Iraqi Journal of Biotechnology ISSN 1815-4794

Global Impact Factor 0.714

IRAQI JOURNAL OF BIOTECHNOLOGY

VOLUME 14 - NUMBER 2 - 2015

Published by the Institute of Genetic Engineering and Biotechnology for Postgraduate Studies

University of Baghdad

Iraqi Journal of Biotechnology, 14 (2): 2015

Editorial Board

Prof. Abdul Hussein M. AL-Faisal	**Institute of Genetic Engineering and Biotechnology for Postgraduate Studies/ Baghdad University**	**Editor –in -Chief**
Assist. Prof. Ayad J. Kubba	**Institute of Genetic Engineering and Biotechnology for Postgraduate Studies/ Baghdad University**	**Editorial Manager**
Prof. Noria A. AL-Khafagi	**Institute of Genetic Engineering and Biotechnology for Postgraduate Studies / Baghdad University**	**Member**
Prof. Mohammed I. Nadir	**Institute of Genetic Engineering and Biotechnology for Postgraduate Studies / Baghdad University**	**Member**
Assist Prof. Ismail Hussein Aziz	**Institute of Genetic Engineering and Biotechnology for Postgraduate Studies / Baghdad University**	**Member**
Assist Prof. Shurook M. K. Saadedin	**Institute of Genetic Engineering and Biotechnology for Postgraduate Studies / Baghdad University**	**Member**
Assist Prof. Majid Shaii Hamdallah	**College of Agriculture / Baghdad University**	**Member**
Assist Prof. Bushra Mohammed Jaber	**College of Science for Women / Baghdad University**	**Member**
Assist Prof. Muhsin Abd AL-Mousawi	**College of Science / Karbala University**	**Member**
Assist Prof. Ibrahim Ismail AL-Mashhadani	**Research Center for Biotechnology/ AL-Nahrain University**	**Member**

International Editorial Board

Prof. Khalid Tobal	**Guys Hospital / London/ UK**	**Member**
Prof. May R. Talha	**Belgium**	**Member**

Advisory Board from the country

Prof. Ali H. Idhaya	**Tropical Diseases Research Unit / Baghdad University**	**Member**
Prof. Kadhim M. Ibrahim	**College of Applied Biotechnology / AL-Nahrain University**	**Member**
Prof. Saad S. AL-Dijaily	**High Institute for Infertility Diagnosis and Assisted Reproductive Technology / AL-Nahrain University**	**Member**
Prof. Dhuha S. Salih	**College of Science / Baghdad University**	**Member**
Prof. Saad Mohammed AL-Nada	**Biotechnology Research Center / AL-Nahrain University**	**Member**
Prof. Ali M. Al-Shaibani	**College of Education for Women /Baghdad University**	**Member**
Prof. Nidhal A. Al-Mohymen	**College of Medicine / AL-Nahrain University**	**Member**
Prof. Abdul Kareem A. AL-Kazaz	**College of Science / Baghdad University**	**Member**
Assist Prof. Khder AL-Jourani	**College of Science / AL-Mustansiriya University**	**Member**

Advisory Board from Abroad

Prof. Yalçin Kaya	**Trakya University / Turkey**	**Member**
Prof. Natthida Weerapreeyakul	**Faculty of Pharmaceutical Sciences/ Khon Kaen University / Thailand**	**Member**
Prof. Sahapat Barusrux	**Faculty of Associated Medical Sciences/ Khon Kaen University / Thailand**	**Member**
Prof. Paula Row	**College of Medicine / Swansea University/ United Kingdom**	**Member**

Secretary

Zainab H. Hussein	**Institute of Genetic Engineering and Biotechnology for Postgraduate Studies / Baghdad University**	**Interpreter**

Instructions for authors

Iraqi Journal of Biotechnology was founded in 2001, it was first issued in 2002, it is a semi-annual refereed scientific journal issued by the Institute of Genetic Engineering and Biotechnology in Baghdad University in fields of biology, environment, agricultural sciences, medicine and researches specialized in bioinformatics.

1- The author should present a written document containing his address and e-mail.
2- Articles should be typed into two columns and presented in three copies with CD, single spaced, in Times New Roman,10 points font for the abstract and 12 points font for the rest of the article.
3- Margins should be 3 cm for all sided of the page.
4- Manuscript must have the following sections:
Abstract, Introduction, Materials and Methods, Results and Discussion, and a list of References.
5- Manuscripts should not exceed 15 pages.
6- Author's full name and current affiliations must be given immediately below the title of the article, if there is more than one author you have to put numbers (1,2,3) which is depended on the number of the authors.
7- The abstract should not exceed 150 words and not less than 100 words.
8- Key words should be placed at the end of the abstract (five words is enough).
9- Figures, graphs, tables should be included within the text and should be written in one column.
10- References should be arranged in the text as numbers.
References arranged as in examples:

Yoshimoto, M.; Yamaguchi, M.; Hatano, S. and Watanabe, T. (1984). Configurational changes in liner nuclear chromatin caused by Azo dyes. Food and Chemical Toxicology, 22(5): 337-334.

11- The article should be presented after making all the notes of the referee and put it on a CD with a draft copy.
12- The fees of publication 100000 Iraqi Dinars for Iraqi authors.
13- Fees of Publication 100$ for foreign authors.
14- You can contact us on this address:

Institute of Genetic Engineering and Biotechnology

Baghdad University

Baghdad-AL-Jadiriya-P.O.box 12074

Phone number:07728433486

E-mail:journal@ige.uobaghdad.edu.iq

Website: www.iqjb.net

Iraqi Journal of Biotechnology

Contents

Iraqi Journal of Biotechnology

Contents

Iraqi Journal of Biotechnology

Contents

Iraqi Journal of Biotechnology, 2015, Vol. 14, No. 2 , 200-216

Methylation status of *MLH1* and *BNIP3*genes in Iraqi Colorectal Cancer Patients

Wafaa Sabri Mahood [1], Mohammed Ibraheem Nadir[2] and Khalid Tobal[3]

[1]College of Education for Pure Science (Ibn Al-Haitham), Baghdad University
[2]Institute of Genetic Engineering and Biotechnology for Post Graduate Studies, Baghdad University
[3]Guy 's and St Thomas Hospital / King's College / London / UK

Abstract: Epigenetic silencing of genes, mostly mediated by aberrant DNA methylation, is another mechanism of gene inactivation in patients with colorectal cancer. Many genes have been reported with methylation including the *MLH1* gene which is considered as a mismatch repair gene, the methylation in promoter regions of this gene is a frequent event in CRC patients as it associated with gene silence . Other studies indicate loss of expression of *BNIP3* protein due to methylation effect. The purpose of this paper was to elucidate the potential methylation status of *MLH1* and *BNIP3* genes which is cancer-associated gene concerned with mismatch repair and apoptosis-related genes respectively of colorectal cancer and to describe the correlations between the methylation and clinical parameters in Iraqi colorectal cancer (CRC) patients.

The methylation status of those two genes were examined in 35 samples of Iraqi colorectal cancers (primary carcinomas) and the five samples of Iraqi bowel inflammation patients using Methyl Sensitive High Resolution Melting (MS-HRM) and the correlation between the methylation status and the clinicopathological findings was evaluated. The results show aberrant methylation of the MLLH1gene, the methylation were successfully determined in 24 out of 35 samples by using MS-HRM The abnormal methylation status of 5' CpG island of the MLH1gene was detected in 19/24(79.61%) primary colorectal carcinomas and 46.42% of 13(28) in BNIP3 gene primary colorectal carcinomas. The abnormal methylation status of 5' CpG island of the MLH1gene also were detected 3/5 (60%) in DNA samples extracted from inflammatory tissue, and 2/5(40%) inflammatory disease were methylated in BNIP3 gene, suggesting that the aberrant methylation of MLH1 and BNIP3 observed in Iraqi colorectal carcinomas. The clinicopathological data were then correlated with these results. Significant differences were observed with age (p=0.0026) ,tumour location (p=0.0042) , moderately differentiated(p=0.0036) and (T3) lymphatic invasion (p=0.0027). This study provides evidence for hypermethylation status *MLH1* and *BNIP3* genes in CRC patients, which may serve as useful information to understand CRC cancer progression.

Key words: MLH1 , BNIP3 , MS-HRM ,Colorectal ,Iraqi

ميثلة الجينين *MLH*1 و *BNIP*3 في مرضى سرطان القولون-مستقيم العراقيون

وفاء صبري ماهود[1] و محمد ابراهيم نادر[2] و خالد طوبال[3]

[1] كلية التربية للعلوم الاساسية-جامعة بغداد
[2] معهد الهندسة الوراثية و التقانات الاحيائية-جامعة بغداد
[3] مستشفى كايز-لندن-بريطانيا

الخلاصة: كبت الجينات فوق الوراثي غالبا ما يتوسط شذوذ مثيلة الجينات وهي ميكانيكية اخرى لثبيط الجينات عند مرضى اورام القولون والمستقيم. وقد عرفت المثيلة في العديد من الجينات منها *MLH* وهو احد جينات اصلاح عدم تطابق الدنا فقدان التعبير عن بروتين الذي يشفر له *BNIP*3 والذي يلعب دورا في الموت المبرمج للخلايا . هدفت الدراسة الى التحري عن حالة المثيلة لجيني *MLH*1 و *BNIP*3 ذات العلاقة باصلاح الخطا والموت المبرمج على التوالي في مرضى اورام القولون والمستقيم .

تم التحري عن حالة المثيلة في 35عينة من مرضى عراقيين مصابين اورام القولون والمستقيم وخمس عينات من مرضى التهابات معوية باستعمال تقنية Methyl Sensitive High Resolution Melting (MS-HRM) .

بينت النتائج شذوذ المثيلة في جين *MLH*1 إذ نجح الكشف عن المثيلة في 24من 35 عينة في منطقة ال 5CpG من بادئ ال *MLH*1 وكانت حالة شذوذ المثيلة في 24/19 بنسبة 79و61% و 28/13 بنسبة 46,42% لكل من جيني*MLH*1 و *BNIP*3على التوالي عينات الأورام المدروسة وكذلك وجد شذوذ المثيلة في 5/3وبنسبة 60% و5/2 بنسبة 40% لجيني *MLH*1 و*BNIP*3 في عينات التهابات المعوية .تم دراسة الصفات السريرية عند المرضى مع هذه النتائج فقد وجد اختلاف معنوي مع العمر (P=0.0026) ومع موقع الورم (P=0.0042) و Moderately differentiated (P=0.0036) وكذلك T3 Lymphatic invasion (P=0.0027) . تشير الدراسة الى زيادة حالة المثيلة في مرضى اورام القولون والمستقيم العراقيين قد تفيد معلومات هذه الدراسة في فهم تقدم وتفاقم اورام القولون والمستقيم .

Introduction:

Colorectal cancer (CRC) is one of the leading causes of deaths in the world. It is the second most common cause of cancer related deaths in Western countries including the United States. CRC was reported to be responsible for 9% of new cancer cases and 10% of cancer deaths in 2010 in the United States alone (1) .

Epigenetic alterations of specific genes have been reported to be related to colorectal cancer (CRC) transformation and would also appear to be involved in the early stages of colorectal carcinogenesis(2).The first report was indicate to methylation of *MLH*1were done by Kane *et al*., they have examined the expression of hMLH1 in sporadic colon tumors and found that lack of expression of *hMLHI*in correlates with methylation of the hMLHI promoter region. These results indicate that DNA methylation is likely to be a common mode of mismatch repair gene inactivation in sporadic tumors (3). Since that time there are many studies were concluded that hypermethylation of promoter CGIs can prevent transcription of tumor suppressor or mismatch repair genes, such as MutL homolog 1 (*MLH1*), and occurs at an early stage of colorectal carcinogenesis. Methylation of promoter CGIs followed by transcriptional silencing of *MLH1* is present in ~70% of sporadic MSI CRCs(4,5).The DNA methylation of apoptosis-related genes in various cancers contributes to the disruption of the apoptotic pathway and results in resistance to chemotherapeutic

agents. Among the genes, Bcl-2/adenovirus E1B 19 kDa protein interacting protein 3 (*BNIP*3), a Bcl-2 family pro-apoptotic protein (6) . The expression of *BNIP*3 is induced by hypoxia, such as that which occurs during cardiac ischemia and in the hypoxic regions of tumors, and it acts against pro-survival proteins, including Bcl-2 and Bcl-xl (7,8,9) . This study was conducted in Iraqi patients to investigate the mathylation status of *MLH1* and *BNIP3* genes and their relation clinicopathological characteristics.

Materials and Method

Patients' selection:

A total of 40 colorectal cancer Iraqi patients who fulfilled the Modified Dukes classification of Astler and Coller, 1954(10). Those patients underwent to the Gastroenterology and Hepatology Diseases Center (Baghdad) between October, 2011 and June,2012. Samples include 15 male and 14 female with average age 52.5 years range (between 25 to 80 years). Ethical permission to conduct the research was obtained from these hospitals and from all participants in this study. Patients treated with radiotherapy or chemotherapy were excluded.

Two specialized consultant histopathologist examined the H & E sections with heamatoxylin and eosin (H & E) for histological typing, stage grouping, and grading of the colorectal carcinoma.

DNA isolation and sodium bisulphite treatment:

Genomic DNA was extracted from tissue samples using a QIAamp DNA Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol . The extraction of DNA were done in National Center for Early Detection of Cancer in Baghdad.The resulting DNA was sodium bisulphite modified in the Molecular Oncology Unite Guys Guys Guys and St Hospital / King's College / London / UK using an (EpiTect® 96 Bisulfite, Qiagen). Methylated DNA control for Methyl sensitive PCR assays was generated using (M.SssI) CpG Methyltransferase (New England Biolabs).

Methylation-Sensitive HRM assay:

Detection of methylation were done in the Molecular Oncology Unite Guys Hospital / King's College / London / UK.The methylation status of each gene was verified by, Methylation-Sensitive HRM assay as described previously by Wojdacz and Dobrovic (11) .

The primers for is *MLH1* is F-TGCTCCTATTGGCTGGATATTT-3 'and R-CCCGCTACCTAGAAGGATATG-5'.While primers for *BNIP*3 are F-GGTTGCGGGATGTGTTTTAGTTG-3'and R-GGTTGCGGGATGTGTTTTAGTTG-3'

A specific CpG-rich sequence in the *MLH1* promoter region (from -330 to 220 bp)on chromosome 3 relative to the translation start site for human *MLH1*, a 110 bp fragment containing 9 CpG sites) was analyzed by designing primers . This region is thought to be strongly associated with *MLH1* silencing (12,13). The primers of *MLH1* were designed by Primer 3-plus software. A specific CpG-rich sequence in the A specific CpG-rich sequence in the *BNPI3* promoter region 128 bp from (133,645,340-133,645,467)fragment on chromosome 10 (11) . Primers were supplied from sigma- Aldrich / Germany. Analyses were run according to the following conditions: 1 cycle of 95 ºC for 10 min, 2 cycles of 95 ºC for 10 s,60 ºC for 15 s,70 ºC for 20 s .2 cycles of 94 ºC for 10 s,58 ºC for 15 s,72 ºC for 20 s. 2 cycles of 94 ºC for 10 s,57 ºC for 15 s,72 ºC for 20 s. 45 cycles of 94 ºC for 10 s,56 ºC for 15 s,72 ºC for 20 s . Followed by an HRM steps, to prepare melting step of 95 ºC for 1min with Ramp Rate of temperature 4.4 and curve acquisition step of 40 ºC for 1 min with Ramp Rate 2.2.The melting data acquisition began step of 65 ºC for 1 s with Ramp Rate 1.The last step is melting data acquisition ended 95 ºC for 1 s with Ramp Rate 0.02. PCR reaction was performed in a 20 ml total volume 2µl Buffer, 3.2µl $MgCl_2$(25 mmol), 0.4 µl of the four (10mM dNTPs) , 0.2 µl Taq polymerase , 10.7 µl of distilled water,1µl of the Syto9 dye, primer mix 0.5 µl and 2 µl of bisulphite modified DNA, the work conducted in a Thermal Cycler (Light cycler 480 Rocche).Followed by sequencing reaction using Big Dye Terminator kit (Applied Biosystems, Foster City, CA) and the same forward primers . The sequencing reaction was analyzed using the (Verti 96 thermal cycler 9 Applied Biosystems). After product cleanup the sequencing analysis was done using the sequencer 3730.

Results and Discussion:

The *MLH1* Gene methylation:

Aberrant hypermethylation of CpG islands in *hMLH1* promoter regions has been well known to play an important role in the tumorigenesis of human sporadic colorectal carcinoma (14) .The frequency of *MLH1* promoter methylation in sporadic CRC varied from 0.0% (15) to 68% (16).

In the first step, colorectal cancers from 35 Iraqi patients were evaluated for methylation status in *MLH1* . A
stretch of 110 bases incorporating (9) CpG sites in the *MLH1* proximal promoter was analyzed by designing primers which shown in figure (1). *MLH1* methylation were successfully determined in 24 out of 35 samples by using MS-HRM. Twelve samples failed to amplify

when using MS-HRM, that may due to degrade of DNA in FFPE (17).

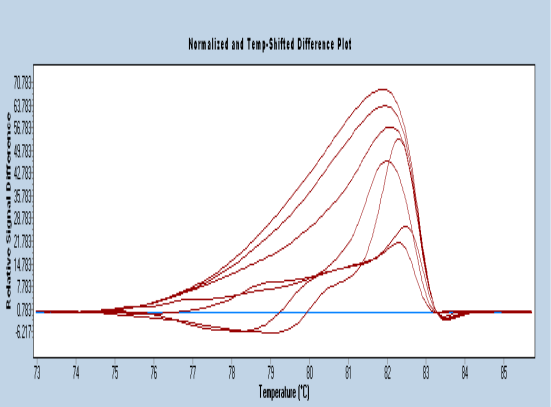
The abnormal methylation status of 5' CpG island of the *MLH1*gene was detected in 19/24(79.61%) colorectal tumor samples,5/24(20.83%) were unmethylated.

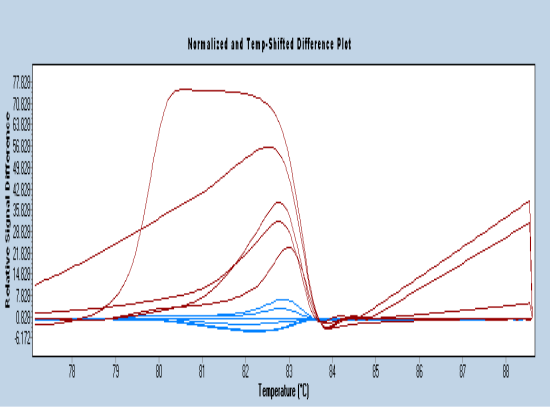
cagatcacctcagcagaggcacacaagcc**CG**gttc**CG**gcatctc*tgctccattggctggatattt*
CGtattccc**CG**agctcctaaaaa**CG**aaccaataggaagagCGgacagCGatctctaaCGCGcaagCG

1 2 3 4 5 6 7 8
*catatccttctaggtagCGgg*cagtagcCGcttcagggagggaCGaagagacccagcaacccacaga
9

Figure (1): Map of the *MLH1* promoter region and primer positions. The sequence numbered relative to the transcription start site for human *MLH1*. Characters in red indicate the forward primer binding sites for methylation-specific MS-HRM,and character in blue indicate the reveres primer the black letter indicate the CpG dinucleotid .

Figure (2,A) shown the normalize melting curve for *MLH1*gene indicating the abnormal methylation in CRC patients. After that cleanup the amplified product of some samples was done to confirm the result by the sequencing analysis.





A: *MLH1* **B:*BNIP3***

Figure (2): MS-HRM assay (normalized melting curves)methylated status are indicated in red color ,unmethylaed in blue color
A:*MLH1*and B:*BNIP3*,isolated from colorectal tissue.

Figure (3) has shown the sequencing analysis for positive and negative cases revealing the methyl site in CpG island ,the methylated case contain cytocine base, which resist the bisulfite treetment as a result of binding of methyl group with cytocine base in CpG dinucleotide in promoter, while the unmethylated case has been change from cytocine to uracil in CpG dinucleotide (promoter region) by bisulfite treatment, and when it amplified it has been change to thymin. Present study indicate that CRC Iraqi patients is more frequency in methylation status than other studiesin *MLH1*gene, *hMLH1* methylation ranged between 21% to 53.4% of CRCs for (18,19). The results indicated that methylation of *MLH1* play important role in pathogenesis of colorectal cancer in Iraqi patients as there is high frequency of methylation in this gene. The *hMLH1* methylation seem to be occur as early event during tumourgenesis that can concludes from many publication ,one study has been reported that CpG methylation of *MHL1* in ~15% of intestinal bowel disease-associated CRC , suggesting that hypermethylation of individual genes, might play a role in inflammation-mediated CRC pathogenesis (20).While other report by Psofaki *et al*., were published that methylation of *hMLH1* might be an initial step strongly associated with MSI-mediated carcinogenesis (21). Methylation of the human DNA mismatch (*hMLH1*) is the principal mechanism underlying the pathogenesis of sporadic high-level MSI (MSI-H) colorectal cancer (CRC) (21,22). Recently, Miyakura and his colleagues referred that ,

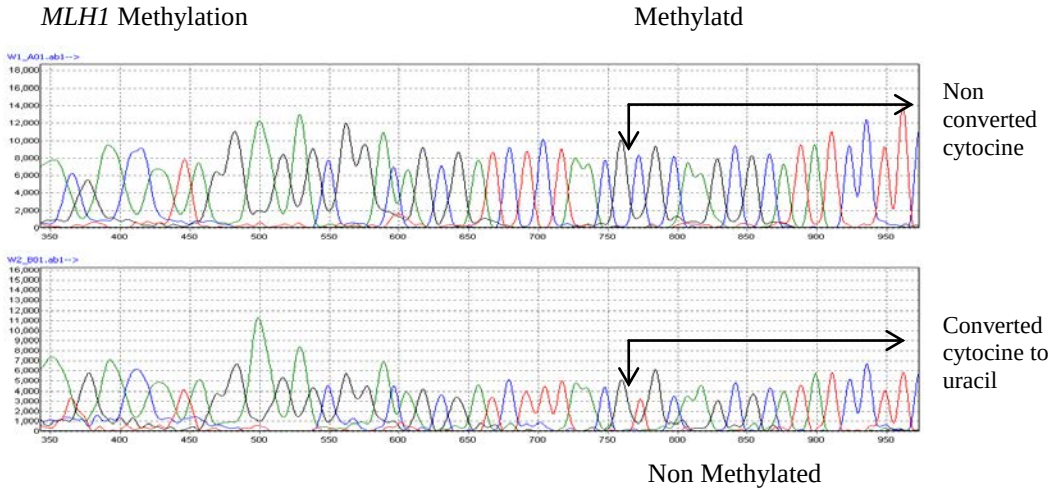


Figure (3): Sequencing from case no. 45 showing MLH1 gene methylation site, upper figure indicated to methylated site compared with un methylated site in lower figure.(Green-A, Red -Thymin , Blue-Cytocine. Black-Guanine).

single-nucleotide polymorphism (SNP) in the *MLH1* promoter region (MLH1-93G/A; rs1800734) has been proposed to be associated with *MLH*1 promoter methylation, loss of MLH1 protein expression and MSI-H tumors. there results indicate that individuals, and particularly females, carrying the A-allele at the MLH1-93G/A SNP, especially in association with the A-allele of rs2276807, may harbor an increased risk of methylation of the MLH1 promoter region (23).Li *et al*., investigate the association between CpG island methylator phenotype (CIMP) and the overall survival of sporadic colorectal cancer (CRC) in Northeast China . may be a predictor of a poor prognosis of CRC in Northeast China patients (24).

Relation of *MLH1* methylation with clinicopathological characteristic:

Clinicapathological parameter association with *MlH1* is shown in table (1). Depend on gender, there was no association relation with *MLH1*methylation while there were high association relation with all other parameter as follow: high frequency of *MLH1* gene methylation in age over fifty years 16/18(88.88%). In respect to tumour location there were distal site high percent of frequency 10(11) 90.90% in comparison with right site 4/5(80%) and 4/7(57.14%) for rectal site. The relation depend on the tumour differentiated,there were little different between the moderately and well differentiated 15 / 16 (93.75%) 5/5(100%) respectively although there was high significant association ($P \leq 0.01$).

Dukeś B and T3 were the most frequency with *MLH1* gene methylation 7/8(87.5%) , 8/9(88.88%) respectively, there were high increase significant ($P \leq 0.01$) regarded all parameter which available. Results in this study in line with Li *et al*.,(25) whose screened large cohort of patients, they found that the *MLH1* promoter methylation may be significantly associated with gender, tumor location, tumor differentiation, MSI and MLH1 protein expression, but they reported lower frequency of *MLH1* promoter methylation in unselected CRC, it was 20.3%. High methylation levels of *MLH1* and *p16* were found in elderly patients with age (73.8 , 65.7) years respectively. Proximal tumours were more often associated with microsatellite instability and higher level of methylation of *hMLH1*, *p16* , while tumours with poor differentiation tended to have higher methylation of the *p16* gene. Local tumour invasion was correlated with the level of methylation of *hMLH1* (26). Compared to MMR-proficient CRCs, MMR-deficient tumors occurred in older patients, and were

more commonly proximally-located. Despite the presence of distinct biological and histopathological characteristics, both tumor types produced similar CFD blood levels (27).

Table (1): Methylation status for *MLH1* in relation to clinicapathological parameter in 24 colorectal cancer Iraqi patients

Variable		no. %	*MLH1* meth.%	*MLH1* un meth.	P Value
Total no.		24			
Gender	Male	11(45.83)	9(81.81)	2(9.09)	0.355 NS
	Female	13(54.16)	8(61.53)	3(23.07)	
Age	≤ 50	6(25)	3(50)	3(50)	0.0028 **
	>50	18(75)	16(88.88)	2(11.11)	
Tumour site	Right	5(21)	4(80)	1(20)	0.017 **
	Left	11(46)	10(90.90)	1(9.09	
	Rectal	7(29)	4(57.14)	3(42.85)	
	unknown	1(4)	1(100)	0	
Differen.	Mod.	16(66.66)	15(93.75)	1(6.25)	0.0043 **
	well	5(21)	5(100)	0	
	Tu.Vi.	2(8.33)	2(100)	0	
	unknown	1(4.16)	0	1(100)	
Dukes Stage		13			
	B	8(61.53)	7(87.5)	1(12.5)	0.0144 **
	C	5(38.46)	5(100)	0	
Frozen		11			
Invasion		13			
	T2	2(15.38)	2(100)	0	0.0058 **
	T3	9(69.23)	8(88.88)	1	
	T4	2(15.5)	2(100)	0	
Frozen		11			

**** (P≤0.01).,meth. -methylated, unmeth.-unmethylated, Differen.-Differentiated ,Tu.Vi Tumour villus.**

***BNIP3* gene methylation:**

BNIP3 is also known to play an important role in the regulation of apoptosis. An increased *BNIP3* expression induces cell death through mitochondrial dysfunction, membrane depolarization, mitochondrial permeability transition pore opening and increased production of reactive oxygen species. A reduced *BNIP3* expression has been identified in a wide range of cancer cells and primary malignancies. A number of studies showed that methylation of the *BNIP3* promoter may play an important role in silencing expression in a range of tumor types (28,29) .

In present study colorectal cancer samples from 35 Iraqi patients were evaluated for methylation status of *BNIP3* gene. The region of 128 bases within the *BNIP3* gene promoter contained 13 CpG sites, analyzed by MS-HRM technique (11) , *BNIP3* amplified were successfully determined by using MS-HRM of 28 samples. Seven samples failed to amplify when using MS-HRM in FFPE**.** The methylation status of 5'CpG island of the *BNIP3*gene was detected, in 15/28(53.57%) colorectal tumor samples,13/28(46.42%) were wild type. Figure (2B) shown the normalize melting curve for *BNIP3* gene indicating the abnormal methylation for CRC Iraqi patients.

The amplified fragments of methylated samples were sequenced using an API 3730 sequencer automated to confirm the result by the sequencing analysis. Figure (4) shown comparable between two sequencing analysis for methylated and non methylated samples. The figure indicated to methylated sites with non converted cytocine in upper figure, the lower figure show the un methylated site with thymin base (red) which results from bisulfite converted cytocine to uracil that amplified to thymin. The results show high frequency of methylation status of *BNIP3*.

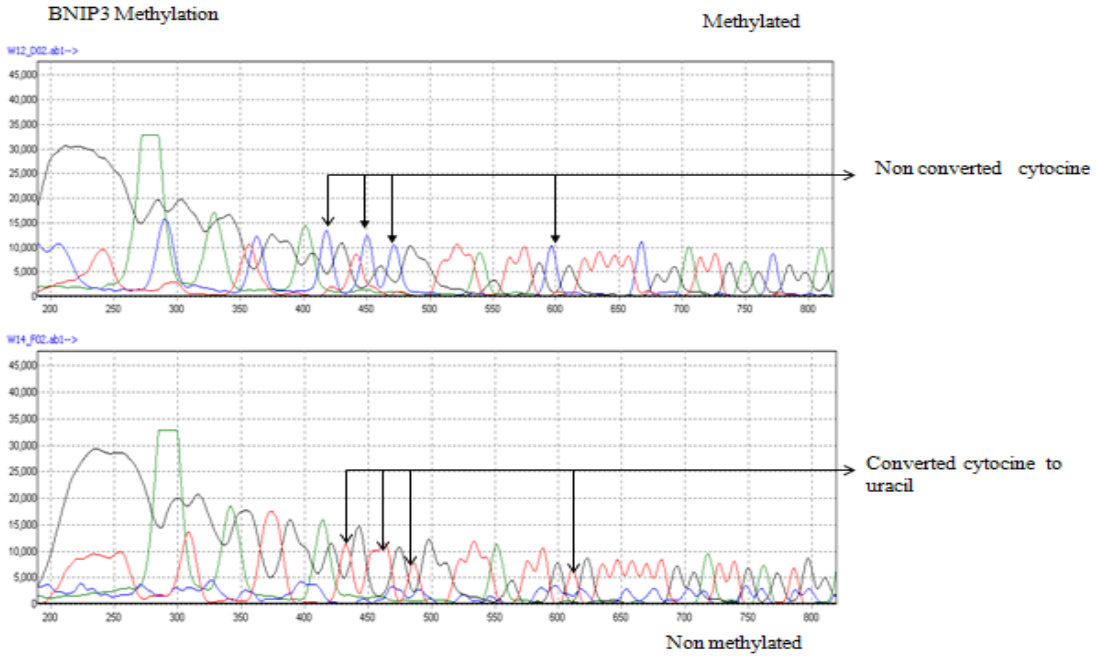


Figure (4): Sequencing analysis from case 42 showing *BNIP3* methylation at sites as pointer. Comparable between two samples, the upper non methylated sequencing and the lower revealing methylated CpGs sites,(Green-A, Red - Thymin , Blue-Cytocine. Black-Guanine).

Gene in Iraqi CRC patients in line with study of Shimizu *et al*., (6) whose reported that *BNIP3* was detected in 58% of the 112 patients, the relatively high frequency of *BNIP3* methylation in primary CRC suggests that this gene is involved in carcinogenesis of the colon and rectum and associated with poor clinical outcome and chemoresistance.

Other study found that *BNIP3* was detcted methylated in 40 (66%) of 61 primary colorectal and 36 (49%) of 73 primary gastric cancers examined, and methylation was closely associated with silencing the gene.

No methylation was detected in samples of normal colorectal and gastric mucosal tissue collected from areas adjacent to the tumors, indicating that *BNIP3* methylation is cancer specific (29). Although solid tumors often contain hypoxic regions, the cells in those regions survive and continue to grow. One way in which this is accomplished is through induction of angiogenesis mediated in part by hypoxic induce factor -1(HIF-1). Because HIF-1activates prosurvival as well as proapoptosis signaling, it has been hypothesized that during progression, cancer cells acquire the ability to escape apoptosis (30).

Colorectal tumour tissue also loses *BNIP3* expression relative to matched normal samples hypoxic (31). *BNIP3* gene methylation is a possible marker predicting a poor response to the S-1/CPT-11 combined therapy in colorectal cancer (32).Silencing *BNIP3* by methylation may be important in tumorigenesis processes in the colon and rectum. Given the potential ability of HIF-1 to evoke apoptosis through its target gene, the down-regulation of *BNIP3* by methylation may disrupt the HIF-1-BINP3 apoptotic pathway and permit cancer cells with high malignant potential to survive, since CRC patients with *BNIP3* methylation had a poorer outcome than those without methylatio (6)

The relation of *BNIP3* methylation and clinicopathological feature:

Looking at table (2) give the relation between methylation status of *BNIP3* gene and clinicopathological feature, there were no association depend on gender, depend on age although there methylation was high frequency in age over fifty years ,14/22(63.63) against 1/6(16.66) in younger age. Respect tumour site, differentiated, Dukes' stage and invasion: The most frequency rate of methylation was found at left site** ($P \leq 0.01$).,meth.-methylated, unmeth.-unmethylated,Differen.-Differentiated 10/13(77.9%),moderately10/19(52.63%) , Dukes' B 9/9(100%) and T310/11(90.9%) respectively, there was significant increase ($P \leq 0.01$) between *BNIP3* metylation with Dukes' and T3 . This results disagree with Shimizu *et al*.,(6) and Iida *et al*.,(19) , they did not find significant correlations were found between *BNIP3* methylation and clinicopathological factors such as age, gender, tumor depth, vessel invasion, lymphatic invasion, lymph node metastasis and stage in patient with colorectal cancer.CRC Patients with *BNIP3* methylation also exhibited a significantly shorter overall survival time (OS) compared to those without methylation (6). Similar the survival time of patients with *BNIP3* methylation was shorter than in absence of methylation in gastric tumors, whereas no such association could be found in breast tumors (33). Since CRC patients with *BNIP3* methylation had a poorer outcome than those with without, it was reported that *BNIP3* silencing induces metastatic growth of breast cancer in the liver, lung and bon (34) .

Table (2): Methylation status for *BNIP3* in relation to clinicapathological parameter in 28 colorectal cancer Iraqi patients

Variable		no. %	*BNIP3* meth.	*BNIP3* unmeth.	P-Value
Total no.		28	15(53.57)	13(46.42)	
Gender	Male	13(46.42)	7(53.84)	5(38.46)	0.355 NS
	Female	15(54)	8(53.33)	7(46.66)	
Age	≤ 50	6(21.42)	1(16.66)	5(83.33)	0.0028**
	>50	22(78.57)	14(63.63)	8(36.36)	
Tumor site	Right	4(14)	1(25)	3(75)	0.0036**
	Left	13(46)	10(76.9)	3(23.07)	
	Rectal	10(36)	3(30)	7(70)	
	unknown	1(4)	1(100)	-	
Differen.	Moderate	19(68)	10(53)	9(47.36)	0.0045**
	well	5(18)	2(40)	3(60)	
	T. Villous	2(7)	1(50)	1(50)	
	unknown	2(7)	2(100)	-	
Duk`s Stage		15			
	B	9(60)	9(100)	-	0.0144**
	C	6(40)	1(16.66)	5(83.33)	
Frozen		13			
Invasion		16			
	T2	3(18.75)	2(66.66)	1(33.33)	0.0059**
	T3	11(68.75)	10(90.9)	1(9.09)	
	T4	2(12.5)	1(50)	1(50)	
Frozen		12			

**** (P≤0.01)., meth. -methylated, unmeth.-unmethylated, Differen.-Differentiated, Tu. Vi Tumour villus.**

CpG site-specific analysis of *MLH1 and BNIP3* in normal tissue:

Five samples from bowel inflammation patients also were evaluated for methylation, Although those samples did not take from colorectal patients but it have methylation in 3/5 (60%) in *MLH1*gene and 2/5(40%) in *BNIP3* . A number of reports have confirmed that methylation is also observed in normal tissue (35,36,37). It has been suggested that methylation in normal tissue may be associated with increasing age (36,38). In conclusion the frequency of *MLH1*

and *BNIP3* promoter methylation in CRC Iraqi patients

were (79.61%) and (53.57%) respectively. There is associated relation between the methylation and clinicopathologic characteristics except the gender in *MLH1* and *BNIP3* , this study provided the groundwork for future large-scale methylation studies in Iraq and suggest recruiting high-risk individuals, for whom colorectal carcinogenesis has not yet occurred and for patients with bowel inflammation disease.

References

1. Hassen, S.; Ali, N. and Chowdhury, P. (2012). Molecularsignaling mechanisms of apoptosis in hereditary non-polyposis colorectal cancer. World J. Gastrointest. Pathophysiol. 3: 71–79.
2. Rengucci ,C.; De Maio, G.; Casadei Gardini, A.; Zucca, M.; Scarpi ,E.; Zingaretti, C.; Foschi, G, *et al*., (2014). Promoter methylation of tumor suppressor genes in pre-neoplastic lesions; potential marker of disease recurrence. J. Exp. Clin. Cancer, Res. 5:65.
3. Kane, M. F. ; Loda, M.;. Gaida,G.M.; Lipman, J.; Mishra,R Goldman, G. J.; Jessup,J.; and Ko1odner, R. (1997). Methylation of the hMLHJ Promoter Correlates with Lack of Expression of hMLH1 in Sporadic Colon Tumors and Mismatch Repair-defective. Cancer Res. 57(5):808-811.
4. Arnold, C.N.; Goel, A.; Compton, C. *et al*., (2004). Evaluation of micro¬satellite instability, hMLH1 expression and hMLH1 promoter hypermethylation in defining the MSI phenotype of colorectal cancer. Cancer Biol. Ther. 3: 73- 78.
5. Samowitz ,W.S.;Albertsen, H.; Herrick, J.; *et al*.,(2005). Evaluation of a large, population based sample supports a CpG island methylator phenotype in colon cancer. Gastroenterology, 129: 837 84
6. Shimizu, S.; Iida, S.; Ishiguro, M.; Uetake ,H.; Ishikawa, T.; Takagi, Y.; Kobayashi, H.; Higuchi, T.; et al.,(2010). Methylated BNIP3 gene in colorectal cancer prognosis. Oncol Lett. (5):865-872.
7. Strasse, A.(2005). The role of BH3-only proteins in the immune system. Nat. Rev. Immunol. 5: 189-200.
8. Willis, S.N. and Adams, J.M.(2005). Life in the balance: how BH3-only proteins induce apoptosis. Curr .Opin. Cell Biol. 17: 617-625.

9. Labi,V.; Erlacher, M.; Kiessling, S. and Villunger, A.(2006). BH3-only proteins in cell death initiation, malignant disease and anticancer therapy. Cell Death Differ. 13: 1325-1338.

10. Astler, V. B. and Coller, F. A.(1954). The prognostic significance of direct extension of carcinoma of the colon and rectum. Ann. Sur. 139:846-852.

11. Wojdacz, K. and Dobrovic,A.(2007).Methylation -sensitive high resolution melting (MS-HRM): a new approach for sensitive and high-throughput assessment of methylation CpG site-specific analysis of MLH1. Nucleic Acids Research, 35: e41 .

12. Capel ,E1.; Fléjou, J.F.; Hamelin, R.(2007).Assessment of MLH1 promoter methylation in relation to gene expression requires specific analysis. Oncogene. 29:7596-600.

13. Wu, P.; Zhang, Z.; Wang, J.; Guo,W.; Xiao,N.; He, Q.; Wang, Y.and Fan,Y. (2012). Germline promoter hypermethylation of tumor suppressor genes in gastric cancer. World J Gastroenterol. 18: 70-78.

14. Wong, J.J.; Hawkins, N.J. ;Ward, R.L. and Hitchins, M.P. (2011). Methylation of the 3p22 region encompassing MLH1 is representative of the CpG island methylator phenotype in colorectal cancer. Mod. Pathol. 3:396-411.

15. Belshaw, N.J.; Elliott, G.O.; Williams, E.A.; Bradburn, D.M.; Mills, S.J.; *et al*., (2004). Use of DNA from human stools to detect aberrant CpG island methylation of genes implicated in colorectal cancer. Cancer Epidemiol. Biomarkers Prev. 13: 1495– 1501.

16. Lin, C. H.; Lin, J. K.; Chang, S.C.; Chang, Y. H. Chang, H. M.; Liu, J.H.; Li, L. H. *et al*.,(2011). Molecular profile and copy number analysis of sporadic colorectal cancer in Taiwan. Biomed Sci. 18: 36.

17. Tournier, B.; Chapusot ,C. ; Courcet , E.; Martin , L.; Lepage , C.; Faivre,J. and Piard , F. (2012).Why do results conflict regarding the prognostic value of the methylation status in colon cancers? the role of the preservation method. B.M.C. Cancer.12:12.

18. Miladi- Abdennadher I.; Abdelmaksoud - Damak, R.; Ayadi, L.; Khabir, A.; Frikha,F.; Kallel, L.; Frikha, M.; *et al*.,(2011). Aberrant methylation of hMLH1 and p16INK4a in Tunisian patients with sporadic colorectal adenocarcinoma. Biosci Rep. 31:257-264.

19. Iida , S. ; Kato, S.; Ishiguro, M. ; Imatsuyma,T.; Ishikawa, T.; Kobayashi, H.; Higuchi, T.; Uetake, H.; Eenomoto, M. and . Sigihara, K. (2012).PIK3CA mutation and methylation influences the outcome of colorectal cancer. Oncol. Lett, 3: 565-570.

20. Antelo, M.; Balaguer, F.; Shia, J.; Shen, Y.; Hur, K.; Moreira, L.; Cuatrecasas, M.; Bujanda, L.; Giraldez, M.D.; Takahashi, M.; et al.(2012). A high degree of LINE-1 hypomethylation is a unique feature of early-onset colorectal cancer. PLoS. One, 7: e45357.

21. Psofaki,V.; Kalogera, C.; Tzambouras, N.; Stephanou, D.; Tsianos, E.; Seferiadis,K. and Kolios,G.(2010). Promoter methylation status of hMLH1 , MGMT , and CDKN2A/p16 in colorectal adenomas. World J. Gastroenterol.16: 3553-3560.

22. Rashid, A.;Shen, L.; Morris, J.S.; Issa, J.P. and Hamilton, S.R.(2001). CpG island methylation in colorectal adenomas. Am. J. Pathol.159: 1129-1135.

23. Miyakura ,Y.; Tahara, M.; Lefor, A.T.; Yasuda, Y. and Sugano, K.(2014). Haplotype defined by the MLH1-93G/A polymorphism is associated with MLH1 promoter hypermethylation in sporadic colorectal cancers. B.M.C. Res. Notes و24:835.

24. Li ,X .;Hu,F .;Wang ,Y1.; Yao, X1.; Zhang, Z1.; Wang ,F1.; Sun, G2.; Cui,B.B2. Dong, X2. and Zhao, Y1. (2014).CpG island methylator phenotype and prognosis of colorectal cancer in Northeast China. Biomed. Res. Int. 2014:236361.

25. Li, X.; Yao, X.; Wang , Y.; Hu , F.; Wang , F. ; Jiang , L. ; Liu ,Y.; Wang ,D.; Sun ,G. and Zhao, Y. (2013). MLH1 promoter methylation frequency in colorectal cancer patients and related clinicopathological and molecular features. PLoS One, 8: e59064.

26. . Mirchev, M.B.; Kahl, P.; Friedrichs, N.; Kotzev, I.A. and Buettner, R.(2010). DNA methylation in patients with colorectal cancer-correlation with some clinical and morphological features and with local tumour invasion. Folia Med. 52:22-30.

27. Lazarev, I.; Leibovitch, L.; Czeiger, D.; Sion-Vardi ,N.;Geffen, D.B.; Douvdevani ,A.and Ariad, S.(2014). Cell-free DNA blood levels in colorectal cancer patients do not correlate with mismatch repair-proficiency. In Vivo. 28:349-354.

