pure Xp-USA) and submitted to sequencing using the same primers *Leishmania* used in the PCR (Macrogen EZ- Sequence-Amsterdam,

Netherlands) in both directions and nucleotide data was alined with nuclotides deposited in the NCBI database through the use of BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST/).

2.3- Data Analysis:

Statistical analysis of epidemiological study was carried out using t-test and Chi-square (X2) test to determine the probability value (p-value) using the graph pad (6.1). A total of 58 samples were sequenced to determine the intraspecific genotype. The sequencing was done using the National Center for Biotechnology Information BLAST programs and databases (Accelrys\Accelrys Gene 2.5) (http://accelrys-gene.software.informer.com/).

3- RESULTS:

Fifty-eight patients were diagnosed with cutaneous leishmaniasis. The highest rate of infection was found in males, which was 60.34% (35/58) versus 39.66% (23/58) in females. Furthermore, the highest rates of lesions/or scars were found on the upper extremities (Arms) which was 41.38% , followed by the face (34.48%) and the lowest rates was found on the lower extremities (legs) which was 24.14%,as shown in Table (1). These differences were statistically nonsignificant (P>0.05). On the other hand, the distribution of human CL cases according to age and residency was shown in Table (2). It is indicated that the highest rate of CL was found in the age group 1-9 years which was 27.59% (16/58 cases) and then the rate slightly declined at older ages, but still was high in the age group 30-39 years which was 22.41% (13/58 cases). The lowest rate of the infections was recorded among ages over 40 years, which was 13.79% (8/58 cases). Regarding the residency, the overall rate of CL in the inhabitants of the rural area was higher as compared to that of urban inhabitants (68.97% and 31.077%, respectively).

Table 1: Prevalence of CL lesions and scars among both sexes in Erbil Province

Lesion location	Sexes with Percentage					
	No. of Male (35)	% (60.34)	No. of Female (23)	% (39.66)	Total No. (58)	%
Face	11	31.43	9	39.13	20	34.48
Arms	15	42.86	9	39.13	24	41.38
Legs	9	25.71	5	21.74	14	24.14

P =0.8285

Table 2: Prevalence of CL according to age and residency in Erbil Province

Age group (years)	Residency					
	Urban (18)	% (31.07)	Rural (40)	% (68.97)	Total (58)	%
1-9	7	12.07	9	15.52	16	27.59
10-19	4	6.90	7	12.07	11	18.97
20-29	4	6.90	6	10.34	10	17.24
30-39	1	1.72	12	20.69	13	22.41
Over 40	2	3.45	6	10.34	8	13.79

All PCR products were sequenced with primer LITSR (forward) and L. 5.8S (reverse), the accuracy of the sequencing data was confirmed by sequencing in both directions. The sequence alignment was compared with the previously recorded sequences under the following accession number {*L. tropica* (GU561644, GU561643, FJ948456, FN395025, FN395026, EU683618, EU683617, FJ940894, FJ940895 and AY045763), *L. aethiopica* (GQ920674), *L. major* (HF586399), *L. infantum* (AJ000289) and *L. donovani* (GQ332356)}. The neighbor-joining method was used depending on the sequence sample alignments with CL reference for the classification. Thirty three human samples(1, 2, 6, 8, 9, 11, 13, 15, 16, 18, 19, 22, 23, 24, 25, 29, 30, 31, 34, 35, 36, 37, 39, 41, 43, 46, 47, 48, 50, 54, 55, 56 and 57) were clustered within *L. tropica* which previously described by Toz *et al*.(2009) (FJ940894) in Turkey and Mahdy *et al*.(2010) (GU561644) in Yemen, while 15 human samples (3, 4, 5, 10, 17, 20, 26, 27, 38, 40, 42, 49, 51, 52 and 58) has one nucleotide substitution (C to G) at position 132 with previous records (FJ940894 and GU561644) and they were clustered within (GU561643) Mahdy *et al*. (2010). Furthermore, ten human samples(7, 12, 14, 21, 28, 32, 33, 44, 45 and 53) have more than one nucleotide substitution (G to T) at positions 246 and 276 and (G to A) at position 281 with previous records (FJ940894 and GU561644) and were cluster within (EU683618) Nasereddin *et al*. (2008) as shown in figure (2).