28. Okami, J.; Simeone, D.M. andLogsdonm, C.D.(2004).Silencing of the hypoxia-inducible cell death protein BNIP3 in pancreatic cancer. Cancer Res. 64: 5338-5346.

29. Murai, M.; Toyota, M.; Suzuki, H. Satoh,A.; Sasaki,Y.; Akino, K.;Ueno,M.; *et al*., (2005). Aberrant methylation and silencing of the BNIP3 gene in colorectal and gastric cancer. Clin. Cancer Res. 11: 1021-1027.

30. Kothari, S.; Cizeau, J.; McMillan-Ward. E.; Israels, S.J.; Bailes, M.; Ens, K.; Kirshenbaum, L.A. and Gibson, S.B. (2003). BNIP3 plays a role in hypoxic cell death in human epithelial cells that is inhibited by growth factors EGF and IGF. Oncogene. 22:4734–4744.

31. Bacon, A.L.; Fox, S.; Turley, H. and Harris, A.L.(2007). Selective silencing of the hypoxia-inducible factor1 target gene BNIP3 by histone deacetylation and methylation in colorectal cancer.Oncogene , 26: 132–141.

32. Hiraki, M.; Kitajima, Y.; Nakafusa, Y.; Nakamura, J.; Hashiguchi, K.; Sumi, K.; Noshiro, H. and Miyazaki, K.(2010). CpG island methylation of BNIP3 predicts resistance against S-1/CPT-11 combined therapy in colorectal cancer patients. Oncol. Rep. 23:191-197.

33. Sugita, H.; Iida, S.; Inokuchi, M.; Kato, K.; Ishiguro, M.; Ishikawa, T.; Takagi, Y.; Enjoji, M.; Yamada, H.; Uetake, H.; et al.(2011). Methylation of BNIP3 and DAPK indicates lower response to chemotherapy and poor prognosis in gastric cancer. Oncol. Rep. 25, 513–518

34. Manka, D.; Spicer, Z.and Millhorn, D.E. (2005). Bcl-2/Adenovirus E1B 19 kDa interacting protein-3 knockdown enables growth of breast cancer metastases in the lung, liver, and bone. Cancer Res.65:11689–11693.

35. Yoshino, M.; Suzuki, M.; Tian, L. Moriya, Y. ; Hoshino, H.; Okamoto, T.; Yoshida, S.; Shibuya, K. and Yoshino, I. (2009). Promoter hypermethylation of the p16 and Wif-1 genes as an independent prognostic marker in stage 1A non-small cell lung cancers. Int. J. Oncol. 35:1201–1209.

36. Steinmann, K.; Sandner, A. ; Schagdarsurengin, U. and Dammann, R. H.(2009). Frequent promoter hypermethylation of tumor-related genes in head and neck squamous cell carcinoma. Oncol Rep. 22:1519–1526.

37. Shima, K.; Nosho, K.; Baba, Y.; Cantor, M. Meyerhardt, J.A.; Giovannucci, E.L.; Fuchs, C.S. and Ogino, S.(2011). Prognostic significance of CDKN2A (p16 promoter methylation) and loss of expression in 902 colorectal cancers: Cohort study and literature review. Int. J. Cancer.128:1080–1094.

38. Santini, V.; Kantarjian, H. M. and Issa, J.P. (2001).Changes in DNA methylation in neoplasia: pathophysiology and therapeutic implications. Ann Intern Med. 134:573–586.

Iraqi Journal of Biotechnology, 2015, Vol. 14, No. 2 , 217-222

The Relationship Between *Chlamydia pneumonia*e infection and Reactive Arthritis

Dunya Fareed Salloom[1], Rana Saadi Aboud[1], mohammed hadi[2] , Ali Hafedh Abbas[3], Emad Resen[4], AnmarSaadi Aboud[5]

[1]University of Baghdad, Science of College, Biology Department
[2]University of Baghdad, Medical College
[3]University of Baghdad, Science College, Tropical Biological research unit
[4]Al-Mustansiriyah University, Science College, Biology Department

Abstract: Reactive arthritis is aseptic arthritis that can be caused by various infectious agents, *Chlamydia pneumoniae*was associated witharthritis .Eighteen patientswith reactive arthritis of age (20-40) years ,from each subject blood samole was collected to measure anti- Chlamydia Abs, IgM and IgG by ELISA test.tha percentage of IgG,IgMwas(27.7%,0%). with significant differences ($p<0.05$) in compared with studied group.thesereaults indicated that infection with *Chlamydia pneumonia*e may play an important role as triggering factor for reactive arthritis.

Key words: Chlamydia, Reactive arthritis, bacterial infections

العلاقة بين الاصابة ببكتريا الكلاميديا الرئوية والتهاب المفاصل التفاعلي

دنيا فريد سلوم[1] رنا سعدي عبود[1] علي حافظ عباس[2] عماد رسن[1] انمار سعدي عبود[3]

[1]قسم الأحياء، كلية العلوم، جامعة بغداد
[2] كلية الطب، جامعة بغداد
[3]وحدة البحوث البيولوجية، كلية العلوم، جامعة بغداد
[4]قسم الأحياء، كلية العلوم، الجامعة المستنصرية

الخلاصة: يعد التهاب المفاصل التفاعلي من الامراض المتسببة بواسطة عوامل خمجية مختلفة . وتشكل الكلاميديا الرئوية اكثر الاخماج شيوعا وتعرف بالتهاب المفاصل المرنبط بالكلاميديا . تم التحري عن 18 شخص مصاب بالتهاب المفاصل التفاعلي تراوحت اعمارهم 20-40 سنة . خضعت عينات الدراسة لقياس اضداد الكلاميديا الرئوية IgMوIgG باستخدام تقنية الامتزاز المناعي المرتبط بالانظيم ، كانت نسبة اضداد الاضداد الكلاميديا الرئوية IgMوIgG (0 ، 27.7 %) على التوالي وقد لوحظ هنالك فروق معنوية ($P<0.05$) عند المقارنة ما بين مجاميع الدراسة .تشير الدراسة بأن الخمج في بكتيريا الكلاميديا الرئوية يلعب دور مهم كعامل محث في التهاب المفاصل التفاعلي.

كلمات مفتاحية: إلتهاب المفاصل التفاعلي، إصابات بكتيرية الكلاميديا الرئوية

Introduction

Reactive arthritis (ReA) is characterized by a non-purulent joint inflammation that develops in response to infections, often with some latency, at joints distant from the site of the primary infection[1,2] .Certain bacterial infections have been demonstrated to be causative of reactive arthritis , *Chlamydia pneumoniae* comprise the most frequent causative pathogens that elicit reactive arthritis (ReA)[3,4]. ReA due to infection is called *Chlamydia*-associated arthritis (Chl-AA). In addition, mRNA as well as DNA has been detected in the synovial tissue, suggesting that *Chlamydia* is viable in inflamed joints. Thus, the notion that ReA is a sterile inflammation should be reconsidered [5]. Although *Chlamydia*-induced reactive arthritis will often spontaneously remit, approximately 30% of patients will develop a chronic course. Modern medicine has provided rather remarkable advances in our understanding of the *Chlamydia*, as these organisms relate to chronic arthritis and the delicate balance between host and pathogen. *C. pneumoniae* has a remarkable ability to disseminate from the initial site of infection and establish persistently viable organisms in distant organ sites, namely the synovial tissue [3]. Several studies have found that a large proportion of patients with ReA show evidence of present or past chlamydial infection [6,7]. Chlamydial infections may be sub-clinical and, therefore,laboratory identification of the triggering agent is fundamental[8].Recently, the role of infective agents such as *Chlamydia* in the pathogenesis of ReAhas been confirmed by molecular, cultural and serological examinations. In particular, diagnostic methods, such as Real Time Polymerase Chain Reaction (RT-PCR), have made it possible to establish a relationship between chlamydial infection and rheumatologicaldiseases [9]. The detection of a microbe or its components at the site of the primary infection or at the joint is optimal to confirm the infectious etiology of ReA[9]. The aim of present study was to determine the relationship between Chlamydia infection and reactive arthritisby determine the level of antibody.

Patients and methods

Eighteen patients suffering from reactive arthritis were enrolled who attended to Baghdad hospitalThe ages of the total patients were ranged from (20-40) years(9male and 9female). And 18 sample were consider as control Blood samples (5 ml) were collected by disposable syringe into gel plaintubes and stand at room temperature until the coagulant was form. Then the samples centrifuged at 3000 rpm for 5 minutes .Serum

samples were dispended on Ependroff tubes. All samples were marked by the name, day and numbering and stored at (-20C) until carried out to immunological examinations.

Immunological examination

The study groups were carried out to measure anti-*chlamydia pneumonia*e antibodies IgM and IgG by ELISA test(Aeskulisa, Germany) according to the instructions of manufacture company. [10].

Statistical analysis

The statistical analysis system-SAS [11] was used to effect of different factors in study parameters .Chi-square test was used to significant comparison between percentage and least significant difference (LS.D) test was used to significant compression between means in this study.

Results and discussion:

The present study show five patients out of eighteen was positive of ChlamydiaIgG (27.7%) while negative result for IgM antibody in compare to control (0%) for IgM and IgGas in figure 1.Some studies confirm an ongoing *C. pneumonia* infection [positive immunoglobulin IgG and IgM, respectively by enzyme –linked immunosorbent assay (ELISA) [1].Other study describe four ReA patients with serological evidence for recent *C. pneumoniae* infection by microimmunofluorescence MIF test [12].Another studies reported five of seventy patients with acute ReA after an infection with *C. pneumoniae*[13]. Some studies have raised the possibility that *C. pneumoniae* may be a causative agent in ReA and as are often asymptomatic the causative trigger is less clinically apparent in many cases [14]. Bacterial degradation products and even DNA of several microorganisms have been detected in joint fluid/tissue from patients with ReA[15].Some evidence suggests that *Chlamydia* may enter the articular cavity during bacteremia or within monocytes and can survive, probably with intermittent periods of replication sustained by as yet unknown phenomena .The lifecycle of an infectious chlamydial organism probably includes at some stage an arrest in its development that makes it viable but non-cultural [16,17]. Indeed, macrophagesor dendritic cells would seem the most likely carriers of organisms into the joint,since they will be able to take up bacteria at the site of infection, enter the circulation, and be recruited to the synovial membrane. In support of this idea chlamydia have been detected in peripheral blood leucocytes of reactive arthritispatients and dendritic cells

in the joint stimulate chlamydia specific T cells [18]. CD4+ T cells play the major part in controlling chlamydial infection, probably through their production on interferon gamma, but protective CD8+ T cells have also been described in mice, and more recently in humans. The relative importance of responses by these subsets in humans has not yet been established, and Chlamydia specific CD8+ T cells have not yet been isolated from human joints. Nevertheless, CD8+ T cells are activated in the joint and also produce a similar set of cytokines as CD4+ T cells and the failure to isolate Chlamydia specific T cells may simply reflect technical difficulties in working with these cells [19]. As a number of investigators have pointed out, in many instances the initial elicitation of disease by a pathogen during primary infection is simply a preliminary for establishment of a longer-term habitation of the host.*In vitro* and *in vivo* studies of persistence did identify one modulation of expression for a set of genes of particular interest in the biology of chlamydial persistence in the joint. Transcript analyses targeting the three Hsp60-encoding genes demonstrated high levels of expression for each of them during normal active infection, with expression levels of the CT604 and CT755 genes exceeding that of the authentic original *groEL* (CT110) gene [20,21].

Conclusion

The result of present study indicate that infection with *Chlamydia* may play important role as triggering factor for reactive arthritis.

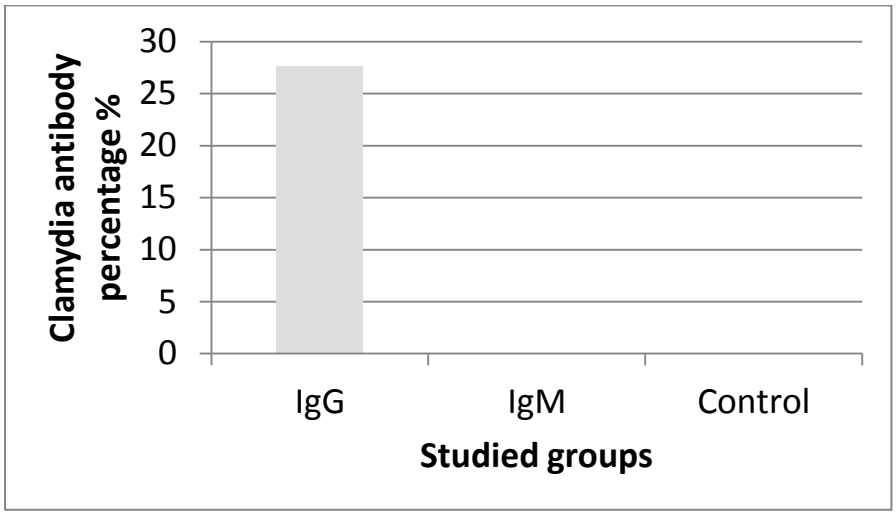


Figure 1: *Chlamydia pneumonia* antibody percentage in studied groups

References

1. Rizzo,A.; Di Domenico,M.; Carratelli,C.R.andPaolillo,R.(2012).The Role of *Chlamydia* and *Chlamydophila*Infections inReactiveArthritis.Intern.Med.51:113-117.
2. Hannu,T.; Puolakkainen,M. and Leirisalo-Repo,M.(1999). *Chlamydia pneumoniae* as a triggering infection in reactive arthritis.Rheumatol.38:411-414.
3. Carter, J.D.; Inman, R.D.; Whittum-Hudson, J. and Hudson,A.P.(2012). *Chlamydia* and chronic arthritis. Ann.Med.44(8):784-792.
4. Zeidler, H.and Hudson, A.P.(2014).New insights into Chlamydia and arthritis. Promise of a cure? Ann. Rheum. Dis. 73(4):637-644.
5. Kotake , S. and Nanke, y.(2011) .[Chlamydia-associated arthritis and enteropathic arthritis-tyo important spondyloarthritides].Nih.Rin.Men.Gakk.Kai.34(3):121-130.
6. Hervé, C.G.; Judith, A.; Whittum-Hudson, J.D.C.andAlan,P.H. (2010).The Pathogenic Role of *Chlamydia* in Spondyloarthritis**.**CurrOpinRheumatol. 22(4):363-367.
7. Carter, J.D.; Espinoza, L. R.; Inman, R.D.; Sneed, K. B.; Ricca, L. R; Vasey, F.B.; Valeriano, J.; Stanich, J. A.; Oszust, C.; Gerard, H. C. and Hudson, A. P. (2010). Combination antibiotics as a treatment for chronic Chlamydia-induced reactive arthritis:A double-blind,placebo-controlled prospective trial. *Arthritis & Rheumatism*.62(5):1298-1307.
8. Brzank,M. and Wollenhaupt, J.(2013). Infection-induced reactive arthritis : etiopathogenesis, clinical spectrum, therapy].Rheumatol.72(10):977-985.
9. Zeidler, H.; Kuipers, J.and Kohler, L.(2004). Chlamydia-induced arthritis. CurrOpin. Rheumatol. **16**: 380-392.
10. Villegas, E.; Sorlozano, A. and Gutierrez, J.(2010). Serological diagnosis of *Chlamydia pneumoniae* infection: limitations and perspectives.J.Med.Microbiol.59 (11).1267-1274.
11. SAS. **(**2010**).** Statistical Analysis System, User's Guide. Statistical. Version 9.1th ed. SAS. Inst. Inc. Cary. N.C. USA.
12. Hamidreza Honarmand. (2012). Pathogenesis of Chlamydia pneumonia Persistent Illnesses in Autoimmune Diseases, Chlamydia, Prof. Mihai Mares (Ed.), ISBN: 978-953-51-0470-4, InTech, Available from http://www.intechopen.com/books/chlamydia/chlamydia-pneumoniae-and-autoimmune-diseases.

13. John, D.C. and Alan, H.(2010). The evolving story of Chlamydia-induced reactive arthritis.Curr.Opin.Rheumatol.22 (4):424-430.

14. Carter, J.D.; Gerard, H.C and spinoza, L.R. (2009).Chlamydiae as etiologicAgents in chronic undifferentiated spondylarthritis. ArthritisRheum **60**: 1311-1316, 2009.

15. Lad, S.P.; Fukuda, E.Y.; Li, J.; de la Maza, L.M. and Li, E. (2005).Up-regulation of the JAK/STAT1 signal pathway during Chlamydia trachomatis infection.JImmunol**174**: 7186-7193.

16. Kuipers, J.G.; Zeidler, H. and Kohler, L.(2003). How does Chlamydia cause arthritis? Rheum. Dis .Clin. North. Am **29**: 613-629, 2003.

17. Zhang, X.; Glogauer, M.; Zhu, F.;Kim, T.H.; Chiu, B. and Inman, R.D.(2005). Innate immunity and arthritis: neutrophil Rac and toll-like receptor 4 expression define outcomes in infection-triggered arthritis. Arthritis. Rheum.**52**: 1297-1304.

18. Gracey ,E. and Inman,R.D.(2012) Chlamydia-induced ReA: immune imbalances and persistent pathogens.Nature.Rev.Rheumatol.8:55-59.

19. Contini , C.; Grilli, A; Badia , L.;Guardigni, V.; Govoni , M. and Seraceni,S.(2010). Detection of Chlamydophila pneumoniae in patients with arthritis: significance and diagnostic value.Rheumatol.Inter.31(10):1307-1313.

20. Gérard, H.C.; Whittum-Hudson, J.A.; Schumacher, H.R. and Hudson, AP.(2004).Differential expression of the three *Chlamydia trachomatis* hsp60-encoding genes in active *vs* persistent infection. Microb. Pathogen. 36:35–39.

21. Carter, J.D.; Gerard, H.C.; Whittum-Hudson, J.A.; Hudson, A.P.(2012). The molecular basis for disease phenotype in chronic *Chlamydia*-induced arthritis.Int J ClinRheumtol. 7(6): 627–640.

Iraqi Journal of Biotechnology, 2015, Vol. 14, No. 2 , 223-237

Study The Effect Of Purified G*oat* Milk Lactoferrin And Bovine Lactoferrin on Cancer Cell Growth (ANG) *In vitro*

Kifah S .Doosh[1] , Zainab H.Abbas[2], Nahi Y. Yaseen[3]

[1] **College of Agriculture / Baghdad University, Baghdad**
[2] **College of Agriculture / Karbala University, Karbala**
[3] **Iraqi center for Caner and Medical Genetic Research / Al-Mustansiriyah Uni.**
E-mail: oum_zain@yahoo.com

Abstract: Lactoferrin is an important protein in many biological applications as a potential cancer treatment agent . In this study, lactoferrin was purified from goat colostrum by Ion exchange chromatography by using CM-Sephadex G-150 column and gelfiltration by using Sephadex G-200 column .To know its ability as anticancer agent the study utilized an *in vitro* evaluation for the cytotoxic effect of the purified goat milk lactoferrin (gLf) on two cell lines, ANG (Ahmed Nahi Glioblastoma Multiform (ANG) cell line and Rat Embryo Fibroblast (REF) cell line at different concentrations and different exposure time of treatment. The purified gLf concentrations ranging (19.53 to 5000) µg/ml for 24,48 and 72 hours was test . The effect of gLf was evaluated by employing MTT assay. The results revealed significant cytotoxic effect at levels ($P<0.05$) for all concentrations and for all exposure time of gLF on ANG cell line as compared to untreated control cells, The inhibition rate IR% increased with raising of gLF concentration and incubation period The highest inhibitory growth was at the concentration (5000 µg/ml) after 72hrs of exposure time (76.06%) ,while only the highest concentration gave significant inhibitory effect ($P< 0.05$) with normal cell REF.

Keywords: goat lactoferrin, ANG, REF, MTT assay ,Anticancer

دراسة مقارنة لتأثير اللاكتوفيرين المستخلص من لبا الماعز وحليب الابقار على كل من خط خلايا ورم الدماغ البشري متعدد الاشكال وخط خلايا الليفية الطبيعية لأجنة الفأر

كفاح سعيد دوش [1] زينب هادي عباس [2] ناهي يوسف ياسين [3]

[1]كلية الزراعة - جامعة بغداد
[2]كلية الزراعة - جامعة كربلاء
[3]المركز العراقي لبحوث السرطان والوراثة الطبية- الجامعة المستنصرية

الخلاصة: يعد اللاكتوفيرين من المواد البروتينية ذات التطبيقات البيولوجية المتعددة والمهمة لاسيما دوره الفعال في علاج العديد من انواع السرطان .في الدراسة الحالية نقي بروتين اللاكتوفيرين من لبأ الماعز بطريقة التبادل الأيوني باستخدام عمود-CM-Sephadex G 150 ثم الترشيح الهلامي باستخدام عمود Sephadex G-200 . تم التحري عن التأثير السمي الخلوي لبروتين اللاكتوفيرين المنقى من لبأ الماعز goat milk Lactoferrin (gLF) في نوعين من خطوط الخلايا هي خط خلايا سرطان عنق الرحم البشري(ANG) وخط الخلايا الليفية الطبيعي لأجنة الفار (REF) باستخدام تراكيز مختلفة منه تراوحت (53،19 - 5000) ميكروغرام / مل ولفترات تعرض مختلفة (48،24 و 72) ساعة. عدا خط الخلايا الطبيعية REF فدرس تأثيره عليها بعد مرور 72ساعة فقط ، تم تقييم تأثيرgLF بحساب نسبة تثبيط نمو خلايا ANG و REF من خلال الفحص باستعمال صبغة MTT أظهرت النتائج فعل gLf المضادة للسرطان يزداد بزيادة التركيز ووقت التعرض وان اعلى نسبة تثبيط بلغت 76.06 %كانت عند تركيز 5000 ميكروغرام / مل بعد فترة تعرض72 ساعة ، في حين كان تاثير gLf محدود على الخلايا الطبيعية REF الا في التراكيز العالية منه.

الكلمات المفتاحية:لاكتوفيرين حليب الماعز ، خلايا ANG ، خلايا REF ، صبغة MTT ، Anticancer

Introduction

Cancer is a group of diseases characterized by uncontrolled growth and spread of abnormal cells cancer, in which normal cells start multiplying uncontrollably ignoring

Cancer is the second leading cause of mortality worldwide as it still takes millions of people lives every year around the world. In 2008, almost 12.7 million people were diagnosed with cancer and more than 7.5 million of them were dead (2). The world health organization (WHO) estimated that if unchecked, annual global cancer deaths rates could rise to 15 million by 2020 (3). Recently in Iraq there is a terrible number of unpublished cancer cases beside the published cases by the Iraqi Cancer Council in 2008 were 19.9 thousand and more than 15.4 thousand of them were died (4). The current conventional cancer treatment options for localized tumors and advanced disease are typically associated with risks and side effects (5). The discovery of anticancer drugs remains a highly challenging endeavor and cancer a hard-to-cure disease (6). The new protocols for cancer therapy include biological natural products. An increasing interest has been

reported on the use of biologically active substances from food (7). Milk and dairy products have become recognized as functional foods , suggesting their use has a direct and measurable effect on health outcomes, namely that their consumption has been related with a reduced risk of numerous cancers (8). *Invivo* studies showed that oral administration of bovine LF to rodents significantly reduces chemically induced tumorigenesis in different organs (breast, esophagus ,tongue, lung, liver, colon and bladder) and inhibits angiogenesis (9,10). Bovine LF are likely to exert various physiological effects in the digestive tract. Moreover, subcutaneously administration of LF inhibited the growth of implanted solid tumors and exerted preventive effects on metastasis (11).These activities of LF have been attributed to its immunomodulatory potential and ability to activate T and NK cells (12) Furthermore, LF was found to induce apoptosis in several human cell lines, as for example A459 lung cells, CaCO-2 intestine cells and HTB9 kidney cells (13) .Moreover, LF was effective against melanoma cells (14), head and neck cancer cells (15).There is no study about the effect of goat milk Lactoferrin on any cancer cell line in Iraq ,Therefore this project was designed to study the effect of a range of LF concentrations at different exposure times on cell viability by using cancer cell line as follows:

1. Isolation and purification of Lactoferrin from goat colostrum by ion exchange chromatography and gel filtration.
2. Study the cytotoxic effect of purified goat Lactoferrin on the growth of cancer cell lines (ANG), and on normal cell line (REF) *in vitro*.

Material and Methods

Goat colostrums

Goat colostrum was obtained from Ruminants researches station , Directorate for agricultural researches-Ministry of Agriculture ,Abu-Grip -Baghdad. The samples were collected within the first five days after goat parturition and were immediately frozen and stored at -18°C until use.

Preparation of acid colostrum whey

The colostrum was skimmed by centrifugation in Sigma MA3-18 centrifuge at 4000 g/min for 30 min at 4°C. Colostrum whey was prepared by precipitation of the casein from skimmed colostrum by addition 1N HCl until pH reached to 4.6, the precipitated casein was removed by centrifugation at 10000g/min for 15 min at 4°C. The supernatant (whey) was adjusted to pH 6.8 with 1N NaOH and dialyzed

against distill water for 18 hr, and then stored at −18°C until use. (16).

Isolation and Purification of lactoferrin

Isolation and purification procedures by (17) were used to separate lactoferrin from other proteins in goat colostrums .The procedure involved cation exchange chromatography using cation exchanger CM-Sephadex G-50 and gel filtration chromatography by using Sephadex G-200

Cell Growth

Ahmed Nahi Glioblastoma Multiform (ANG) cell line and Rat Embryo Fibroblast (REF) cell lines were kindly provided from Iraqi Center of Cancer and Medical Genetic Researches. The cell lines cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mmol/L glutamine, Streptomycin (100 U/ml), penicillin (100 U/ml), and incubated in 5% CO2 at 37 ºC for 24 h. Cell counts determined using 0.2 ml of trypan blue solution and 1.6 ml PBS , then subculture when monolayer's cells were confluent. Afterwards, 200 µl of cells in growth medium were added to each well of a sterile 96-well microtiter plate. The plates were sealed with a self-adhesive film, lid placed on and incubated in 5% CO2 at 37°C. When the cells are in exponential growth, *i.e.* after lag phase, the medium was removed and serial dilutions of purified goat milk lactoferrin in SFM (5000 µg/ml, 2500 µg/ml, 1250 µg /ml, 625 µg/ml, 312.5 µg /ml,156.25 µg /ml,78.125 µg /ml,39.625 µg /ml and 19.53 µg/ml) were added to the wells. four replicates were used for each concentration. The middle two columns as control (cells treated with SFM only). Afterwards, the plates re-incubated under the same condition for the selected exposure times (24, 48, 72 hrs).

Cytotoxicity assay

200µl of cell suspension (Confluent monolayer's) of both ANG and REF were seeded into wells of a 96-well plate. After 24 hrs of incubation 200 µl of glf dilutions were added. Four replicates were used for each concentration of gLF Afterwards, the plates were re-incubated at 37°C for the selected exposure times (24, 48, 72 hrs).The cytotoxicity test was determined by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. In brief, 50 µl of MTT was added to the wells, the cells were cultured for additional 4 hrs at 37ºC. Then 100 µl of DMSO was added to the wells. The solubilized formazan was measured at 550 nm using microplate spectrophotometer (Multiskan, Finland). The % Inhibition were

calculated with the following formulae

% Inhibition = 1-(OD of sample / OD of control) x 100

Results and discussion

Isolation and Purification of lactoferrin

Two steps were used to purify lactoferrin from colostrum whey, ion exchange chromatography and gel filtration were applied respectively.

Fig (1) shows one protein peak appeared in the washing step belong to unbounded proteins ,while two protein peaks appeared in the elution the first protein peak with green color refers to the lactoperoxidase enzyme ,while the second protein peak was given pink color which shows high value when read on the wave length of 465nm Special detects for protein lactoferrin (19). Results indicated that the second peak which has LF that appeared in fraction (165-175) and eluted by using 0.5M of NaCl. The fractions of second peak were pooled and desalted by dialyzing against distilled water overnight, and then concentrated by sucrose. The results of this study compatible with several other studies (20) purified LF from goat milk by using CM-Toyopearl 650M column followed with AF-Heparin Toyopearl column, and they found two protein peaks in elution the first peak belonged to lactoperoxidase enzyme and the second peak belonged to LF . (21) isolated LF from Carabo's *(Bubalus bubalis* L.*)* milk whey by ammonium sulfate precipitation and cation exchange chromatography using carboxymethyl cellulose (CMC), (22) purified LF from goat colostrum by CM-Sephadex G-50 ion-exchange column and affinity chromatography on *Hi-Trap* Heparin HP. Moradian (23) enabled purified LF from colostrum of cow's milk with one step by using CM-sephadex G-50, a cation exchange chromatography and they determined LF concentration by Bradford assay which was about 2.4 mg/ml, with good biological activity and purification efficiency was about 90%.

Gel filtration chromatography

Results in Fig (2) showed one protein peak with light pink color appeared in the eluted fractions (20 to 30) had the LF protein which shown high value when read on the wave length of 465nm Special detects for protein Lactoferrin . Gel filtration method was used widely in extra (24) purified LF from human milk by Sephadex G-200 column, while (25) applied Sephadex G-100 column in purification of LF from mare milk .

Cytotoxic activity of lactoferrin on several cell lines

Growth inhibitory effect

The effect of different concentrations of purified goat milk lactoferrin (gLF) from (19.53 to 5000 µg/ml) on (ANG) tumor cell lines after (24, 48 &72) hrs of exposure was present in Table (1) and Figures (3 and 4) respectively. The results revealed significant cytotoxic effect at levels ($P<0.05$) for all concentration and for all exposure time of gLF on ANG cell line . The inhibition rate IR% increased with rising of gLF concentration and incubation period , The IR% values after 24h of exposure to gLF at concentration dependent (19.53, 39.06 ,78.12, 156.25, 312.50, 625.00, 1250.00, 2500.00, 5000 µg /ml) were (12.23, 17.71, 22.60, 29.94,34.32,42.09,45.90,52.62and 56.20%) respectively . whereas after 48hrs the rates increased and reached to (22.71, 35.32, 36.40, 49.21, 54.00,58.22, 60.35, 61.76 and 64.41%) respectively. and after 72 hrs they reached to (45.55,47.45, 57.32, 57.64, 67.71,68.62,71.30,73.92 and 76.07%) respectively .The highest inhibitory growth was shown at concentration 5000 µg/ml after 72hrs of exposure time it was 76.07%. To determine the effect of standard bovine lactoferrin bLF as anticancer toward ANG a comparative study was done between goat and cow milk LF on ANG tumor cell lines ,Table 2 shows the effect of different concentrations of purified gLF and standard bLF after (24, 48 &72) hrs of exposure time. The results revealed that the bLF has an effect on inhibiting the growth of ANG tumor cells line and this effect increased with the increase in the time of exposure, as well as with the rise in the bLF concentration . The inhibition rate after 24 hrs were (2.99,3.28,11.23,14.25,12.05, 16.07, 21.31 , 33.53 and 40.89 %) respectively. when the exposure time increased to 48 hrs the IR% increased and reached to (9.36,7.53,10.43 ,11.32, 13.25, 19.12, 30.24 and 45.53 %) respectively, whereas the values after 72hrs reached their highest (11.34, 10.94, 11.01, 9.57, 18.43, 20.59, 27.44, 32.13 and 47.63%) respectively., The results in Table 2 also indicates that there are significant differences between the two kinds of LF in their effect on the growth of ANG tumor cell line , gLF possess a highly growth inhibition than bLF which appeared clearly from the first four concentrations (19.53, 39.06, 78.125 and 156.25 µg/ml) after 72 hrs of exposure the gLF had IR% five times more than that for bLF and it was (45.55,47.45,57.32 and 57.64%) for gLF and (11.34,10.94,11.01 and 9.57%) for bLF. while the differences in IR%

were reduced in high concentrations reached to three fold at concentration 312.50 to 1250 µg/ml (67.71 & 18.43%) and two fold at 2500 µg/ml (73.92 &32.13%), while at the high concentration 5000 µg/ml after 72hr of exposure time IR% values were (76.07 and 47.63 %) for gLF and bLF respectively .The effect of different concentrations of gLF on the growth of normal cell line REF was studied after exposing for 72 hrs .The result showed a slight effect on the viability of normal cell line the inhibition rates were (6.34 ,10.45 , 10.59 , 10.72, 11.08, 11.14, 17.28, 17.59 and 19.22%) when nine concentrations (19.53, 39.06 ,78.12, 156.25, 312.50, 625.00, 1250.00, 2500.00,5000) µg /ml of gLF were used respectively .The inhibition rate values gradually increased with increasing of gLF concentration ,These results indicate that gLF have slight effect on the viability of a normal mammalian cells line so the results encouraging using gLF as anticancer agent. In order to know the cytotoxicity of bLF on the growth of normal cell line REF cell lines exposured to nine concentration of bLF for 72 hrs the results in Table (3) and Fig (5 and 6) show a slight effect of bLF on the viability of normal cell line, the inhibition rates are (2.93,8.11, 11.74, 16.92, 19.68, 23.66, 30.56,31.38 and 33.67%) respectively when the same nine concentrations of (19.53, 39.06 ,78.12, 156.25, 312.50, 625.00, 1250.00, 2500.00,5000) µg /ml of bLF are used.The effect of bLF on growth of normal cell line (REF) more than that for gLF. Lactoferrin is an iron binding minor glycoprotein present in bovine milk. A number of physiological roles have been suggested for Lactoferrin (26) , but it is likely to be the iron-binding properties that contribute to anticancer properties of this whey protein, It is thought that LF may bind iron locally in tissues, therefore reducing the risk of oxidant-induced carcinogenesis (27). (28) looked at the influence of bLF on colon carcinogenesis in rats exposed to carcinogens. The incidences of colon adenocarcinomas in rats receiving LF were significantly lower than those in control groups, In addition to that there were also fewer aberrant crypt foci. (29) showed that rats fed a soyabean-based diet supplemented with LF or with β-lactoglobulin had significantly fewer aberrant colonic crypt cells (cancer precursors) than animals which consumed the normal (non-supplemented) diet. In addition to its effect in dietary inclusion, there is some evidence that LF administered by a parenteral route may have important anticancer properties. (30) explained the molecular mechanism of LF-

induced cell growth inhibition. by Studied head and neck cancer cells which treated with hLF, growth arrest in three of four cell lines tested was observed this growth arrest was caused by cell cycle inhibition at the G0-G1checkpoint. Lactoferrin-induced growth inhibition was associated with a large increase in p27 protein, accompanied by decreased phosphorylation of retinoblastoma protein, and suppression of cyclin E. Decreased levels of phosphorylated Akt were also observed in LF-sensitive cell lines after treatment. These findings suggest that in head and neck cancer cells the growth inhibitory effects of LF are mediated through a p27/cyclin E-dependent pathway that may be modulated in part by changes in Akt phosphorylation.

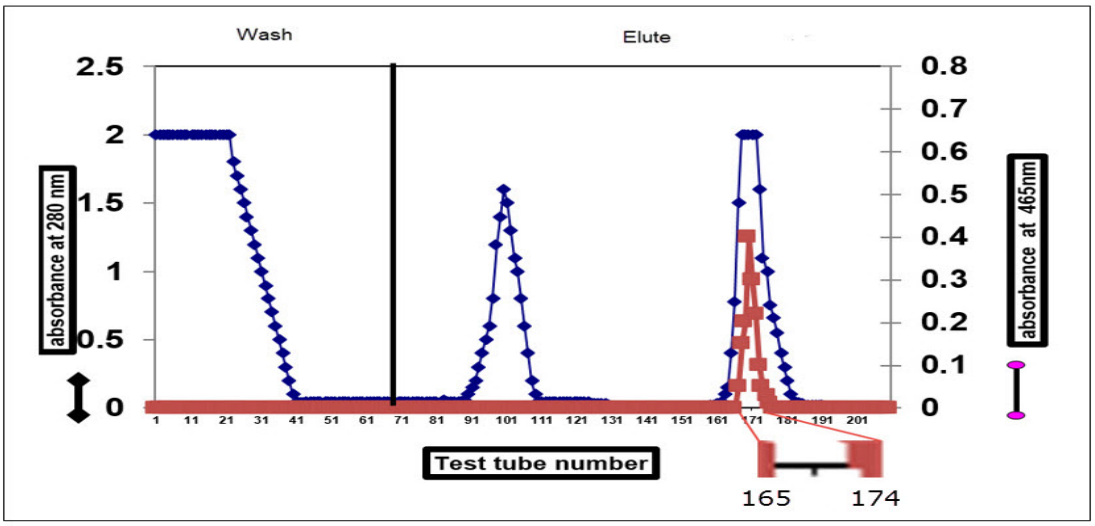


Figure 4-1: Ion exchange chromatography for purification gLF by using CM-Sephadex C-50 (2×24 cm) column, equilibrated with Tris-HCL buffer (0.05M, pH7.5), eluted with Tris-HCL buffer with NaCl gradient 0.2 and 0.5 M in flow rate 18 ml/hr.,3ml for each fraction.

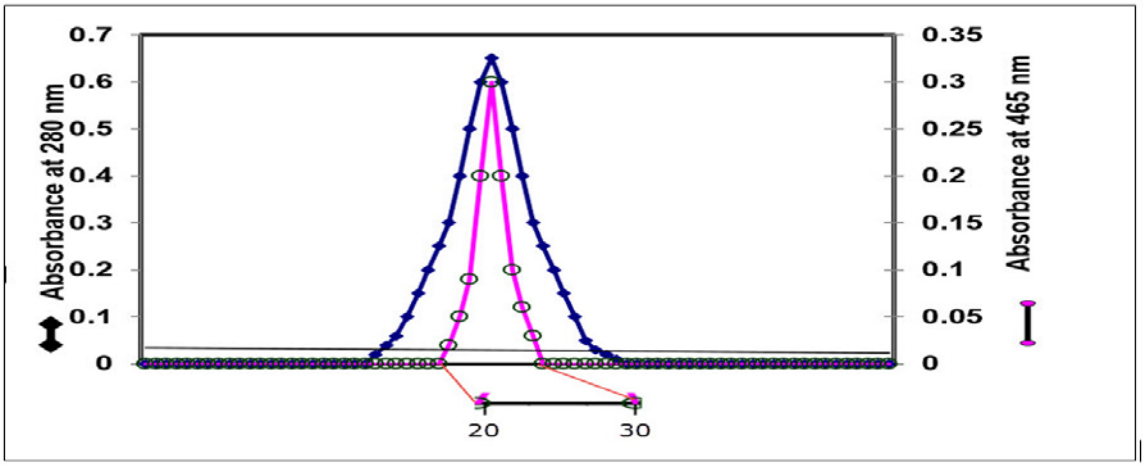


Figure 2 : Gel filtration chromatography for purification gLF by using Sephadex G-200 column (1.5 × 60 cm), equilibrated with 0.5 M phosphate buffer containing 0.01 M NaCL ,pH 7.4 with a flow rate of 18 ml/hr ,3ml for each fraction .

Table1: Mean values of inhibition rate percentage (IR %) of ANG cell lines after treatment with different concentrations of goat milk LF for (24, 48 &72) hours.

Concentration (µg/ml)	IR%			LSD value
	24hrs	48hrs	72hrs	
19.53	12.23	22.71	45.55	9.552 *
39.06	17.71	35.32	47.45	9.063 *
78.125	22.60	36.40	57.32	10.318 *
156.25	29.94	49.21	57.64	8.642 *
312.50	34.32	54.00	67.71	10.025 *
625	42.09	58.22	68.62	8.947 *
1250	45.90	60.35	71.30	8.043 *
2500	52.26	61.76	73.92	7.939 *
5000	56.20	64.41	76.07	8.516 *
LSD value	10.893 *	9.358 *	9.625 *	---
*** (P<0.05), NS: Non-significant.**				

Table 2: Mean values of inhibition rate percentage (IR %) of (ANG) cell lines after treatment with different concentrations of goat and cow milk LF for (24, 48 &72) hours.

Concentration (µg/ml)	Goat			LSD value	Cows			LSD value
	IR%				IR%			
	24hrs	48hrs	72hrs		24hrs	48hrs	72hrs	
19.53	12.231	22.71	45.55	9.552 *	2.99	9.36	11.34	5.42 *
39.06	17.711	35.32	47.45	9.063 *	3.28	7.53	10.94	5.06 *
78.125	22.598	36.40	57.32	10.318 *	11.23	10.43	11.01	4.38NS
156.25	29.943	49.21	57.64	8.642 *	14.25	11.32	9.57	5.13NS
312.50	34.322	54.00	67.71	10.025 *	12.05	13.04	18.43	5.38 *
625	42.090	58.22	68.62	8.947 *	16.07	13.25	20.59	5.44 *
1250	45.90	60.35	71.30	8.043 *	21.31	19.12	27.44	5.29 *
2500	52.259	61.76	73.92	7.939 *	33.53	30.24	32.13	5.75NS
5000	56.20	64.41	76.07	8.516 *	40.89	45.53	47.63	5.94 *
LSD value	10.893 *	9.358 *	9.625 *	---	8.43*	9.52*	8.77*	---

*** (P<0.05), NS: Non-significant.**

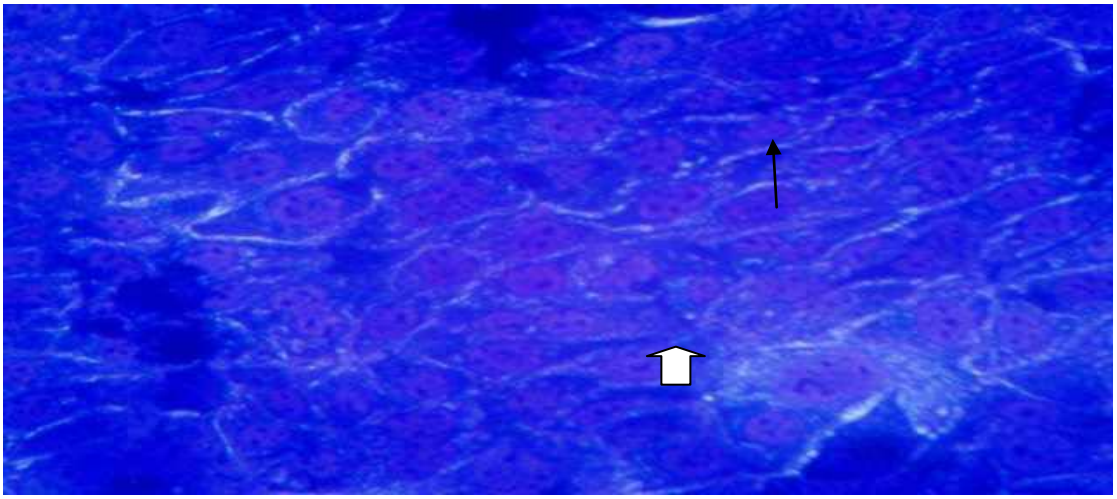


Figure 3: Figure (4.1): ANG cell line shows confluent monolayer (↑), no empty spaces (⇧), cohesive malignant cell control 100X, crystal violet.

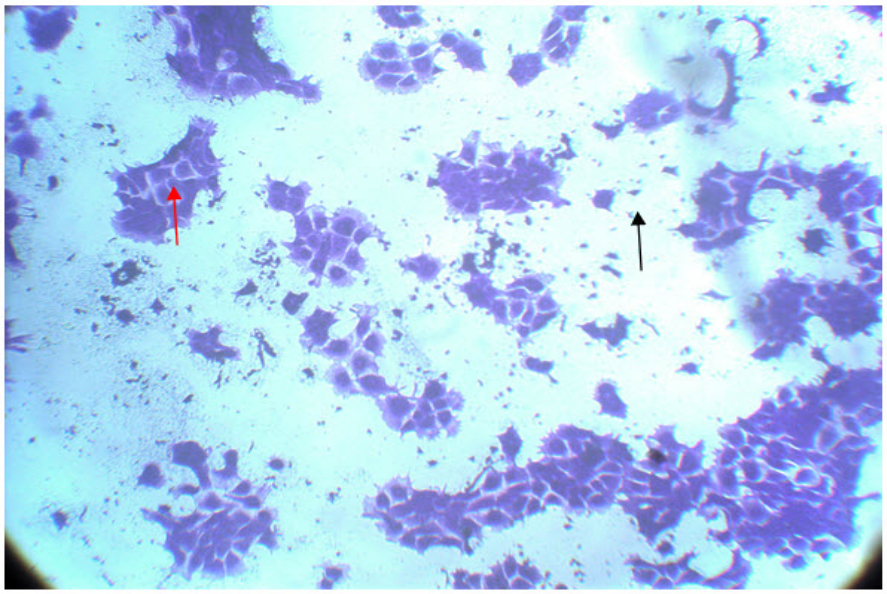


Figure 4-12 : AMGM cell line reveals great loss of cellular features (↑), and large number of dead cells (↑) after exposure to GLF at 72 hr., 100X, crystal violet.

Table (3): cytotoxicity of gLf on REF after 72h exposure.

Concentration (µg/ml)	IR%
	72h
19.53	6.34
39.06	10.45
78.125	10.59
156.25	10.72
312.50	11.08
625	11.14
1250	17.28
2500	17.59
5000	19.22

*** (P<0.05).**

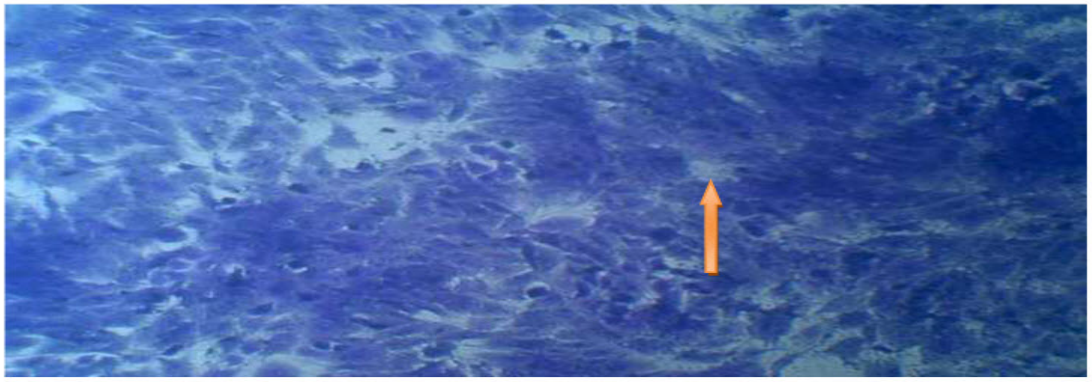


Figure (4.19): REF cell line shows confluent monolayer ( ), no empty spaces, (control) 40X, crystal violet.

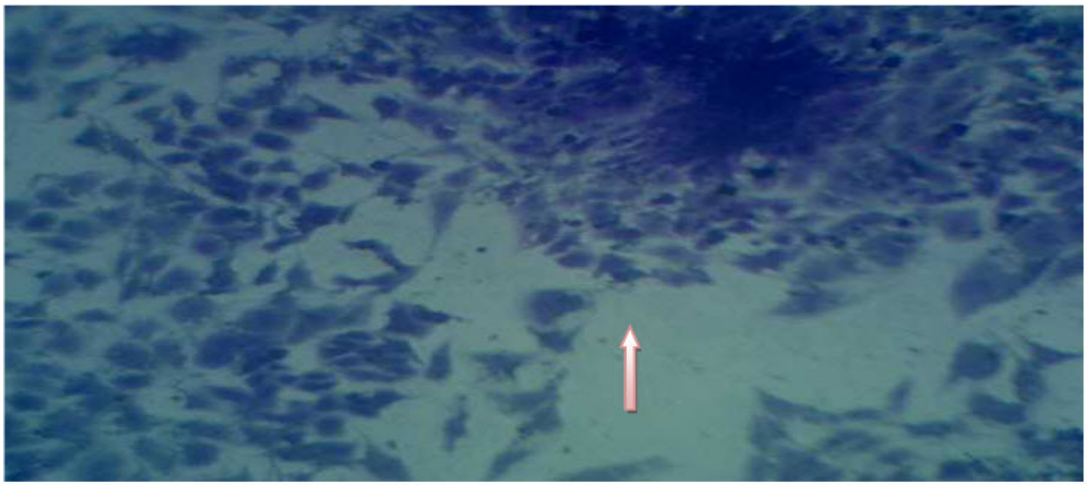


Figure (4.20): REF cell line shows space between cells ( ) after exposure to gLF for 72hr. 40X, crystal violet.

References

1. American Association for Cancer Research (2013). AACR Cancer Progress Report 2013. Clin. Cancer Res., 19 (Supplement 1): S1-S88.
2. Siegel, R., Naishadham, D. and Jemal, A. (2012), Cancer statistics, 2012 CA: A Cancer Journal for Clinicians, 62: 10–29.
3. Rastogi, T., Hildesheim, A. and Sinha, R. (2004). Opportunities for cancer epidemiology in developing countries. Nature Rev. cancer, 4:909.
4. IARC (International Agency for Research on Cancer), (2011) Section of Cancer Information. Cited by: Yousif, R. A. (2012). Effect of Cordia myxa L. crude extract on growth of cancer and normal cell lines. M. Sc. Thesis. College of science for women, University of Baghdad, Iraq.
5. Ronald ,R.W.; Sherma , Z. and Vector, R.B.(2010). Dietary Components and Immune Function. Humana press Springer New York Dordrecht Heidelberg London Library of Congress Control Number: 2010931836.
6. Hatzimichael, E. and Crook, T. (2013). Cancer Epigenetics: New Therapies and New Challenges. Journal of Drug Delivery. Volume 2013 (2013), Article ID 529312, 9 pages.
7. Comstock, S.S.; Reznikov ,E.A.; Contractor ,N. and Donovan, S.M. (2014). Dietary bovine Lactoferrin alters mucosal and systemic immune cell responses in neonatal piglets.J Nutr.4:525-32.
8. Esmat, A.; Gaspar ,R. and Carmen ,F.(2013). Structure and Functions of Lactoferrin as Ingredient in Infant Formulas. J of Food Res; (2):25-36.
9. Tsuda ,H.; Sekine, K.; Fujita ,K. and Ligo ,M . (2002) . Cancer prevention by bovine lactoferrin and underlying mechanisms – a review of experimental and clinical studies. Biochem Cell Biol 80:131–136.
10. Iigo, M.; Alexander,D.B.; Long,N.;Xu,J.; ukamachi,K.; futakuchi, M. ; Takase, M. ; tsuda, H. (2009). Anticarcinogenesis pathways activated by bovine Lactoferrin in the murine small intestine.Bio-Chimi.91(1):86-101.
11. Bezault , J.; Bhimani , R.; Wiprovnick ,J. and Furmanski ,P. (1994). Human lactoferrin inhibits growth of solid tumors and development of experimental metastases in mice. Cancer Research, 54, 2310-2312.
12. Damiens ,E.; El -Yazidi ,I.; Mazurier ,J.; Duthillie, I.; Spik, G. and Boilly- Marer, Y. (1999). Lactoferrin inhibits G1 cyclin-dependent kinases during growth arrest of human breast carcinoma cells. J Cell Biochem 74:486–498.

13. Hakansson, A.; Zhivotovski, B.; Orrenius, S.; Sabharwal, H. and vanbory , C.S. (1995). Apoptosis induced by a human milk protein. Proc. Natl. Ac. Sci. 92:8064-8068.

14. Pan, Y.; Rowney , M.; Guo ,P. and Hobman ,P. (2007). Biological properties of Lactoferrin: an overview. Aust. J. Dairy Technol. 62:31-42.

15. Xiao ,Y.; Monitto ,C.; Minhas ,K.and Sidransky ,D .(2004). Lactoferrin down-regulates G1 cyclin-dependent kinases during growth arrest of head and neck cancer cells. Clin Cancer Res 10:8683–8686.

16. Al-Mashikhi, S. A. and Nakai, S.(1987).Isolation of bovine immunoglobulins and lactoferrin from whey protein by gel filtrations techniques.J of Dairy Science, 70:2486-2492.

17. Yoshida, A.; Wei, Z.; Shinmura, Y.; Fukunaga, N.(2000). Separation of Lactoferrin-a and -b from bovine colostrum. J. Dairy Sci: 83, 2211-2215.

18. Bonner, P.L.R. (2007). Protein purification (the basics) ,Nottingham Trent University. First edition by Taylor and Francis group.

19. Shimazaki, K. (2000). Lactoferrin: a marvelous protein in milk?. *Animal Science Journal*, 71: 329-347.

20. Nam, M. S.; Shimazaki, K.; Kumura, H.; Kyung Lee, K. K. and Yu, D.Y (1999). Characterization of korean native goat Lactoferrin. *Comparative Biochemistry and Physiology, Part B*, 123:201–208.

21. Angeles A.A.; Merca ,F.E. and Roxas ,N.P. (2008) Isolation and characterization of Lactoferrin from CARABAO'S *(Bubalus bubalis* L.*)* milk whey. *Philippine J. Vet. Anim. Sci.* 2008.*(delete) 34(1): 43-54*.

22. Younghoon, K.;Mae ,J.;Kyoung , S.H.; Jee ,Y.I.;Sejong, O. and SAE, H.K(2009). Anticancer activity of Lactoferrin isolated from caprine colostrum on human cancer cell lines. Intern Journal of Dairy Techn.62:277–281.

23. Moradian ,F. (2014) Lactoferrin, Isolation ,Purification and Antimicrobial Effects . Journal of Medical and Bioengineering. 3:203-206.

24. Legrand, D, Pierce A, Elass E, Carpentier M, Mariller C, Mazurier J (2008) Lactoferrin structure and functions. Adv Exp Med Biol 606:163–194.

25. Kim, K. S.; Kim, J.S.; Shin, M.S.; Noh, H. W.; Lim, S.D.; Suvd, D. and Alimaa , J .(2009).Purification and characterization of Mongolian mare lactoferrin. Journal of Dairy Science, 29(2): 164-167.

26. Mulder,A.M and Morris, C.A. (2010).Lactoferrin in Immune Function , Cancer and Disease Resistance. Dietary Components and Immune Function. Chapter 17 / Humana press Springer New York.

27. Mohamed, M. E and Mohamed, B.M .(2011). Lactoferrin in relation to biological functions and applications : A Review.Int J of dairy Sci .6 (2): 79-111.

28. Tsuda, H., T. Kozu, G. Iinuma, Y. Ohashi, Y. Saito, D. Saito, T. Akasu, D. B. Alexander, M.Futakuchi, K. Fukamachi, J. Xu, T. Kakizoe, and M. Iigo.(2010). Cancer prevention by bovine lactoferrin: from animal studies to human trial. Biometals 23:399-409.

29. Smithers,, G.W. (2008). Whey proteins-From 'gutter-to-gold'. International Dairy Journal 18(7):695-704.

30. Yan ,X.; Monitto ,C.; Minhas ,K.and Sidransky ,D .(2004). Lactoferrin down-regulates G1 cyclin-dependent kinases during growth arrest of head and neck cancer cells. Clin Cancer Res 10:8683–8686.

Iraqi Journal of Biotechnology, 2015, Vol. 14, No. 2 , 238-248

Diversity and geographical distribution of Freshwater Gastropods in Diyala River Basin , Iraq

Khansaa. S. Farman ; Emaduldeen A. Almukhtar

Department of Biology , Collage of Science for Women , Baghdad university.

Abstract: A few studies have focused on aquatic Gastopods in Diyala River in Iraq .

Despite their importance in aquatic habitats, Monthly measurement from April 2013 to January 2014 at 10 sites were carried out on many environments parameters the results showed that average temperature was (10 -33) ºC., Dissolved Oxygen concentration (6 -10.7)mg/l. The calcium concentration was (46-211) mg/l) .The Organic matter ranged from 0.6 - 4.01 % . pH values ranged between (6.6-8.2) . Electrical Conductivity ranged between (470 - 1530)µS/cm and current velocity (0.09 -1.21) m/sec.

Also a total of 12 species belong to 8 families were recorded, including *:Theodoxus jourdani* , *Neritina mesopotamica* , *Valvata saulcyi* , *Bellamya bengalensis* ,*Melanopsis nodasa* ,*Melonopsis costata*, *Melanopsis preamorsa* , Melanopsis *subtingitana* , *Melanoides tubercula* , *Lymneae natalensis*, *Physa acuta* and , *Planorobis gibbonsi* . The highest population density was recorded in November and the lowest was at January .

Key words : Freshwater Gasteropods , Diversity , environmental factors , Diyala river

التنوع والانتشار الجغرافي للقواقع بطنية القدم المائية في حوض نهر ديالى، العراق

خنساء سلمان فرمان ؛ عمادالدين عبدالهادي المختار

قسم علوم الحياة / كلية العلوم للبنات / جامعة بغداد

الخلاصة: بالرغم من اهمية قواقع بطنيه القدم للبيئة المائية الا ان هناك القليل من الدراسات التي اجريت عليها في حوض نهر ديالى في العراق . خلال فترة الدراسة التي امتدت من نيسان 2013 الى كانون الثاني 2014 ولعشرة مواقع تم قياس العديد من المؤشرات البيئية ، فقد تراوحت درجة الحرارة (10-33) مº، تركيز الأوكسجين المذاب (6- 10.7) ، اما تركيز الكالسيوم فقد تراوحت بين (46 – 211) ملغم / لتر ، تركيز المادة العضوية لرواسب القاع تراوحت بين (0.6 – 4.01) % ، وبلغت اقل قيمه للأس الهيدروجيني (6.6 - 8.2) وقيم التوصيلية تراوحت بين 470 – 1530 مايكروسمنس / سم اما سرعة التيار المائي فقد تراوحت بين (0.09 - 1.21) م/ ثا .

كما تم خلا الدراسة ايضا تسجيل 12 نوع يعود الى 8 عوائل وقد شملت الانواع : *Theodoxus jourdani* ، *Neritina mesopotamica* ، *Valvata saulcyi* ، *Bellamya bengalensis* ، *Melanopsis nodasa* ، *Melonopsis costata* ، *Melanopsis subtingitana* ، *Melanoides tuberculata* ، *Lymneae natalensis* ، *Melanopsis preamorsa* و *Physa acuta* و *Planorobis gibbonsi* . وقد تم تسجيل أعلى كثافة في تشرين الثاني واقلها في كانون الثاني .

Introduction

Diyala river is one of the most important tributaries of River Tigris , and it is one of the main water bodies of Iraq. Many cities are situated on its banks. as well as, wastes fluids of agricultural and industrial activities in these cities are also outflow directly to this river. It runs through Iran and Iraq and drains an area of 32600 km^2 and covers a total distance of 445 km. The river basin is widely varied through the entire catchments area from semi-arid plain north of Baghdad to mountainous area of western Iran[1] .

Gastropods are the most successful class occupying nearly all aquatic habitats [2] ,and they play an important role in the food chain , Survival and geographical distributions of Gastropods depends on their abilities to colonize a habitat and survive there [3] and that controlled by physico-chemical factors such as calcium salts[4] , dissolvedoxygen,water conductivity, pH and temperature [5].

In Iraq, many researchers conducted studies on different fresh water Gastropods in different parts of the country, but no work has been done on its distribution in Diyala river basin. Especially, Diyala River is characterized by different of natural aquatic environments ranging from rivers to lakes . Therefore the present study aimed to provide an inventory of the freshwater Gastropods fauna in that region .The study also provided an information on the abundance and distribution of Freshwater Gastropods and investigated the relationship between freshwater Gastropods and the environmental factors.

Materials and Methods

Monthly measurements were made from April 2013 to January 2014 at 10 sites as shown in Fig 1. The parameters included water temperature, pH, dissolved oxygen and electrical conductivity which were determined by using field digital meter (TRANS instrument Model WalkLAB) , current velocity measured by Rubber Bag Method which has been described by [6]. Calcium concentration measured by complexometric titration method , Organic matter Determined by Loss on Ignition (LOI) method that was described in [7] .Sediment texture determined by the hydrometer method as described in [8]

A total of 252 samples were collected from the sites of Diyala River Basin lying between latitudes(33^o 13 -35^o 50) and longitudes(44^o 30 - 47^o 50) (Fig. 1). The samples were collected from the mostly gravel covered sites (Darbandikhan , ALwand , Jalula and Himreen) by Cylinder 70 cm and diameter 50 cm . Eckman Dredge was used to collect samples from sites (Kalar , Al sodur ,

Mahroot , Khalis , Al saria and Baqubah).

The samples of gastropods were put into separate polyethylene containers and brought to the laboratory for examination where they were washed in the laboratory and the isolated gastropods preserved with 70% ethyl alcohol. Preserved samples of Molluscs were identified mainly according the shell with the help of [9], [10] ,[11], and confirmed by comparing with the British Natural History Museum's preserved sample .

Results and Discussion

Physico-chemical parameters:

As shown in figure (1) temperature variation during study period ranged between (10-33)ºC and this wide range of the variation may be due to the length of the day period, air temperature

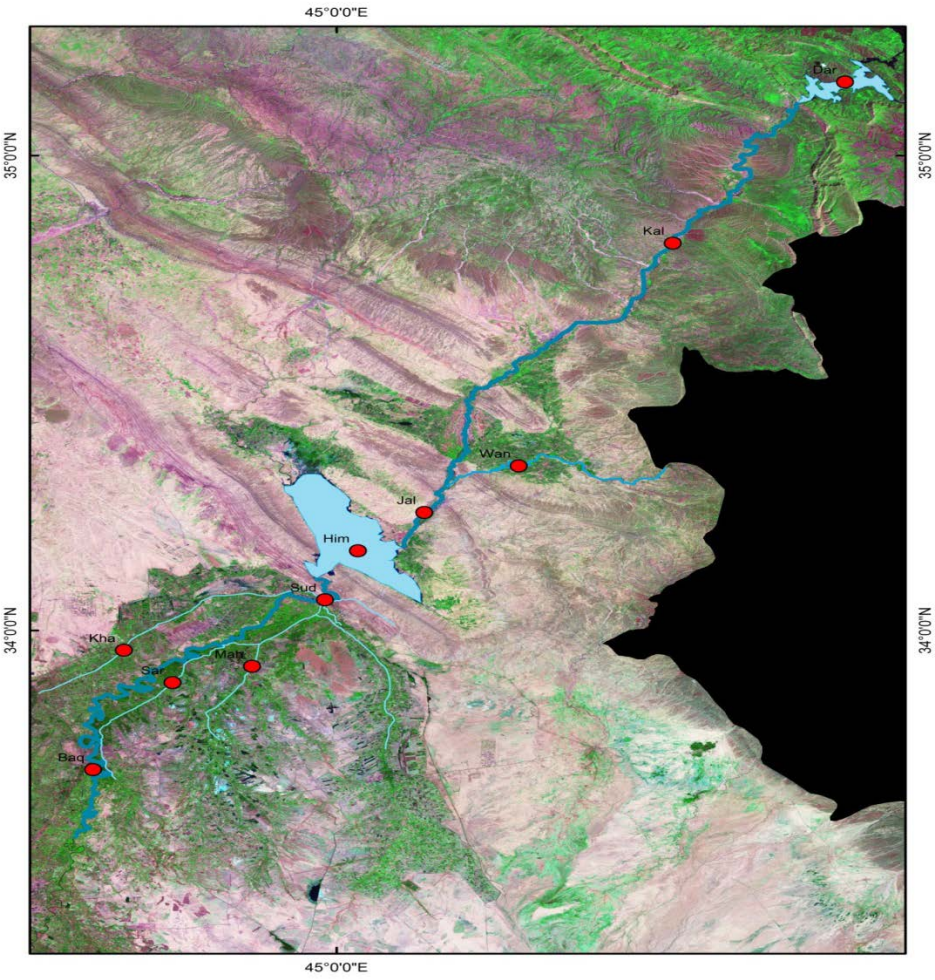


Fig. (1) Diyala River Basin with idicated study sites [Dar = Darbandikhan lake ; Kal = Kalar ; Wan = Wand tributary ; Jal =Jalula ; Him = Himreen lake ; Sud =Sudor ; Mah = Mahroot stream ; Kha = Khalis stream ; Sar = Saria stream ; Baq = Baqudah] .

and location [12] . Also The investigation IS shown that the pH ranged between weak acidic to alkaline (6.6 -8.2) but it trend to weak alkaline as it noted in most of the readings of the months , the study recorded no significant difference between the sites where the changes in pH values are few and within a tight range may be due to the fact that water flows on the land with limestone nature so that the presence of carbonate and bicarbonates and silicates ions that act as buffering and reserve at ion pH values [14] .

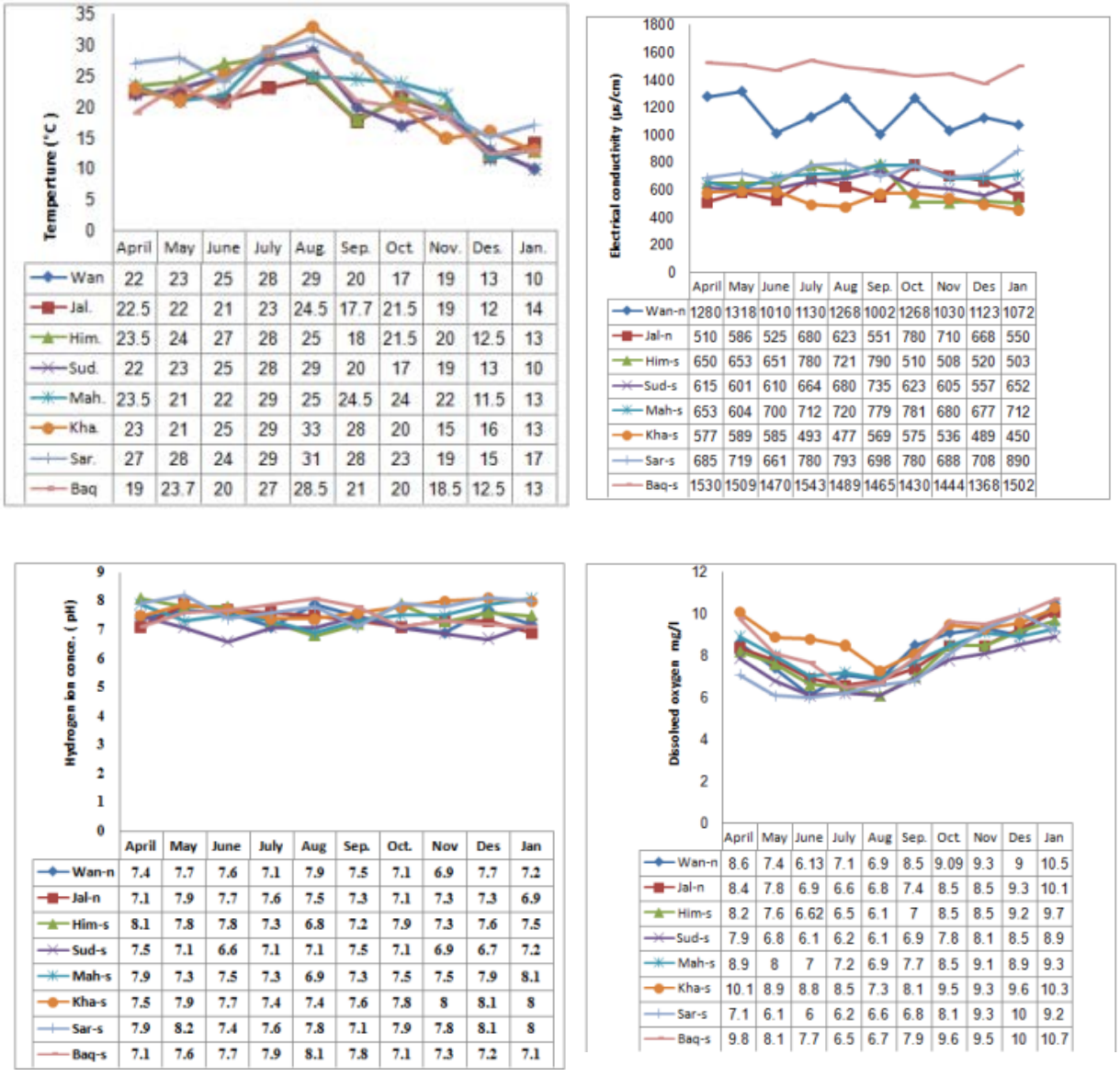
For dissolved oxygen the investigation recorded it arranged between (6-10.7) mg/l with by high values of dissolved oxygen in the winter and spring months due to low temperatures and increase the speed of the current because of rising water levels as a result of raining.

The Electrical conductivity ranged between (1530–477) µs/cm ,where the highest values recorded in Baqubah site where the river pass through are as containing water puncture from farms and orchards in addition to the discharge water from the residential area to it. In general as observed from the results the EC values increased in summer months may be due to the high temperatures, which lead to increased evaporation and low flows when the capacity of the stream is at its lowest then lead to increased salt concentration [15]

The changes in the speed of the current affected by drainage , river view , the depth , the nature of the bottom and the degree of slope land . The results showed increasing in flow in April and May also in September that because of the increasing the amount of discharge from Darbandikhan lake through the spring and winter months [16] in addition to the rain that drop at that months , while it decrease in summer months when the level of the water decrease . As it can be seen from (fig. 1) fluctuation in the velocity in Mahroot and Khalis sites due to it used as irrigation projects that are subject to a program prepared by the Directorate of irrigation that controlling the drainage level for the purpose of organizing irrigation of agricultural land in the surrounding areas.

Results showed that there are variations in calcium ion concentration between study sites where the highest rates recorded in Al wand and Baqubah sites may be due to what goes in to the river from the neighboring territory and is growing with the course of the river as well as the geological nature of area in high Diyala river that contain Gibson and Anhydrate rock that increasing the Ca concentration through melting processes for that rocks [17] . Also divine rain in Autumn and Winter that cause wash soil and discharged into the

water body cause increased Ca concentration. . About the organic matter the higher values were recorded in August in Baq site reach to 4.01% and that high value was caused by decreasing the water level in summer month and death and of organisms and accumulated in the bottom also because of organic fertilizer that add by dunging in neighboring area and accumulated in stream through puncture [18].



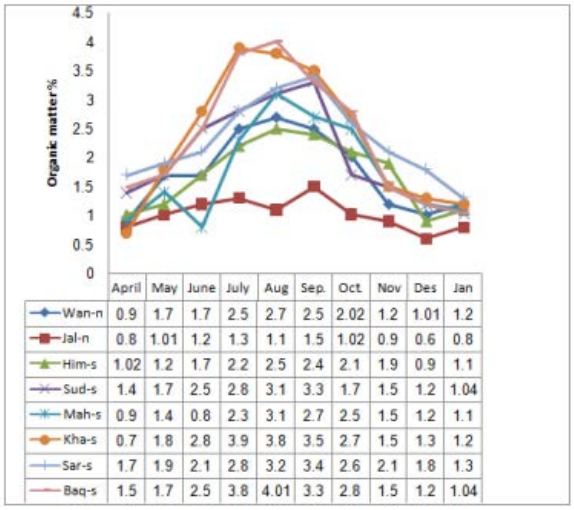
	April	May	June	July	Aug.	Sep.	Oct.	Nov.	Des.	Jan.
Wan	22	23	25	28	29	20	17	19	13	10
Jal.	22.5	22	21	23	24.5	17.7	21.5	19	12	14
Him.	23.5	24	27	28	25	18	21.5	20	12.5	13
Sud.	22	23	25	28	29	20	17	19	13	10
Mah.	23.5	21	22	29	25	24.5	24	22	11.5	13
Kha.	23	21	25	29	33	28	20	15	16	13
Sar.	27	28	24	29	31	28	23	19	15	17
Baq	19	23.7	20	27	28.5	21	20	18.5	12.5	13

	April	May	June	July	Aug	Sep.	Oct.	Nov	Des	Jan
Wan-n	1280	1318	1010	1130	1268	1002	1268	1030	1123	1072
Jal-n	510	586	525	680	623	551	780	710	668	550
Him-s	650	653	651	780	721	790	510	508	520	503
Sud-s	615	601	610	664	680	735	623	605	557	652
Mah-s	653	604	700	712	720	779	781	680	677	712
Kha-s	577	589	585	493	477	569	575	536	489	450
Sar-s	685	719	661	780	793	698	780	688	708	890
Baq-s	1530	1509	1470	1543	1489	1465	1430	1444	1368	1502

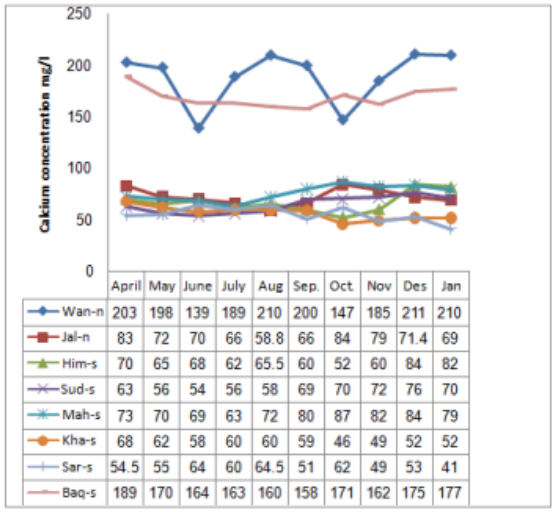
	April	May	June	July	Aug	Sep.	Oct.	Nov	Des	Jan
Wan-n	7.4	7.7	7.6	7.1	7.9	7.5	7.1	6.9	7.7	7.2
Jal-n	7.1	7.9	7.7	7.6	7.5	7.3	7.1	7.3	7.3	6.9
Him-s	8.1	7.8	7.8	7.3	6.8	7.2	7.9	7.3	7.6	7.5
Sud-s	7.5	7.1	6.6	7.1	7.1	7.5	7.1	6.9	6.7	7.2
Mah-s	7.9	7.3	7.5	7.3	6.9	7.3	7.5	7.5	7.9	8.1
Kha-s	7.5	7.9	7.7	7.4	7.4	7.6	7.8	8	8.1	8
Sar-s	7.9	8.2	7.4	7.6	7.8	7.1	7.9	7.8	8.1	8
Baq-s	7.1	7.6	7.7	7.9	8.1	7.8	7.1	7.3	7.2	7.1

	April	May	June	July	Aug	Sep.	Oct.	Nov	Des	Jan
Wan-n	8.6	7.4	6.13	7.1	6.9	8.5	9.09	9.3	9	10.5
Jal-n	8.4	7.8	6.9	6.6	6.8	7.4	8.5	8.5	9.3	10.1
Him-s	8.2	7.6	6.62	6.5	6.1	7	8.5	8.5	9.2	9.7
Sud-s	7.9	6.8	6.1	6.2	6.1	6.9	7.8	8.1	8.5	8.9
Mah-s	8.9	8	7	7.2	6.9	7.7	8.5	9.1	8.9	9.3
Kha-s	10.1	8.9	8.8	8.5	7.3	8.1	9.5	9.3	9.6	10.3
Sar-s	7.1	6.1	6	6.2	6.6	6.8	8.1	9.3	10	9.2
Baq-s	9.8	8.1	7.7	6.5	6.7	7.9	9.6	9.5	10	10.7

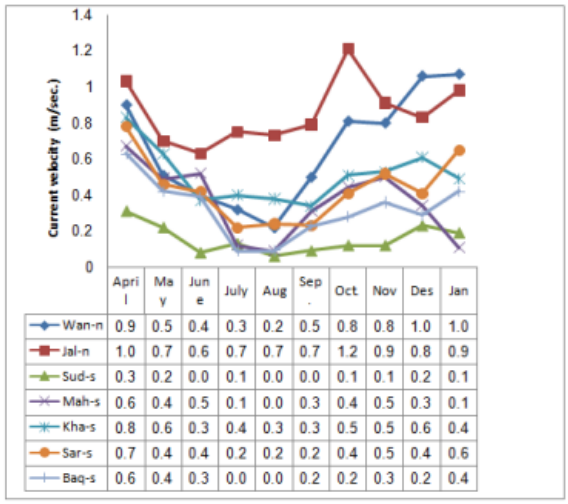
Fig. (2): Monthly Averages of Physico-chemical parameters in the studied sites for the period from April 2013 to January 2014



	April	May	June	July	Aug	Sep.	Oct.	Nov	Des	Jan
Wan-n	0.9	1.7	1.7	2.5	2.7	2.5	2.02	1.2	1.01	1.2
Jal-n	0.8	1.01	1.2	1.3	1.1	1.5	1.02	0.9	0.6	0.8
Him-s	1.02	1.2	1.7	2.2	2.5	2.4	2.1	1.9	0.9	1.1
Sud-s	1.4	1.7	2.5	2.8	3.1	3.3	1.7	1.5	1.2	1.04
Mah-s	0.9	1.4	0.8	2.3	3.1	2.7	2.5	1.5	1.2	1.1
Kha-s	0.7	1.8	2.8	3.9	3.8	3.5	2.7	1.5	1.3	1.2
Sar-s	1.7	1.9	2.1	2.8	3.2	3.4	2.6	2.1	1.8	1.3
Baq-s	1.5	1.7	2.5	3.8	4.01	3.3	2.8	1.5	1.2	1.04



	April	May	June	July	Aug	Sep.	Oct.	Nov	Des	Jan
Wan-n	203	198	139	189	210	200	147	185	211	210
Jal-n	83	72	70	66	58.8	66	84	79	71.4	69
Him-s	70	65	68	62	65.5	60	52	60	84	82
Sud-s	63	56	54	56	58	69	70	72	76	70
Mah-s	73	70	69	63	72	80	87	82	84	79
Kha-s	68	62	58	60	60	59	46	49	52	52
Sar-s	54.5	55	64	60	64.5	51	62	49	53	41
Baq-s	189	170	164	163	160	158	171	162	175	177



	April	May	June	July	Aug	Sep.	Oct.	Nov	Des	Jan
Wan-n	0.9	0.5	0.4	0.3	0.2	0.5	0.8	0.8	1.0	1.0
Jal-n	1.0	0.7	0.6	0.7	0.7	0.7	1.2	0.9	0.8	0.9
Sud-s	0.3	0.2	0.0	0.1	0.0	0.0	0.1	0.1	0.2	0.1
Mah-s	0.6	0.4	0.5	0.1	0.0	0.3	0.4	0.5	0.3	0.1
Kha-s	0.8	0.6	0.3	0.4	0.3	0.3	0.5	0.5	0.6	0.4
Sar-s	0.7	0.4	0.4	0.2	0.2	0.2	0.4	0.5	0.4	0.6
Baq-s	0.6	0.4	0.3	0.0	0.0	0.2	0.2	0.3	0.2	0.4

Fig. (3): Monthly Averages of Physico-chemical parameters in the studied sites for the period from April 2013 to January 2014

Table (1): The percentage of sediment components with the texture of sediment of each studied sites and LSD values for differences between the sediment components in the studied sites

Site	Silt %	sand %	clay %	Sediment texture
Dar-n	18.1	50.5	31.4	Sandy clay loam
Kal-n	45	15	40	Silty clay
Wan-n	42.1	18.1	39.8	Sandy clay loam
Jal-n	23	52.7	24.3	Sandy clay loam
Him-s	12.3	77.96	9.74	Sandy loam
Sud-s	55	15	30	Silty clay loam
Mah-s	48.21	38.19	13.6	loam
Kha-s	32.1	16.8	51.1	clay
Sar-s	33.2	16.4	50.4	clay
Baq-s	49.2	41.2	9.6	loam
LSD	11.96*	15.09*	11.40*	

Distribution and Monthly variation of the recorded Gastropeds species .

Results showed in table 2 and 3 reveal that total catch of snails reached highest value in October and November flowed by April , that probably due to the fact that temperatures in these months are optimal for breeding and reproduction these results are agree with [19] , [20], [21] and [22] it don't mean that lower temperatures in winter eliminate the species but it restrict its reproduction and therefore its densities[23] . In addition population densities of the species were affected by the nutritional conditions which in turn affected by production of phytoplankton and increasing temperature which is reflected on density of species specially in (transitional months) as April and October[24]

T. jordani was the highest in numbers , it reached its maximum density in September and October the highest record was in (Mah), (Him) and (Kha) sites which were lined by gravels and different size of rocks and characterized by sandy-loam and clay-loam which differ with that recorded by [25] that showed it preferred clay bottom , while [24] mentioned that *T. jordani* living on gravel bottom and solid bank or vegetation .

Also *N. mesopotamica* recorded in stony substratum in (Mah) site and reached highest density in November and December . While sample of (Dar) site recorded *v.saulcyi* in two months, the density in September was higher than in October in (Dar)Site probably because the site was characterize by sandy clay loam bottom texture and lined by gravels and rocks that close to that recorded by [24] in the study of Lake Gölbaşı in Turkey, also it was close to the description of [26] with muddy or fine sandy bottoms .

For Melanopsidae family which represented in current study by : *M.nodosa* and *M.costata* were found on stones and boulders in shallow water and reached it maximum density in April which is agreed with [24]while *M. preamorsa* reached its maximum density in October and *M.subtingitana* in June and November .

M. tuberculata shown rare abundance and almost of samples collected were empty shell and according to [27] this species is active at night and hide during day but it widely rang distributed as current study showed it precence in almost southern sector and reached its maximum density in April and May , [21] recorded peak density in October .

L.natalensis were collected in low densities, with the exception of sites (Dar. and Baq.) (22.2 , 2.7 ind./m^2) respectively , with peak density in May followed by April

which was close to what was recorded by [20] who mentioned that maximum density of this species in spring month but differed from the study of [22] in Iran when they recorded it in Autumn and early winter .

P.acuta were collected in all studied sites over the study period , its high abundance and density in different sites due to *physa* spp being hermaphrodite and its ability to self-fertilization in addition of large rate of reproduction so it can produce large number of offspring through the year [29] it appeared associated with edge-water habitats of shallow depths, which are characterized by fluctuating temperatures and water levels , in current study it reached maximum density in April and May and that agreed with what [30] who reported that the population of *P. acuta* followed a seasonal pattern as its reproduction reached optimum value between the Autumn and spring season with a high peak reproduction in the spring months , While [31] have mentioned that *P. acuta* frequently occurring in warm water and found in high density in summer , Also he referred to that *Planarobis* spp can tolerate substantial changes in different environmental variables so it was collected in all season with highest density in spring and summer months and that is what has been reached in the current study .

Table 2 Species density ind /m² , Relative abundance and Occurrence of the species in the sites of the Northern sector during the studied period April 2013 – January 2014 .

D : Density , RA : Relative abundance , OC : Occurrence

Site / Species	Dar-n			Kal-n			Wan-n			Jal-n		
	D	RA	OC	D	RA	OC	D	RA	OC	D	RA	OC
T.jordani	0	0	0	0	0	0	0	0	0	1.5	31.9	16
V.saulcyi	8.3	15	66	0	0	0	0	0	0	0	0	0
M.preamorsa	0	0	0	0	0	0	0	0	0	0.2	5.6	13
L.natalensis	22.2	42.1	100	0.5	6.8	33	0.5	11.1	36	0	0	0
P.acuta	13.3	25	100	5.5	75	83	4.1	86.1	96	2.9	62.4	83
P.gibbonsi	5.3	10	66	0	0	0	0	0	0	0	0	0

Table 3 Species density ind /m² , Relative abundance and Occurrence of the species in the sites of the Southern sector during the studied period April 2013 –January 2014
D : Density , RA : Relative abundance , OC : Occurrence

Site / Species	Him-s			Sud-s			Mah-s			Kha-s			Sar-s			Baq-s		
	D	RA	OC	D	RA	OC	D	RA	OC	D	RA	OC	D	RA	OC	D	RA	OC
T.jordani	4.2	27.3	63	0	0	0	4.7	28.4	63	2.4	14.4	63	0	0	0	0	0	0
N.mesopotamica	0	0	0	0	0	0	0.8	4.8	30	0	0	0	0	0	0	0	0	0
B.bengalensis	4.9	31.8	56	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
M.nodosa	0.5	2.6	13	0.5	6.6	16	0	0	0	1.16	7.04	50	0	0	0	0.4	4.6	16.6
M.costata	1.9	12.3	33	0.46	6.2	10	3.6	30.2	60	8.6	52.1	66	0	0	0	0.3	3.8	10
M.preamorsa	0.3	1.9	13	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
M.subtingitana	0.6	3.9	13	0	0	0	0.5	3.2	26	0.06	0.4	3	0	0	0	0	0	0
M.tuberculata	0.4	2.6	20	0.3	4.4	10	0.6	3.8	43	0.1	0.6	6	0	0	0	0.3	3.4	13
L.natalensis	0.1	0.6	6	0.7	9.3	26	0.6	3.8	36	0.6	3.6	40	0	0	0	2.7	31	63
P.acuta	1.9	12.3	36	4.4	59.5	73	4.3	25.8	93	3.6	21.7	83	9.3	96.5	96	2.3	26	56
P.gibbonsi	0.6	3.9	20	1.03	13.7	46	0	0	0	0	0	0	0.3	3.4	16	1.03	11.9	53

Acknowledgment

The authors would like to extend their deep gratitude to the staff of the British Natural History Museum / London , especially to Mr. Jonthan Ablett for allowing the first author to visit the museum and allow free access to examine and compare preserved material , which benefited not only the completion of this research but also the conservation of biodiversity in Iraq.

Refrences

1. Al Adili ,A. and Q.Al Suhaili.2010. Spectral analysis of some selected hydrochemical parameters of Diyala River , Iraq. fourteenth international water technology conference ,IWTC142010, Cairo .Egypt.
2. Haszprunar G, and A. Wanninger .2012. Primer: Mollusca. Current Biology 22: R510– 514.
3. Thorp J. and A. P. Covich .2010. Ecology and Classification of North American Freshwater Invertebrates 3rd edition. Academic Press. (Elsevier)
4. Range B. R.2003. Size and environmental calcium requirements of British freshwater gastropods..Global .Ecol. Bio.Geogr. 12: 47-51.
5. Pyron M, L..2004. Hydrological variation and fish assemblage structure in the middle Wabash River. Hydrobiologia . 525: 203-13.
6. Hynes, H.B. 1972. The ecology of running water . University of Toronto press.

7. Sutherland, R.A.(1998). Loss-on-ignition estimates of organic matter and relationships to organic carbon in fluvial bed sediments. Hydrobiologia . 389 (1-3): 153-167 .

8. Page, A.I. ,Miller,R.H. and Kenney,D.R. 1982.Methods of soil analysis.2nd ed . Soil science society of Amarica , Madisin .WI.

9. Ward, H.B. and G. C. Whipple**.**1959. Fresh-Water Biology .2nd ed. Wiley, New York. Chapman and Hall, London.

10. Burch, J. B. 1982. Freshwater snails (Mollusca: Gastropoda) of North America. U.S. Environmental Protection Agency Publication 600/3-82-026.

11. Brown, D.S (1994). Freshwater Snails of Africa and Their Medical Importance, 2nd edition. Taylor and Francis, London.

12. Wasfi ,M.K.2008.Ecological study in some water bodies in Diyala province . Al fatih Journal.vol.33.(In Arabic).

13. Hassan, F. M.(1997) . A Limnological study on Hilla river . Al Mustansiryia J. Sci., 8(1): 22-30.

14. Al saadi ,H.A. Basic of Ecology and Pollution , 2006.Al yazordy.Amman ,Jordan.

15. Delincé, G. 1992. The Ecology of the Fish Pond Ecosystem: with special reference to Africa. Library of congress of cataloging, published by kluwer Academic publisher.