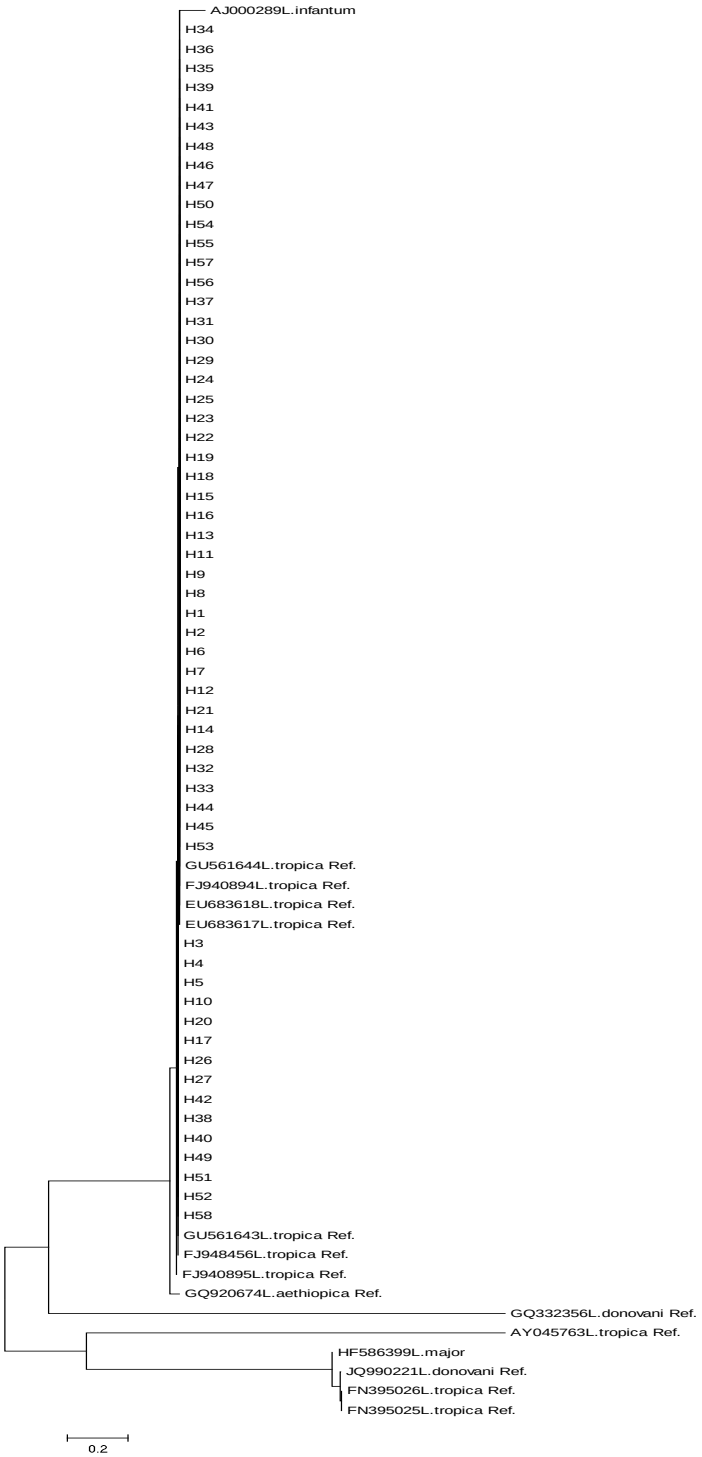

Figure (2): Phylogenetic Tree of the Representative Sequences of CL and Reference Sequences of *L. tropica, L. aethiopica, L. major, L. infantum* and *L. donovani* Deposited in GenBan

4- DISCUSSION:

Cutaneous leishmaniasis represents a major public health problem in Iraq: from 2008-2015, the incidence rate was 2.9-10.5 cases per 10,000 (Al-Samarai and Al-Obaidi, 2009 and Al-Obaidi, *et al*., 2016). Regarding gender differences, CL have been reported more frequently in males (60.34%) in comparison to females (39.66%). The reason is frequently related with migrants, seasonal and sex workers, truck drivers and employment. Furthermore, males usually work and sleep in open areas in addition to less coverage of their bodies than females, therefore, have more chances of exposure to the infected sand flies. This result is in agreement with the results of Al-Samarai and Al-Obaidi(2009); Curti *et al*. (2011); Soares *et al*. (2014); Bsrat *et al*. (2015); Al-Obaidi *et al*.(2016) ; Hijjawi *et al*. (2016) and Traore *et al*.(2016). The present result disagree with those of Kahime *et al*. (2016) in Morocco, who stated that leishmaniasis affects both genders with an equal distribution of cases between them (56% female and 44% male). This may be attributed to the fact that women suffering from the disease seek medical advice more often than men.

The highest rates of lesions/or scars were on the arms or upper extremities (41.38%) and the lowest rates (24.14%) on the legs (lower extremities) because the arms are more exposed to insect bites than the legs. The present results are in agreed with other studies performed in Iraq (Al-Samarai and Al-Obaidi, 2009 and Khalaf *et al*., 2016), who also reported that the predominant part of the body was arms (57% and 52.94%, respectively). On the other hand, the result disagree with those of Rahi *et al*. (2013) who represented that most affected part of the body was the face (64.3%) with single lesion (46.4%). Furthermore, Bsrat *et al*. (2015) also, showed that the majority of active lesions were observed on the face in which the cheeks and the nose were most affected. A similar pattern was observed regarding the distribution of the scars on different parts of the body.

All ages were affected by different forms of the disease, with a higher incidence (27.59%) in young ages (1-9 years) this may be due to several factors, like, children's spending more time outdoors for playing, sleeping outdoors during hot season, thus the chances of their contact with the insect vector will be increased, the lower rates in older ages may be due to better protection from flies or to a higher immune status (Arroub *et al*., 2012; Rahi *et al*., 2013; Bsrat *et al*., 2015; Al-Obaidi *et al*., 2016 and Kahime *et al*., 2016). While the present result disagree with those of Al-Samarai and Al-Obaidi (2009) and Khalaf *et al*. (2016) in Iraq and Talari *et al*. (2006) in Iran, who reported higher rate of CL infection (57%, 52.94% and 38%, respectively) in patients aged 10-20 years. The higher rate (68.97%) in rural inhabitants as compared to urban (31.07%) one, may be due to agricultural

activities and the long duration of work expose them to insects bites (Rahi *et al*., 2013).

The usual diagnosis of leishmaniasis in Iraq is the clinical features and the examination of stained smears prepared from lesions to look for amastigotes stage (Al-Hamash, 2012). This classical method is not able to differentiate between *Leishmania* species due to their homologous morphology, this may pose a problem particularly in the chronic phase of CL where parasite levels in skin lesions are very low (Rahi *et al*., 2013 and Khalaf *et al*., 2016).Therefore, it is necessary to apply a sensitive and specific method such as, the molecular approach which led to more precise diagnosis and differentiation between different *Leishmania* species, and improve therapy (Laskay *et al*., 1995; Mohammed, 2006; Qader *et al*., 2009 and Al-Nahhas and Kaldas, 2013).