16. Hussien , H.A. , 2010. Dependable discharge of the upper and the middle Diyala basin . Journal of Engineering . 16(2) : 4960-496 .

17. Nsief ,R.M., A.Abdullah and A.A.Al Azawy .2012.Study of Water Quality of Diyala River in some regions of Middle-Lower Basin. Scientific Journal of Karbla University . 10(2) : 221-234.

18. Arzayus, K.M. ·and E. A. Canuel .2005. Organic matter degradation in sediments of the York River estuary: Effects of biological vs. physical mixing Geochimica et Cosmochimica Acta . 69(2-69):455-464.

19. Al-Abbad, M.Y., S. D. Salman and I. H. Al-Qarooni .2015. Biodiversity of the macroinvertebrates in the Southern Iraqi Marshes, with a special reference to oligochaeta .Journal of Biodiversity and Environmental Sciences (JBES) 7(1): 61-71.

20. Ali, A.H., N.M. Aziz and H.A. Hamza . 2007. Abundance, occurrence, seasonal changes and species composition of Macroinvertebrates in the restored Iraqi southern marshes. Marsh Bulletin 2(1) : 80-95.

21. Hussein, M.A. , A. L.Obuid-Allah , A.A.Mahmoud and H.M. Fangary . 2011. Population dynamics of freshwater snails (Mollusca: Gastropoda) at Qena Governorate, Upper Egypt . Egypt. Acad. J. Biolog. Sci., 3(1): 11 -22 .

22. Karimi, G. R., Derakhshanfar, M. and Paykari, H. (2004): Population density, trematodal infection and ecology of Lymnaea snails in Shadegan, Iran. Arch. Razi Inc., 58: 125-129.

23. Grac¸a , M.A.S., S . Q. Serra and V. Ferreira. 2012. A stable temperature may favour continuous reproduction by *Theodoxus fluviatilis* and explain its high densities in some karstic springs. Limnetica, 31 (1): 129-140 .

24. Şereflisan H, M. Z. Yidemer and M .Şereflism. 2009.The gastropod fauna and their abundance, and some physicochemical parameters of Lake Gölbaşı (Hatay, Turkey). Turk . j. Zool. 33: 287-296 .

25. Al raei ,H.A.2014. The correlation between Aquatic snails and worms in aquatic environment of mid of Iraq. Thesis, Collage of Science for Women ,Baghdad university.

26. Wasylikowa, K., A. Witkowski, A. Walanus, A. Hutorowicz ,S. W. Alexandrowicze ,and J. J. Langer . 2006. Palaeolimnology of Lake Zeribar, Iran, and its climatic implications. Quaternary Research 66: 477–493.

27. Amr .Z.S. and M.Abu Baker . 2004 . Freshwater snail of Jordan . Landesmuseen. Neue Serie 2 : 237-244.

28. Poznan´ska , M. ,D Goleniewska , T. Gulanicz, T.Kakareko , Ł. Jermacz , and J. Kobak .2015. Effect of substratum drying on the survival and migrations of a freshwater pulmonate snail *Planorbarius corneus* (Linnaeus, 1758). Hydrobiologia. 747:177–188

29. Moore A.2003. Taxonomic morphological evolution of an endemic freshwater Gastropods, thesis , University of Michigan .

30. Maqboul A, R. Aoujdad, M. Fadli and M. Fekhaoui . 2014. Population dynamics of Physaacuta(Mollusca: Pulmonata) in the lakes of Rif mountains (Northern Morocco, Ouergha watershed). Journal of entomology and zoology studies. 2 (6): 240-245.

31. Şahin,S.K.2012.Gastropod Species Distribution and it Relation with some Physico-chemical Parameters of the Malatya's Streams (East Anatolia, Turkey) . Acta zool. bulg., 64 (2), 2012: 129-134.

Iraqi Journal of Biotechnology, 2015, Vol. 14, No. 2 , 249-269

Determination of Optimal Conditions on Biofilm Production by *Escherichia coli* Isolated from Fecal and Milk Samples of some Animal

Hassan A. Abdul-Ratha [1], Ban N. Nadhom [2]

[1] University of Baghdad/College of Agriculture, Iraq
[2] University of Baghdad / College of Veterinary medicine, Iraq

Abstract: Diarrheal disease are major problem in third world countries which are responsible for death of millions of people and animals each year(1,2,3).Ecoli mastitis remains one of the most costly disease in farm animal affected many high producing cows in dairy herds and may cause several cases of death per year in most severe cases with economic losses to the dairy industry (4).
This study was conducted to detect the ability of *E.coli* isolated from diarrhea and mastatic milk to produce Biofilm and study the optimal pH and temperature on the thickness of biofilm.
Two hundred and eighty four samples were collected (182 mastitic milk samples and 102 fecal samples) from Salahaddin governorate ,College of veterinary medicine - university of Baghdad , College of Agriculture - university of Baghdad , Radhwanya , Dora and Abu-Ghraib zones . Samples were cultured on MacConkey agar and Eosin Methylene Blue agar, after purification of cultured bacteria, cultural, Microscopical, biochemical characteristic, API 20 E System and RapID ^{TM} ONE System kit were done.

heT results showed that 54 out of 182 milk samples were positive for *E.coli* and 91out of 102 fecal samples were also positive for *E.coli*. The ability of these *E.coli* isolates to produce biofilm was detected and the results showed that 50 out of 54 *E.coli* isolated from milk samples produced biofilm (92.59%)and,38 out of 49 *E.coli* isolated from fecal samples of cow produced biofilm (77.55%) In addition.39 out of 42 *E.coli* isolated from fecal samples of sheep produced biofilm (92.85%) with different thickness ranged between (0.2-2)mm, whereas 4 isolated from milk samples ,11 isolated from 49 fecal samples of cow and calves and 39 isolated from 42 fecal samples of sheep did not produce biofilm.

Optimal pH for biofilm production was studied, the result showed that optimal pH was (7), also optimal temperature for biofilm production was studied and ranged between (37-45) ºC.

Keywords: Fecal Samples; Milk Samples; *E.coli* ; Biofilm; Optimal Conditions.

تحديد الظروف المثلى لانتاج الغشاء الحيوي من بكتريا الاشريكية القولونية المعزولة من عينات البراز والحليب لبعض الحيوانات

حسن علي عبدالرضا /كلية الزراعة –جامعة بغداد –العراق

بان نمير ناظم/كلية الطب البيطري-جامعة بغداد –العراق

الخلاصة: الاسهال من الامراض التي تسبب مشاكل كبيرة خاصة في دول العالم الثالث اذ انه مسؤول عن موت الملايين من البشر والحيوانات سنويا (1,2,3) . ان التهاب الضرع المتسبب بوساطة الاشريكية القولونية يعد من الامراض ذات الاضرار الاقتصادية لحيوانات المزرعة لما يسببه من انخفاض حاد في انتاج الحليب وموت العديد من الحيوانات عند الاصابات الشديدة.(4)

استهدفت الدراسة الحالية تحديد قابلية بكتريا الاشريكية القولونية المعزولة من عينات الاسهال والحليب لبعض الحيوانات المصابة بالتهاب الضرع في انتاج الغشاء الحيوي وتحديد درجة الحموضة والحرارة المثلى لسمك هذا الغشاء.

تم جمع 284 عينة(182 عينة حليب من حيوانات مصابة بالتهاب الضرع و102 عينة براز لحيوانات مصابة بالاسهال) من محافظة صلاح الدين وجامعة بغداد-كلية الطب البيطري، جامعة بغداد -كلية الزراعة ،مناطق الرضوانية ،الدورة وابوغريب.زرعت جميع هذه العينات على وسطي الماكونكي الصلب والايوسين مثلين الازرق الصلب وبعد تنقية العزلات البكتيرية النامية درست خصائصها الزرعية والكيموحيوية ،تم استخدام نظام API20E,RapID لغرض تاكيد تشخيص هذه العزلات البكتيرية.اظهرت النتائج ان 54عينة من اصل 182 عينة حليب كانت موجبة لوجود بكتريا الاشريكية القولونية و91 عينة من اصل 102 عينة اسهال كانت موجبة ايضا لوجود هذه البكتريا.

تم اختبار قدرة عزلات الاشريكية القولونية المعزولة في انتاج الغشاء الحيوي اذ اظهرت النتائج ان 50 عزلة من اصل 54 من عزلات الحليب كانت منتجة للغشاء الحيوي وبنسبة(92.59%) و38 عزلة من اصل 49 مستحصل عليها من براز الابقار(77.55%) و39 عزلة من اصل 42 مستحصل عليها من براز الاغنام(92.85) كانت منتجة للغشاء الحيوي وقد تراوح سمك الغشاء الحيوي المنتج بين (0.2-2ملم) في حين لم تتمكن بقية العزلات من انتاج الغشاء الحيوي.

اظهرت النتائج ان افضل درجة حموضة لانتاج هذا الغشاء هي 7 في حين كانت افضل درجة حرارةلانتاجه قد تراوحت بين 37-45 درجة مئوية.

Introduction:

E.coli is a normal inhabitant of the intestines of most animals, including humans. Some *E.coli* strains can cause a wide variety of intestinal and extra–intestinal diseases, such as diarrhea, urinary tract infections, septicemia, mastitis and neonatal meningitis. (5). Generally,the formation of biofilms in Ecoli in a host based on the current evidence,that it is an intra cellular event 6)

The diseases caused by a particular strain of *E. coli* depend on the distribution andthe expression of many virulence determinants such as adhesion ,biofilm formation, production of haemolysin, enterotoxin, shiga toxin ,endotoxin and capsules formation (7).

Biofilms are not easily defined as they vary greatly in the structure and the composition from one environmental niche to another Microbial biofilms are extremely complex microbial ecosystems consisting of microorganisms attached to a surface and embedded in an organic polymer matrix of microbial origin , the in addition to microbial components , non–cellular materials such as mineral crystals , corrosion particles, clay or silt particles , or blood components , may also be found in the biofilm matrix , Biofilms , particularly in water systems ,can be highly complex , whilst others such as those on medical devices , may be simpler , and composed of single , cocciod or rod shaped organisms, Given these differences , it does not seem plausible to suggest that a true "biofilm model " can be considered to be industrial ogyecol and medical situation, there for the definition of a biofilm has to be kept in general and may redefined to microbial cells immobilized in a matrix of extra cellular polymers acting as an independent functioning ecosystem,and homeostatically regulated" (8).

Diarrheal diseases are major problem in the third world countries which are responsible for death of millions of people and animals each year, diarrhea is an alteration in normal bowel movement and it is characterized by an increase in the water content, volume, or frequency and decrease of dry matter of feces (1,2,3). It can be either acute or chronic (9).

Diarrhea accounts of 46% of calves and lambs mortality (10).The most common causes of acute diarrhea are bacterial and viral infections (11,12).Infections with *Escherichea coli* being one of the major causative agents (13).

E.coli mastitis remains one of the most costly disease in farm animal , and this disease affected many high producing cows in dairy herds and may cause several cases of death per year in most severe cases with economic losses to the dairy industry (4).

Research on biofilm formation from *E.coli* and *Streptococcus uberis* shed an interesting light on the whole dynamic process of bacterial invasion , adherence ,persistence , invasive strategies of these bacteria and reveal parallels and differences between these bacteria and *Staphylococcus aureus* , The search for a successful treatment of biofilm infections that can prevent and eradicate biofilms in the clinical environment is still ongoing (14,15).

Materials and Methods:

1-Sample Collection

102 fecal samples (52 from cows, calves and 50 from sheep, goat) which were collected from Salahaddin governorate, university of Baghdad-College of veterinary medicine, university of Baghdad-College of Agriculture,Radhwanya, Dora and Abu-Ghraib zone from animals (sheep , goats, cows and calves) suffering from diarrhea.

2-Diagnostic tests:

All specimens were streaked on MacConkey agar and Eosin Methylene blue agar and incubate over night at 37C.In next day,recoverd and identified according to standard bacteriological and biochemical criteria, in addition to Api 20 E system and RapID ^{TM} ONE System were used to diagnos these isolates , A qualitative assessment of biofilm formation was determined by tube method (16). Thickness of biofilm which produced by these isolates was measured by a ruler.

3-Statistical analysis:

The data were analysed statistically using the Microsoft program(SPSS).Statistical analysis of data was performed on the basis of Analysis of Variance(ANOVA),and specific group differences were determined using least significant differences(L.S.D)

Results and Discussion:

Microscopical, cultural characteristics, biochemical and confirmatory tests :

The results showed differences between the various zone in the growth of fecal samples and milk samples on MacConky agar and EMB with different morphological characteristics of *E.coli* on different media, after incubation at 37°C for 24hours. Isolated bacteria were appeared as Gram negative rods, non spore forming under light microscopic lense and these results are agreed with that recorded by (17). The red /pink color on MacConkey agar was occurred due to the utilization of the lactose that available in the medium with surrounding areas of precipitated bile salts, While EMB agar was used for selection and isolation purposes, and was considered as a rapid and accurate method for distinguishing *E. coli* from other Gram-negative pathogens. The visible colonies were appeared as green metallic sheen that indicating vigorous fermentation of lactose and acid production which precipitates the

green metallic pigment .This result agree with (*18*).

The Biochemical identification results showed that these isolates belong to *E.coli* , positive for catalase, indol,methyl red test and motility test with the ability to produce gas on kligler iron test while gave negative results for oxidase, simmon citrate test, urease test and voges proskaure test .

The results of Api 20 E system and RapID ^{TM}ONE System were confirmed that these 91 isolates out of 102 fecal samples were belong to *E.coli* and 54 isolates out of 182 milk samples were belong to *E.coli* .

Results of bacterial isolation showed that out of total 182 milk samples were collected from cows suffering from acute mastitis (104 samples) and apparently normal cows (78 samples), 54 milk samples (29.67%) showed positive results for the presence of *Escherichia coli* after culturing one EMB agar. Table (1).(19), has Found that 31 out of 110 milk samples collected from cows infected with acute and subclinical mastitis of cows distributed between Baghdad and Diyala governorate showed positive results for *E.coli* (28.18%) and this is in agreement with the present results.(20) reported has that 36 out of 206 raw milk samples collected from various cow farms in Kermanshah zone showed positive results for *E.coli* (17.47%). (21) also found that 11 samples out of 135 milk samples from cows with mastitis were positive for *E.coli* (8.14%) from different places of Pantnagar city.(22) demonstrated that 80 out of 96 milk samples were collected from local market in Baghdad contaminated with *E.coli* (83.9%) and this disagrees with the present results.

On the other hand out of 102 fecal samples (52 fecal samples from cows and 50 fecal samples from sheep), 91 fecal samples (89.21%) showed positive results for the presence of *E.coli* after culturing on EMB Table (2), There are many researches reporting the colonization of the gastroenteritis tract of both large and small ruminants with O157 and non O157 *E.coli*(23. rMoreve(24)has found that 36 out of 41 fecal samples of sheep and goats in Duhok governorate were positive for *E.coli* (87.80%) and this is in agreement with the present results Furthermore .(25)has found that only 22 out of 326 fecal samples from diarrheic dairy calves collected from some private dairy farms in the vicinity of Najaf city were positive for *E.coli* (6.74%) and this is disagreed with the present results.

Table (1): Collected data of *E.coli* isolated from milk samples

city of collected samples	Total No.	Number of acute mastitis	Number of apparently normal cows	Positive samples on	
				MacConkey agar	EMB agar
Salahaddin Governoratete	105	77	28	56	31
College of veterinary-medicine university of Baghdad	10	2	8	7	2
College of agriculture-university of Baghdad	40	3	37	24	3
Radhwanya zoon	20	16	4	18	16
Abu- Ghraib zoon	7	6	1	5	2
Total No.	182	104	78	110	54

Table (2): Collected data of E.*coli* isolated from fecal samples

city of collected samples	Total No.	Positive samples on	
		MacConkey agar	EMB agar
Abu- Ghraib zoon	35	33	32
College of veterinary-medicine university of Baghdad	28	26	23
College of agriculture-university of Baghdad	17	16	16
Dora zoon	22	20	20
Total No.	102	95	91

Biofilm production results

Detecting the ability of *E.coli* isolates to produce biofilm was done by using Christensen tube method (16). The results showed that 50 out of 54 *E.coli* isolates from milk samplesewer produce biofilm (92.59%)and 38 out of 49 *E.coli* isolates from fecal samples of cow produce biofilm (77.55%). Furthermore 39 out of 42 *E.coli* that isolatedfrom fecal samples of sheepshowed to were produce biofilm (92.85%), whereas 4 isolates from milk samples ,11 isolates from 49 fecal samples of cow and calves and 39isolates from 42 fecal samples of sheep were not produce biofilm. table (3,4,5), These results are in agreement with(26) who showed that 46 of total 56 isolate of *Staphylococcus aureus* produced biofilm (82.14%) while only 10 isolates gave negative results (17.85) ,(27) who demonstrated that 32 of 35 *S. aureus* isolates were produced biofilm (91.42). also these results in agreement with the current study whereas(28) found that a lower percentage (12%) of biofilm-positive producer strainshad been tested in 92 bovine strains, this finding is disagreement with the current study.

Table (3) Biofilm production of different *E.coli* isolates from milk samples:

No. of isolates	Result	No. of isolates	Result	No. of isolates	Result	No.of isolates	Result
1	+	15	+	29	+	43	+
2	-	16	+	30	+	44	+
3	+	17	+	31	+	45	+
4	+	18	+	32	+	46	+
5	+	19	+	33	+	47	+
6	+	20	+	34	+	48	+
7	+	21	+	35	-	49	+
8	+	22	+	36	+	50	+
9	+	23	+	37	+	51	+
10	+	24	+	38	+	52	-
11	+	25	+	39	+	53	+
12	+	26	+	40	+	54	+
13	+	27	-	41	+		
14	+	28	+	42	+		

Table (4) Biofilm production of different *E.coli* isolates from fecal samples (cow):

No. of isolates	Result	No. of isolates	Result	No. of isolates	Result	No. of isolates	Result
1	-	14	-	27	+	40	+
2	+	15	+	28	+	41	+
3	+	16	+	29	+	42	-
4	-	17	+	30	+	43	+
5	+	18	+	31	+	44	+
6	+	19	+	32	+	45	+
7	+	20	+	33	+	46	+
8	+	21	+	34	+	47	+
9	-	22	-	35	+	48	-
10	+	23	+	36	+	49	-
11	-	24	+	37	-		
12	+	25	+	38	+		
13	+	26	+	39	-		

Table (5) Biofilm production of different *E.coli* isolates from fecal samples (sheep):

No. of isolates	Result	No. of isolates	Result	No. of isolates	Result	No. of isolates	Result
1	+	12	+	23	+	34	+
2	+	13	+	24	+	35	+
3	+	14	+	25	+	36	+
4	+	15	+	26	+	37	+
5	+	16	+	27	+	38	+
6	+	17	-	28	+	39	+
7	-	18	+	29	+	40	+
8	-	19	+	30	+	41	+
9	+	20	+	31	+	42	+
10	+	21	+	32	+		
11	+	22	+	33	+		

+ Biofilmproduction **- non biofilmtionproduc**

Biofilm thickness results

The results showed that these isolates differed in its biofilm productionefficiency. The thickness of biofilm was measured in these isolatesit is ranged between (0.2-2) mm (29) has found that the thickness of biofilm produced by *Pseudomonas aeruginosa* ranged between (1.1-6.5) mm ,these results are disagreed with the presentdata, whereas (26) showed that the thickness of biofilm produced by *S.aureus* is ranged between (0.2-1.5) mm and this is in agreement with the present results Table (6,7,8).

Table (6): Thickness of biofilm produced by *E.coli* isolates from milk samples:

No. of isolate	Thickness of biofilm (mm)	No. of isolate	Thickness of biofilm (mm)
1	0.6	28	0.2
3	1	29	1
4	0.3	30	1
5	0.2	31	2
6	1	32	1.5
7	0.2	33	0.2
8	0.7	34	0.3
9	0.2	36	1
10	1.5	37	0.4
11	0.2	38	0.2
12	0.5	39	0.6
13	1.5	40	1
14	0.4	41	0.2
15	0.7	42	0.8
16	2	43	0.2
17	1	44	0.2
18	0.4	45	0.6
19	0.3	46	0.8
20	0.2	47	0.2
21	0.6	48	0.2
22	2	49	1
23	1.5	50	0.4
24	0.2	51	0.3
25	0.2	53	0.6
26	0.2	54	0.2

Table (7) Thickness of biofilm produced by *E.coli* isolates from fecal sample of cows:

No. of isolate	Thickness of biofilm (mm	No. of isolate	Thickness of biofilm (mm
2	0.2	26	0.2
3	2	27	0.2
5	0.7	28	0.2
6	1.5	29	0.5
7	0.8	30	1
8	0.2	31	0.9
10	0.2	32	0.2
12	1	33	1
13	0.4	34	0.5
15	0.2	35	0.2
16	0.2	36	0.9
17	1.5	38	0.5
18	1	40	0.5
19	1	41	0.6
20	0.4	43	0.5
21	0.5	44	0.3
23	0.3	45	0.7
24	0.5	46	0.2
25	2	47	0.4

Table (8) Thickness of biofilm produced by *E.coli* isolates from fecal samples of sheep:

No. of isolate	Thickness of biofilm (mm	No. of isolate	Thickness of biofilm (mm
1	0.8	24	2
2	0.2	25	0.3
3	0.9	26	0.2
4	0.2	27	2
5	0.8	28	0.4
6	0.4	29	0.2
9	0.3	30	0.5
10	0.6	31	0.2
11	0.2	32	0.5
12	0.3	33	0.2
13	0.2	34	0.2
14	0.2	35	0.6
15	0.4	36	0.5
16	0.2	37	0.6
18	0.4	38	0.2
19	0.2	39	0.6
20	0.4	40	0.3
21	0.2	41	0.2
22	0.4	42	0.3
23	0.2	43	0.6

Optimal pH for biofilm production:

Tffecte eh of different pH values of tryptic soya broth on biofilm production in *E.coli* was studied,35 isolates were selected for this study؛ (10 isolated from fecal sample of sheep؛ 10 isolated from fecal sample of cow and 15 isolates from milk sample .

Ten samples of cow fecal are corresponding to number 3,5,6,7,12,25,30,34,40,and 45, ten samples of sheep fecal are belong to number 1,3,5,10,12,18,27,30,36 ,39 and 15 isolates of milk samples refer to number 1,3,5,7,15,17,19,22,29,31,32,39,42,46 in addition 49 isolates were used to study the effect of pH medium on biofilm production .

E.coli isolates have the ability to produce biofilm at pH ranged from (6 -9) whereas no biofilm production was obtained at pH (4).The means of bioflim thickness of *E.coli* isolated from cow fecal samples at pH (5,6,7,8,9) were (0.12, 0.72 , 1.07, 0.78,0.63), (fig ;1)Table 9 . The means of bioflim thickness of *E.coli* isolated from sheep fecal samples at pH (5,6,7,8,9) were (0.15, 0.4 , 0.74, 0.49,0.27) (fig ; 2)Table 10 and the means of bioflim thickness of *E.coli* isolated from milk samples at pH (5,6,7,8,9) were (0.14, 0.36 , 0.91, 0.5,0.52) (fig ;3)Table11.(26) has showed that all *S.aureus* isolates have the ability to produce biofilm at pH ranged from (6-9) while no biofilm production has been observed at pH (1-4) This result is in agreement with the present results . pH has a remarkable influence on enzyme activity, each enzyme activity has an optimal pH (29, 30,31) has found that in many biofilm-forming bacteria, the differentiation of planktonic cells into sessile bacteria is associated with environmental stress factors at as acidic pH level (pH 1-4) all the tested isolates were non biofilm producers.

In this study, optimal pH of biofilm formation for all of these isolates was **7**.

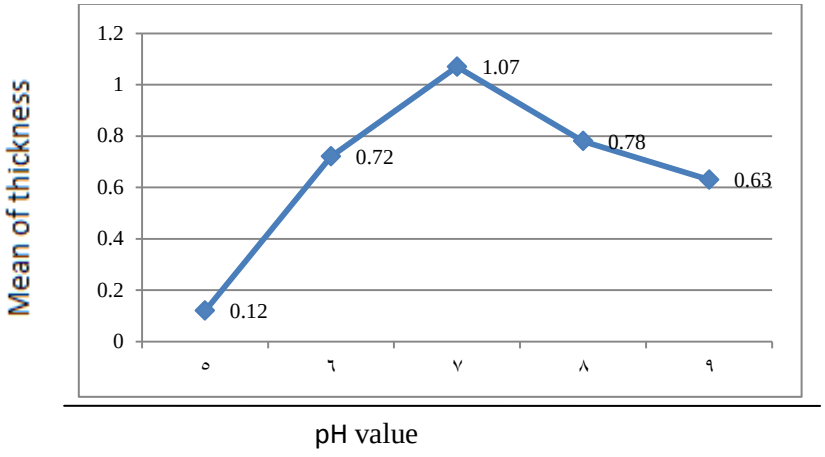


Figure (1): Mean of bioflim thickness of *E.coli* isolated from cow fecal samples

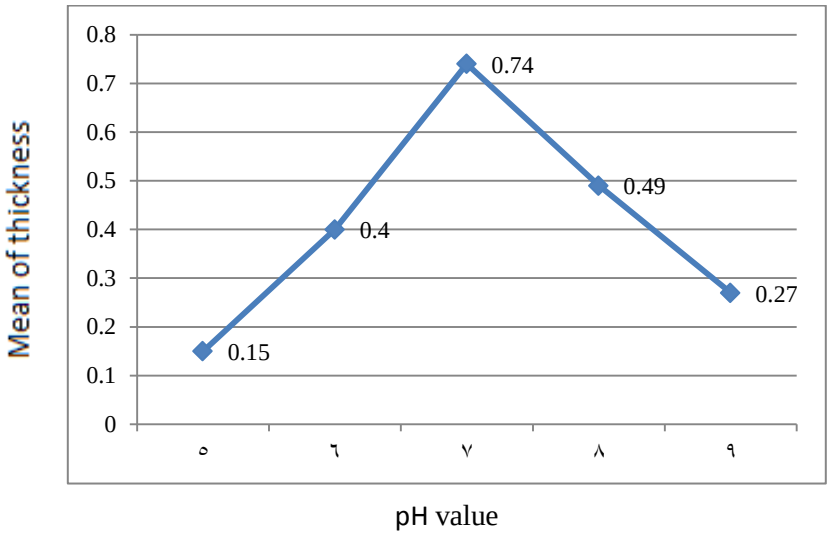


Figure (2): Mean of bioflim thickness of *E.coli* isolated from sheep fecal samples

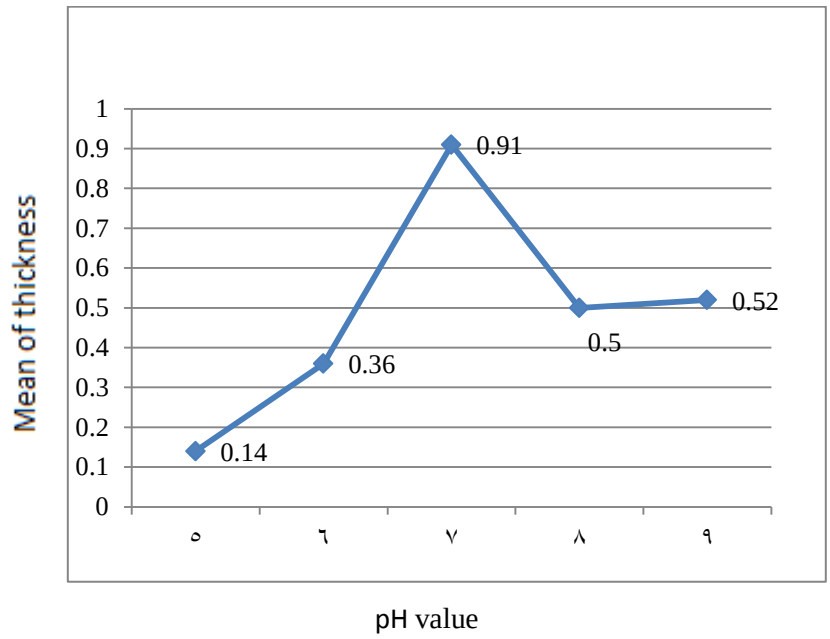


Figure (3): Mean of bioflim thickness of *E.coli* isolated from milk samples

Table (9): Optimal pH for biofilm production (Thickness) for cow fecal samples

pH=4	pH=5	pH=6	pH=7	pH=8	pH=9
C Mean ±S.E 0±0	C Mean±S.E 0.12±0.05	AB Mean±S.E 0.72±0.18	A Mean±S.E 1.07±0.17	AB Mean±S.E 0.78±0.18	B Mean±S.E 0.63±0.19

Values represent mean±S.E
L.S.D=0.36
Different capital letters mean significant differences between isolates (P<0.05).

Table (10): Optimal pH for biofilm production (Thickness) for sheep fecal samples

pH=4	pH=5	pH=6	pH=7	pH=8	pH=9
E Mean ±S.E 0±0	D Mean ±S.E 0.15±0.06	BC Mean ±S.E 0.4±0.05	A Mean ±S.E 0.74±0.14	B Mean ±S.E 0.49±0.12	C Mean ±S.E 0.63±0.19

Values represent mean ±S.E
L.S.D±19
Different capital letters mean significant differences between isolates (P<0.05).

Table (11):Optimal pH for biofilm production(Thickness) for Milk sample.

pH=4	pH=5	pH=6	pH=7	pH=8	pH=9
E Mean ±S.E 0±0	D Mean ±S.E 0.15±0.03	C Mean ±S.E 0.36±0.05	E Mean ±S.E 0.91±0.14	E Mean ±S.E 0.51±0.07	E Mean ±S.E 0.54±0.06

Values represent mean ±S.E
L.S.D±0.17
Different capital letters mean significant differences between isolates (P<0.05).

Optimal Temperature on biofilm production:

The isolates differ on its biofilm production under different temperatures, All isolates produced biofilm at (25-45) ºC, whereas no biofilm production for all isolates was obtained at (20) ºC . The optimumtemperature for biofilm production for all isolates was at 37 ºC as shown in Figure: (4,5,6) . The mean of thickness of biofilm *E.coli* isolates isolated from cow fecal samples at temperature (25,30,37,45) were (0.37, 0.61 , 1.07, 0.82)respictivlyTable12. The mean of thickness of biofilm in *E.coli* isolates that isolated from sheep fecal samples at temperature (25,30,37,45) were (0.38, 0.6 , 0.74, 0.71)respictivlyTable13، and the mean of thickness of biofilm *E.coli* isolates isolated from milk samples at temperature (25,30,37,45) were (0.28, 0.47 , 0.91,0.43

.respictivly Table14

(26)has showed that the optimal temperature for biofilm production by *S.aureus* was at 35 ºC with the mean of thickness around (0.934) mm and this finding is in disagreement with the present results. *E.coli* can grow in temperature ranged from 7 ºC to 50ºC, with an optimum temperature of 37 ºC (32) and this is in agreement with the present study.(26)has also showed that the best temperature of biofilm production by *P. aeruginosa* was 35 ºC with the mean of thickness 4.967 mm .

All these results suggested that optimal temperature for bioflm is production ranged between (30-45) ºC.

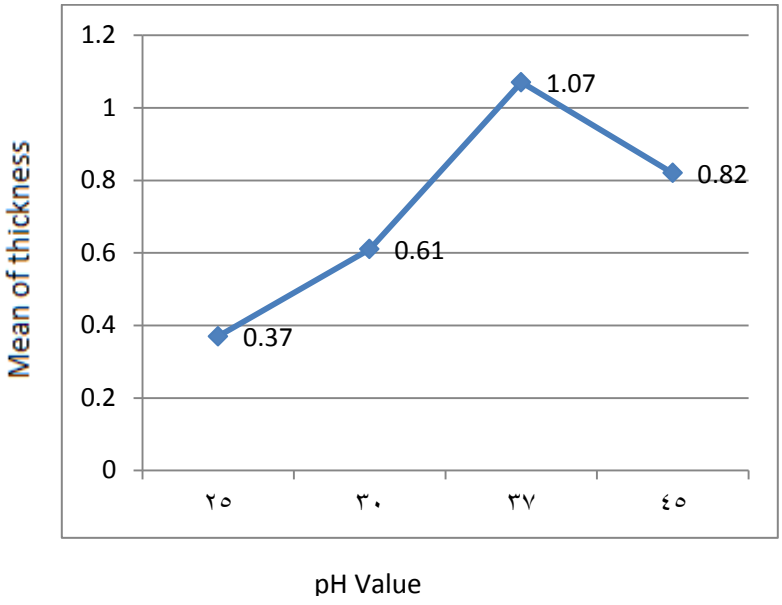


Figure (4): Mean of bioflim thickness of *E.coli* isolated from cow fecal samples at different temperature.

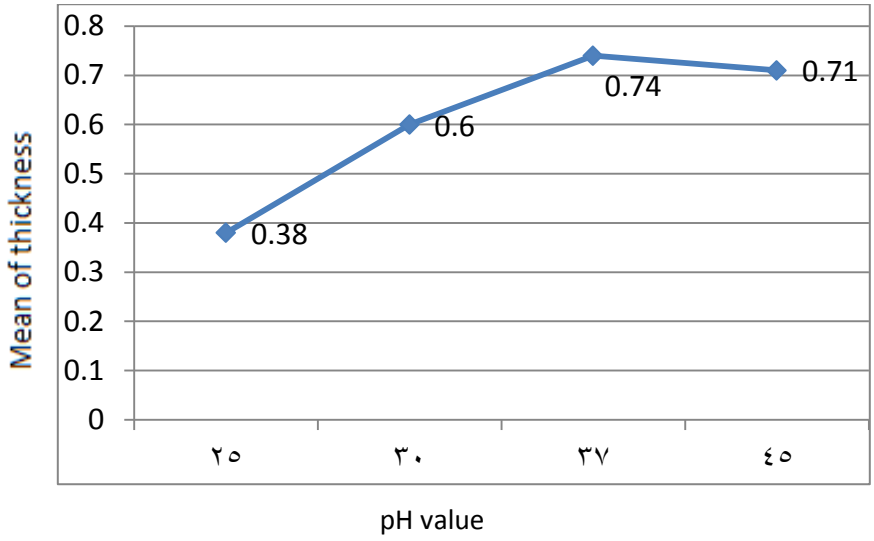


Figure (5): Mean of bioflim thickness of *E.coli* isolated from cow fecal samples at different temperature

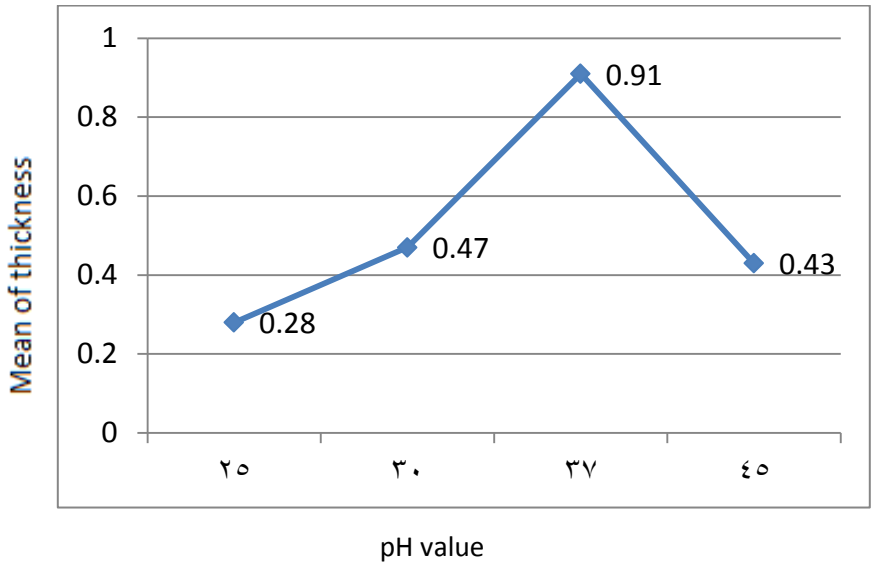


Figure (6): Mean of bioflim thickness of *E.coli* isolated from milk samples at different temperature

Table (12): Optimal temperature on biofilm production (Thickness) for cow fecal sample.

	Temperature			
20 ºC	**25 ºC**	**30 ºC**	**37 ºC**	**45 ºC**
D Mean ±S.E 0±0	D Mean ±S.E 0.37±0.08	C Mean ±S.E 0.61±0.1	B Mean ±S.E 1.07±0.18	A Mean ±S.E 0.82±0.18

Values represent mean ±S.E

L.S.D=0.32

Different capital letters mean significant differences between isolates (P<0.05).

Table (13): Optimal temperature on biofilm production (Thickness) for sheep fecal sample.

	Temperature			
20 ºC	**25 ºC**	**30 ºC**	**37 ºC**	**45 ºC**
D Mean ±S.E 0±0	D Mean ±S.E 0.38±0.75	C Mean ±S.E 0.6±0.16	B Mean ±S.E 0.74±0.21	A Mean ±S.E 0.71±0.13

Values represent mean ±S.E
L.S.D±14
Different capital letters mean significant differences between isolates (P<0.05).

Table (14): Optimal temperature on biofilm production (Thickness) for Milk sample.

	Temperature			
20 ºC	**25 ºC**	**30 ºC**	**37 ºC**	**45 ºC**
D Mean ±S.E 0±0	D Mean ±S.E 0.28±0.87	C Mean ±S.E 0.47±0.87	B Mean ±S.E 0.91±0.14	A Mean ±S.E 0.43±0.06

Values represent mean ±S.E L.S.D±11
Different capital letters mean significant differences between isolates (P<0.05).

References:

1. Abed Al-Rhada M.H. , Aseel . (2014) Antimicrobial effect of alcoholic and water extracts of Thymus Vulgaris and Hibiscus sabdariffa on different bacteria isolated from raw cow milk. International journal of advance research (March 2014) Vol. 2 Issu 4, 163-167.
2. Al-Charrakh & Al-Muhana (2010) Prevalance of verotoxin-producing *Escherichia coli* (VTEC) in a survey of dairy cattle in Najaf, Iraq. Iranian journal of microbiology Vol 2 No. 3 (September 2010)128-134.
3. Al-Ithawy, A. A. (2010). Affected factors on *Pseudomonas aeruginosa* Isolated from deferent environmental sources on biofilm formation, M.Sc. Thesis submitted to the university of Anbar/ college of science.
4. Anderson GG,Goller CC, Justice S, Hultgren SJ, Seed PC(2010) Polysacchride capsule and sialic acid–mediated regulation promote biofilm–like intracellular bacterial communities durin cystitis . Infect Immun 78(3):963-975.
5. Ansaruzzaman, M.; Albert, J.; Nahar, S.; Byun, R.; Katouli, M.; Kuhn, I. and Mollby, R. (2000). Clonal groups of enteropathogenic isolated in case control studies of diarrhea in Bangladesh. J. Med. Microbiol.
6. Bannerman, D.D; Paape,J.M.; Weilee, J.; Zhao,X. ; Hope, C. J. and Rainard, P.(2004). Escherichia coli and Staphylococcus aureus elicit differential innate immune response following intramammary infection .Clinical and Diagnosis Laboratory Immunology., 11(3): 463 -472.
7. Baselga, R.; Albizu, I.; De La Cruz, M.; Del Cacho, E.; Barberan, M. and Amorena, B. (1993). Phase variation of slime production in *Staphylococcus aureus*: implications in colonization and virulence. Inf. Imm. 61, 4857–4862.
8. Bradley AJ , Green MJ (2001) Adaptation of *Escherichia coli* to the bovine mammary gland . J Clin Microbial 39(5) :1845-1849
9. Brandt, L.J. and Greenwald, D. (2001). Acute and chronic diarrhea: a primer on diagnosis and treatment. Available at : http://www.acg.gi.org/acg-dev/physicianforum/gifocus/diarrhea.html.
10. Christensen, G .D.; Simpson, W .A.; Bisno, A. L.and Beachey, E .H. (1982). Adherence of slime producing strains of *Staphylococcus epidermidis* to smooth surfaces. Infect Immun; 37:318–26.

11. Costerton, J. W.; Stewart, P. S. and Greenberg, E. P. (1999). Bacterial biofilms: a common cause of persistent infections. Science 284:1318–1322.

12. Guerrant, R.L.; Van Gilder, T.; Steiner, T.S.; Theilman, M.N.; Slutsker, L. and Tauxe, R.V. (2001). Practice guidelines for the mangement of infectious diarrhea. Clin. Infect. Dis., 32: 331-35.

13. Holland, R.E. (1990). Some infectious causes of diarrhea in young farm animals. Clinical Microbiology Reviews.345-375

14. Kaper, J .B ., Nataro , J. P . And Mobley, H.L.T. (2004). Pathogenic *Escherichia coli*. Nat. Rev. Microbial 2:123- 140.

15. Kumar R, Prasad A. (2010) Detection of *E.coli* and *Staphylococcus* in Milk and Milk Products in and around Pantnagar Vet World J 3(11):495-496.

16. Mohammadi P, Abiri R, Rezaei M (2013) Isolation of Shiga toxin-producing *Escherichia coli* from raw milk in Kermanshah, Iran. Iranian J. of Microb, Sep Vol.5,Issue 3,p233.

17. Nielsen, E. M.; Scheutz, F. ; and Torpdahl, M. (2006). Continuous surveillance of Shiga toxin-producing *Escherichia coli* infections by pulsed-field gel electrophoresis shows that most infections are sporadic. Foodborne Pathog. Dis. 3:81–87.

18. Percival , S.L. ; Derek , C.k.; Christine, A.C.(2011) "Biofilm and veterinary medicine " London: 42.

19. Percival SL , Walker J, Hunter P (2000) Microbiological aspects of biofilms and drinking water . CRC press , New York.

20. Quinn, P.J.; Markey, B.K.; Carter, M.E.; Donnelly, W.J. and Leonard, F.C. (2004). "Veterinary Microbiology and Microbial Diseases Textbook". Printed and bound in Great Britain by International Ltd. Mosby, London, Padstow-Cornwall:118-126.

21. Radostits, O.M.; Gay, O.M.; Hincheliff, K.W. and Coststable, K.W. (2007).Veterinary Medicine . A text book of the cattle ,horses, sheep, pigs and goats. 8th edition. Slsevier. USA. Pp:163.

22. Rahim S.A, (2014). Study the effect of bacterial extracts of filamentous bacteria (*Streptomyces chromofucus* /NS-38) on the bacterium *Staphylococcus aureus* isolated from cases of mastitis in cows. M .Sc .Thesis – College of Veterinary Medicine – Baghdad University .

23. Saeed , A.Y., Ibrahim, Kh. S., (2013) Identification of *Escherichia coli* O157 in sheep and goats using PCR Technique. JOSR Journal of Agriculture and Veterinary Science Vol.6 Issue 2 (Nov.-Dec. 2013) PP 30-32.

24. Sandler, R.S.; Stewart, W.F.; Liberman, J.N.; Ricci, J.A. and Zorich, N.L. (2000). Abdominal pain, bloating and diarrhea in the United States: prevalence and impact. Dig. Dis. Sci., 45(6): 1166-71.

25. Schoenian, S. (2007). Diarrhea (scours) in small ruminants. Maryland cooperative Exttension. Sheep and *G*oat specialist Western Maryland Research and Eduction Center.

26. Schulze, J.; Schiemann, M. and Sonnenborn, U. (2006). 120 years of *E. coli* Its importance in research and medicine. 6th ed. Alfred-Nissle- Gesellschaft, Germany: 11-13.

27. Shapiro J.A. (1998). Thinking about bacterial populations as multicellular organisms. Annu. Rev. Microbiol. 52, 81-104.

28. Shoba, F.G. and Thomas. M. (2001). Study of antidiarrheal activity of four medicinal plants in castor oil-induced diarrhea. J. Ethnopharmacol., 76:73-76.

29. Tenaillon , O., Skurnik, D., Picard , B., and Denamur , E.(2010). The population genetics of commensal *Escherichia coli*. Nat Rev Microbial 8: 207- 217.

30. Vasudevan, P.; Nair, M.K.M.; Annamalai, T. and Venkitanarayanan, K.S. (2003). Phenotypic and genotypic characterization of bovine mastitis isolates of *Staphylococcus aureus* for biofilm formation. Vet. Microbiol. 92, 179–185.

31. White, A., Handler, P., Smith, E.L. (1978). Enzymes I, nature, classification, kinetics, metabolic inhibitors: control of enzymatic activity. In: White A. Ed. Principles of Biochemistry, McGraw-Hill, Tokyo. 196-230.

32. WHO (2011) Enterohaemorrhagic *Escherichia coli* , Media center Printed on Dec. 2011.

33. Yarwood, J. M.;Bartels, D. J.; Volper, E. M. and Gereenberg, E. P. (2004). Quorum sensing in *Staphylococcus aureus* biofilms. Journal of Bacteriology 186: 1838-1850.

Iraqi Journal of Biotechnology, 2015, Vol. 14, No. 2 , 270-283

The study of Diabetic nephropathy disease and its relashionshipe with GSTM1and GSTT1 genes deficients and the risk factor of age of patients in the sample province of Dhi-Qar

Hassan Ressan AL-Rikabi , Huda Taher Faisal

Department of Biology – College of Education for Pure Science - University of Thi- Qar Nassiriyah-Iraq

Abstract: The experimental group of this study contains 100 blood samples collected from patients with diabetic nephropathy infraction admitted to AL-Hussein Teaching Hospital , the center of the industrial kidney and some civil medical laboratories in the province of Thi-Qar who ranged in age between 18-89 years old and 90 samples for people who are not infected with diabetic nephropathy infraction as a comparison group . The samples have been preserved in tube containers with EDTA under tempreture (-20c^{o}) DNA was extracted and then GSTM1 and GSTT1 were amplified which are responsible for detoxification and Albumin as an internal control.

The results showed that61% of the patients were Femals while 39% were males, as well as to age that the maximum cases in the age group of (60-69) year (26%) but the minimum cases in the age group of (20-29) year (11%) and patients who have family history are 51% compare to 49% for those who does not have family history .

The results indicate that the GSTT1 null genotype increased 17-fold in age of at least 50 years old with an (OR= 17.6;95%CI= 2.108-146-919). While the deletion in GSTM1 null genotype and both genes (GSTM1 and GSTT1 null genotypes)was increased (OR= 2.4) and (OR= 1.67) respectively.

showed there was very clear association between the risk of the disease and the older ages of 50 years when GSTT1 null genotype (OR=23.78) and the deletion in GSTM1 null genotype was also increased (OR= 4.82)

Key word : Diabetic nephropathy, Genes, GSTM1, GSTT1 , PCR

دراسة مرض اعتلال الكلى السكري وعلاقته مع جينات الحذف GSTT1,GSTM1 وعامل خطورة العمر لدى عينة من مرضى محافظة ذي قار

حسن ريسان الركابي ، هدى طاهر فيصل

قسم علوم الحياة – كلية التربية للعلوم الصرفة – جامعة ذي قار / الناصرية – العراق

الخلاصة: جمعت 100 عينة دم بحجم 2,5 مل من اشخاص تتراوح اعمارهم بين 18-89 سنة من كلا الجنسين لآشخاص مصابين باعتلال الكلى السكري (مجموعة المرضى) يراجعون مستشفى الامام الحسين التعليمي – مركز الكلى الصناعيه وبعض المختبرات الطبيه الاهليه في محافظة ذي قار و 90عينة دم من غير المصابين للمرضى أعتمدت كسيطره من غير المصابين بهذا المرض من مختلف الشرائح الاجتماعيه . حفظت العينات بانابيب حاويه على الـ EDTA بدرجة حراره(-20 ^{o}c) لمستخلص DNA وعدها ضخمت الجينات GSTT1,GSTM1 المسؤولة عن ازالة السميه والالبومين كسيطره داخليه .

أظهرت نتائج الدراسة أن 61% من المرضى كانوا من الاناث بينما كانوا 39% من الذكور، أضافة الى االعمر حيث كانت اعلى نسبة اصابه عن المرضى الذين تتراوح اعمارهم بين (60-69) حيث كانت النسبه 26% واقل نسبة اصابه للمرضى الذين تتراوح اعمارهم مابين (20-29) حيث كانت النسبه 11% ، ونسبة المرضى الذين يملكون تأريخآ عائليا كانوا 51% والذين لايملكون تأريخا عائليا 49%.

اظهرت النتائج بان فقدان الجين GSTT1 يزداد 17 مره في العمر اقل من 50 سنه (OR= 17.6;95%, CI=2.108) بينما فقدان الجين GSTM1 والجينين GSTT1,GSTM1 معآ يكونا في زياده مرتين تقريبآ (OR= 1.67 , 2.4) بينما ظهرت علاقة ارتباط واضحه بين خطر الاصابه بالمرض في الاعمار الكبيره 50 سنه عند ازدياد فقدان الجينين GSTT1,GSTM1 .

الكلمات المفتاحيه: اعتلال الكلى السكري ، جينات ، PCR, GSTT1,GSTM1

Introduction:

Diabetes mellitus (DM) a term describes a metabolic disorder due to many pathogens such as excessive sugar, chronic blood disorders in the metabolism of carbohydrates, fats and proteins resulting from a defect in insulin secretion or insulin action, or both that triggered diabetes in the long term include damage and failure in various tissues and patients diabetes appear to have characteristic symptoms such as thirst and frequent urine and blurred vision, loss of weight, and when you get a coma disease progression or death in the absence of effective treatment. [1]

DM prevalence increases continually around the world ; it became one of the major global problems for the developing as well as the developed countries .It is affecting millions of peoples, about 6-7% of the world's population [2] [3] [4].

Diabetic nephropathy (DN) is defined as urinary albumin excretion equals to or more than 300 mg/24hr and more commonly represented by persistent

albuminuria which is detected by various dipsticks [5].

DN with diabetic chronic renal insufficiency (CRI) is a leading cause of end stage renal disease worldwide [6]. The genesis of DN involves myriad of factors including older age, sex, hyperglycemia, and hyperlipidaemia. Ethnicity is the other major risk factor, with African, Americans, Asians, and native Americans being more prone to develop DN than Caucasians.

Nearly 30% of the cases of end-stage renal disease in India are due to diabetes, and this group is more likely to develop this complication than the Caucasians [7] [8] [9]. is rapidly becoming the leading cause of end – stage renal disease (ESRD), Particularly in the industrialized countries of the world [10].

Glutathione S-transferaseare (GSTs) genes that belong to the phase II gene super family encodes enzymes play an important role in cellular protection and resistance to cellular compounds Almtaadh of the drug, since these genes contribute to the detoxification of the outputs of enzymes catalyzed reactions Phase I and the Phase II other enzymes to facilitate disposal [11].

Materials and Methods:

1. Specimens Collection :

100 blood samples of diabetes nephropathy patients were collected from Al-Hussien teaching Hospital in Al-Nasiryiah city , Artificial Kidney Center and some civil medical laboratories and 90 blood samples of non-diabetes nephropathy and type 2 diabetes as control group were collected from volunteers , their age range between 9 to 89 years old. Two ml of peripheral blood was drawn from the two groups then it have been kept in EDTA tubes at – $20c^0$ for DNA extraction.

Data were collected according to Questionnaire form which includes age, sex, place of residence , family history of case , diabetes and blood pressure .

2 . DNA Extraction:

Method was used (12) in DNA extraction from blood samples, according to the following steps:

500µ was added of a blood sample in the pipes (Eppendorf tube) capacity (1.5 ml) and then 600µ added solution (RBC buffer) after the expulsion of centrally quickly 5000 cycle for 15 minutes to a centrifuge and then poured the filtrate was retained sludge and repeated the process more than once note until the

white precipitate at the bottom of the tube.

3. **Electrophoresis:**

The electrophoresis tank has filled with 1X- TBE buffer about 3mm above the gel.Added 9 µof DNA undiminished oxygen to 3 µ of the Bromophenol blue dye, or by (1:3) then it was loaded in wells of agarose gel.

Electrophoresis was done at 60V and 120 mA then the loading dye was left to migrate from the wells toward the other side.After electrophoresis , the gel was placed on a UV light and a digital photograph of the fluorescent Ethidium Bromide-stained DNA separation pattern was taken.

4. Multiplex Polymerase Chain Reaction (PCR):

Three primer pairs were included in the multiplex PCR for simultaneous amplification of fragments in the GSTM1 and GSTT1 and Albumin genes according to the protocol of [13) (14).

Table (1): Oligonucleotide primer sequences used for amplification of GSTT1 & GSTM1 genes.

Primers		Primer sequences	Length	Tm	TA
GSTM1	*F	5-GAA CTC CCT GAA AAG CTA AAG C- -3	22	64°C	59°C
	*R	5- GTT GGG CTC AAA TAT ACG GTG G- 3	22	64°C	59°C
GSTT1	F	5- TTC CTT ACT GGT CCT CAC ATC TC- 3	23	64°C	59°C
	R	5- TCA CCG GAT CAT GGC CAG CA -3	20	64°C	59°C
Albumin	F	5-GCC CTC TGC TAA CAA GTC CTA C- 3	22	64°C	59°C
	R	5- GCC CTA AAA AGA AAA TCG CCA ATC- 3	24	64°C	59°C

Table (2): PCR reaction for amplication of GSTM1 & GSTT1 genes.

materials	Volume
Master Mix	5 µl
Primer Forward	1 µl for one gene
Primer Reverse	1 µl for one gene
DNA	5 µl
D.W.	8 µl
Total	20 µl

Table (3): PCR condition for amplication of GSTM1 ,GSTT1 genes.

No. of Steps	Steps	Temperature	Time	No. of Cycle
1	Denaturation 1	94 °C	3 min	1 Cycle
2	Denaturation 2	94 °C	1 min	30 Cycles
3	Annealing	58°C	1 min	
4	Extension 1	72°C	1 min	
5	Final Extension 2	72°C	5 min	1 cycle

5.

Detection outputs of the technique (PCR):

Followed the same method of deportation electrode for the detection of (DNA), but with the use of (DNAmarker) and Alagaros concentration (2%).

After the detection of packets to a UV results were recorded as follows: - The emergence of the package when the pair baseband bp 215 means the presence of the gene GSTM1 and the emergence of a package when the pair baseband 350 bp mean the presence

of the gene, which was used Albumin xatrh interior. While the existence of a package when the pair baseband 480 bp, it means the presence of GSTT1 gene after comparison with DNA Marker [13] [14].

6. Statistical Analysis:

T Test was used and (OR) using a statistical program (SPSS ver .8) and the level of probability $P < 0.05$ and confidence interval 95% CI for comparison between patients and control samples to study the effect of deletion mutations in the genes GSTM1 and GSTT1 in the incidence of the disease.

Results:

The image (1) shows the migration of packages the DNA extracted from samples compared to patients.

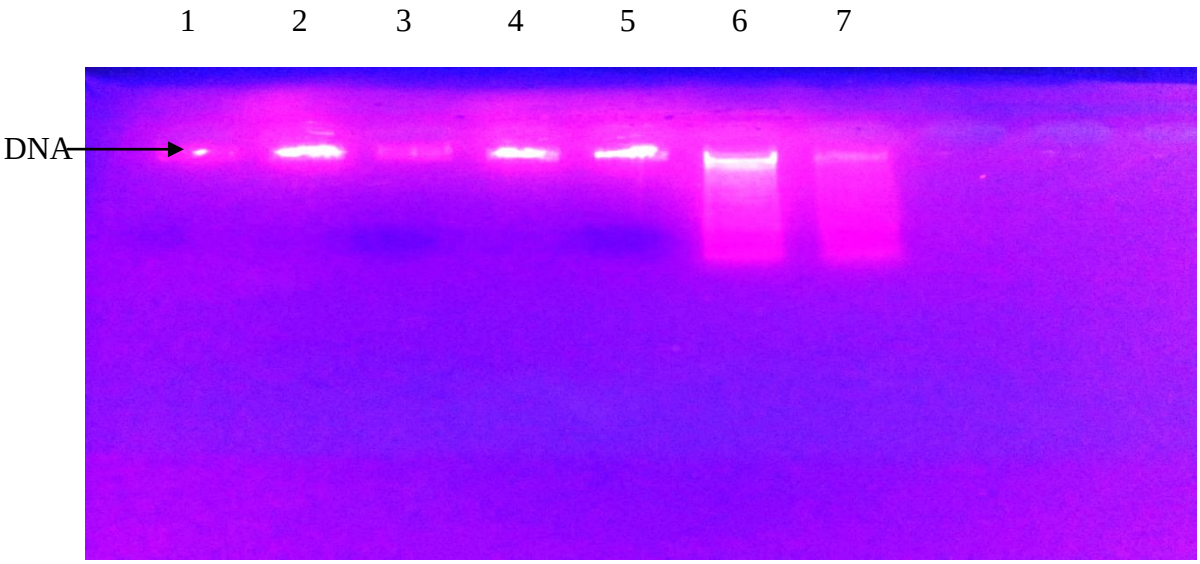


Figure (1): The Electrophoresie for total DNA 0f agarose gel 0.8%

Lane 1,2,3 Patients samples of Diabetic Nephropathy

Lane 4,5 Patients samples of Diabetes Mellitus

Lane 6,7 Control samples .

The image (2) shows the electrophoresis for PCR results on agarose gel 2%

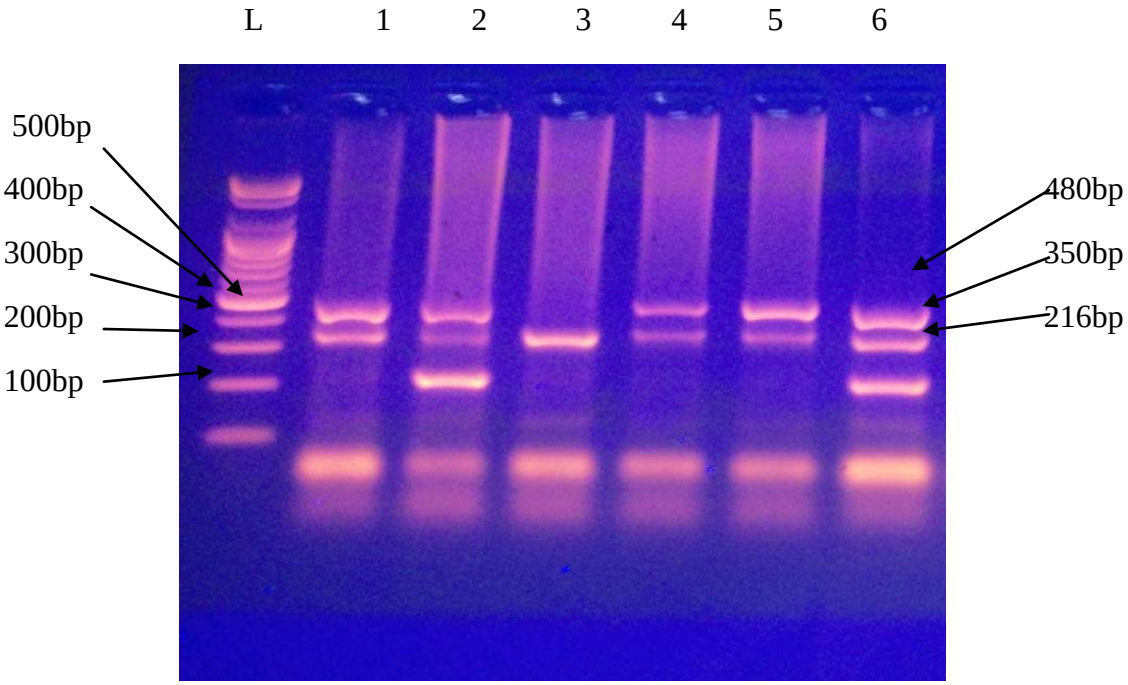


Figure (2): The Elactrophoresie for PCR technique results on Agarose gel 2%

L –measure DNA (1500 bp)

Lane 1,4,5 Loss of GSTM1 genes

Lane 2 Loss of GSTT1 genes

Lane 3 Loss of together genes GSTM1,GSTT1

Lane 6 Natural (contain the three genes).

This study showed:

1.1 The loss of gene GSTM1 and risk of diabetic nephropathy disease.

Found that loss of the gene GSTM1 was (48.49%) in the control samples, while (31%) in patients samples, through statistical analysis showing no significant differences between the loss of the gene GSTM1 and the risk of diabetic nephropathy impairment compared to the control group in possession of this gene (OR= 0.469 ; 95%CI= 0.259 – 0.849).Table (1)

1.2 . The loss of gene GSTT1 and risk of diabetic nephropathy disease.

The percentage of risk for the disease by three times in patients when compared to the loss of gene GSTT1 control group with significant differences (OR= 3.017 ; 95%CI= 1.574 – 5.785).Table (1)

1.3. The loss of GSTM1, GSTT1 genes togathers and risk of diabetic nephropathy disease.

The percentage by almost twice the risk in patients when compared to the loss of the two genes together control group that they had a loss of genes at a rate of (18.19%) (OR= 1.883 ; 95%CI= 0.734 – 4.838) . As in the table below (4).

Table (4): combarison between models for genetic control samples and patients

Genetic models	Control groups%	Patients groups%	OR*	95% CI	P value*
GSTM1 (+)*	46 (51.11%)	69 (69%)			
GSTM1 (-)*	44 (48.49%)	31 (31%)	0.469	0.259 – 0.849	0.0123
GSTT1 (+)	72 (80%)	57 (57%)			
GSTT1 (-)	18 (20%)	43 (43%)	3.017	1.574 – 5.785	0.0009
GSTM1, GSTT1 (+)	36 (81.81%)	43 (70.49%)			
GSTM1, GSTT1 (-)	8 (18.19%)	18 (29.50%)	1.883	0.734 – 4.838	0.188

***(+)Presence of geenes *(-) loss of genes *P= 0.05**
*** OR Odd Ratio**
***95%CI Confidence Interval**

2. . Age distribution of the cases and controls.

The results of this study found that the maximum cases in the age group of (60-69) year (26%) following by the age group (50-59) year (22%), but the minimum cases in the age group of (20-29) year (11%) . as shown in table (5).

Table (5):The distribution of the two groups of patients and control according to age groups.

Age	Control		patients	
	n	%	n	%
20-29	7	7.77%	11	11%
30-39	4	4.44%	8	8%
40-49	10	11.11%	12	12%
50-59	12	13.33%	22	22%
60-69	30	33.33%	26	26%
70-79	23	25.55%	16	16%
80-89	4	4.44%	5	5%
Total	90	100%	100	100%

X^2 = 4.29 df=7 P<0.05

The impact of age and deletion genes in the incidence of diabetic nephropathy disease for both sexes.

A- Ages of at least 50 years old.

The results of our study indicate that the GSTT1 null genotype increased 17-fold in age of at least 50 years old with an (OR= 17.6;95%CI= 2.108-146-919). While the deletion in GSTM1 null genotype and both genes (GSTM1 and GSTT1 null genotypes)was increased (OR= 2.4) and (OR= 1.67) respectively.

Table (6): Genotype of *GSTM1*and*GSTT1* genes of control and patients samples by ages of at least 50 years old.

Genotype	Controls group Least 50= 33	Patients group Least 50= 53	OR	95% CI
GSTM1 (+)	30 (33.33%)	25 (25%)	1.0	
GSTM1 (-)	3 (3.33%)	5 (5%)	2.000	0.434-9.205
GSTT1 (+)	32 (35.55%)	20 (20%)	1.0	
GSTT1 (-)	1 (1.11%)	11 (11%)	17.6	2.108-146.919
GSTM1, GSTT1 (+)	19 (21.11%)	17 (17%)	1.0	
GSTM1, GSTT1 (-)	2 (2.22%)	3 (3%)	1.67	0.249-11.260

B- Older ages of 50 years.

Table (7) showed there was very clear association between the risk of the disease and the older ages of 50 years when GSTT1 null genotype (OR=23.78) and the deletion in GSTM1 null genotype was also increased (OR= 4.82).as in table (7).

Table (7):Genotype of *GSTM1*and *GSTT1* genes of control and patients samples by ages of at older 50 years old.

Genetic models	Control groups Older=57	Patients groups Older= 69	OR	95% CI
GSTM1 (+)	51(56.66%)	44 (44%)	1.0	
GSTM1 (-)	6 (6.66%)	25 (25%)	4.82	1.816-12.843
GSTT1 (+)	55 (61.11%)	37 (37%)	1.0	
GSTT1 (-)	2 (2.22%)	32 (32%)	23.78	5.37- 105.331
GSTM1, GSTT1 (+)	23 (25.55%)	27 (27%)	1.0	
GSTM1, GSTT1 (-)	11 (12.22%)	15 (15%)	1.161	0.446-3.023

The statistical analysis showed relationship between least and older 50 years old and *GSTM1* null genotype and *GSTM1* and *GSTT1* null genotypes (OR= 2.367), (OR= 3.148).as in table (8).

Table (8): Genotype of *GSTM1* and *GSTT1* genes by age.

Genetic models	Patients groups at least 50years old= 31	Patients groups at older 50 years old= 69	OR	95% CI
GSTM1 (+)	25 (25%)	44 (44%)	1.0	
GSTM1 (-)	6 (6%)	25 (25%)	2.367	0.856-6.548
GSTT1 (+)	20 (20%)	37 (37%)	1.0	
GSTT1 (-)	11 (11%)	32 (32%)	1.572	0.656-3.771
GSTM1, GSTT1 (+)	17 (17%)	27 (27%)	1.0	
GSTM1, GSTT1 (-)	3 (3%)	15 (15%)	3.148	0.792-12.516

Discussion:

Glutathione S- transferase (GSTs) genes encoding the large gene family of enzymes that play an important role in protecting cells against free oxygenic species [(15)], there are many studies have pointed to the existence of a relationship between genes (GSTs) and deletions in the emergence of many diseases that are thought to be one of the causes is the oxidative stress such as cancer, [(13)] and bronchial asthma [(15),] vitiligo[(16)] and Myocardial infarction [(17).]

The results of the current study as shown in table (4) that for people with missing genes together GSTM1,GSTT1 and also missing the gene GSTT1 are more susceptible to diabetes, renal impairment was found that the loss of the two genes together was increased by almost twice (OR= 1.883; 95%CI= 0.734-4.838) the loss of the gene was increased by three times (OR= 3.017; 95%CI= 1.574-5.785).

(17) found that the loss of Gene GSTT1 increased by three times (OR= 3.172; 95%CI= 1.595- 6.308), while the loss of the GSTM1gene did not show any significant difference (OR= 0.651; 95%CI= 0.341- 1.243) either loss of the two genes together

GSTT1,GSTM1 did not show significant treatment effect (OR= 0.294; 95%CI= 0.160- 0.538).

(18) pointed out that the loss of the two genes together (GSTM1andGSTT1) had increased six times (OR=6.80; 95%CI= 1.17-29.5) and a half ago and the loss of the gene GSTT1 was increased by two and a half (OR= 2.70; 95%CI= 0.80- 9.19) while the gene GSTM1 was twice (OR= 2.06; 95%CI= 1.21-3.46).

The reason may be due to increasing expression of GSTs in epithelial cells of the proximal tubule during the early stage of diabetes , likely in response to oxidative triggered by hyperglycemia or other toxic effects of glucose [(19).]

In this study, high significant difference in the frequencies of both *GSTM1*,*GSTT1* genes polymorphism by age , this results is in agreement with (Farouk,H .*et al*., 2013) [(20)] because genetic predisposition substantially determines the occurrence and severity of diabetic nephropathy (21). This likeliness of diabetic nephropathy is higher in siblings and children of parents with diabetic nephropathy , independently of the type of DM (22).

Present study showed high significant association between *GSTT1* null genotype and *GSTT1* null , *GSTM1* null genotype and ages of at least 50 years old and older ages of 50 years. Addition , this study showed high significant in DN in comparison the age with*GSTT1* null genotype (OR= 17.6;95%CI= 2.108-146.919) and GSTM1 null (OR= 23.78;95%CI= 5.37-105.331), (OR=4.82;95%CI= 1.816-12.843).that agreement with (162). may be to genetic predisposition substantially determines the occurrence and severity of diabetic nephropathy (18, 23) .

Conclusions:

Results of the present showed that there were a significant relationship between age and loss of deletion GSTM1,GSTT1 genes with increase of diabetic nephropathy infection.

References:

1. World Health Organization (1999): Definition , Diagnosis andclassification of Diabetes Mellitus and its Complication . Report of a WHO Consultation ;Geneva: World Health Organization.

2. Adeghate E, Schattner P, and Dunn E (2006): An Update on the Etiology and Epidemiology of diabetes mellitus . Annual New York Academy of Sciences ; 1084:1-29.

3. American Diabetes Association (2006): Diagnosis and Classification of diabetes mellitus . Diabetes Care 2004;29 (1): s43-s4

4. IDF (2008): Diabetes and facts sheets on Diabetes . International Diabetes Federation website, at IDF http//www.idf.org.2006 (Accessed on: 14 October, 2008) .

5. Woodrow DA (1987): Introduction to Clinical Chemistry .First edition , London , Butterworths; P: 3-118 .

6. American Diabetes Association (2002): Diabetic nephropathy. Diabetes Care, 25, S85-S89.

7. Young, B. A., Maynard, C., &Boyko, E. J. (2003): Racial differences in diabetic nephropathy, cardiovascular disease, and mortality in a national population of veterans. Diabetes care, 26, 2392-2399

8. Viswanatham, V., Snehalatha, C., Mathai, T., Jayaraman, M., &Ramachandran, A.(1998): Cardiovascular morbidity in proteinuric south Indian NIDDM patents. Diabetes Research and Clinical practice, 39, 63-67.

9. Erale, K. K., Porter, K. A., Ostberg, J., &Yudkin, J. S. (2001). Variation in the progression of diabetic nephropathy according to racial origin. Nephrology, Dialysis, Transplantation, 16, 286-290.

10. Ritz E et al. End- stage renal failure in type 2 diabetes (1999): A medical catastrophe of worldwide dimensions. American journal of kidney disease, 34(5): 795-808

11. Hayes, J.D.; Flanagan ,J.U. ; Jowsey, I.R. (2005): Glutathione transferase . Annu Rev PharmacolToxicol 45:51-88.

12. Sambrook,K.J.; Fritsh,E.F.&Maniatis ,T.(1989): Molecular cloning laboratory manual, 2nd ed. ,cold spring Harbor laboratory press.U.S.A.

13. Al-Badran ,AI.(2003): Studies on the molecular genetics of urinary bladder cancer. Ph.D.thesis. Punjab University . India.

14. Arand, M.; Muhlbaur,R.; Hengstler,J.; Fuch ,J.; Winckler,I.&Oesch,F.(1996): A multiples polymerase chain reaction protocol for the simultaneous analysis of the glutathione S-transferase GSTM1 and GSTT1 polymorphism. Anal.Biochem.236: 184-186.

15. Al- Guzi , N.O. (2013): Study the relationship between the null genotype of GSTM1,GSTT1 genes and Bronchial Asthma in Thi-Qar province.Thesis , Thi-Qar university , Iraq.P (82).

16. AL- Zaidy, H.H.(2013): The relationship between genes GSTT1,GSTM1 and the loss of some of the factors leading to the occurrence of vitiligo in the province of Thi –Qar. Thesis, Thi-Qar University, Iraq. P (86).

17. AL- Safii.M.T.(2013): Myocardial infarction and its relationship to smoking and loss of genes in patients in the province of Thi – Qar. Thesis. Thi-Qar University ,Iraq.P (87).

18. Amavizca BE.G, Castellanos R.O, Orozco R.O, et al (2013) :Contribution of GSTM1, GSTT1 , and MTHFR polymorphisms to end – stage renal disease of unknown etiology in Mexicans. Genetic , University Center of Health Science., 23(6): 438- 443.

19. Fujita H, Haseyama T, Nozaki J, Wada Y, Ito S, et al(2001): Increased expression of glutathione S-transferase in renal proximal tubules in the early stages of diabetes (2001): A study of type 2 diabetes in the Akita mouse model.ExpNephrol;9:380-6.

20. Farouk,H, Kandil D, Kamel S, Elghoroury EA, Elshamaa MF Sabry S, Galal A .(2013): Effect of GSTM1 and GSTT1 deletions in the development of Oxidative stress in children with Chronic Kidney Disease. Journal of Clinical and Basic Cardiology 2013 ;16(1-4),1-5.:

21. Alev E A.; Ilhan Y, et al (2003): Homocystein levels in Type 2 diabetic patients with nephropathy and coronary artery disease. Turkish Journal of Endocrinology and Metabolism 4:159-164.

22. Wang,L. Zhang,Q. Li,2006. Genetic polymorphisms of GSTT1, GSTM1, and NQO1 genes and diabetes mellitus risk in Chinese population . Biochemical and biophysical research communications, 341:310-313.

23. National Institutes of Health . (2001). Incidence and prevalence of ESRD. Excerpts from the United States Renal Data Systems 2000 annual data report ;atlas of end –stage renal disease in the United state . AM. J. Kidn. Dis . 38(3):38-52.

Iraqi Journal of Biotechnology, 2015, Vol. 14, No. 2 , 284-295

Comparison of three methods for detection of *Brucella* infection in some Baghdad hospitals

Inas Saad

Environmental Research Center, , University of Technology. Microbiology and Toxicology Laboratory

Abstract: Diagnosis of brucellosis from patients suspects of infection and their casualty for brucellosis by serological method (Rose Bengal test) and culture method and also used DNA extraction technique in diagnosis of human brucellosis. Blood and serum samples were took from suspected and casualty brucellosis patients (female and male) and obtained from some hospitals in different city for the province of Baghdad (Karkh and Rusafa) and which include: {General hospital Mohammad Baqir Al-Hakim, Al-Shaheed Al-Sadder hospital, hospital of Al-Imam Ali (peace be upon him)}, during the duration from (October 2014 to June 2015).The study was conducted on 117 peripheral blood samples were from patient's suspect of infection and their casualty for brucellosis and which include: 53 (45.2%) female and 64 (54.7%) male. The diagnosis of brucellosis was established on the clinical findings confirmed by serological test (Rose Bengal test), while the isolated colonies confirmed by used Gram staining and different biochemical tests. A total of 117 peripheral blood sample and which include: female and male, the result appeared: 70 (59.82%) samples were positive result by RBT and which comprise: 20 (28.5%) female and 50 (71.4%) male, and also 59 (50.42%) samples were positive result for culture was applied to patient's blood and which comprise: 39 (66.1%) female and 20 (33.8%) male. DNA extraction was carried out using a commercial kit and the results of all patients were positive for DNA extract. These results indicate that patients were used milk or milk product or their contact with infected livestock or suspected infection of *Brucella* like: sheep, goat, cow and buffalo located in epidemiological regions in Baghdad province and showed blood culture method is important for the detection of brucellosis compared with serological method (Rose Bengal test) for the diagnosis of brucellosis.

Key Words: Human, Brucellosis, Patients, Rose Bengal test, Culture, DNA extraction.

مقارنة بين ثلاثة طرق للكشف عن عدوى البروسيلا في بعض مستشفيات بغداد

ايناس سعد

مختبر الأحياء المجهرية والسموم ، مركز بحوث البيئة ، الجامعة التكنولوجية

الخلاصة: الهدف: تشخيص داء البروسيلا في المرضى (أنثى وذكر) المشتبة إصابتهم والمصابين بداء البروسيلا بواسطة الطريقة المصلية (إختبار الروز بنكال) وطريقة الزرع وكذلك تقنية إستخلاص الدنا في تشخيص داء البروسيلا في الأنسان.
مدة ومكان الدراسة: تم الحصول على عينات الدم والمصل من المرضى المشتبه إصابتهم والمصابين بداء البروسيلا (أنثى وذكر) من بعض المستشفيات في مدن مختلفة لمحافظة بغداد (كرخ ورصافة) والتي تشمل: {مستشفى محمد باقر الحكيم العام، مستشفى الشهيد الصدر، ومستشفى الأمام علي (عليه السلام)}، للمدة من شهر تشرين الأول 2014 لغاية حزيران 2015.

طريقة العمل: أجريت الدراسة على 117 عينة دم من المرضى المشتبه إصابتهم والمصابين بداء البروسيلا. والتي تشمل 53 (45.2%) أنثى و 64 (54.2%) ذكر. إعتمد التشخيص على العلامات السريرية المؤكدة بواسطة الأختبار المصلي (إختبار الروزبنكال) ، بينما المستعمرات المعزوله تم تأكيدها بواسطة صبغة كرام ومختلف الأختبارات الكيميائية.
النتائج: من مجموع 117 عينة دم، والتي تشمل: أنثى وذكر، ظهرت النتائج: 70 (59.82%) عينة موجبة لأختبار الروز بنكال، والذي يشمل: 20 (28.5%) أنثى و 50 (71.4%) ذكر، وكذلك 59 (50.42%) عينة موجبة للزرع المستعمله دم المرضى، وتشمل: 39 (66.1%) أنثى و 20 (33.8%) ذكر. إستخلاص الدنا بأستخدام العدة المختبرية، وكانت النتيجة لكل المرضى موجبة لأستخلاص الدنا.
الأستنتاجات: النتائج تشير أن المرضى كانوا يستعملون الحليب أو منتجاته أو اللذين على إتصال بالماشية المصابة أو المشتبه إصابتها بالبروسيلا مثل: الأغنام، الماعز، الأبقار والجاموس الموجودة بالمناطق الوبائية في محافظة بغداد، ولاحظنا إن طريقة زرع الدم مهمة للكشف عن داء البروسيلا مقارنةً مع الطريقة المصلية (إختبار الروز بنكال) في تشخيص داء البروسيلا.

الكلمات الأساسية: الأنسان، داء البروسيلا، المرضى، إختبار الروز بنكال، الزرع، إستخلاص الدنا.

Introduction

Brucellosis is among the most widespread zoonotic infections causing human suffering and economic losses in livestock [1-3].

Brucellosis is a disease of domestic and wild animals that can be transmitted to humans (zoonosis). Brucellosis can be transmitted from animals to humans in many ways: ingestion of infected meat or unpasteurized dairy products; direct contact of broken skin or mucous membrane with infected animal tissues; and inhalation of infectious aerosols. Except for possible cases of transmission after blood transfusion, parenteral drug use and bone marrow transplant [4], brucellosis is still considered not to be transmissible from person to person via close contact [5].

Congenitally infected infants can exhibit low birth weight, failure to thrive, jaundice, hepatomegaly, splenomegaly, respiratory difficulty, and general signs of sepsis (fever, vomiting). Some cases are asymptomatic [6].

Milk and milk products are common sources of infection. The survival of *Brucella* may be prolonged in milk stored at optimal conditions to prevent souring. Milk may also neutralize the gastric acid and in turn protects ingested bacteria in the stomach. The raw milk, clotted cream, and unevenly heated

milk can harbor live *Brucella* organisms [7].

Brucellae are capable of prolonged survival in the environment, so that viable organisms inhaled in dust may be infective. Blood transfusion, bone marrow, transplantation, and possible kidney transplantation are sources of infection. Sexual transmission in semen may occur [8, 9].

Diagnosis of brucellosis based on the clinical picture alone is difficult due to similarity with clinical presentations of other infections [3, 10]. Symptoms and signs are non-specific and several other febrile illnesses, for example glandular fever, influenza, malaria and enteric infections may be simulated [11, 12, 13]. When an unusual complication is present, it may be overlooked [14]. Therefore, laboratory testing is an absolute prerequisite for proper diagnosis through blood culture and isolation of the causative organisms or serological testing [14].

However, culture requires special media, takes several weeks of incubation and has low sensitivity. Serological tests including the serum agglutination test (SAT or STAT), anti-human globulin test (Coombs test), complement fixation test (CFT) and Enzyme-linked Immunosorbent Assay (ELISA), therefore, are indispensable for an accurate diagnosis [10].

The disease exists worldwide, particularly in the Mediterranean basin, the Arabian Peninsula, the Indian subcontinent, and in parts of Mexico, Central, and South America. Consumption of contaminated foods and occupational contact remain the main sources of infection [15].

The aim of the study is to analyze available data and present the frequency and distribution of brucellosis in human at different hospitals in Baghdad for the period from October 2014 to June 2015 especial for patient which suffered from typhoid fever because the disease interferes with brucellosis because of cross-reactions with antigen from other organism especially Salmonella, as well as the patient which suffered from arthritis especially ESR. In addition, the study confirmed the isolation and identification of the *Brucella* species in human blood for both male and female and compared with culture and different biochemical test,

Materials and Methods

A total of 117 peripheral blood specimens were collected from patients with high suspected and casualty of brucellosis which include: 53 (45.2%) females and 64 (54.7%) males referred to General hospital Mohammad Baqir Al-Hakim, Al-Shaheed Al-Sadder, Al-Imam Ali (peace be upon him)

from patient suspected to be with brucellosis before and after adequate antibiotic treatment and from casualty patients for brucellosis, during the period from October 2014 to June 2015.

The diagnosis of brucellosis was established by the presence of a compatible clinical picture [16] including undulant fever, night sweat, arthritis, headache, fatigue, erythrocyte sedimentation rate (ESR), decrease in white blood cells (WBCs) and serological diagnosis was carried by positive Rose Bengal test titer of ≥1:160 and culture method, moreover demographic, occupational, clinical and risk factor details were recorded for each patient and in addition to DNA extraction.

The Statistical Analysis System-SAS [17] was used to know the effect of different factors of study parameters. Chi-square test was used to know the variation among the percentages in this study.

Serological tests - Rose Bengal test (RBT)

The RBT was performed following the procedure described by Alton *et al*. [18]. Briefly, 30 µL of RBT antigen and 30 µL of the test serum were placed on the plate and then mixed thoroughly. The plates were shaken for 4 min and any agglutination that appeared within this time was recorded as a positive reaction.

Traditional test Culture and biochemical test

All media were prepared according to the manufacturing company instructions; *Brucella* agar or Trypticase soy agar were used sterilized by autoclaving at 121ºC for 15 min, after cooling the media to 56 ºC, they were brought to antibiotics with 5% of fetal calf serum for *Brucella* nutrition and mixture with media [19] and put in petri dish. Otherwise the media were incubated at 37 ºC for 24 hours to ensure sterility. On the other hand, they were brought media (Blood agar) and brought Trypticase soy broth were prepared according to the manufacturers company instructions; and then sterilized by autoclaving at 121 ºC for 15 min.

Five milliliters of blood were taken from each patient and divided into identical parts. One part was collected in EDTA and the serum was separated from the second part, was aliquot and store at -20°C until processing. The first part of the blood with anticoagulant was inoculated into: Blood agar, *Brucella* agar, trypticase soya agar and trypticase soya broth culture medium containing both a solid and a liquid phase [20]. Then it was subculture on duplicate agar plates and incubated one in air and the other in an atmosphere at 37°C in the presence of 5-10% CO_2 .After 7-30 days, colonies grown in the solid phase, were identified by

inoculation into *Brucella* agar or trypticase soya agar and taken the growth of colonies by loop and spreaded on the surface of plates containing blood agar media and performance of biochemical tests [21]

DNA extraction from blood sample

Genomic DNA was extracted from blood and colonies grown on solid media (bacterial culture) of *Brucella* spp. using a Wizard Genomic DNA Purification Kit/Promega – company (USA).

The diagnosis of brucellosis was established by clinical findings and used different tests like serological test:- Rose Bengal test and culture and confirmed by Gram stain and different biochemical test and also used DNA extraction.

The main serological test used for diagnosis of brucellosis is the Rose Bengal test (RBT), total of 117 samples, 70 (59.82%) samples were positive RBT which comprise: 20 (28.5%) female and 50 (71.4%) male, and 47 (40.17%) samples were negative RBT, (Table 1).

Results

Table 1: Relation between the different Baghdad hospitals and serum of patients using RBT

**** (P<0.01) = highly significant.**

No.	Hospitals in Baghdad	Number of sample	Positive		Negative		Chi square-χ^2
			Female (%)	Male (%)	Female (%)	Male (%)	
1	Al-Shaheed Al-Saader	11	4 (36.36)	2 (18.18)	2 (18.18)	3 (27.27)	**6.227 ****
2	Al-Imam Ali (peace be upon him)	90	15 (16.66)	45 (50.00)	18 (20.00)	12 (13.33)	**10.648 ****
3	General hospital Mohammad Baqir Al-Hakim	16	1 (6.25)	3 (18.75)	3 (18.75)	9 (56.25)	**9.207 ****
Total	-	117	20 (59.27)	50 (86.93)	23 (56.93)	24 (96.85)	**9.614 ****
**** (P≤0.01).**							

Out of 117 (89%) serum samples were detected by RBT revealed 70 (59.82%) positive, whereas 59 (50.42%) samples were positive using conventional culture method which comprise: 39 (66.1%) female and 20 (33.8%) male. (Figure 1)

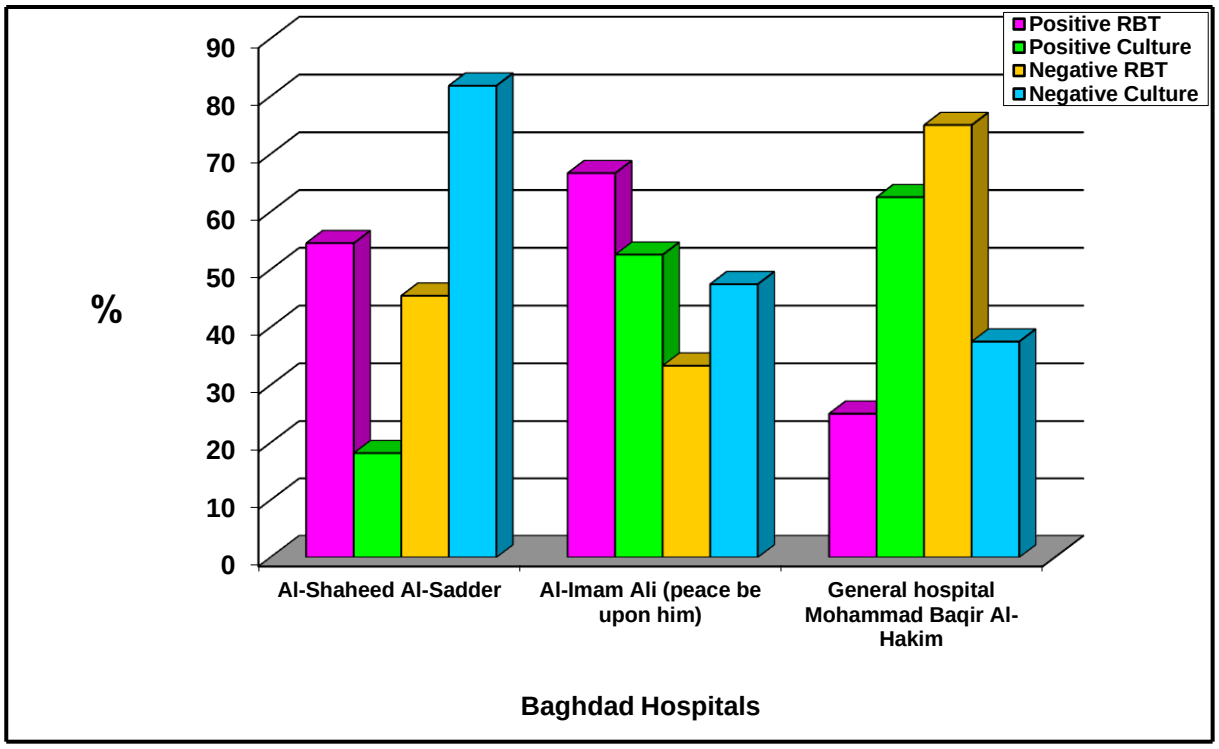


Figure1: The Comparison of *Brucella* antibody titer (RBT) and conventional culture Result.