Several PCRs against polymorphic regions, such as the spliced leader mini-exon (10 parasites/ reaction), heat shock protein 70 (30 parasites/reaction), RFLP and kDNA PCR have been developed (Toz *et al*., 2009; Fraga *et al*., 2010; Al-Nahhas and Kaldas, 2013; Auwera *et al*., 2013; El-Beshbishy *et al*., 2013; El-Badry *et al*., 2016 and Hijjawi *et al*., 2016). They can identify the infecting *Leishmania* species, the sensitivity of these assays are not comparable to sequencing techniques used for strains detection (Almeida *et al*., 2011). The results shed light on the presence and the distribution of *Leishmania* spp. for the first time, in Erbil Province. Furthermore, this study reported the existence of three isolates of *L. tropica*. The expansion of this species in Erbil could be due to the movements of immigrants from Mosul, Anbar, Tikriert, and from Baghdad and Syrian refugees. The result in agreement with Serin *et al*., 2005 and Mahdy *et al*., 2010, identify the *Leishmania* species causing CL based on sequencing.

5- CONCLUSIONS:

The PCR-based assays increased the speed and sensitivity of species-specific leishmaniasis diagnosis compared to the conventional techniques and is considered the method of choice for identifying the causative species of CL. The current sequencing results confirmed that CL is mainly caused by *L. tropica* in Erbil Province which is anthroponotic transmission. The test may be used as a new test for the detection of parasites in suspected patients with negative microscopic examination and/or culture results. Species identification is a vital target in the control strategies, therapy, in determining the epidemiology and dynamics of leishmaniasis

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Assessment of some hematological, biochemical parameters and cardiac biomarker levels in patients with ischemic heart disease in Erbil city

Kalthum A. Maulood

Biology Department, College of Education, Salahaddin University-Erbil /Kurdistan region- Iraq

A R T I C L E I N F O

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***Corresponding Author:**

Kalthum A. Maulood
Kalthum.maulood@su.edu.krd

A B S T R A C T

The present study was designed to investigate some hematological, biochemical parameters and cardiac biomarker level in male and female patients with ischemic heart disease and healthy person. This study was carried out in the center of heart diseases in Erbil city, one hundred twenty people participated, and sixty of them were suffering from ischemic heart disease (IHD) with both male and female sex in age range 44-72 years. Other sixty healthy people with both sex and same age participate as a control group. The results showed that the white blood cell (WBC)count, increased significantly($P<0.05$), hemoglobin (Hb) and packed cell volume (PCV)level, red blood cell (RBC)count, mean corpuscular volume(MCV) and mean corpuscular hemoglobin(MCH) decreased significantly, mean corpuscular hemoglobin concentration(MCHC) decreased nonsignificantly, and platelet count changed nonsignificantly in male patients with IHD when compared with control group. According to biochemical test the level of serum urea, creatinine, cholesterol, triglyceride increased significantly, low-density lipoprotein (LDL), creatine kinase (CK-MB) isoenzyme and troponin- T level increased nonsignificantly and high-density lipoprotein(HDL) level decreased significantly in male patients with IHD when compared with control group. In female patients with IHD, the WBC count increased significantly, Hb concentration, MCV, MCH and MCHC level decreased significantly, in PCV level, RBC count and platelet count no significant changes observed when compared with control group. There was a significant increase in serum urea, creatinine, cholesterol, triglyceride, LDL and creatine kinase(CK-MB) isoenzyme level, and a significant decrease in HDL level, while there was no significant increase in troponin- T level in female patients when compared with control group. In conclusion, a consequence of ischemic heart disease and other risk factors, increment in white blood cells, decrement in blood cellular and alter cardiac biomarker through elevation of CK- MB isoenzyme and Troponin-T in male and female patients was observed.