For 59 patients (50.42 %), the diagnosis of brucellosis was established by isolated the pathogen in blood cultures. (Table 2 and Figure 2, 3).

Table 2: The conventional culture method to *Brucella* result for both female and male.

No.	Hospitals in Baghdad	Number of sample	Positive		Negative		Chi square-χ^2
			Female (%)	Male (%)	Female (%)	Male (%)	
1	Al-Shaheed Al-Saader	11	2 (18.18)	0	5 (45.45)	4 (36.36)	8.327 **
2	Al-Imam Ali (peace be upon him)	90	32 (35.55)	15 (16.66)	25 (27.77)	18 (20.00)	9.681 **
3	General hospital Mohammad Baqir Al-Hakim	16	5 (31.25)	5 (31.25)	4 (25.00)	2 (12.50)	5.048 *
Total	-	117	39 (84.98)	20 (47.91)	34 (98.22)	24 (66.86)	9.593 **

* (P<0.05), ** (P≤0.01).

**** (P<0.01) = highly significant.**

Figure 2: *Brucella* Culture on Blood Agar

The genus characterization were performed using Gram staining and identification by different biochemical tests. (Table 3).

Table 3: Biochemical Characters of *Brucella* Isolates.

No. of Test	Name of Tests	*Brucella* Isolates
1.	Oxidase	+
2.	Catalase	+
3.	Urease test	+
4.	Indole test	+
5.	Motility	-
6.	Production of H_2S	-

+ = Positive, - = Negative.

In present study DNA extracted from 117 human blood samples, 117 (100%) samples were positive results by the Wizard Genomic DNA Purification Kit (Promega – company –USA) was used figure (4).

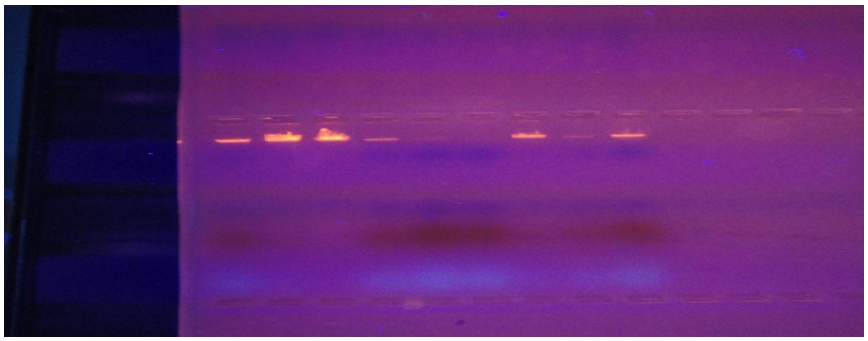


Figure 3: Total DNA Extracted from Human Blood. The seven bands of the *Brucella* Chromosomes.

Separated by Electrophoresis on 0.8% Agarose Gel stained with Ethidium Bromide at 70 volts for 90 min.

Discussion

Brucellosis continues to be a serious public health problem in Iraq, especially in epidemiological region because some people used unpasteurized dairy products like: - milk, cheese, etc. and also people who contact with infected animals. High, endemic level ever since. Brucellosis has a worldwide distribution with different rate of focal disease involving any organ. Clinical manifestations and severity of symptoms may vary depending on geographic areas with respect to pathogenic species of *Brucella* and the host.

Although the definite diagnosis of brucellosis requires the isolation of the organism from blood or other body fluids, since *Brucella* are slow growing organisms and require special culture conditions [22] owing to
the delay in the isolation, serologicalmethods serological methods are required for a rapid diagnosis. The antibodies detected by serological testing are directed against the lipopolysaccharide of the bacterial cell wall [23].

However, although the presence of *Brucella* in both vaginal secretions [24] and semen [25] has been described, sexual transmission of *Brucella* species has rarely been reported in humans. Moreover, in all previous case reports, definite proof for the existence of *Brucella* species in the semen has been lacking.

Statistical analysis showed that the 70 (59.82%) patients revealed positive result by RBT, which comprise: 20 (28.5%) female and 50 (71.4%) male and 47 (40.17%) patients negative result for RBT out of 117 patients.

In this study occurs in the epidemiological region and patient who suffer from different disease and related with brucellosis. The prevalence found in children, men, women and also pregnant women RBT of < 1/160 is problematic in areas of endemicity in patients suffering joint pain and an increase in Erythrocyte Sedimentation Rate (ESR) and also for presence of appropriate signs and symptoms can also include these clinical signs: headache, weakness, arthralgia, depression, weight loss, fatigue, and liver dysfunction., a presumptive diagnosis of brucellosis is usually defined serologically as a RBT titer of 1/160 or greater [26]. It is crucial to be able to differentiate *Brucella* from *Salmonella*, which could also be isolated from blood cultures. Hence statistic showed that seropositive of brucellosis by RBT 70 (59.82%) and it is increased comprised with culture, thus 59 (50.42%) samples reported that culture was positive, which comprise: 39 (66.1%) female and 20 (33.8%) male. The explanation for

the low yield of conventional culture in present study appears patient's used of different antibiotic treatments for various diagnostic suspicions in the other clinical sector, before samples are taken from hospitals and health centers, than to the technical difficulty of isolation *Brucella* spp. from clinical samples.

In the laboratory, biochemical tests can be diagnostic [27]. Oxidase, catalase, urease, indole, production H_2S tests and motility are positive for most members of the genus *Brucella*.

Although most investigators prefer using commercial kits for extraction of *Brucella* DNA [28-30). We used a laboratory extraction procedure according to Wizard Genomic DNA Purification Kit / Promega – company –USA. Using comparable methods *Brucella* DNA was successfully extracted from the cultures by [31-34] and from whole blood for human.

As well as the statistical analyses in this study are aware that the incidence in certain cities of Baghdad, more than others and also in certain province of Iraq more than others.

Considering the difficulties mentioned above, it is clear that the association of direct and indirect laboratorial tests with clinical and epidemiological data is essential to perform a definitive diagnosis of brucellosis.

References

1. Ayyildiz, A. 2004. Survey of Brucellosis Seroprevalence in Risk Groups in Erzurum, Turkey. Prague/ Czech Republic: 14th European Congress of Clinical Microbiology and Infectious Diseases.
2. Nakavuma, J.K.S. and Opuda-Asibo, J. 1999. Serostudy of *Brucella abortus* in Cattle and Goat in Central and Southern Uganda. Uganda Agric Sc, 4:13–18.
3. WHO. 2006. Zoonoses and Food Safety, in World Health Organization. Geneva, Switzerland.
4. Naparstek, E., Block, C.S. and Slavin, S.1982. Transmission of Brucellosis by Bone Marrow Transplantation. Lancet. I:574– 5.
5. Peter, G.editor. 1991. Report of the Committee on Infectious Diseases. 23 rd edn. Elk Grove: American Academy of Pediatrics. Brucellosis; pp. 143–4.
6. Poester, F. and Nielsen, K. 2010. "Diagnosis of Brucellosis". The Open Veterinary Science Journal, 4, 46-60.
7. Wright, S. G. 2001. "Brucellosis," in *Manson's Tropical Diseases*, G.Cook, Ed., pp. 886 – 891, Harcourt (India) Private Limited, New Delhi, India, 20th edition.

8. Mantur, B. G., Mangalgi, S. S. and Mulimani, M. 1996 . "*Brucella melitensis* - a Sexual Transmissible Agent?" *Lancet*, vol. 347, no. 9017, p. 1763.

9. Wright, S. G. 2000. "Brucellosis," in Hunter's Tropical Medicine and Emerging Infect Diseases, G. T. Strickland, Ed., pp. 416 – 420, WB Saunders, Philadelphia, Pa, USA, 8th edition.

10. Henk, L.S. and Cutler, S.J. 2004. Contributio Biotechnology to the Control and Prevention of Brucellosis in Africa. Afr J Biotechnol, 3(12):631–636.

11. Jennings, G.J., Rana, A.H., Fouad, Y.G., Moustafa, A.F., Mohamed, A.M., Momtaz, O.W., Nasr El-Sayed, Padmini,S., Stephen, P.L., Kenneth, E. and Francis, J.M. 2007. Brucellosis as a Cause of Acute Febrile Illness in Egypt. Trans R Soc Trop Med Hyg, 101:707–713.

12. Mukhtar, F. and Kokab, F. 2008. *Brucella* Serology in Abattoir Workers. J Ayub Med Coll Abbottabad , 20 (3):57–61.

13. Badiaga, S., Imbert, G., la Scola, B., Jean, P., Delmont, J. and Brouqui, P. 2005. Imported Brucellosis Associated with Plasmodium falciparum malaria in a traveler returning from the tropics. *J Travel Med*. 12(5) 282– 284.

14. Afifi, S., Ken, E., H.El-Sakka, Momtaz, W., Fouad, G., Hoda, G., M.Adel Azab, Frank, M. 2005. Hospital-based surveillance for acute febrile illness in Egypt: a focus community- acquired bloodstream infections. *Am J Trop Med Hyg*,73 (2):392–399.

15. Mathur, T. N. 1954. "Chronic Brucellosis *Indian Medical Gazette*, vol. 89, pp. 288–294.

16. World Health Organization. (1998). Surveillance of Communicable Diseases: a Traninig Manual. Geneva: Regional Office for the Eastern Mediterranean. WHO-EMm /CDS /52 /E /L.

17. SAS. 2012. Statistical Analysis System, User's Guide. Statistical. Version 9.1th ed. SAS. Inst. Inc. Cary. N. C. USA.

18. Alton, G.G., Jones, L.M., Angus, R.D. and Verger, J.M. (1988). Techniques for the Brucellosis Laboratory. INRA, Paris. Ch. 2 p.114.

19. Corbel, M.J., Gill, K.P.W. and Thomas, E.L. (1978). Methods for Identification of *Brucella*, Ministry of Agriculture, Fisheries and food. RVc 22, published.

20. Yagupsky, P. (1999). Detection of *Brucella* in Blood Cultures. J. Clin. Microbiol .37: 3437-42.

21. Bricker, B.J. and Halling, S.M. (1994). Differentiation of *Brucella abortus* bv. 1, 2, and 4, *Brucella melitensis*, *Brucella ovis* and *Brucella suis* bv. 1 by PCR. J. Clin Microbiol. 32:2660-26.

22. Pascual, E. 1993. "Brucellar Arthritis," in *Oxford Textbook of Rheumatology*, P. I. Maddison,D. A. Isenberg, P. Woo, and D.N.Glass, Eds., pp. 581–589, Oxford University Press, Oxford UK.

23. Diaz, R., Jones, L.M., Leong, D. and Wilson J.B. 1968. "Surface Antigens of Smooth *Brucellae*," *Journa of Bacteriology*, vol. 96, no. 4, pp. 893–902.

24. Young, E.J., Corbel, M.J. and Williams E. 1989. Chapter 2—The Mediterannean Fever Commission-its origins and achievements. In: Young EJ, Corbel, MJ, editors. Brucellosis - clinical and laboratories aspects. Boca Rat Raton: FL: CRC Press. P. 11-24.

25. Vandercam. B., Zech, F., de Cooman, S., Bughin, C., Gigi, J. and Wauters, G. 1990. Isolation of *Brucella melitensis* from human sperm. Eur J Clin Microbiol Infect Dis; 9:303-304.

26. Munoz, P.M., Marin, C.M., Monreal, D., Gonzalez, D., Garin- Bastuji, B., Diaz, Mainar-Jaime, R.C., Moriyon, I. and Blasco, J.M. (2005). Efficacy of Several Serological Tests and Antigens for Diagnosis of Bovine Brucellosis in the Presence of False-Positive Serological results due to Yersinia enterocolitica O: 9. *Clin*. Diagn. Lab. Immunol; 12: 141-151.

27. Hofer,E.2009."Microbiological diagnosis of *Brucella* spp. and Austrian Epidemiology Brucellosis (*B. suis* biovar 2)", Institute for Veterinary Disease Control, Mödling.

28. Nimir, L.F. (2003) Diagnosis of Recent and Relapsed Cases of Human Brucellosis by PCR assays. BMC Infect Dis 3:5. 29.

29. Navarro, E., Escribano, J., Fernandez, J.A., and Solera, J. (1999). PCR of Human assay forDiagnosis Brucellosis. J.Clin Microbiol 37 (5): 1654-1655.

30. Morata, P., Queipo- Ortuno, M.I., Reguera, J.M.,Garcia-Ordonez, M., Cardenas, A. and Colmenero, J. (2003). Development and Evaluation of a PCR- Enzyme Linked Immunosorbent Assay for Diagnosis of Human Brucellosis. J. Clin. Micro. Vol. 41, No. 1p: 144-148.

31. Leal-Klevezas, D.S., Martinez-Vazquez, I.O. and Martinez-Soriano, J.P. (1995). Single-Step PCR for Detection of *Brucella* spp. from Blood and Animals of Infection Animals. J. Clin. Microbiol. 33: 3087-3090.

32. Romero, C., Pardo, M., Grillo, M. J., Diaz, R., Blasco, J.M. and Lopez- Goni, I. (1995). Evaluation of PCR and Indirect Enzyme-linked Immunosorbent Assay on Milk Samples for Diagnosis of Brucellosis in Dairy Cattle. J. Clin. Microbiol. 33:3198-3200.

33. Navarro, E., Escribano, J., Fernandez, J.A. and Solera. (2002). Comparison of three Different PCR Methods for Detection of *Brucella* spp. in Human Blood sample. FEMS Immunology and Medical Microbiology 34:147-151.

34. Kanani, A.N. (2007). Srological Culture and Molecular Detection of *Brucella* Infection in Breeding Bulls. Ph. D. Thesis. College of Vet. Med. Anands agricultural University Gujarat, India.

Iraqi Journal of Biotechnology, 2015, Vol. 14, No. 2 , 296-311

Association of the HLA-DRB1*0301, HLA-DRB1*0304 and HLA-DQA1*0502 alleles with thyrotoxicosis in Thi – Qar city population

Majida Ghazi. M.[1] , Jaber Hameed [2] , Alia Eesam [3]

[1]Biology department /college of science, Thi-Qar University

[2]Department of clinical laboratory sciences ,college of pharmacy, Al-Mustansiria University.
[3]Biology department /college of science, Al-Mustansiria University

Abstract: Thyroid is gland composed of two encapsulated lobes, located on either side of the trachea in the front of neck, releases hormones triiodothyronine (T3) and thyroxine (T4) are active hormones that control metabolism secreted under the control of (Thyroid Stimulation Hormone) TSH from adenohypophysis of pituitary gland.

Associations between thyroid disorders and antigens of the major Histocompatibility complex (MHC) have long been recognized especially Human leukocyte antigen (HLA) HLA-DQ and HLA- DR .

This study was carried out to estimate immunogenetic parameters of some Iraqi patients in Thi-Qar city with thyrotoxicosis.

It consisted 72 patients 87.5% of them females and 12.5% were males who have been referred to the endocrine and diabetic Medical center in Nassiryhia and 10 healthy control during the period from May 2014 till September 2014.The age of patients and healthy individuals ranged between (23-50) years. The HLA-class II typing was conducted for groups patients and healthy group by using molecular methods Polymerase chain reaction-sequences specific primers (PCR- SSP)for HLA-Typing in college of veterinary medicine / university of al-qadisiya and the results showed that a high frequencies of HLA-DQA1*0502 have 77.14% $_{vs0}$%,OR = 61.8649,RR =16.4247) ,and HLA- DRB1 0301 RR= 5.575,OR =7.1284 and have 25 % $_{vs\ 0}$ % among patients as compared to control group. as well as the following allele DRB1*0304 was presented with frequencies 18% $_{vs\ 0}$ % ,OR=4.7647 ,RR=4.068 in patients when compared with control group. The results showed that anti-TPO, was the most associated with HLA-Typing HLA-DQA1*0501, DRB1*0304, DRB1*0301 (28,7,6) Respectively positive result (RR :1.37, 2.72 , 1.70) Respectively in patients.

Keywords: Thyroid disorder , HLA-Typing, genetic Thyrotoxicosis.

الارتباط بين الاليلات لمستضد الكرية اليبضاء البشري (HLA-DQA1*0502 DRB1*0301, HLA-DRB1*0304 ومرض افراط الغدة الدرقية في محافظة ذي قار

ماجدة غازي مكطوف [1]، جابر حميد حسن[2] ، عالية عصام [3]

[1]كلية العلوم \علوم الحياة\ جامعة ذي قار
[2]كلية الصيدلة \التحاليل السريرية\الجامعة المستنصرية
[3]كلية العلوم \علوم الحياة \الجامعة المستنصرية

الخلاصة : الغدة الدرقية غده تتكون من فصين محاط بكبسولة تقع أمام القصبة الهوائية تحت ألرغامي تفرز هرمونا T3,T4وبسيطرة من الغدة النخامية بواسطة هرمون TSH.

يعد مستضد الكريه البيضاء البشري (HLA) من العوامل الوراثية المهمة في بدء أو تنظيم الاستجابة المناعية.حيث ان وجود هذه المستضدات DQ، DR يساهم في دراسة مستوى الإصابة او الاستعداد للإصابة بالمرض.

جاءت هذه الدراسة لتقييم بعض المؤشرات المناعية الوراثية لدى عينة من المرضى المصابين في مدينة ذي قار. وأجريت ل 72 مريض87,5% منهم إناثا و12,5% ذكورا يعانون من إفراط إفراز الغدة الدرقية من الذين تم فحصهم في مركز الطبي للغدد الصم والسكري في مدينة الناصريه و 10 أشخاص أصحاء تم اتخاذهم كمجموعة سيطرة وذلك للفترة من ايار2014 إلى أيلول 2014 تراوحت أعمار المرضى والأصحاء من (23- 50) سنة .

وقد اشتملت الدراسة الجزيئية استخدام تقنيةPCR-SSP لتنميط مستضد الكريه البيضاء البشري للصنف الثاني (Class-II-HLA-DRB1 , -DQA1) في المرضى و مجموعة السيطرة في كلية الطب البيطري – جامعة القادسية . وأظهرت النتائج بأن النسب المئوية لتكرار الاليلات هي 77.14% و 25% و 18% للمستضدات HLA- DRB1 0301 , DQA1*0502 . DRB1*0304 على التوالي لدى المرضى مقارنة بمجموعة السيطرة0% ، فضلا عن وجود علاقة خطورة بين الاليلات قيد الدراسةو TPO الضد المناعي الذاتي HLA-DQA1*0501, DRB1*0304, DRB1*0301 بالتعاقب (RR::1.37, 2.72, 1.70).

Introduction:

The thyroid gland is an organ composed of two encapsulated lobes located in the front of neck and releases hormones that control metabolism, breathing, heart rate, nervous system, weight, body temperature, and many other functions in the body.

Many studies were reported that the majority of thyroid disorders are common in adults age between 30 to 50 years old and the incidence was in females more than males (1; 2) and that confirmed by previous local studies in Iraq which performed by Al- ramahi in2011 and Jwaid in2011 which found that the thyroid disorders are more common in women than men(3,4) .This diseases are very common types of endocrine disorder, thyroid disease involve two types either the decreased production of thyroid hormone (TH) (hypothyroidism), or conversely a rise in the production of TH (hyperthyroidism) (5). The most common forms of hyperthyroidism include diffuse toxic goiter (Graves disease), toxic multinodular goiter and toxic adenoma.

Graves disease is the most common form of hyperthyroidism in

the United States, causing approximately 60-80% of cases of thyrotoxicosis (6) .

The Major Histocompatibility Complex (HLA) is the most polymorphic genetic system in the human being, with numerous alleles contains more than 200 genes which are situated on the short arm of chromosome 6 at 6p21.3 spans a DNA segment of about 4000 kbp, These genes are the most polymorphic genes in vertebrates (7).

Class II molecules are glycoproteins encoded by the MHC Class II genes, are found only on a few specialized cell types, including macrophages, dendritic cells and B cells, all of which are professional APC_S. the spectrum of HLA molecules is much more restricted. There are between 50 and 100 different DR molecules, and a much smaller number of DQ and DP molecules, all coded on Chromosome 6, in the human genome . The amino acid sequences of the DR molecule determine the shape of the antigen presenting cleft, and peptides formed from protein antigens fit into the cleft with greater or lesser affinity (8).

An association between HLA-Ags and a GD disease can be studied in population or in families , and the first study in Iraq it by AL-Zubaidi in1974 .

Aims of this study was to measure the association between certain HLA-DR and HLA-DQ alleles, and incidence of Thyrotoxicosis in Thi-qar population.

Material and method:

The study patients consisted of 72 patient and 10 healthy control with age (23-50) year, during the period from May 2014 till September 2014.

5 ml of blood collected from each patient or control , 3 ml was left to clot for 1-2 hour at room temperature and then centrifuged at 3000 rpm for 10 minutes, ,serum was stored at – 20C until was used .Tested for the determination of thyroid hormone levels: (T3, T4, TSH) using a miniVidas system. (Biomerieux ,France). Thyroid anti-TSH receptor immunoglobulin (anti-TSH), anti-thyroperoxidase (anti-TPO) and anti-thyroglobulin (anti-TG) antibodies were measured in all the samples using electrochemiluminescent"ECL" with Modular Analytics cobas e 411 analyzer (Roche Diagnostics, England). in the endocrine and diabetic Medical center in Nassiryhia city.

The remaining 2ml of blood were transferred into EDTA tube remaining kept at -20 Cº for the genotyping of DRB and DQA

antigens .Using ***Polymerase Chain Reaction –sequence specific primers (PCR-SSP)*** system provided from Mygen / Bioneer Korea **.**The test procedure was done by using SSP(9). This method was conducted in college of veterinary medicine / university of al-qadisiya and based on the fact that primer extension and hence successful PCR relies on an exact match at the 3-end of both primers .Therefore ,only if the primers entirely match the target sequence the amplification obtained which is subsequently visualized by agarose gel electrophoresis . The composition of individual primer mixtures makes clear of identification of the HLA types indicate in the respective evolution diagrams.

MHC HLA alleles specific primers that used in this study were design by using HLA-alleles specific sequence from NCBI-GenBank database and Batch primer design online, where provided from Bioneer company, Korea as following table:

Primer	Sequence	size
DRB1*0301	CAGCCTTGATGTAAGGCACA ATCCAGGCAGCATTGAAGTC	698bp
DRB1*0301 allele	CATTCCACTGTGAGAGGGC AGCACCCAAGCGTGACA	
DQA1*0502	CCAGTCTTACGGTCCCTCTG AAGAGCCCTCCTACCAGCAT	554bp
DQA1*0502 allele	CCCTTCATACCAAGTTTCATTATTTTA ATCTGGGGACCTCTTGGAA	
DRB1*0304	AACCCCGTAGTTGTGTCTGC GATCCTCCTCCAGCTCCTG	531bp
DRB1*0304 allele	AGAAACGTGCTGCGGG TGACCGGATCCTTCGTGT	

PCR Thermocycler conditions

PCR step	Temp.	Time	repeat
Initial Denaturation	**95C**	**5min**	**1**
Denaturation	**95C**	**30sec.**	**30 cycle**
Annealing	**58C**	**30sec**	
Extension	**72C**	**45sec**	
Final extension	**72C**	**7min**	**1**
Hold	**4C**	**Forever**	**-**

Statistical analysis:

Odds ratio (OR) was used assessed by a special X^2 formula. The relative risk(RR) is the real measure of association between exposure to a certain factor and having the disease .

Result:

This PCR-SSP method was used for the determination of individual HLA-DRB1*0301 , HLA-DRB1*0304 , HLA-DQA1*0502 alleles in patients with thyrotoxicosis as well as control. Successful amplification was resulted in the generation of a DNA fragment of defined length as a positive internal control band in a lanes except the negative control lane , when there was no amplification the band was missed, a positive specific amplification was resulted in the generation of a positive specific amplification band in addition to an internal positive control band .

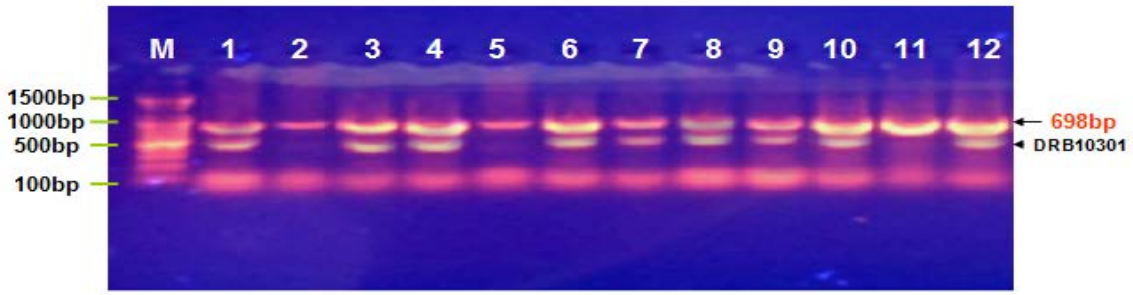


Figure (1): Agrose gel electrophoresis image that show the SSP-PCR results for HLA-DRB10301 allele. Where M: Marker (1500-100bp), Lane (1,3,4,6,7,8,9,10,& 12) some positive HLA-DRB10301 allele samples and Lane (2,5,&11) some negative HLA-DRB10301 allele, whereas, all samples lanes (1-12) were show internal positive control 698bp SSP-PCR product bands.

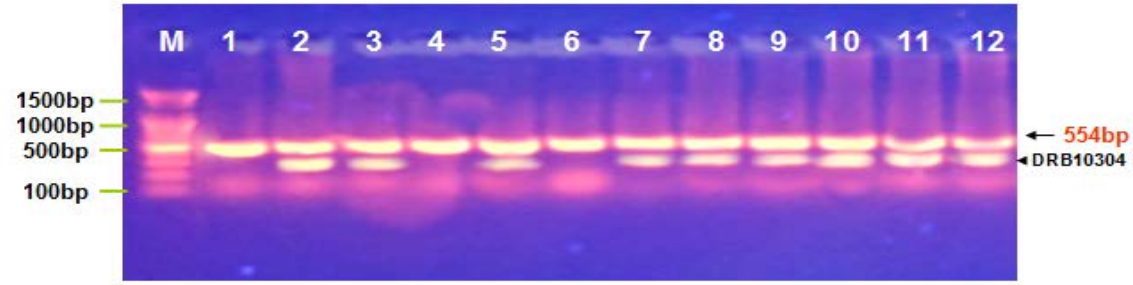


Figure (2): Agrose gel electrophoresis image that show the SSP-PCR results for HLA-DRB10304 allele. Where M: Marker (1500-100bp), Lane (2,3,5,7,8,9,10,11& 12) some positive HLA- DRB10304 allele samples and Lane (1,4&6) some negative HLA- DRB10304 allele, whereas, all samples lanes (1-12) were show internal positive control 554bp SSP-PCR product bands.

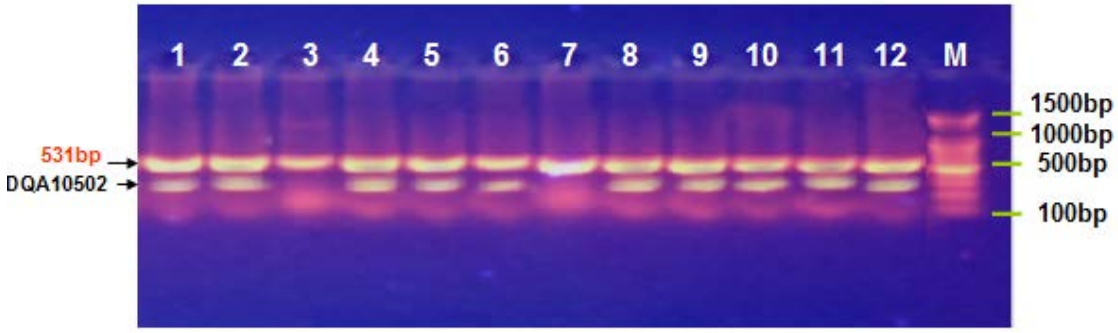


Figure (3): Agrose gel electrophoresis image that show the SSP-PCR results for HLA-DQA10502 allele. Where M: Marker (1500-100bp), Lane (1,2,4,5,6,8,9,10,11& 12) some positive HLA-DQA10502 allele samples and Lane (3&7) some negative HLA-DQA10502 allele, whereas, all samples lanes (1-12) were show internal positive control 531bp SSP-PCR product bands.

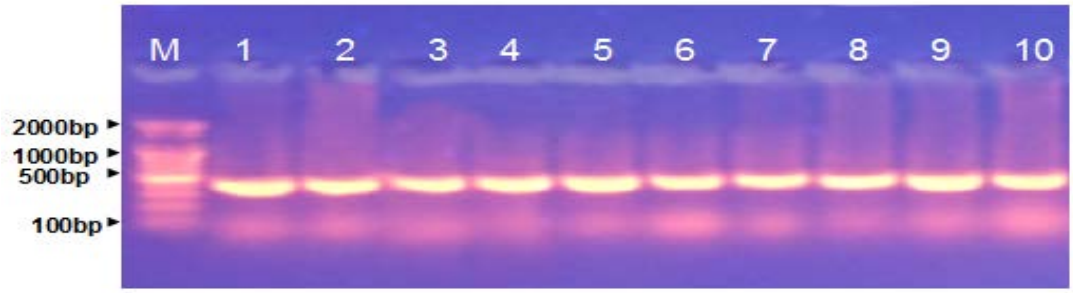


Figure: (4) Agrose gel electrophoresis image that show the SSP-PCR results for control samples. Where M: Marker (2000-100bp), Lane (1-10) some negative HLA allele that show internal positive control 554bp SSP-PCR product bands.

Table (1):The relative risk and odds ratio of positive HLA- DQA1*0502Genotype among patients group

DQA1*0502	Patient %	Control	P value
Positive	54 **77.14%**	0	
negative	18	10	
Total	72	10	
RR	16.4247		0.0431
OR	61.8649		0.0051

The distribution of HLA-DQA1***0502 77.14 %** is shown in Table(1). which has elevated (OR) equal to (61.86) but it did not reveal high significance .The risk factor (16.4247) not significance .

Table (2): The relative risk and odds ratio of positive HLA- DRB1* 0301 genotype among patients group.

DRB1* 0301	Patient %	Control	P value
Positive	18 **25%**	0	
negative	54	10	
Total	72	10	
RR	5.575		0.218
OR	7.1284		0.1822

The distribution of HLA-DRB1***0301 25 %** is shown in Table(2). which has (OR) equal to (7.12) but it did not reveal significance .The risk factor (5.57) and not significance .

Table (3): The relative risk and odds ratio of positive HLA -DRB1*0304 genotype among patients group.

DRB1*0304	Patient %	Control	P value
Positive	13 **18%**	0	
negative	59	10	
Total	72	10	
RR	4.068		0.3173
OR	4.7647		0.29

The distribution of HLA-DRB1***0304 18 %** is shown in Table(3). which has (OR) equal to (4.76) but it did not reveal significance .The risk factor (4.06) and not significance .

Comparison of HLA –Allelic frequency in patients with different autoantibodies .

Table (4) :The relative risk and odds ratio of positive HLA DQA1*0502Genotype among patients group with positive outoantibodis

Anti-TPO	**Patient**	**Control**	**P value**
Positive	28	0	
negative	26	10	
Total	54	10	
RR	1.3722		0.0029
OR	22.5849		0.0342
Anti-TG	**Patient group**	**Control group**	**P value**
Positive	21	0	
negative	33	10	
Total	54	10	
RR	1.2836		0.0058
OR	13.477		0.0776
Anti-TSH	**Patient**	**Control**	**P value**
Positive	26	0	
negative	28	10	
Total	54	10	
RR	1.3431		0.0034
OR	19.5263		0.0436

The result showed that auto anti-body TPO more than others auto anti-bodies OR and RR (22.584,1.37) respectively table(4) .

Table (5): The relative risk and odds ratio of positive HLA DRB1*0301Genotype among patients group with positive outoantibodies

Anti-TPO	Patient	Control	P value
Positive	6	0	
negative	12	10	
Total	18	10	
RR	1.7086		0.0140
OR	10.92		0.1172
Anti-TG	**Patient**	**Control**	**P value**
Positive	4	0	
negative	14	10	
Total	18	10	
RR	1.5517		0.0521
OR	6.5172		0.225
Anti-TSH	**Patient**	**Control**	**Pvalue**
Positive	5	0	
negative	13	10	
Total	18	10	
RR	1.6296		0.0251
OR	8.5556		0.1615

The result showed that auto anti-body TPO more than others auto anti-bodies OR and RR (10.92 ,1.708) respectively table (5) .

Table (6): The relative risk and odds ratio of positive HLA DRB1*0304Genotype among patients group with positive outoAB outoantibodis

Anti-TPO	Patient	Control	P value
Positive	7	0	
negative	5	10	
Total	13	10	
RR	2.7273		0.0050
OR	28.636		0.0307
Anti-TG	**Patient**	**Control**	**P value**
Positive	3	0	
negative	10	10	
Total	13	10	
RR	1.7500		0.0526
OR	7		0.2162
Anti-TSH	**Patient**	**Control**	**P value**
Positive	4	0	
negative	9	10	
Total	13	10	
RR	1.8947		0.0217
OR	9.9474		0.14

The result showed that auto anti-body TPO more than others auto anti-bodies OR and RR (28.636, 2.727) respectively table (6).

In all results of tables showed that anti-TPO, was the most associated with HLA – typing HLA-DQA1*0502, DRB1*0304, DRB1*0301 (28,7,6) Respectively . OD : **(28.636 ,22.5849 , 10.92**) DRB1*0304, HLA-DQA1*0502, DRB1*0301 Respectively in patients. And RR:(**1.3722** , **1.7086** , **2.7273 significantly)** DRB1*0304, DRB1*0304 ,DQA1*0502 Respectively in patients .

(The RR of > 1 means the event is more likely to occur in the experimental group than in the control group). That is mean ,these alleles had the strongest effect in

the development of this antibody in susceptible person. **Tables (4,5,6)**.

Discussion :

HLA genes have been known for a long time and the whole of the HLA complex has been sequenced , but the reasons for doing was to identify disease-specific susceptibility (risk) and protective markers that can be used in immunogenetic profiling ,and risk assessments .

The results of different works show few reproducible results because there are important differences in the expression of the different HLAs, depending on the geographical area to which reference is made (10). This is due to that the frequency of presentation of the different HLA alleles which determined by the dominant pathogens of each geographic region in particular, and because these genes are highly polymorphic.

Characterized autoimmune thyroid disease (AITD) has demonstrated clear differences in association within the HLA class II region, differences in HLA class II genotype may, in part, contribute to the different immunopathological processes and clinical presentation of these related diseases.(11)

However , associations of HLA class II (DRB1*1602-DQA1*0102-DQB1*0502) might serve as a marker for genetic susceptibility to GD in Asian population (12) . In other study provide among the class II genes, HLA-DRB1*0301 (DR3) has long been well recognized as a susceptibility gene between Caucasians population,(13 , 14). Important development in this field especially in Caucasians population with (15 ; 16 ; 17 ; 18) the results of these studies provide association between thyrotoxicosis and certain high risk (HLA-DQA1*0501 genotypes and DRB1,DR3)

In our study HLA -DQA1 (DQA1*0502) was the most frequent in thyrotoxicosis patients (77.14% vs.0% in healthy subjects, OR :(61.864), the RR:16.424 same probability continue after adjustment, that is mean ,these alleles had the strongest effect in the development of the disease in susceptible person in our area .The second alleles that ,had mid frequency ,was DRB1(DRB1*0301), 25% VS.0% in healthy subjects OR:7.1284 and RR:5.57 .

In contrast ,the ferquency rate of DBR1*0304)alleles was decreased in patients,13% VS. 0% in healthy subjects OR: 4.7647 with RR:4.06 .

The current work compared with abroad studies ,the highest risk. In Brazilian patients are HLA-DRB1*0301 allele and HLA-DQA1*0501 allele susceptibility conferred by two alleles was independent (19) It is well established that in HLA-class II

region were associated with various degree of predisposition and protection for GD, and conclude, that HLA-DQA1*0501 are strongly associated with Graves disease in Spain and Germany populations. (20) . Philippou *et al* ., suggest that HLA DQA1*0501 and DRB1*0301 antigens are not independent risk factors for the development of Graves' disease. but, the presence of both these alleles results in a significant increase in the RR for the development of Graves' disease in the Greek population, particularly in females.(21), other proposed that DQA1*0501 was associated with GD independently of the DR3 haplotype as a whole. (22) DRB1*08 showed by Chen et al.,associated with GD in a North American Caucasian cohort (OR=3.2) (23).

And then expanded upon in a study by (24) Saied the main etiological variant within the HLA class II region was contained in DRB1 or DQA1 further confirmed the association of the DRB1*03 allele with GD not only an association of a single copy of DRB1*03 (OR 1.32 ; 95%) but that individuals homozygous for DRB1*03 showed even stronger association (OR 3.49; 95%.). Simmonds et al again showed ssociation of rs2076530 to be secondary to the previously established DRB1 exon 2 encoded position beta74 effect although a rare haplotype effect, including both loci, and added the importance of an amino acid substitution at position 74 of the DR beta 1 chain of HLA-DR3 (DRB1-Arg74), in susceptibility to Graves disease (25,26). Mechanistically, the presence of an arginine at position 74 elicits a significant structural change in the peptide binding pocket of HLA-DR, and attractive mechanism is by which amino acid variants in Tg could predispose to AITD is by altering Tg peptide presentation by APC’s to T-cells within HLA class II molecules (27), there exist an interaction between Tg variants and HLA-DR variants predisposing to AITD. Sawai and DeGroot (2000) refer to DR molecules which best fit epitopes derived from the TSH receptor, are most effective in presenting the epitope to the T cell to induce immunity, they found that TSH-R epitopes with aspartic or glutamic acid in positions 71 and 74 in the DR sequence bind more strongly to DRB1*0301 are more stimulatory to GD patients, (28).

the main etiological variant within the HLA class II region was contained in DRB1 or DQA1 may lead to an accelerated replicative senescence of CD4+ T cells and granulocytes resulting in a decrease in cell turnover rates. This change in the nature of the T cell population may reduce the efficacy of the immune system in response to

antigenic stimuli. It may be that the T cells entering senescence are unable to prevent autoreactive cells from expanding. Alternatively may be the senescent T cells, which are less responsive to controlling stimuli, attack the hosts own cells (29 ,30 ,31).

In Iraq, radioactive contamination was and still an environmental pollution problem since its levels raised after both Gulf wars I and II, (32). Therefore, determine individual predispositions to the development of certain pathological processes under the influence of hazardous factors, this was based on the detection of individual genetic markers, the great number of associations of the HLA-antigens with various diseases has recently been estimated and documented .(33)

Conclusions:

The frequency of HLA-DQA1*0502 allele was higher than other HLA- alleles in patients, this may indicate the presence of at least one necessary genetic factor for susceptibility to disease , and indicator for risk factor.

Reference:

1. Nordyke R. A.; Gilbert, F. I. And Harada, A.s. (1988). Graves disease: influence of age on clinical findings. Arch. Intern. Med., 148: 626–631.
2. Gabriel B.; Thomas, A.; Kent, F. ; Talavera, M. (2009) . Thyroid Disease. Staff Neurologist, Neurology Service, Philadelphia Veterans. Affairs. Medical Center. Me Rep., 8(1): 16-19.
3. AL- Ramahi , I. J. (2011). Molecular and Genetic Study in samples of Iraqi patients with Thyroid Disorders.Ph.D. Thesis. Genetic Engineering and Biotechnology Institute for Postgraduate Studies. University of Baghdad.
4. Jwaid,S.M., (2011).Detection of some hormonal and immunological markers related in infertile female with thyroid disease .M.Sc thesis. College of Health and Medical Technology Foundation of technical education.
5. Longmore, M. (2007). Chapter 6 in Oxford Handbook of Clinical Medicine. 7th Edition. Oxford: Oxford University Press.; 200-205.
6. Davies TF and Larsen PR. (2003)Thyrotoxicosis. Williams Textbook of Endocrinology. 10thed. Philadelphia: Saunders; 374-421.
7. Eizaguirre, C.; Lenz, L.; Martin,K.; and Milinski,M . (2012). Rapid and adaptive evolution of MHC genes under parasite selection in experimental vertebrate populations. Nature communications, 3:621 :1-7.

8. Trowsdale J; Ragoussis J; Campbell RD ; (1993) The map of the human MHC is a compilation of physical mapping and cloning data from a number of laboratories. Immunol Today 14:349-352,

9. Olerup O and Zetterquist H.(1992) HLA-DR typing by PCR amplification with sequence specific primers (PCR-SSP) in 2 houres : an alternative to serological DR typing in clinical practice including donor – recipient matching in cadaveric transplantation Tissue Antigens,;39(5):225-35 .

10. Lavado , R.; Benavides, M.; Villar, E.; Ales, I.; Alonso, A and Caballero, A. (2005). The HLA-B7 allele confers susceptibility to breast cancer in Spanish women Immunol Lett, 101:223-5.

11. Jang H .;Shin H.;Cho H .; Kim H.;Lee J.,Kim S.; KimJ.; ChungJ.. (2011)Identification of HLA-DRB1 alleles associated with Graves' disease in Koreans by sequence-based typing. Immunol. Invest. 40 (2): 172-82.

12. Wongsurawat T.; Nakkuntod J; Charoenwongse P; Snabboon T; Sridama V; Hirankarn N.(2006) The association between HLA class II haplotype with Graves' disease in Thai population.Tissue Antigens. 67(1):79-83.

13. Flynn JC, Rao PV; Gora M; Alsharabi G; Wei W; Giraldo AA; David CS;BangaJP; Kong YM. (2004) Graves' hyperthyroidism and thyroiditis in HLA-DRB1*0301 (DR3) transgenic mice after immunization with thyrotropin receptor DNA.Clin Exp Immunol. 135(1):35-40.

14. Messaaoui M.,Sylvie Messaaoui, M.;Sylvie T.; Bart ,V.Christian M. and HarryD.(2012).Autoimmune Thyroid, Celiac and Addison's Diseases Related toHLA-DQ Types in Young Patients with Type 1 Diabetes in Belgium Open Journal of Endocrine and Metabolic Diseases.2, 70-73.

15. Barlow AB; Wheatcroft N; Watson P; Weetman AP.(1996) Association of HLA-DQA1*0501 with Graves' disease in English Caucasian men and women. Clin Endocrinol. 44:73–7.

16. Cuddihy RM and Bahn RS. (1996) Lack of an independent association between the human leukocyte antigen allele DQA1*0501 and Graves' disease. J Clin Endocrinol Metab. 81:847–9.

17. Yanagawa T; Mangklabruks A; DeGroot LJ. (1994) Strong association between HLA-DQA1*0501 and Graves' disease in a male Caucasian population. J Clin Endocrinol Metab. 79:227–9.

18. Heward JM; Allahabadia A; Daykin Jet.(1998) Linkage disequilibrium between the human leukocyte antigen class II region of the major histocompatibility complex and Graves' disease: replication using a population case control and family-based study. J Clin Endocrinol Metab. 83:3394–7.

19. Maciel LM.; Rodrigues SS; Dibbern RS; Navarro PA ; Donadi EA. (2001).Association of the HLA-DRB1*0301 and HLA-DQA1*0501 alleles with Graves' disease in a population representing the gene contribution from several ethnic backgrounds. Thyroid. (1):31-5.Tissue Antigens. 67(1):79-83.

20. Ramos- Lopez E ; Fernandez ;BalsellsM.; Kahles H.; Seidi C.; Ferrer J.; Badenhoop K;(2007) HLA-DQ haplotypes in Spanish and German families with Graves' disease: contribution to DQA1*0501-DQB1*0301 mediated genetic susceptibility from fathers. Thyroid 17 (11): 1131-5**.**

21. PhilippouG; KrimitzasA; Kaltsa sG.; AnastasiouE.; Souvatzoglo u A. ; Alevizaki M. (2001). HLA DQA1*0501 and DRB1*0301 antigens do not independently convey susceptibility to Graves' disease. J. Endocrinol. Invest. 24 (2): 88-91.

22. Yanagawa T.and degroot LJ. (1996) HLA class II associations in African-American female patients with Graves' disease. Thyroid. Feb;6(1):37-9

23. Chen QY.; Huang W.; She JX.; Baxter F.; Volpe R.; Maclaren NK. (1999). HLA-DRB1*08, DRB1*03/DRB3*0101, and DRB3*0202 are susceptibility genes for Graves' disease in North American Caucasians, whereas DRB1*07 is protective. J. Clin. Endocrinol. Metab. 84 (9): 3182-6.

24. Simmonds ,MJ. and Gough, SC. (2005). Unravelling the genetic complexity of autoimmune thyroid disease: HLA, CTLA-4 and beyond.Clin Exp Immunol, 136(1): 1–10.

25. Simmonds, M. J.; Heward , J. M.; Barrett, J. C.; Franklyn, J. A and Gough, S C. (2006). Association of the BTNL2 rs2076530 single nucleotide polymorphism with Graves' disease appears to be secondary to DRB1 exon 2 position beta74. Clin Endocrinol (Oxf).; 65**:**429-32.

26. Hsiao JY; Hsieh MC; Tien KJ, Hsu SC; Lin SR; Ke DS; (2008) Exon 33 T/T genotype of the thyroglobulin gene is a susceptibility gene for Graves' disease in Taiwanese and exon 12 C/C genotype protects against it. Clin Exp Med. (1):17–21.

27. Ban Y. ; Davies T.; Greenberg D.; Concepcion E. ; Osman R.; Oashi T. (2004). Arginine at position 74 of the HLA-DRb1 chain is associated with Graves' disease. Genes Immun. 5:203–208.

28. Sawai Y. and DeGroot LJ.(2000) Binding of human thyrotropin receptor peptides to a Graves' disease predisposing human leukocyte antigen Class II molecule. J Clin Endocrinol Metab 85:1176-1179,

29. Sakaguchi, S. (2000). Regulatory T cells: key controllers of immunologic self-tolerance. Cell.; 101**:** 455-8

30. Schonland, S. O.; Lopez, C.; Widmann, T.; Zimmer, J.; Bryl, E.; Goronzy, J. J and Weyand, C. M. (2003). Premature telomeric loss in rheumatoid arthritis is genetically determined and involves both myeloid and lymphoid cell lineages. Proc. Natl. Acad. Sci.; 100**:** 13471-6.

31. Davies , E.T. (2012) .Ex vivo alloanergization with belatacept: a strategy to selectively modulate alloresponses after transplantation. Cell Transplantation.

32. International Atomic Energy Agency IAEA (2010). Radiological Conditions in Selected Areas of Southern Iraq with residues of Depleted Uranium: Report by an international group of experts. Vienna.

33. Al – Janabi , M. (2013) Immunogenetic Study of Thyroid Disorders and Its Relation with Radiation Exposure thesis in Genetic Engineering and Biotechnology. Bagdad University.

Iraqi Journal of Biotechnology, 2015, Vol. 14, No. 2 , 312-321

Association of Glutathione –S-Transferase (GSTP1) gene polymorphism with Acute Myeloid leukemia in Iraqi patients

Kh.Ibraheem, Batool A. Shihab, Nagham E. Al-Essa, Mayadah

Department of Biology,College of Science for Women,University of Baghdad.

Abstract: GSTP1 gene polymorphism is correlated with elevated risk of many cancers including (Acute myeloid leukemia).In this study we investigated the inherited GSTP1 polymorphism and the susceptibility to AML in Iraqi (60) patients and (60) healthy controls using PCR-RFLP assay.We found that the mutant allele (G) was the most prevalence in male 0.38 than in female 0.17.The mutant genotype Val\Val was highly significant difference in male.The percentage of AML patients of 85.71 %while the percentage of female was 14.29% P<0.01 with OR 1.648.Age group less than 30% years old with mutant genotype Val\Val was significantly difference than other age groups.Our results suggest that mutant allele and sex may be associated with increased risk of AML.

Key words: GSTP1 polymorphism, AML, PCR-RFLP.

العلاقة بين تعدد النمط الوراثي للجين (GSTP1) الثاني ومرض ابيضاض الدم النخاعي الحاد لمرضى عراقيين

مياده خالد ابراهيم[1] ، بتول علي شهاب [2]، نغم عيسى العيسى[3]

جامعة بغداد / كلية العلوم للبنات

الخلاصة: تعدد النمط الوراثي للجين GSTP1 ارتبط بتقيم عدد من الامراض السرطانيه ومنها سرطان الدم النخاعي الحاد. في هذه الدراسة بحث عن توارث النمط الوراثي GSTP1وقابليه الاصابه بسرطان الدم النخاعي الحاد في (60) عينه لمرضى عراقيين و(60) من الاصحاء باستخدام تقنية تحاليل التضاعف التسلسلي (polymerase chain reaction)وتباين اطوال قطع الدنا المقيدة (Restriction Fragment Length Polymorphism).اضهرت النتائج ان الاليل الطافر (G) كان اكثر وجودا في الذكور 0,38مقارنة بالإناث 0.17. التركيب الوراثي المطفر Val\Val كان في الذكور المرضى بنسبه اعلى 85.71٪ من الإناث 14.29٪ وبفارق معنوي عالي 1.648 . الفئة العمرية أقل من 30 سنة كان النمط الوراثي المطفر هو الاكثر شيوعا وبفارق معنوي عن بقيه الفئات العمرية . الدراسه افترضت ان الاليل المطفر والجنس ممكن ان يكونا عاملين خطوره مرتبطه بمرض ابيضاض الدم النخاعي الحاد.

Introduction

Acute leukemia is divide into acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML)[1].The last one is the most common affection in adult[2].The real reason are still un known but like other cancers, AML is a complex disease caused by the combination effect of environmental factors such as chemical exposure,ionizing radiation and genetic factors[3].The mechanism of this disease is mediated by accumulating of abnormal white blood cells (WBC)in the bone marrow and distrusting the formation of normal blood cell[4].Many studies reported that DNA damage by reactive oxygen species (ORS) in hematopoietic precursor cells influence the carcinogenesis to several cancers including acute leukemia.

The glutathione S transferase (GST) family groups of enzymes which play an important role in the detoxification of carcinogens by forming the conjugation of glutathione (GSH)to electrophilic compound[5].Among these enzyme groups GSTP1 which is the major GST expressed in many tissue and has been shown to be over expressed in several malignant tissues compared with normal tissues.Single nucleotide polymorphism (SNPs)in GSTP1 resulting in amino acid substitution that effect enzyme activity function is A313G substitution resulting in an lle 105 val amino acid change in exon 5[6].

The A313G variant of GSTP1has been studied in many different population which they occurs at frequencies of 14-20 % among Asian and 28-32%among Caucasians which appear these are ethnically different[7][8].

Polymorphism of GSTP1 gene decrease the ability to conjugate electrophiles material with glutathione and thus sensitize cells to damage that caused by free radical.The GSTP1 variant has been correlated with susceptibility to different cancers [9] and heart disease [10,11].

There are several studies which have been investigated on the correlation between polymorphism of GSTPI and the susceptibility to many kinds of cancers among them (AML) [12].These results are either from significant correlation by the polymorphisms to AML,or not significant relation[13].The first case and the control studies on the Iraqi people were designed to provide more information about the effects of the polymorphisms of *GSTP1* on AML risk and the complications related to AML.We have found important results regarding the association of GST polymorphisms and patient of AML.Our study is the

first one on Iraqi people with AML cancer in order to provide information about the effect of the GSTP1 polymorphism on AML risk.

Material and Methods:

The study consisted of 60 clinically diagnosed AML and 60 healthy as a control peers.Their ages rang were (15-70) years.All samples have been taken from Center for Hematology\AL-Mustanseria University,hematology clinic of Baghdad Hospital and Alkadhymian medical city teaching hospital.The following detailed information were obtained:Age, sex, Hb,WBC.

Collection of Blood Samples:

Five milliliters of blood of each patient and healthy human were obtained by vein puncture using 5 ml.The blood sample was put into EDTA tube,this blood was mixed gently and put on shaker for(5 min).All blood samples were placed in a cool – Box under aseptic condition and this tube was stored in the freezer(-20C°)\and then used for DNA extraction.

Genomic DNA extraction and genotyping:

DNA was isolated using 5 mL whole blood collected in tubes of EDTA using purification kit of the Wizard genomic DNA (promega , USA).All samples showed bands which represent the genomic DNA when the electrophoresis of the gel.The polymorphism of the GSTP1 gene was detecting using a PCR – RFLP according to the method detailed by Harries *et al* [14]

PCR amplifications were performed in a total volume of 30 µL consisted of 5 µL genomic DNA,8 µL D.W.15 µL master mix and 1 µL of each primer. GSTP1 forward were 5′-ACC CCA GGG CTC TAT GGG AA-3′;and GSTP1reverse were 5′ TGA GGG CAC AAG AAG CCC CT-3′.The conditions were as follows: 95°C for 5 min of an initial denaturation step , 94°C for 30 sec (30 cycles) ,55°C for 30 sec and 72°C for 30 sec, and 72°C for 5 min of a final extension step.The fragment 176 bp that consisted by PCR was separated on a 2% agarose gel and using ethidium bromide staining to confirm the presence of these fragment.

After amplification, 15 µL of PCR products was digested with 4U of *BsmAI* restriction enzyme (New England Biolabs) in a total volume of 30 µL.The mixture was incubated at 37 °C for 24 hour using an incubator.The digestion products separated on a 3% agarose gel staining by ethidium bromide and visualized under UV source.

Statistical Analysis

The Statistical Analysis System-SAS (2012) was used to explore the effect of different factors in studied parameters.Chi-square test was

applied to compare differences in clinical parameters between patients and controls.GSTP1 was classified as homozygous wild type Ile/Ile,heterozygous mutant Ile/ Val,mutant Val/Val.P-values were a value of ≤ 0.01, 0.05 was considered statistically significant.Least significant difference LSD test has been used to significant comparison between means in this study.

Results

A total of 120 blood sample of (60 patient, and 60 controls) were enrolled in this study.Genomic DNA extracted were isolated figure (1).

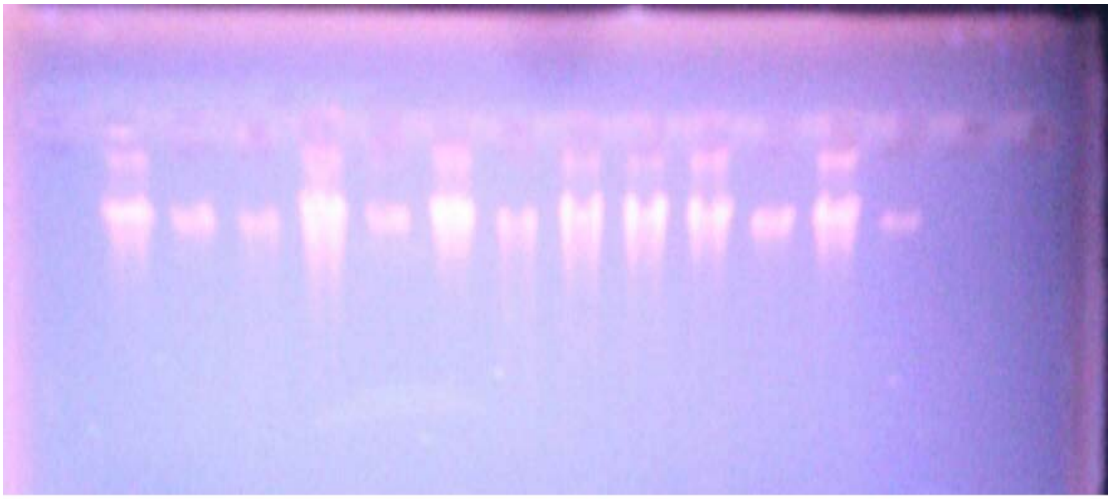
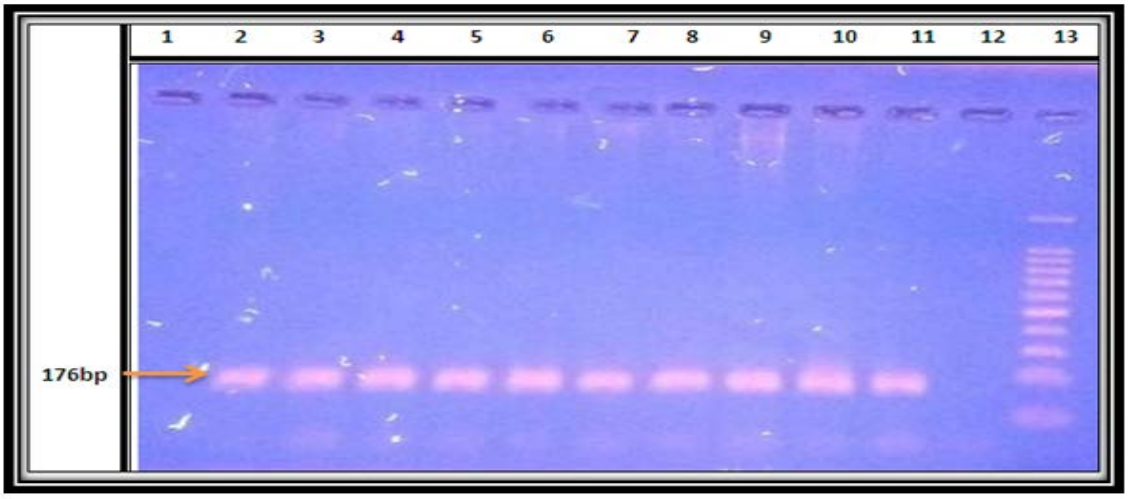


Figure (1): Agarose gel electrophoresis of DNA extracted from blood sample. The extracted DNA was run on 0.3 agarose at 70 voltages for one hour, 1X Tris-borate buffer and stained with ethidium bromide before visualized by UV transilluminator.

Gel electrophoresis of amplified DNA products showed the band of GSTP1 gene at size of 176 pb. Figure (2).



Figure(2):Agarose gel electrophoresis of PCR product of the GSTP1 gene.The PCR product resolved by 2% agarose gel electrophoresis(70 volt/ 75 min).Lanes (2-11)samples from patients.Lane12,negative control A176 bp DNA fragments corresponding to the GSTP1 gene,and lane 13 DNA molecular weight marker.

Products of amplified DNA were digested with *BsmA1* enzyme and the samples were then run in 3% agarose by gel electrophoresis. Homozygous wild type genotype Ile\Ile was indicated by 176 bp band while the presence of 91 band and 85 bp band were indicated the homozygous mutate type val\val. The heterozygous type exhibited all the three band 176 pb,91pb 85pb band Figure (3)

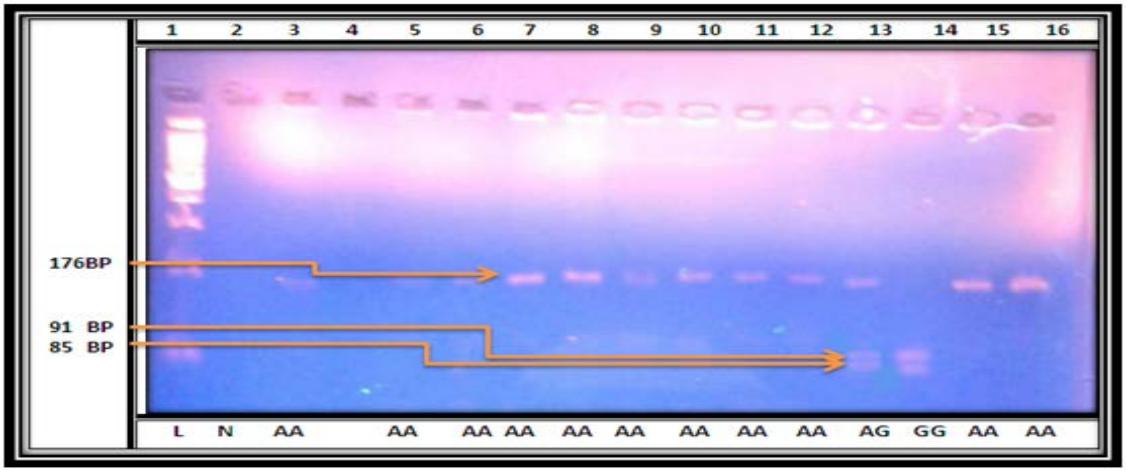


Figure (3): Photograph of the PCR products of the GSTP1 gene after *BsmAI* enzyme digestion and on a 3% agarose gel.Lane L shows the 100 bp DNA ladder marker ;lanes3,5,6,7,8,9,10,11,12,15and16 showed individuals with the Ile/ Ile genotype (176 bp).lanes 13 show the Ile/Val genotype and (176bp, 91 bp, 85bp) Lane 14 shows the Val/Val genotype (91bp, 85bp).

The result of GSTP1 gene polymorphism distribution in both patients and controls was showed in table (1):

Table (1): Genotype distribution of GSTP1 polymorphism in AML patients and control.

Genotype	Control	Patients	Chi-square	O.R
AA	39(65.00%)	34(56.67%)	4.623*	0.594
AG	16(26.67)	19 (31.67%)	1.297 NS	0.173
GG	5(8.30%)	7(11.67%)	0.906 NS	0.082
*(P<0.05), NS :Non – significant .				

The percentage of Ile\Ile genotype was 65% in controls and 56% in patients with AML with significant difference P<0.05.

The heterozygous and homozygous mutant the percentage were 26.7%, 31.7% and 8.3% , 11.7% respectively in controls and patients but not statistically significant.

The association between the GSTP1 polymorphism and sex in AML group was showed in table (2).

Table 2: GSTP1polymorphism and sex in AML group

Genotype	Male	Female	Chi-square	O.R.
AA (34)	13 (38.24%)	21 (61.76%)	9.271 **	1.184
AG (19)	11 (57.89%)	8 (42.11%)	6.318 **	0.928
GG (7)	6 (85.71%)	1 (14.29%)	13.59 **	1.648
Allele frequency				
A	0.62	0.83	---	---
G	0.38	0.17	---	---
** (P<0.01)				

The lle\lle genotype was highly significant in female than male and the percentage was 61.8%and 38.24% respectively.The lle\val genotype percentage was 57.7% in male and 42.11% in female while in val\val the percentage was 85.71% in male while in female 14.3% and the difference was highly significant P<0.01 between both two groups.

The relationship between GSTP1polymorphism and the age onset in AML group was showed in table (3).

Table 3: GSTP1 polymorphism and age at onset in AML group .

Genotype	Less than 30	30-50	More than 50	Total	p-value
AA	1(38.24%)	11(32.3%)	10(29.41%)	34	0.0417*
AG	5(26.32%)	4(21.05%)	10(52.63%)	19	0.0136**
GG	4(57.14%)	3(42.86%)	0(0.00%)	7	0.0001**
Allele frequency					
A	0.70	0.27	0.75	0.73	---
G	0.30	0.28	0.25	0.27	---
*(P<0.05),**(P<0.01).					

The data was showed that the percentage of patient with Ile\Ile 38.24% in less than 30 years group was the highest and significant than other groups P<0.05 ,but in groups more than 50 with Ile\val genotype the percentage was 52.63% higher significantly than others P< 0.01.

Homozygous genotype val\val was higher significantly percentage 57.14% in less than 30 than other group P <0.01.

Discussion

GSTP1 is critical for protecting cell from oxidative stress because they can utilize a wide variety of products of oxidative stress as substrates.It has catalysis the detoxification of products that arise from DNA oxidation the lacks of this function which is genetically determined might be a risk factor for disease development among these AML.

There were few studies which discuss the relationship between GSTP1gene polymorphism and the risk of variety of common cancers including AML.There are enormous differences in the result of these studies which are either from

significant correlation by the polymorphism or not significant relation.

Our results were indicated that there was no significant difference between two studies groups lle\val , val\val genotypes ,and this in agreement with many studies in different populations.[15]They found that there was no association between homozygous val\val and chronic pancreatic in Serbian patients furthermore,there were no significan differences between all three genotypes in studies samples interestingly, Rasheed [16] found that there were significant different between all genotypes and the homozygous mutant val\val was evaluated in patients only with T_2DM in Iraqi population.

In another study val\val genotype might confer risk to AML and val\val genotype is known to be associated with defective detoxification that arise from DNA oxidation which interrupted with cellular protection against oxidative stress[17].

Our result showed slightly increased risk of AML with G allele of GSTP1/AG-GG but not statistically significant.Ibrahim found that GSTP1 A313G polymorphism is associated with a decrease risk of lymphoma .

[18]found that G allele genotype and the val\val ,lle\val genotype were more distributed in AML patients and controls in position A—G polymorphism for GSTP1. They suggested that the GSTP1 gene polymorphism was associated with the AML and the risk increased in persons caring G allele.

The polymorphism of GSTP1 lle 105 val for pancreatic pathology remains unclear and they found that the frequency of 105 val was less frequent in patients with pancreatitis and controls in comparison to T_2DM patients nevertheless the differences were not statistically significant.With respect to sex of the proband GSTP1 val\val genotype frequency was increased in female AML patients as compared to male patients and val\val genotype was also associated with early on set at AML less than 30 years but in our study we found that val\val more frequent in male than in female patients[19].

The difference in results might due to the sample size and this in turns insufficient for statistical power to detect the effect of GSTP1 gene on AML.

Conclusion

This is the first study to determine the correlation of acute myeloid leukemia with GSTP1 genetic polymorphism in the population of Iraq.Our results suggest that in GSTP1 gene polymorphisms there is no significant difference between

two studies groups lle\val,val\val genotypes,and GSTP1 have no active role in the pathogenesis of AML.

References:

1. Mandegary A, Rostami S, Alimoghaddam K,(2011). Gluthatione-S-transferase T1-null genotype predisposes adults to acute promyelocytic leukemia; a case-control study. *Asian Pac J Cancer Prev*, 12, 1279-82.
2. Jiang L,zhou P,sun A, (2011). Functional variant (21304 T.G)in the MKK4 promoter is associated with decreased risk of acute myeloid leukemia in a southern Chinese population.Cancer Sci , 102,1462-8.
3. Ruddon , R.W. (2007) .Cancer Biology .4thed. Oxford University press, Inc. New York . pp:321-351
4. Zhou L, Zhu YY, Zhang sXD, Li Y, Liu ZG. Risk effects of GST gene polymorphisms in patients with acute myeloid leukemia:a prospective study. Asian Pac J Cancer Prev 2013;14:3861–4.
5. Hayes J.D. ; Flanagan, J.U. and Jowsey, I.R. (2005).Glutathione transferases. Annu. Rev .Pharmacol. 45: 51-88.
6. Strange RC, Feyer AA. The glutathione S-transferases:influence of polymorphism on cancer susceptibility.IARC Sci pub 1999; 148:231-49.
7. Sprenger, R., Schlagenhaufer ,R., Kerb, R.,bruhn,C., Brockmoller,J.,Roots,I.&Brinkmann,U. (2000) Pharmacogenetics 10,557-565.
8. Seidegard, J., Vorachek, W.R., Pero,W.R.&Pearson,W.R (1988) Proc.Natl.Acad.Sci USA 85,7293-7297.
9. Yoshida, K., Hirokawa, J., Tagami, S., Kawakami ,Y., Urate ,Y. and Kondo,T. 1995.Weakened cellular scavenging activity against oxidative stress in diabetes mellitus regulation of glutathione synthesis and efflux, Diabeteologia. 38(6): 201–210.
10. Buchard A, Sanchez, J.J., Dalhoff ,K., and Morling, N. 2007. Multiplex PCR detection of GSTM1, GSTT1, and GSTP1 gene variants: simultaneously detecting GSTM1 and GSTT1 gene copy number and the allelic status of the GSTP1 Ile105Val genetic variant, J Mol Diagn. 9(6): 612–617.

11. Zimniak, P., Nanduri, B.,and Pikula, S. 1994.Naturally occurring human glutathione S-transferase GSTP1-1 isoforms with isoleucine and valine in position 104 differ in enzymic properties, Eur J Biochem . 224 (3): 893–899.

12. Crump C,Chen C,Appelbaum FR,*et al* (2000).Glutathione S-transferase theta 1 gene deletion and risk of acute myeloid leukemia .Cancer Epidemiol Biomakers Prev ,9457-60.

13. Wilson,M.H.,Grant,P.J.,Hardie, L.J.&Wild,C.P.(2000)FASEB J.14,791-796.

14. Harries, L.W., Stubbins, M.J., Forman, D., Howard, G.C.and Wolf,C.R. 1997.Identification of genetic polymorphisms at the glutathione S-transferase Pi locus and association with susceptibility to bladder, testicular and prostate cancer, Carcinogenesis. 18(4): 641-644.

15. Nikolic, A.; Stankovic, M.; Nisevic, I.; Lukic, S.; Andelic-Jelic, M.; Popovic, D. and Radojkovic,D. (2011). GSTP1 Ile105Val Polymorphism in Serbian Patients with Pancreatic Diseases. J Med Biochem; 30(2): 121–125.

16. 16.Raghad, H. Rasheed,. (2015). Association of Glutathione–S-Transferase (GSTP1) Genetic Polymorphism in Iraqi Patients with Diabetes Mellitus Type 2.

17. 17.Dunna, N.R; Vuree, S.; Kagita, S.; Surekha, D.; Digumarti, R.; Rajappa, S.and Satti,V.(2012). Association of *GSTP1* gene (I105V) polymorphism with acute leukaemia. Journal of Genetics; 91(4): 60-63.

18. Xu, C.; Li, C. Y. T. and Kong, A. N. T. (2005). Induction of Phase I, Ii and Iii Drug Metabolism/Transport by Xenobiotics. Arch Pharm Res; 28 (3): 249-268.

19. Nikolic, A.; Stankovic, M.; Nisevic, I.; Lukic, S.; Andelic-Jelic, M.; Popovic, D.and Radojkovic, D. (2011). GSTP1 Ile105Val Polymorphism in Serbian Patients with Pancreatic Diseases. J Med Biochem; 30(2): 121–125.

Iraqi Journal of Biotechnology, 2015, Vol. 14, No. 2 , 322-329

In vitro cytotoxicity of myriocin against *Leishmania tropica* promastigotes

Hayder Zuhair Ali and Fatima Mohsin Mousa

Dept. of Biology, College of Science, University of Baghdad (h.z.ali@scbaghdad.edu.iq)

Abstract: *Leishmania tropica* is a protozoan parasite causes cutaneous leishmaniasis. Sphingolipids (SLs) are main constituents of eukaryotes cell membranes, including *Leishmania*. In this study, we have focused on the *de novo* sphingolipid synthesis pathway of *Leishmania* to examine its ability to survive and proliferate after treatment the promastigotes by the drug, myriocin, which is a potent inhibitor of the serine palmitoyltransferase (SPT), the first key enzyme in the sphingolipid biosynthesis. Myriocin with different concentrations (100, 50, 25, 12.5, 6.25, 3.125, 1.56, and 0.78) µM were adopted for three times of follow up, 4, 12 and 24 hours. The results showed that promastigotes maintain healthy culture after myriocin treatment with a minimum cell viability of 79% in foetal bovine serum (FBS) media and was 86 % in FBS-free media. This is to conclude that the Iraqi strain of cutaneous leishmaniasis has its own pathway to scavenge SLs to survive and multiply, *in vitro.*

Key words: Leishmania, myriocin, Sphingolipids.

سمية الميريوسين ضد الطور المسوّط لطفيلي اللشمانيا الجلدية خارج الجسم الحي

حيدر زهير علي و فاطمة محسن موسى

كلية العلوم – قسم علوم الحياة – جامعة بغداد

الخلاصة: اللشمانيا الجلدية طفيلي ابتدائي يسبب مرض اللشمانيا الجلدية. الدهون الاسفنجولية مكونات أساسية في الغشاء الخلوي للكائنات حقيقية النواة من ضمنها اللشمانيا. في هذا البحث تم دراسة مسار التخليق الحيوي للدهون الاسفنجولية للطفيلي للتحري عن قدرته في البقاء على قيد الحياة و التكاثر بعد معالجة الاطوار المسّوطة بعقار الميريوسين ، المعروف بقدرته على تثبيط الانزيم الأول في تخليق الدهون palmitoyltransferase (SPT). استخدمت عدة تراكيز من الميريوسين (100, 50, 25, 12.5, 6.25, 3.125, 1.56, and 0.78) مايكروومولار و اعتمدت ثلاثة أوقات للمتابعة 4 و 12 و 24 ساعة. أوضحت النتائج ان الاطوار المسّوطة استطاعت الحفاظ على حيويتها بادنى فعالية خلوية قاربت 79% في الوسط الزرعي المزود بمصل جنين العجل وأدني فعالية خلوية قاربت 86 % في الوسط الزرعي الخالي من مصل جنين العجل. نستنتج من هذه الدراسة ان العزلة العراقية من اللشمانيا الجلدية لديها الية خاصة في الحفاظ على مستوى الدهون الاسفنجولية و البقاء حيّة بعد استخدام الميريوسين خارج الجسم.

Introduction:

Introduction: Leishmaniasis is a disease caused by *Leishmania* parasite; it is transmitted to humans and mammals by the bite of the female of sand-fly (1). About twenty-one species have been known to infect human (2). Sphingolipids (SLs) are important for eukaryotic cells in the formation of lipid rafts, signal transduction, membrane trafficking and programmed cell death (3). It has been known that in *Leishmania*, the primary product in the *de novo* SLs biosynthesis is, inositol phosphorylceramide (IPC) catalyzed by serine palmitoyltransferase (SPT) , while in other parasites, such as *Plasmodium*, the *de novo* SLs pathway synthesize sphingomyelin (SM) by SM synthase (4). In another word, the conformation and function of sphingolipids (SLs) in *Leishmania* is different from those in other eukaryotes, including unicellular parasites (5). Myriocin, [(2S,3R,4R,6E)-2-amino-3,4-dihydroxy-2-(hydroxymethyl)-14-oxo-6-eicosenoic acid], also known as thermozymocidin and ISP-1, is a fungal metabolite isolated from *Myriococcum albomyces, Isaria sinclairi*, and *Mycelia sterilia*; it is found to be a potent inhibitor of serine palmitoyltransferase (SPT) the key enzyme in the *de novo* SLs biosynthesis (6). Myriocin remains the most valuable and widely used chemical probe in SLs; for example, recent studies recent studies using the natural product in whole eukaryotic cells and cell extracts have revealed unexpected structural complexity in the membrane-bound SPTs from higher organisms (7). The surface of extracellular *Leishmania* and other trypanosomatid parasites is characterized

by a high abundance of glycosylphosphatidylinositol (GPI)-anchored glycoconjugates and proteins; and many of these molecules segregate into detergent-resistant membrane fractions that are enriched in sphingolipids and sterols (8,9). The old world *L. major* intra-macrophage amastigotes found to have undetectable expression of subunit 2 of SPT (*LmLCB2*) inside the parasitophorous vacuole (10). In this study, the ability of Iraqi strains of cutaneous *L. tropica* to grow and proliferate, *in vitro*, after myriocin treatment were studied.

Materials and methods:

Parasite isolates: *L. tropica* was isolated from Baghdad hospital-dermatology section from a patient with a skin ulcer, clinically diagnosed with cutaneous leishmaniasis was used in this study.

Culture media: M199 media (Sigma Aldrich) was used in this study for

Leishmania procyclic promastigotes culture, the media was prepared according to the manufacturer's procedure, supplemented with 10% foetal bovine serum (FBS) sigma and 50µg/ml of gentamycin was added and pH was adjusted at 7.4. Culture tubes were incubated at 26^{o}C for daily experiments (11).

Myriocin: the drug was purchased from Sigma and prepared according to the manufacturer's procedure, in brief, 2mg of myriocin powder was dissolved in 5 ml methanol for 100 mM concentration.

Cytotoxicity screening:

Plates of 96 flat bottom well-plate were used in this experiment, two pairs were prepared for each *Leishmania* species. For each plate, 100 µl of parasite suspension of 4×10^5 cell/ml was seeded in each well; myriocin was added starting with 100 micromolar (µM) concentration and serial dilution was made in triplicate to end with 3.125 mM; M199 media was used as complete media supplemented with FBS or FBS-free media (12). Plates were incubated at 26^{o}C for 12 or 24 hours. Control plates were made the same but with methanol added instead of myriocin.

Alamar Blue® assay: After 12 or 24 hours of incubation, alamar blue was added in a ration of 1:10 and incubated at 26^{o}C for 4 hours (according to the manufacturer's) before reading the absorbency by microplate reader (570/630) and cell viability was calculated by plotting the percentage of parasites absorbency against logarithm myriocin concentration (13).

Test was used for statistical analysis.

Results and discussion:

Myriocin cytotoxicity has been screened against procyclic promastigotes of old world Iraqi strains of *L. tropica,* with or without FBS added to the media, in order to examine the effect of myriocin on the *de novo* SLs biosynthesis of the parasite. Absorbency was used to determine cell viability by the ability of living parasites to reduce the alamar blue from blue to red. Results showed that there was no significant differences ($p > 0.05$) between test and control for all plates of *L. tropica* cultured in media with or without FBS, after the different concentrations of myriocin used in this study, in the two period of times (12 and 24) hours. Procyclic promastigotes were grown normally and myriocin did not affect the proliferation and cell viability of the parasites. The percentage of cell viability was above 50% for all plates. For *L. tropica,* the minimum

cell viability of parasites cultured in FBS-media, 12 hours incubation, was 94.21% at concentration of 1.56 µM and was 84.86%, 24 hours incubation at concentration of 3.125 mM; figure 1 and 2. Similar results were noticed in *L. tropica* cultured in FBS-free media, in which the minimum cell viability was 75.42% at concentration of 50 µM after 12 hours incubation and was 86.75% at concentration of 6.25 after 24 hours incubation, figure 3 and 4.

The results of *L. donovani* cytotoxicity with myriocin was not different from *L. tropica* (data not shown).

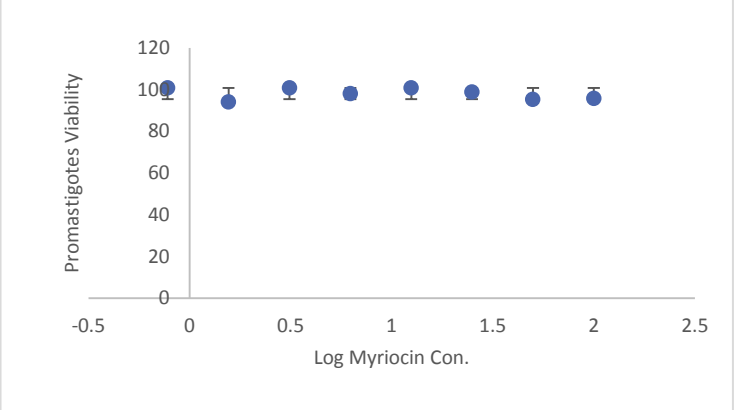


Figure-1: Myriocin cytotoxicity against *L. tropica* promastigotes cultured in FBS-medium, after 12 hours incubation.

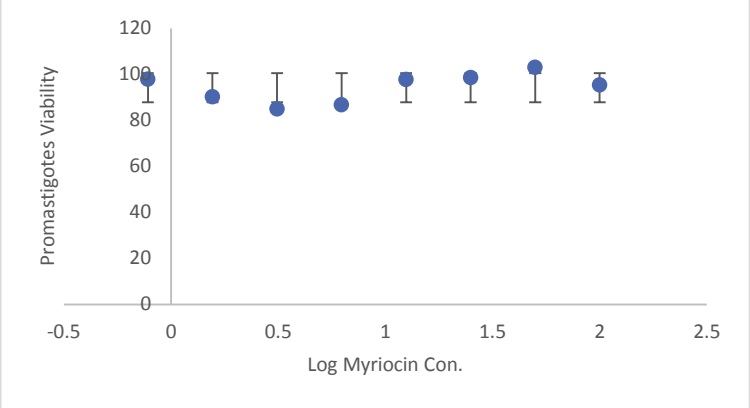


Figure-2: Myriocin cytotoxicity against *L. tropica* promastigotes cultured in FBS- free medium, after 24 hours incubation.

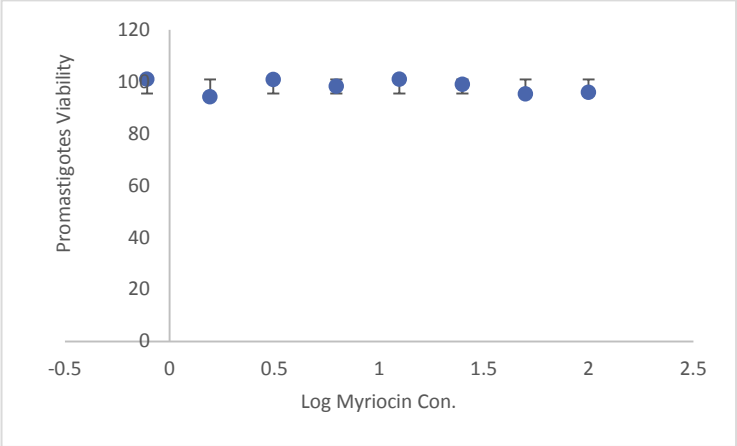


Figure-3: Cell viability of *L. tropica* promastigotes treated with myriocin, cultured in FBS-free medium, after 12 hours incubation.

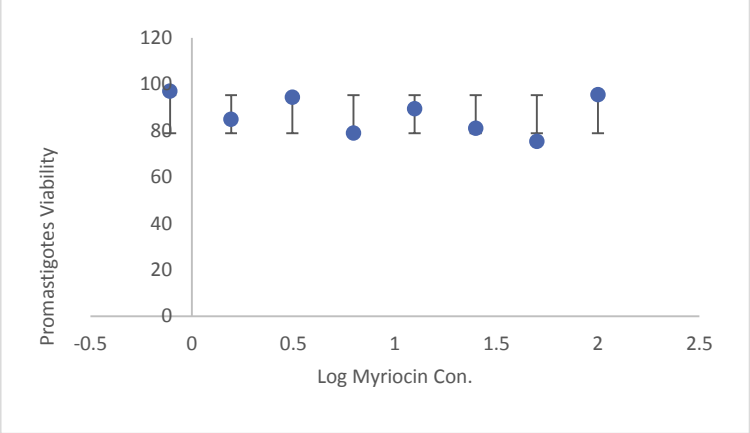


Figure-4: Cell viability of *L. tropica* promastigotes treated with myriocin, cultured in FBS-free medium, after 24 hours incubation.

These results are different than previous findings in yeast and mammalian cells, in which the deletion of *SPT*[2] subunit led to severe growth retardation and death of cells (14). However, like fungi, *Leishmania* synthesizes inositol phosphorylceramide (IPC) instead of sphingomyelin or glycosphingolipids; IPCs account for 5–10% of total cellular lipids in *Leishmania* and are enriched in raft-associated membrane fractions (15, 16). A similar study on the old world *L. major*, found that the inhibition of SPT by myriocin led to aberrant cytokinesis, characterized

by delayed kinetoplast segregation and emergence of cells with abnormal DNA content, followed by cell death (17). Parallel experiments on the old world *L. major* used wild type and *SPT2*$^-$ null mutant parasites cultured in FBS-medium, they showed that wild type and *SPT2*$^-$ null mutant parasites did grow normally in the presence of myriocin and there was no direct effect on the viability or growth during the log phase, unlike yeast and mammalian cells, which they underwent retardation and death, in contrast, the *SPT2*$^-$ parasites lost viability and failed to generate the infective metacyclic promastigotes stage and this may conclude that the null parasites could be rescued by the exogenous sphingoid bases available in the culture media, so the parasites grew normally at the log phase but the *SPT2*$^-$ died in the stationary phase and failed to differentiate into infective metacyclic stage (18, 19).

When *L. major* amastigotes are within the parasitophorous vacuole (PV) of host macrophage, the SPT is down regulated so it is thought that production of sphingolipids is necessary for growth and survival of the intra-macrophage parasites, this is, therefore, suggested that *L. major* amastigotes may scavenge some sphingolipids or sphingolipids precursors from the host to survive within the PV (10). Another study by (20) used SPT mutant *L. major* and followed the mutant parasites in animal model infection, they found that, after some delay than wild type parasites, mice were infected and *SPT2*$^-$ amastigotes went to develop a lesion, they implied that mutant amastigotes acquisitioned of host cells (macrophages) and salvage SLs.

This study can conclude that inhibition of SPT by myriocin have a minor effect on the survival of procyclic promastigotes of *L. tropica*, *in vitro*, this should be followed by similar investigations for *ex vivo* experiments.

References:

1- Cardona-Arias, J.A.; Velez, I.D. and Lopez-Carvajal, L. (2015). Efficacy of thermotherapy to treat cutaneous leishmaniasis. PLOS one. 10(5): e122569.

2- Murray,H.W.; Berman, J. D.; Davies, C. R. and Saravia, N. G. (2005). Advance in leishmaniasis. *The Lancet*. 366 (9496):1561–1577.

3- Zhang, K.; Showalter, M.; Revollo, J.; Hsu, F.; Turk, J. and M.Beverely, S. (2003). Sphingolipids are essential for differentiation but not growth of *Leishmania. The EMBO Journal.* 22(22): 6016-6026.

4- McConville, M.J. and Bacic, A. (1989). A family of glycoinositol phospholipids from *Leishmania major*. Isolation, characterization and antigenicity. *J Biol Chem.* 246:757-766.

5- Zhang, O.; Xu, W.; Pillai, A.B. and Zhang, K. (2012). Developmentally regulated sphingolipids degradation in *Leishmania major*. *PLOS one.* 7(1): e30195.

6- He, Q.; Jhonson, V.; Osuchowki, M.F. and Sharma, R.P. (2004). Inhibition of serine palmitoyltransferase by myriocin, a natural mycotoxin, causes induction of c-myc in mouse liver. *Mycopathol.* 157: 339-347.

7- Han, S.; Lone, M.A.; Schneiter, R. and Chang, A. (2010). Orm1 and Orm2 are conserved endoplasmic reticulum membrane proteins regulating lipid homeostasis and protein quality control. *Proc. Natl. Acad. Sci. USA.* 30(107): 5851-5856.

8- Ralton, J.E.; Mullin, K.A.; and McConville, M.J. (2002) Intracellular trafficking of glycosylphosphatidylinositol (GPI) - anchored proteins and free GPIs in *Leishmania mexicana*. *Biochem.* J. 363: 365–375.

9- Denny, P.W.; Field, M.C.; and Smith, D.F. (2001). GPI anchored proteins and glycoconjugates segregate into lipid rafts in kinetoplastida. *FEBS. Lett.* 491: 148–153.