1. INTRODUCTION

Ischemic heart disease (IHD) is a case in which there is an insufficient supply of blood and oxygen to a part of the myocardium; it occurs when there is an imbalance between myocardial oxygen supply and request (Antman *et al.*, 2012). IHD is a type of heart

disease with primary appearance that result from myocardial ischemia and may lead to myocardial infarction and abnormal functioning of the heart (Liu *et al*., 2011). Anemia has been known as a risk factor for ischemic heart disease (Shulman *et al*., 1997). Leukocytes are implicated in the pathogenesis of atherosclerosis due to proteolysis, oxidative damage, and leukocyte aggregation and adhesion (Jia *et al*., 2005).Linkage between leukocyte counts with coronary artery disease has been shown to be an independent risk factor and prognostic indicator (Sweetnam *et al*.,1997). An increase of leukocyte counts was associated with a 65% increase in the risk of death from ischemic heart disease (Weijenberg *et al*.,1996). Decreased hemoglobin concentration associated with an increased risk of coronary atherosclerosis and noted that one gram decrease in hemoglobin concentration is an independent risk factor for the development of cardiac morbidity and mortality (Zeidman *et al*., 2004). Hemoglobin concentration could affect the cardiovascular system through oxygen supply and blood viscosity (Finch and Lenfant.1972). Some studies reported that high hemoglobin or packed cell volume level could be a risk factor of IHD in men but not in women, but they did not consider the well-known cardiovascular risk factors (Carter *et al*., 1983 and Campbell *et al*.,1985). Decreased hemoglobin concentration is an independent risk factor for cardiovascular disease in general population (Sarnak *et al*., 2002). Hyperlipidemia is distinguished as a strong modifiable risk factor for cardiovascular disease; serum cholesterol and triglyceride were higher while high-density lipoprotein (HDL) levels were lower in individuals with IHD than in healthy subjects (Gupta *et al*.,2002). A higher prevalence of low HDL and an elevated triglyceride level was found in the country than in the civilian population (Chadha *et al*., 1997).Zrari and Mohammed (2016) indicated that the level of cholesterol, triglyceride and LDL level increased significantly and HDL level decreased significantly in patients with cardiovascular disease. Low HDL level is the most predominant lipid abnormality in men with cardiovascular disease patients (Robins *et al*., 2001). It is estimated that about 40% of patients with coronary disease have normal low density lipoprotein (LDL) levels, and most of these patients have low levels of HDL, with or without increased levels of triglycerides (Rubins *et al*., 1999). Measurement of cardiac markers is an index of care standard in the evaluation and diagnosis of cardiovascular disease, two of the major cardiac markers are Creatine Kinase isoenzyme CK-MB and Troponin T, which are commonly used in the diagnosis of heart disease (Higgins *et al*., 1999).They measure intracellular cardiac tissue molecules released into the circulation after the origination of cardiovascular disease (Lehot *et al*., 1998). In patients with myocardial disease, the CK-MB isoenzyme consist approximately 20% of the total CK in this tissue. CK-MB is sensitive for myocardial injury (Jain 1995). Troponin T is a cardiac-specific marker which, many studies have reported it to be highly sensitive in the detection of cardiovascular disease 5-6 hours after the beginning of chest pain (Durfi et al., 1998). Oluleye *et al*.,(2013) reported that the measurements of high-sensitivity cardiac troponin T are independently related with a range of adverse outcomes, include heart failure, coronary heart diseases and all-cause mortality. Cardiac troponins are used to assist in the diagnosis of chest pain patients with non-diagnostic ECG, also used as predictive indicators of a myocardial infarction and to recognize patients having an increased risk from cardiac events end of the death. (Antman *et al*.,1996).The present study aimed to evaluate some hematological, biochemical parameters, CK-MB isoenzyme and Troponin-T level in male and female patients with ischemic heart disease.

2. MATERIALS AND METHODS

2.1. Design of the Study

A total number of 120 people were studied. Sixty of them were hospitalized patients of both sexes(30 male and 30 female) age range 44-72 years with ischemic heart disease; they were admitted at Hawler center for heart diseases in Erbil (Hawler) province, the other sixty healthy of both sexes with age range 44-

72 years were participate as a control group. All subjects did not suffer from obesity, diabetes mellitus or blood pressure.

2.2. Blood Sampling

Ten ml of venous blood samples were drawn from patients and control group after 12 hours of an overnight fast, 2ml of blood collected into EDTA tubes for determination of hematological parameters and 8 ml of blood collected in nonheparinized tubes, Serum was obtained with centrifugation at 3000rpm for 20 minute, and samples were immediately used for determination of biochemical parameters.

2.3. Hematological Analysis

determination of complete blood count which includes hemoglobin concentration, packed cell volume level, red blood cell count and RBC indices, white blood cell count and platelet count by Fully automated hematological analyzer (USA).

2.4. Determination of serum urea and creatinine.

Serum urea and creatinine level determined by colorimetric and kinetic method kit (Bio-lab, France) (Tietz 1999).

2.5. Determination of lipid profile.

Lipid profile (serum cholesterol, triglyceride, high density lipoprotein and low density lipoprotein) determined by kit for each parameters (Amundson and Zhou 1999).

2.6. Determination of CK-MB isoenzyme and Troponin-T.

CK-MB isoenzyme level determined by kit and Troponin-T level by high-sensitivity assay(Cobas E411)(Saenger *et al*.,2011), and the paper questionnaire which included gender, age, smoking, diabetes mellitus, hypertension, type of cardiac heart diseases including those with echocardiography evidence of possible or definite myocardial was reported .

2.7. Statistical Analysis

Data are expressed as mean and standard error; means of the two groups were compared by student's independent t- test. These statistical tests were performed using the Statistical Package for the Social Science (SPSS) version 20. The significance level was $P<0.05$.

3. RESULTS

Hematological parameters in male patients and control group showed in Table (1) white blood cell count increased significantly with a mean value (9.55±0.56) in male patients when compared with control group (7.93±0.24).Significant decrease noted in level of Hb concentration, PCV, RBC count, MCV and MCH with a mean values (14.29±0.28, 40.76±0.72, 4.27±0.05, 81.65±0.49 and 28.51±0.22) respectively in comparison with control group(15.02±0.14, 43.08±0.41, 4.91±0.08, 89.79±0.73 and 31.14±0.32) respectively. MCHC level decreased nonsignificantly with a mean value (29.23±0.25) when compared with control group (34.94±0.19). Platelet count increased nonsignificantly in male patients with mean value (234.77±9.16) when compared with control group (181.27±5.42).

Parameters	**Patient N=30**	**Control N=30**	**P-value**

WBC×10^3/mm^3	**9.55±0.56**	**7.93±0.24**	**0.008**
Hb g/dl	**14.29±0.28**	**15.02±0.14**	**0.001**
PCV %	**40.76±0.72**	**43.08±0.41**	**0.017**
RBC×10^6/mm^3	**4.27±0.05**	**4.91±0.08**	**0.043**
MCV fl	**81.65±0.49**	**89.79±0.73**	**0.015**
MCH pg	**28.51±0.22**	**31.14±0.32**	**0.013**
MCHC %	**29.23±0.25**	**34.94±0.19**	**NS**
Platelet×10^3/mm^3	**234.77±9.16**	**181.27±5.42**	**NS**

Table (1) Mean±S.E. of some hematological parameters in male patients with ischemic heart disease and control group.

According to the kidney function test which includes serum urea and creatinine, both of them increased significantly in male patients with a mean values (34.87±1.69 and 1.07±0.06) respectively in comparison with control group (26.43±1.21 and 0.93±0.03) respectively. Results of lipid profile which includes serum cholesterol and triglyceride level increased significantly with a mean value (155.80±8.06 and 246.67±42.60) respectively when compared with control group (153.10±4.76 and 107.00±7.92) respectively. A significant decrease in level of High-density lipoprotein with a mean value (31.53±1.02) in IHD patients, in control group was (37.93±1.93). Level of low-density lipoprotein, CK-MB isoenzyme and troponin-T level increased nonsignificantly with a mean values (108.17±4.17, 2.232±0.20 and 0.171±0.06) when compared with control group(99.30±2.63, 1.343±0.31 and 0.158±0.08) respectively. Table (2).

Parameters	**Patient**	**Control**	**P-value**
Serum Urea mg/dl	**34.87±1.69**	**26.43±1.21**	**0.048**
Serum Creatinine mg/dl	**1.07±0.06**	**0.93±0.03**	**0.026**
Cholesterol mg/dl	**155.80±8.06**	**153.10±4.76**	**0.002**
Triglyceride mg/dl	**246.67±42.60**	**107.00±7.92**	**0.002**
HDL mg/dl	**31.53±1.02**	**37.93±1.93**	**0.010**
LDL mg/dl	**108.17±4.17**	**99.30±2.63**	**NS**
CK-MB ng/ml	**2.232±0.20**	**1.343±0.31**	**NS**
Troponin-T ng/ml	**0.171±0.06**	**0.158±0.08**	**NS**

Table (2) Mean±S.E. of Serum urea, creatinine, lipid profile, CK-MB and Troponin-T in male patients with ischemic heart disease and control group.

In female patients with IHD, white blood cell count increased significantly with a mean value (8.75±0.43) when compared with control group (8.10±0.29), Hb concentration, MCV and MCH level decreased significantly with a mean values (12.50±0.14, 80.33±1.30 and 28.43±0.52)respectively when compared with control group(13.99±0.21, 86.02±0.66 and 30.81±0.27)respectively. PCV, RBC count and MCHC level decreased nonsignificantly with a mean values (37.25±0.57, 4.09±0.05 and 34.14±0.29) respectively in comparison with control group (40.28±0.74, 4.77±0.06 and 34.85±0.18) respectively, nonsignificant increase in platelet count with a mean value

(260.00±11.52) was observed in female patients when compared with control group (243.53±14.66).Table(3).

Parameters	Patient N=30	Control N=30	P-value
WBC×10^3/mm^3	8.75±0.43	8.10±0.29	0.016
Hb g/dl	12.50±0.14	13.99±0.21	0.030
PCV%	37.25±0.57	40.28±0.74	NS
RBC×10^6/mm^3	4.09±0.05	4.77±0.06	NS
MCV Fl	80.33±1.30	86.02±0.66	0.012
MCH pg	28.43±0.52	30.81±0.27	0.040
MCHC%	34.14±0.29	34.85±0.18	NS
Platelet×10^3/mm^3	260.00±11.52	243.53±14.66	NS

Table (3) Mean±S.E. of some hematological parameters in female patients with ischemic heart disease and control group.

Table(4) revealed that the level of serum urea, creatinine, cholesterol, triglyceride, LDL and CK-MB were increased significantly with a mean values (43.47±5.03, 0.80±0.06, 178.23±11.32, 199.90±26.27, 114.00±10.21 and 2.415±0.32)respectively when compared with control group, level of HDL in control group was(45.87±1.75)this level was decreased significantly to(35.70±1.50) in female patients, troponin-T level increased nonsignificantly with a mean value (0.086±0.02) when compared with control group (0.040±0.03).

Parameters	Patient	Control	P-value
Serum Urea mg/dl	43.47±5.03	26.57±0.91	0.000
Serum Creatinin mg/dl	0.80±0.06	0.66±0.02	0.003
Cholesterol mg/dl	178.23±11.32	163.40±7.95	0.064
Triglyceride mg/dl	199.90±26.27	117.90±8.19	0.001
HDL mg/dl	35.70±1.50	45.87±1.75	0.016
LDL mg/dl	114.00±10.21	102.77±5.35	0.008
CK-MB ng/ml	2.415±0.32	1.175±0.11	0.002
Troponin-T ng/ml	0.086±0.02	0.040±0.03	NS

Table (4) Mean± S.E. of S. urea, creatinine, lipid profile, CK-MB and Troponin-T in female patients with ischemic heart disease and control group.

4.DISCUSSION

The results of the present study showed a significant increase in the level of WBC count in both sexes' male and female patients with IHD when compared with its control group. This result is analogous with other studies which observed that the elevated leucocyte count (even within normal range) is associated with cardiovascular risk and hypoxia induced by anemia is a kind of stress, which increases vascular reactivity to catecholamine by glucocorticoids, which help in raising leucocyte count (Chavan and Patil .2007).Generally Hb concentration, PCV level, RBC count and RBC indices decreased in male and female patients in this study, and the possible mechanism may be related with decreasing production of these elements and the abnormality of cardiovascular may cause these results. When hemoglobin concentration is reduced to less than half the normal level, ventricular function is impaired, because the coronary flow has reach its maximum (Case *et al*.,1955). Reduced hemoglobin concentration

is responsible for inadequate oxygenation of cardiac muscle, increased stroke volume, decreased peripheral resistance and ventricular remodeling. All these mechanisms have the potential factor to produce the ischemia and related symptoms (Shahzad *et al*.,2009). Lowe *et al*.,1980 showed that the high PCV level or Hb concentration in patients with IHD is associated with an increase in blood viscosity, which may lead to vascular occlusion; this result is disagreement with present study. Statistical analysis revealed that serum cholesterol, triglyceride and LDL increased and HDL level decreased in male and female patients with IHD. As it was mentioned previously, Elevated LDL and cholesterol are considered risk factors for coronary heart disease, also a combination of elevated triglycerides, elevated LDL and low level of HDL recognized as the atherogenic lipid which is important predictor of heart diseases (Tripathy and Shah.1998). Increased Triglyceride is a risk factor for atherosclerosis and influences the lipid deposition and clotting pathway (Ghafoorunissa.1994). The LDL increases the risk of atherosclerosis by its longer resident time in blood whereas the low HDL increases the risk by slower removal of cholesterol from blood to liver for the synthesis of various sterol compounds and also for excretion (Maharjan *et al*., 2008). Stampfer *et al*. reported that estrogen have a protective role against ischemic heart disease especially in women of reproduction period, the current results also revealed that CK-MB isoenzyme and Troponin-T level increased in male and female patients. CK-MB was the most common marker of myocardial necrosis used in the determination for myocardial infarction and constitutes 1–3% of the total CK found in skeletal muscle, and is present in smaller quantities in intestine, diaphragm, uterus, and prostate tissues (Roberts and Sobel .1973). Troponins are regulatory proteins that found in contractile structure of skeletal and cardiac muscle tissue; they are part of the thin filaments within the myofibrils and are essential for the calcium-mediated regulation of muscle contraction. The troponin complex consists of three distinct proteins (troponin I, T, and C) (Kittleson and Kienle 1998). The troponin C, I and T are structural components of the myofilaments that regulate muscle contraction and inhibiting the ATPase activity of actomyosin (Cummins *et al*.,1987) Elevated cardiac troponin has a strong association with an adverse prognosis in patients with acute coronary syndromes (Amsterdam *et al*.,2014). An elevated cardiac troponin level indicates an important diagnostic factor in patients with cardiovascular disease, and Troponin-T may remain elevated for 10–14 days (Wells and Sleeper 2008). It has been shown that men have higher cardiac troponin levels than women and that levels increase with age. (Motiwala *et al*.,2014). Everett *et al*.,(2015) indicate that the factors which cause troponin release in patients with ischemic heart disease, comprise hypertension, metabolic abnormalities, chronic injury from small-vessel ischemia and renal dysfunction. The results of Zrari and Muhammed (2016) revealed that the levels of serum Troponin and Creatine kinase-MB activity in patients with myocardial infraction were significantly higher compared with normal healthy subjects.

5. CONCLUSION

From the present study, it can be concluded that ischemic heart disease cause increasing in the level of WBC count and decrease RBC count and its indices, with increasing the level of cholesterol, triglyceride, LDL and decrease in level of HDL, on the other hand CK-MB isoenzyme and Troponin –T have an important role in the patients with IHD by increasing the level of these two parameters.

Conflict of Interest

There is no conflict of interest.

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