10- Denny, P.W.; Goulding, D.; Ferguson, M.A.J. and Smith, Deborah, F. (2004). Sphingolipid-free *Leishmania* are defective in membrane trafficking, differentiation and infectivity. *Mol. Microbiol.* 52(2): 313-327.

11- Patel, N.; Singh, S.B.; Basu, S.K. and Mukhopadhyay, A. (2008). *Leishmania* requires Rab7-mediated degradation of endocytosed hemoglobin for their growth. *PANS.* 15(10): 3980-3985.

12- Mina, J.G; Bacic, Mosely, J.A.; Ali, H.Z.; Shams-Eldin, H.; Schwartz, R.T.; Steel, P.G. and Denny, P.W. (2010). A plate based assay system for analysis and screening of the *Leishmania major* inositol phosphorylcerami -de synthase. *Int. J. Biochem. Cell Biol.* 42: 1553-1561.

13- Ali, H.Z. (2014). Promastigotes viability of *Leishmania major* W.T and *L. major* knockout *LmLCB2* after myriocin treatment. *IJBPR.* 5(6): 486-489.

14- Hanada, K. (2003). Serine palmitoyltransferase, a key enzyme for sphingolipid metabolism. *Biochem. Biophys. Acta*. 1632: 16-30.

15- Denny, Paul W.; Field, Mark C. and Smith, Deborah F. (2001). GPI-anchored proteins and glycoconjugates segregate into lipid rafts in Kinetoplastida. *F.E.B.S. Lett*. 491:148-153.

16- Kaneshiro, E. S.; Jayasimhulu, K. and Lester, R. L. (1986). Characterization of inositol lipids from *Leishmania donovani* promastigotes: identification of an inositol sphingophospholipid. *J. Lipid. Res*. 27(12): 1294-12303.

17- Sutterwala, Shaheen S; Creswell, Caleb H.; Sanyal, Sumana; Menon, Anant K. and Bangs, James D. (2007). *De Novo* Sphingolipid synthesis is essential for viability, but not for transport of glycosylphosphatidyl -inositol-anchored proteins, in African trypanosomes. *Eukaryot. Cell*. 6(3):454-464.

18- Zhang, Kai; Showalter, Melissa; Revollo, Javier; Hsu, Fong-Fu; Turk, John and Beverley, Stephen M. (2003). Sphingolipids are essential for differentiation but not growth in *Leishmania*. *EMBO. J*. 22(22): 6016-6026.

19- Zhang, K.; Pompey, J.M.; Hsu, F.; Key, P.; Bandhuvula, P.; Saba, J.D.; Turk, J. and Beverely, S. (2007). Redirection of sphingolipid metabolism toward *de novo* synthesis of ethanolamine in *Leishmania. EMBO J*. 26(4): 1094-1104.

20- Zhang, K.; Hsu, F.; Scott, D.A.; Docampo, R.; Turk, J. and Beverley, S.M. (2005). *Leishmania* salvage and remodelling of host sphingolipids in amastigote survival and acidocalcisome biogenesis. *Mol. Microbiol*. 55(5): 1566-1578.

Iraqi Journal of Biotechnology, 2015, Vol. 14, No. 2 , 330-339

Measuring Anti-Toxoplasmosis Antibodies, C-Reactive Protein and Rheumatoid Factor in Rheumatoid Arthritis patients treated with Methotrexate

Rasha H. Kuba, Khawla H. Zghair

Department of Biology, College of Science, University of Baghdad, Baghdad, Iraq.

Abstract: This study was conducted to evaluate the prevalence rate of toxoplasmosis among 294 rheumatoid arthritis (RA) patients treated with methotrexate (MTX), 50 RA patients without treatment and 50 samples as healthy control. Blood samples were collected and the presence of *T. gondi*i IgG and IgM antibodies was determined by using Enzyme linked immunosorbent assay (ELISA). C - reactive protein (C-RP) and Rheumatoid Factor (RF) were also estimated in serum of all subjects by using Latex Fixation Test. The percentage of positive RF 140(47.62%) in RA treated with MTX, 39(76%) in untreated patients RA, and 6(12%) in healthy group. While positive percentage of C-RP was 72(76.59%) in RA patients treated with MTX, 43(86%) in untreated RA patients, and 5(10%) in healthy group. The seroprevalence of toxoplasmosis IgM and IgG in RA treated with MTX was 60(20.408%), and 98(33.33%), in RA patients 4(8%), and 18(36%) while, it was 2 (24%), 6(12%) in healthy group, respectivley. The results showed significant difference ($P \leq 0.05$) between studied groups.

Keywords:*Toxoplasma gondii*, auto-immune disease, enzyme linked immunosorbent assay.

قياس الأجسام المضادة لداء المقوسات و بروتين سي التفاعلي والعامل الرثياني في مرضى التهاب المفاصل الرثياني المعالجين بعقار الميثوتركسيت

رشا حسين كبه و خوله حوري زغير

قسم علوم الحياة ، كلية العلوم ، جامعة بغداد ، بغداد ، العراق.

الخلاصة: أجريت هذه الدراسة لتقييم معدل انتشار داء المقوسات الكونيدية في 294 من مرضى التهاب المفاصل الرثياني المعالجين بالميثوتركسيت، و 50 من مرضى المقاصل التهاب الرثياني غير المعالجين و 50عينة سيطرة طبيعيه. تم جمع العينات وتحديد وجود الأجسام المضادة لطفيلي المقوسات الكونيدية من النوع (ج) و (م) باستخدام تقنية الاليزا. كذلك تم قياس بروتين سي التفاعلي والعامل الرثياني باختبار اللاتكس المثبت . وكانت الأجسام المضادة لطفيلي المقوسات الكونيدية منالنوع (ج) و (م) في مجموعة التهاب المفاصل الرثياني المعالجين بالميثوتركسيت(20.408%)60 و ,(33.33%)98 ولمرضى التهاب المفاصل الرثياني غير المعالجين(8%)4 و (63%)18أما في مجموعة السيطرة فكانت (24%)2 و .(12%)6 على التوالي.إن النسبة المئوية الموجبة لبروتين سي التفاعلي كانت (76,59%)72 في المرضى المعالجين بعقار الميثوتركسيت و(86%)43 في مرضى المفاصل الرثياني غير المعالجين و(10%)5 وفي الاشخاص الاصحاء،بينما النسبة المئوية الموجبة للعامل الرثياني كانت (47,62%)140في المرضى المعالجين بالميثوتركسيت و(76%)39 في المرضى غير المعالجين و (12%) 6 وفي الاشخاص الأصحاء.أوضحت النتائج وجود فروق معنوية ($P \leq 0.05$) بين المرضى المعالجين وغير المعالجين.

Introduction

Rheumatoid arthritis (RA) is a common autoimmune disease that is associated with inflammation of joints (Firestein, 2003). RA is one of the complex immune-mediated diseases for which an understanding of the etiology is dependent on the definition of environmental triggers that, in a restricted genetic context, may initiate immune reactions having the potential to contribute the disease development (Stolt *et al.*, 2003).There are several immunological markers had been observed in a variety of inflammatory disease, including RA. Their concentration levels usually reflect disease severity and prognosis (Goronzy and Weyand, 2009). Interlukin-1(IL-1) and Tumor Necrosis Factor- alpha (TNF-α) have been characterized as the major pro-inflammatory cytokines in the inflamed joint in RA (Zwerina *et al.*, 2005). Also RA has been associated with several auto antibodies; it can be present before the disease manifestation (Silman *et al.*, 1992). The autoantibodies that are most frequently found in patients with RA are antibodies against IgG (IgM rheumatoid factor [IgM-RF]) and antibodies against citrullinated proteins (Goldbach-Mansky *et al.*, 2000). The presence of C-Reactive Protein (C-RP) (an abnormal protein occurs at the acute stage of any febrile disease). It's used to detect the progression of RA and described as a passive agglutination reaction (Pisetsky, 2002).

The response has cross reactivity with host tissue, initiating an autoimmune synovitis and subsequent hypertrophy. Synovial hypertrophy is the key factor that leads to cartilage and bone destruction, causing progressive joint damage and disability (Adebajo, 2010).

The diagnosis of RA is easily made in persons with typical established disease. Constitutional features indicative of the inflammatory nature of the disease, such as morning stiffness, support the diagnosis. Demonstration of subcutaneous nodules is a helpful diagnostic feature, additionally, the presence of rheumatoid factor (RF) and C-RP. Inflammatory synovial fluid with increased numbers of polymorphonuclear cells and radiographic findings of juxta articular bone demineralization and erosions of the affected joints substantiate the diagnosis (Fauci, 2010).

Disease modifying drugs are the most effective means of improving the signs and symptoms of RA as well as reducing radiological progression (Donahue *et al.*, 2008). Agents in this class fall into two categories: non-biologics - disease modifying anti-rheumatic drugs such as methotrexate (MTX) (Weinblatt, 1996), and biologic, such as anti-TNF-α antagonists

(Etanercept) (Zalevsky *et al.*, 2007). Despite the good results, anti-TNF-α therapy has a number of side effects. Areas of concern include opportunistic infections, malignancies and acute infectious diseases. Although toxoplasmosis is one of the opportunistic infections, reactivation of latent tuberculosis remains as the most important safety issue of anti-TNF therapies (Sfikakis, 2010).

Toxoplasmosis caused by the ubiquitous obligatory intracellular coccidian protozoan organism, *Toxoplasma gondii* (Negash, *et al.*, 2008). *T. gondii* has a wide range of hosts including humans, mammals, birds and marine mammals. *T. gondii* infection is a global concern, and about one third of the human population has been exposed to this parasite. Humans or animals can acquire *T. gondii* infection postnatal by ingestion of undercooked or raw meat from infected animals, or ingestion of food or water contaminated with oocysts excreted by infected felids (Dubey, 2010). Felids are considered as the only definitive hosts of *T. gondii* playing a crucial role in the transmission of the parasite (Elmore *et al.*, 2010). Cats infected by *T. gondii* may pose a potential threat to public health, because they can shed and excrete environmentally resistant oocysts in their feces (Cenci-Goga *et al.*, 2011). *T. gondii* infection in immune-competent individuals is rarely symptomatic, but toxoplasmosis occurred in fetus and immunocompromised hosts may result in a severe disease or even lethal damage (Elmore *et al.*, 2010). The immune response to *T. gondii* infection is individual and complex (Johnson *et al.*, 2002). *T. gondii* triggers an innate immune response characterized by a rapid recruitment of neutrophils to the site of infection, followed by a strong Th1 protective response associated with the production of proinflammatory cytokines, including interleukin-12 (IL-12) and TNF- α (Bennouna *et al.*, 2003).

Although cell-mediated immunity plays the major role in resistance against *T. gondii*, humeral immunity is also involved in controlling the parasite (Johnson and Sayles, 2002). Patients with primary or acquired immunodeficiency represent a broad group of individuals with various deficits in T cell, monocyte, cytokine and B cell function. Cancer patients with immunosuppressive cancers, including leukemia and lymphoma, experience recrudescence of chronic infection as T cell function declines. Cancer or transplant patients receiving immunosuppressive drugs as part of a chemotherapeutic regimen also present with reactivated toxoplasmosis (Lappaleinen *et al.*, 1998).

Materials and Methods

The samples were collected from September 2013 till the end of February 2014. A total of 294 blood samples were collected from studied groups from both gender, their ages between 20 – 80 years. Samples were collected from Department of Rheumatology, Baghdad Teaching Hospital and Al- Ulwia Hospital in Baghdad, in addition to outpatient clinics. RF antibodies and C-RP were determined by using Latex agglutination test (LAT) (Leaner Chemical Company – Spain); the principle of the test is based on antigen – antibody reaction directly. *Toxoplasma* (IgG) and (IgM) Enzyme Immunoassay Test kit, BioCheck, Europe in human sera also in all subjects sera according to the manufactures instructions.

Calculation and statistical analysis

Statistical analysis system (SAS) program was used for data analysis. Person Chi-square -χ^2 test by using computer program IBM SPSS version (SAS, 2004). P value <0.05 was considered statistically significant.

Results and Discussion

Rheumatoid Factor (RF) had been used as a marker for RA and was included into RA classification criteria for more than half a century (Strupuviene *et al.*, 2005). Among RA groups, the lower percentage of RF was recorded in RA patients treated with MTX 140(47.62%), and untreated RA patients have revealed 39(76%) and 6(12%) in healthy group. There was a significant difference (P< 0.05) between RA (MTX) and other studied groups, as shown in table (1). The present results clarified that patients whom were treated with MTX, their RF antibody titer was decreased in comparison to other untreated RA groups, because MTX is able to induce cell-type-specific apoptosis in the synovial monocyte /macrophage population and decrease the number of these inflammatory cells in rheumatoid joints (Catrina *et al.*, 2005).

Table (1): The percentage distribution of RF Antibodies in studied subjects by Latex agglutination test (LAT).

Test / Subject	RF-Latex test		
	Positive No (%)	**Negative No (%)**	**Total**
RA(MTX)	**140(47.62%)**	**154(52.38%)**	**294**
RA	39(78%)	**11(22%)**	**50**
Healthy	6(12%)	**44(88%)**	**50**

These anti-inflammatory effects may account for the reduction in acute-phase reactants and autoantibody generation (Nozaki *et al.*, 2013). The evaluation of RF may be useful in clinical practice, not only as a diagnostic tool but also for predicting possible clinical improvement with anti-TNF-α therapy (Oewierkot *et al.,* 2012).

Another potential marker for increasing the risk of RA disease is the C-reactive protein (CRP), since CRP is a sensitive marker of systemic inflammation and it is elevated in patients with RA. Additionally, CRP correlates with response to therapy as CRP levels decrease or normalize in RA patients following effective treatment (Otterness, 1994). Among RA patients groups, there was a significant difference ($P<0.05$) among studied groups. Among RA groups the lower percentage of positive CRP was recorded in patients treated with MTX 72(76.59%), while in untreated RA patients 43(86%) of positive CRP compared with 5(10%) in healthy group as shown in fig. (2).

Table (2): The percentage distribution of CRP in studied subjects by Latex agglutination test (LAT).

Test / Subject	C-RP-Latex test		
	Positive No (%)	**Negative No (%)**	**Total**
RA(MTX)	**72(76.59%)**	**32(34.04%)**	**294**
RA	**43(86%)**	**7(41%)**	**50**
Healthy	**5(10%)**	**45(90%)**	**50**

This study was clarified that CRP positivity was decreased in patients who were treated with MTX, because these patients with well-established RA shown to effectively improve clinical, functional and radiographic outcomes. MTX was more effective on CRP level, as is true for any drug (Sesin and Bingham, 2005).TNF inhibitors (e.g. MTX) show substantial efficacy in combination with other drugs, providing rapid and substantial benefit and improvement in patient outcomes (Breedveld and Combe, 2011); this is because of significant reductions in CRP concentrations (Catrina *et al.*, 2002).

The current study tried to estimate the actual recent or past infection of toxoplasmosis in RA patients treated with MTX by using more specific tests ELISA-IgM and ELISA-IgG. The presence of acute toxoplasmosis characterized by the

presence of positive anti-*Toxoplasma* IgM antibodies while the chronic infection characterized by the presence of anti-*Toxoplasma* IgG, as shown in table (3). The results were shown that RA patients treated with MTX has higher percentage of toxoplasmosis IgM and IgG 60(20.4%), and 98(33.3%), positive cases, in RA patients 4(8%), and 18(36%) while it was 0(0%), 6(12%) in healthy group (those with negative toxoplasmosis). The statistical analysis showed significant differences (P< 0.05) between the studied groups.

Table (3): The percentage distribution of studied subjects infected with toxoplasmosis diagnosed by ELISA test (IgG, IgM).

Test / Subject	ELISA test					
	Total	IgM		IgG		P value
		Positive No (%)	Negative No (%)	Positive No (%)	Negative No (%)	
RA+MTX	294	60 (20.4%)	234(79.6 %)	98(33.3%)	198 (66.7%)	0.000
RA	50	4 (8%)	46 (92%)	18 (76.92%)	32 (23.08%)	0.000
Healthy	50	0	100 (100%)	6 (12%)	44 (88%)	0.003
P< 0.05* significant differences						-

The results of RA patients treated MTX were lower in IgM seropositive but higher in IgG. These results were higher than the results that have been obtained by Walle *et al.*, (2013), who used ELISA test in detecting toxoplasmosis in HIV patients. They showed that out of 103 positive result, 11(10.7%) gave anti-*Toxoplasma* IgM antibodies and 90(87.4%) gave anti-*Toxoplasma* IgG antibodies. On the other hand, our results disagreement with study done by Beladi Mosuvi and Faramarzi, (2013) were they showed that anti-*Toxoplasma* IgG antibodies were positive in 45.45% of patients, while anti-*Toxoplasma* IgM antibodies were negative in transplantation recipient patients.

The present study clarified that the RA patients treated with MTX drug were highly infected with acute toxoplasmosis than untreated RA patients. Meanwhile chronic infection was higher in patients treated with MTX. These findings are similar to that of Lassoued *et al.*, (2007), who mentioned that the risk

of serious toxoplasmosis infection occurred during anti-TNF- α therapy.

Chronic infection in patients treated with MTX drug was higher than untreated RA patients that mean the ability of MTX drug decrease patient's immunity. However, numerous studies have demonstrated that TNF-α acts within a complex network of cells and mediators of inflammation (Wong *et al.,* 2008). Inhibition of its action by MTX drug reduces the inflammatory response which is especially useful for treating autoimmune diseases. MTX inhibits the production of proinflammatory cytokines like TNF-α. TNF-α is the most important cytokine in the inflammatory process in RA patients (Ernst et *al*. 2010). MTX drug was administrated in low dose (5-25 mg/week) (Felson *et al.,* 1996). The high percentages of toxoplasmosis infections in this study may happen due to the effect of the drug (type of drug, dose, specific action and mode of action), or to the patient biological behavior (their consumption of contaminated food, under cooked infected meat, contaminated water and dealing with domestic cats or birds). Untreated RA patients infected with toxoplasmosis, their incidence of acute infection was 4(8%), while chronic infection was 18(76.92 %). These results were in line with (Shapira *et al.,* 2012) who recorded that acute toxoplasmosis in RA patients was 5%, while chronic infection was 42 %. RA disease causes an increase in the body's immune response, and hence the *T. gondii* parasite cannot reactivate chronic infections, so the parasite remains in the bradizoite forms. This interprets the higher percentage of chronic than the acute infections while the incidence of acute infection in group A was higher than chronic infections due to the treatment action on immune response.

References

1. Firestein, G. S. (2003). Evolving concepts of rheumatoid arthritis. Nature., 423: 356-361.
2. Stolt, P.; Bengtsson, C.; Nordmark, B.; Lindblad, S.; Lundberg, I.; Klareskog, L. and Alfredsson, L. (2003). Quantification of the influence of cigarette smoking on rheumatoid arthritis. Ann. Rheum. Dis., 62:835-841.
3. Goronzy, J. and Weyand, E. (2009). Developments in the scientific understanding of rheumatoid arthritis. Arthritis Res. Ther., 11: 249.
4. Zwerina, J.; Redlich, K.; Schett, G. and Smolen, J. S (2005). Pathogenesis of rheumatoid arthritis: targeting cytokines. Ann. N. Y. Acad. Sci. 105: 716-729.

5. Silman, A. J.; Hennessy, E. and Ollier, B. (1992). Incidence of rheumatoid arthritis in a genetically predisposed population. Br. J. Rheumatol., 31:365-368.

6. Goldbach-Mansky, R.; Lee, J.; McCoy, A.; Hoxworth, J.; Yarboro, C.; Smolen, J. S.; Steiner, G.; Rosen, A.; Zhang, C.; Ménard, H. A.; Zhou, Z. J.; Palosuo, T.; Van Venrooij, W. J.; Wilder ,R. L.; Klippel, J. H.; Schumacher, HR. Jr. and El-Gabalawy, H. S. (2000). Rheumatoid arthritis associated autoantibodies in patients with synovitis of recent onset. Arthritis Res., 2:236-243.

7. Pisetsky, D. (2002). Tumor necrosis factor blocker in rheumatoid arthritis. NEJM. 40(100): 1189-1190.

8. Adebajo, A. (2010). ABC of Rheumatology. 4th ed. Blackwell Publishing Ltd. U.K.

9. Fauci, A. S. (2010). Harrison's Rheumatology 2nd Ed. The Mc Grow Hill - Companies, Inc. London.

10. Donahue, K. E.; Gartlehner, G.; Jonas, D. E.; Lux, L. J.; Thieda, P.; Jonas, B. L.; Hansen, R. A.; Morgan, L. C. and Lohr, K. N. (2008). Systematic review: Comparative effectiveness and harms of disease-modifying medications for rheumatoid arthritis. Ann. Intern. Med., 148(2):124-34.

11. Weinblatt, M. E. (1996) . Methotrexate in rheumatoid arthritis: toxicity issues. Br. J. Rheumatol., 35:403-406.

12. Zalevsky, J.; Secher, T.; Ezhevsky, S. A.; Janot, L.; Steed, P. M.; O'Brien, C.; Eivazi, A.; Kung, J.; Nguyen, D. H.; Doberstein, S. K.; Erard, F.; Ryffel, B. and Szymkowski, D. E. (2007). Dominant-negative inhibitors of soluble TNF attenuate experimental arthritis without suppressing innate immunity to infection. J. Immunol. 179 (3): 1872-1883.

13. Sfikakis, P. P. (2010). The first decade of biologic TNF antagonists in clinical practice: lessons learned unresolved issues and future directions. Curr. Dir. Autoimmun., 11: 180-210.

14. Negash, T.; Tilahun, G. and Medhin, G. (2008). Seroprevalence of Toxoplasma gondii in Nazareth town Ethiopia. East. Afr. J. Public Health. 5(3):211-214.

15. Dubey, J.P. (2010). Toxoplasmosis of animals and humans, 2nd ed. Boca Raton: CRC Press. 313 p.

16. Elmore, S. A.; Jones, J. L.; Conrad, P. A.; Patton, S.; Lindsayand, D. S. and Dubey, J. P. (2010). *Toxoplasma gondii*: pidemiology, feline clinical aspects, and prevention. Trends. Parasitol., 26:190-196.

17. Cenci-Goga, B. T.; Rossitto, P. V.; Sechi, P.; McCrindle, C. M. and Cullor, J. S. (2011). Toxoplasma in animals, food, and humans: an old parasite of new concern. Foodborne. Pathog. Dis., 8:751-762.

18. Bennouna, S.; Bliss, S. K.; Curiel, T. J. and Denkers, E. Y. (2003). Cross-talk in the innate immune system: neutrophils instruct recruitment and activation of dendritic cells during microbial infection. J. Immunol.,171:6052-6058.

19. Lappalainen, M.; Jokiranta, T. S.; Halme, L.; Tynninen, O.; Lautenschlager, I.; Hedman, K.; Höckerstedt, K. and Meri, S. (1998).Disseminated toxoplasmosis after liver transplantation case report and review. Clin. Infect. Dis., 27: 1327-1328.

20. Johnson, J.; Suzuki, Y.; Mack, D.; Mui, E.; Estes, R.; David, C.; Skamene, E.; Forman, J. and McLeod, R. (2002). Genetic analysis of influences on survival following Toxoplasma gondii infection. Int. J. Parasitol., 32(2):179-188.

21. Johnson, L. L., and Sayles, P. C. (2002). Deficient humoral responses underlie susceptibility to Toxoplasma gondii in CD4-deficient mice. Infect. Immun., 70:185-191.

22. Strupuviene, S.; Lapiniene, G.; Redaitiene, E.; Kirdaite, G. and Dadoniene, J. (2005). Rheumatoid arthritis marker: antibodies against cirullinated peptides. Acta Medica Lituanica., 12(3): 37-41.

23. Catrina, A. I.; Trollmo, C.; af Klint, E.; Engstrom, M.; Lampa, J.; Hermansson, Y.; Klareskog, L. and Ulfgren, A. K. (2005). Evidence that anti-tumor necrosis factor therapy with both etanercept and infliximab induces apoptosis in macrophages, but not lymphocytes, in rheumatoid arthritis joints: extended report. Arthritis Rheum. 52:61-72.

24. Nozaki, Y.; Kinoshita, K.; Funauchi, M. and Matsumura, I. (2013). A response of TNF? Inhibitor treatment outcome in patients with rheumatoid arthritis. OA Immunology., 1(1):1.

25. Oewierkot, J.; Szmyrka-kaczmarek, M.; Korman, L.; Sokolik, R. and Wiland, P. (2012). Effect of methotrexate on serum levels of anti-CCP antibodies and different classes of rheumatoid factors in rheumatoid arthritis patients. Centr. Eur. J. Immunol., 37(3): 253-257.

26. Otterness, I. G. (1994). The value of C-reactive protein measurement in rheumatoid arthritis. Semin. Arthritis. Rheum. 24(2):91- 104.

27. Sesin, C. A. and Bingham, C. O. (2005). Remission in rheumatoid arthritis: wishful thinking or clinical reality? Semin. Arthritis Rheum. 35:185-196.Breedveld, F. C. and Combe, B. (2011). TNF inhibitors show substantial efficacy in combination with MTX, providing rapid and substantial benefit and improvement in patient outcomes. Arthritis Research & Therapy., 13(1):3.

28. Lassoued, S.; Laurent Zabraniecki, L.; Frédéric Marin, F. and Billey, T. (2007). Toxoplasmic chorioretinitis and antitumor necrosis factor treatment in rheumatoid arthritis. Semin. Arthritis Rheum., 36:262-263.

29. Wong, M.; Ziring, D.; Korin, Y.; Desai, S.; Kim, S.; Lin, J.; Gjertson, D.; Braun, J.; Reed, E.; and Singh, R.R. (2008). TNF-alpha blockade in human diseases: mechanisms and future directions. Clin. Immunol., 126:121-136.

30. Ernst, P.; Garrison, J. and Thompson, L. (2010). Much ado about adenosine: adenosine synthesis and function in regulatory T cell biology. J. Immunol., 185:1993-1998.

Iraqi Journal of Biotechnology, 2015, Vol. 14, No. 2 , 340-352

Detection of *Streptococcus pneumoniae* causing Bacterial Meningitis by Molecular ways for Detection of Virulence factor Autolysin *lyt A*

Huda Z., Al-Banae[1]; Khalid A., Habib [2] and Kifah A., Al-Khurki[3]

[1] Department of Biology, College of Science, Al-Mustansiriyah University
[2] Department of Biology, College of Science for women, Baghdad University
[3] The Central Public Health Laboratory, Health Ministry

Abstract: The aim of the present study is to assess the presence of autolysin gene as a virulence factor that may contribute in pathogenicity of *Streptococcus pneumoniae* causing bacterial meningitis in children. Non-culture tests such as latex agglutination test and PCR for *S. pneumoniae* diagnosis were considered for patients who have previously received antibiotics or who need early identification of pathogens or whose initial cerebrospinal fluid Gram's stain is negative with negative culture at (72) hr incubation. There were (303) cases delivered their cerebrospinal fluids samples to the Central Public Health Laboratory . Seventy seven (25.41%) cases were diagnosed as bacterial meningitis , (36) (11.88%) cases as viral meningitis . *S. pneumoniae* was isolated from (16) cases (20.77 %) of bacterial meningitis. DNA from *S. pneumoniae* was extracted then subjected to amplification by simplex PCR leading to detecting of autolysin in (9) out from (16) isolate, while the autolysin was detected in all isolates by Real Time PCR.

Keywords: *S.pneumoniae*, Bacterial meningitis , *lyt A* , Real Time PCR

تشخيص *Streptococcus pneumoniae* المسببة لالتهاب السحايا البكتيري بأستخدام الطرق الجزيئية للتحري عن جين عامل الضراوة Autolysin *lyt A*

هدى زهير البناء[1] ، خالد عبد الرزاق حبيب[2] ، كفاح احمد الكرخي[3]

[1] قسم علوم الحياه ، كليه العلوم ، الجامعة المستنصرية
[2] قسم علوم الحياه ، كليه العلوم للبنات ، جامعه بغداد
مختبر الصحة المركزي ، وزاره الصحة[3]

الخلاصة: الهدف من هذا البحث هو التحري عن جين Autolysin كعامل ضراوه يمكن ان يساهم في امراضيه بكتريا *Streptococcus pneumoniae* المسببه لالتهاب السحايا البكتيري في الاطفال. الاختبارات غير المعتمده على الزرع مثل اختبار التلازن واختبار تضخيم الدنا التضاعفي التسلسلي لتشخيص بكتريا *Streptococcus pneumoniae* له اهميه خاصه بالنسبه للمرضى الذين تناولوا علاجا من المضادات الحيويه مسبقا او الذين يحتاجون الى تشخيص مبكر للممرضات المسببه او المرضى الذين نماذجهم من سائل النخاع الشوكي سالبه للتصبيغ بصبغه كرام مع نتيجه زرع سلبيه بعد (72) ساعه من الحضن. تم تسليم (303) عينه من سائل النخاع الشوكي الى مختبر الصحه المركزي . شخصت (77) حاله (25.41 %) كحالات التهاب سحايا بكتيري و (36) حاله (11.88%) كحالات التهاب سحايا فايروسي. عزلت بكتريا *S. pneumoniae* من (16) حاله (20.77%) من حالات التهاب السحايا البكتيري. تم تضخيم الدنا المستخلص من بكتريا *S. pneumoniae* بتقنيه تضخيم الدنا التضاعفي التسلسلي simplex PCR للكشف عن جين Autolysin ، ولوحظ أن Autolysin موجود في (9) عزلات من اصل (16) عزله بينما استخدام تقنيه Real Time PCR كشفت عن وجوده في كل العزلات.

Introduction

S. pneumoniae (pneumococcus) is a leading human pathogen that usually asymptomatically colonizes the mucosal surfaces of the upper respiratory tract in early childhood (1). Once carriage is established, however, *S. pneumoniae* may invade several sterile sites, leading to what is known as invasive pneumococcal disease. Indeed, the pneumococcus is responsible for episodes of community-acquired bacteremic pneumonia, bacteremia and meningitis, mainly in children, the elderly, and immunocompromised patients (2).

Over recent years non-culture techniques such as specific bacterial nucleic acid amplification, serology and antigen detection have considerably developed and been applied within research studies to clinical samples, significantly increasing pathogen detection. There are promising signs of improved diagnostic yields when using molecular techniques to detect pneumococcal gene sequences especially when pathogens have traditionally been difficult to detect and treatment is usually successful with empiric antibiotics. However, directed antibiotic treatment has significant benefits in terms of antibiotic stewardship and these new technologies make this goal a possibility, though not yet a reality (3).

Lyt A is also responsible for the characteristic autolytic behavior of the pneumococcus, the bacteriolysis caused by β-lactam antibiotics (4). Lyt A belongs to the amidase-2 family of proteins , which includes Zn-dependent NAM-amidases and the peptidoglycan-recognition proteins (highly conserved pattern-recognition molecules of the immune system) (5).

All the isolates from both invasive and ocular infections were *lyt A* positive signifying that irrespective of site of isolation, kind of infection caused and autolysin is an obligate necessity for the *S.pneumoniae* isolates (6).

So the aim of this study was to compare between conventional and Real Time PCR of *lyt A* in *S.pneumoniae* isolated from bacterial meningitis in children.

Materials and Methods :

The study included a total of 303 samples of CerebroSpinal Fluid (CSF) collected at different hospitals which included : Al-Kadhymia Pediatric Hospital , Central Child Hospital, Children Welfare Teaching hospital, Elwiyah Pediatric Hospital, Basrah General Hospital) from April to September,2010.

Once the CSF has arrived at the microbiology laboratory, it was centrifuged for 20 minutes at 2000 rpm. The supernatant was drew off with a Pasteur pipette. One or two

drops of sediment were used to prepare the Gram stain and 1 drop were used to streak the primary culture media (Blood agar and Chocolate agar plates).

The agar plates were incubated at 37ºC in 5% CO_2 for 18-24 hours. The α-haemolytic colonies with morphology indicative of *S.pneumoniae* in Gram staining were subcultured to a fresh plate with optochin disk (5 mg) .

Detection of Bile solubility was done by preparing each isolate as a suspension of bacterial cells from fresh growth on agar plate which were suspended in 1 ml of saline to make heavy suspension (McFarland 2.0 or greater). The suspension was divided in half in two glass tubes, and 0.5 ml of saline was added to one tube and 0.5 ml of 10% deoxycholate was added to the other. The tubes were incubated up to 30 minutes at 35°C. Lysis of the cells in the tube with deoxycholate indicates a positive test (7). Isolates that exhibited an inhibition zone of 14 mm or more around the optochin disk and those showing bile-solubility were identified as *S.pneumoniae.*

A- Simplex PCR for detection of autolysin gene

1- DNA Preparation

DNA isolation from *S.pneumoniae* isolates was performed according to (8):

1- Isolates from which DNA was to be extracted were grown for an overnight at 37 ºC on blood agar plates. Bacteria were resuspended in 500 µl SET Buffer.

2- 10 µl of lysozyme solution was added and mixed in vortex , then incubated for (30-60) min at 37 ºC.

3- 14 µl of Protease solution was added and mixed in a vortex , 60 µl of 10 % SDS was added and mixed by inversion , then incubated for 2 hr at 55 ºC in incubator or 55 ºC overnight in water bath.

4- 200 µl of 5 M NaCl was added and mixed thoroughly by inversion and let cool to 37 ºC.

5- 500 µl of Chloroform was added and mixed by inversion for 30 min at 20 ºC. Then it was centrifuged for 15 min at 4500 rpm at 20 ºC. Supernatant was transferred to fresh tube .

6- 600 µl of Isopropanol was added and mixed by inversion and then centrifuged at 4500 rpm at room temperature for 15 min.

7- DNA was rinsed in 200 µl 70 % Ethanol, centrifuged 14000 rpm for 2 min , air dried and dissolved in 50 µl of TE Buffer.

2- Primers preparation:

Lyophilized forward and reverse primers mentioned in Table (1) were suspended with distilled water as recommended by Alpha- DNA company protocol (USA).

Table (1) Primers of PCR

Gene	Sequence of primers (5´⟶3´)	Gene size (bp)	Reference
Lyt A	Forward primer CAA CCG TAC AGA ATG AAG CGG Reverse primer TTA TTC GTG CAA TAC TCG TGC G	319	(6)

3- PCR working solution:

PCR reaction was conducted in 20 µl of a reaction mixture containing DNA , Gotaq Green Master , $MgCl_2$, forward and reverse primer and distilled water . Optimization of polymerase chain reaction was accomplished after several trials, thus the following mixture were adopted as mentioned in Table (2) and amplification was conducted using a master cycler (Eppendorf) programmed as mentioned in Table (3).

Table (2) The mixture of Simplex PCR working solution

Working Solution for simplex PCR of *lyt A*	
Distilled Water	4 µl
Gotaq Master Mix	10 µl
Forward Primer	2 µl
Reverse Primer	2 µl
$MgCl_2$	-
DNA	2 µl
Final volume = 20 µl	

Table (3) PCR Programs

Gene	Initial denature-tion	No. of cycles	Final extension
Lyt A	5 min at 94 ºC	30 cycles , each cycle consist-ing of : 30 sec at 94 ºC 30 sec at 53 ºC 30 sec at 72 ºC	10 min at 72 ºC

PCR end products were analyzed on 1 % agarose gel for *Lyt A* stained with ethidium bromide and the bands were visualized under UV illumination (260 nm).

B- Real Time PCR for detection of autolysin gene

Briefly, during real-time PCR, the fluorogenic probe and PCR primers first hybridize to their DNA targets. With the fluorogenic probe still intact, the emission of the reporter dye is quenched, but during the PCR extension phase the probe is cleaved by the 5´-exonuclease activity of the Taq DNA polymerase (9).

This cleavage interrupts the fluorescence resonance energy transfer and permits the reporter dye to fluoresce, with the level of fluorescence produced being in proportion to the level of PCR product accumulation. Furthermore, the increment in the signal from the degraded fluorogenic probe can be continuously monitored throughout the course of gene amplification. The background fluorescence is often determined from cycles 3 to 15, and an automated software feature calculates 10 times the standard deviation to produce a threshold value. The cycle threshold (CT) value is defined as the cycle at which the reporting dye fluorescence first exceeds the calculated background level identifying amplification of the target sequence. A low CT value thus corresponds to a high target concentration. The CT value can also be set manually to be equivalent across experiments (9).

Specific primers selected for Real Time PCR analysis of *lyt A* gene are shown in Table (4) .

Table (4) Primers and probe sequences for Real Time PCR of *lyt A* gene

Sequence of primer (5´ ⟶3´)	**Reference**
Forward primer ACG CAA TCT AGC AGA TGA AGC A Reverse primer TCG TGC GTT TTA ATT CCA GCT Probe FAM-GCC GAA AAC GCT TGATAC AGG GAG-BHQ1	(10)

The Real Time PCR amplifications were performed in 25 µl reaction volumes as mentioned in Table (5) . All reactions were performed in duplicate. Standard amplification parameters were used with the Applied Biosystem 7500 Real Time PCR System device as mentioned in Table (6).

Table (5) Working solution for Real Time PCR of *lyt A* gene

Working solution for Real Time PCR of *lyt A* gene	
Distilled Water	8.5 µl
Gotaq master mix	12.5 µl
Forward Primer	0.5 µl
Reverse Primer	0.5 µl
Probe	0.5 µl
DNA	2.5 µl
Final volume 25 µl	

Table (6) Program for Real Time PCR of *lyt A* gene

Thermal Cycling	
1 cycle	95 ºC for 10 min
40 cycle	95 ºC for 15 sec
	60 ºC for 1 min

To determine the sensitivity and the detection range of the Real Time PCR assay, a standard curve for *S. pneumoniae* was generated according to (9) as follows:

1- *S. pneumoniae* was grown aerobically on Blood agar medium at 37 ºC for 4 hr to reach the logarithmic phase.

2- This culture was diluted with Normal saline until it reached a McFarland tube No. 0.5.

3- Starting from this concentration, 10-fold serial dilutions in Normal saline were prepared, One milliliter of each dilution was used for DNA extraction

4- Amplification *in vitro* as described above was done. The calculated cycle threshold (CT) values were then plotted against the numbers of microorganisms.

5- Each of the 10-fold serial were diluted again by taking 100 µl of

each dilution to 900 µl 0f Normal saline , then plating 100 µl of each dilution onto Blood agar plates and then aerobic incubation overnight at 37 ºC in order to determine the number of CFU in each dilution

Results and Discussion

There were (303) cases comprised of (183) males (60.39 %) and (120) females (39.60 %) . Cases were diagnosed by physician as bacterial meningitis by (77) cases (25.41%) , viral meningitis by (36) cases (11.88 %). *S. pneuminae* appeared from (16) CSF samples (20.77 %) , Patient´s information form showed that antibiotics were taken by patients before lumbar puncture in (124) cases (40.92 %) .

DNA extraction was done according to (8) as shown in Figure (1).

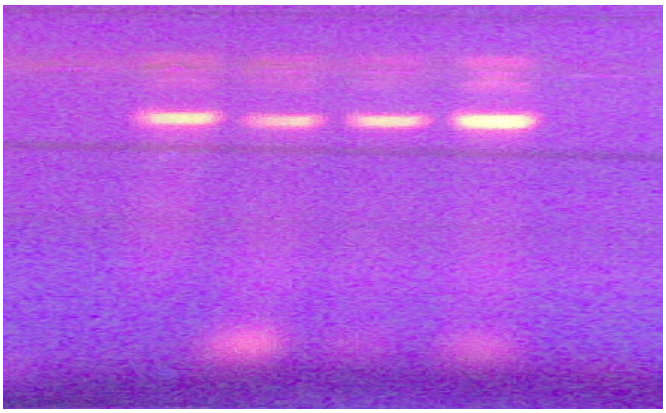


Figure (1) Extracted DNA from *S. pneumoniae*, using 1 % Agarose for 1 hr at 5 volt/cm in TBE Buffer

Conventional simplex PCR of the most important virulence gene *lyt A* was used to identify *S.pneumoniae* . The primers of gene was mentioned in Table (1) . After desolation of primers, mixtures of PCR working solutions mentioned in Table (2) were prepared , then PCR device was programmed depending on program mentioned in Table (3).

Gene *lyt A* appeared in 9 out of 16 isolates for *S.pneumoniae* as shown in Figure (2).

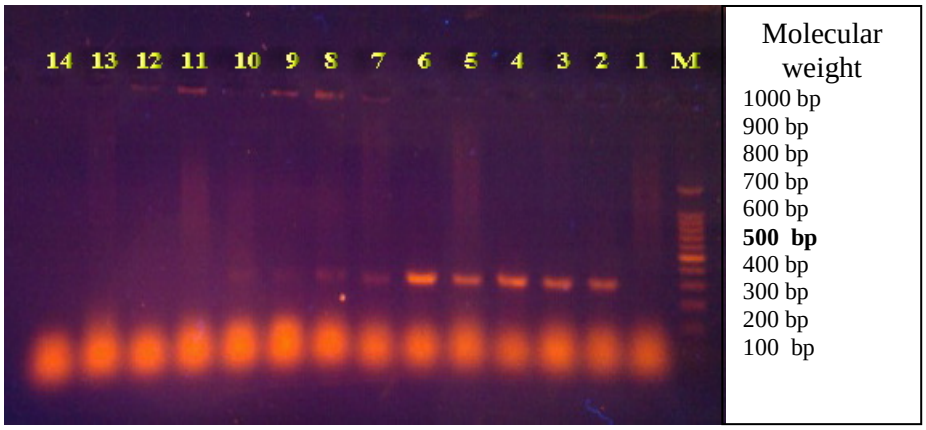


Figure (2) Presence of autolysin gene detected by the amplification of the 319 bp fragment of the *lyt A* gene ,using 1 % Agarose for 1 hr at 5 volt/cm in TBE Buffer

Lane M- 100 base pair Molecular marker. Lane 1- Negative control. Lane 2-10 positive *lyt A S.pneumoniae.* Lane 11-14 negative *lyt A S.pneumoniae*

Real Time PCR was used to identify *S.pneumoniae* by the most important virulence gene *lyt A* . The primer and probe sequence of the gene were mentioned in Table (4) . Mixtures of Real Time PCR working solution mentioned in Table (5) were prepared , then Real Time PCR device was programmed depending on program mentioned in Table (6). The first trial of Real Time PCR was done by using positive control of *S. pneumoniae* in order to insure of kit validity .

The second trial of Real Time PCR was done by using (16) isolates of *S. pneumoniae* in duplicate with a negative control depending on thermal cycling shown in Table (6).

The amplification curve of isolates of *S. pneumoniae* showed positive amplification of *lyt A* gene in all isolates as shown in Figure (3).

Figure (3) Amplification curves of positive *lyt A S. pneumoniae*

Results of cases diagnosis by cultural and molecular ways can be summarized as shown in Table (7).

Table (7) Diagnosis of *S.pneumoniae* causing bacterial meningitis

Cases	**No.**	303
Bacterial meningitis	**No.**	77
	%	25.41
S.pneumoniae	**No.**	16
	%	20.77
S.pneumoniae* diagnosed by simplex PCR for *lyt A	**No.**	9
	%	56.25
S.pneumoniae* diagnosed by Real Time PCR for *lyt A	**No.**	16
	%	100

Streptococcus pneumoniae is the leading cause of pneumonia and meningitis in children and older adults. Mortality among children with pneumococcal meningitis is at least double the rate of mortality of meningococcal meningitis and survivors have a higher incidence of sequelae (11).

Molecular methods have the advantage of providing timely and accurate results with automation, are independent of the effects of previous antibiotics and are characterized by high specificity for their targets as shown in this and other studies (12).

The sensitivity of Real Time PCR is double that of culture in the diagnosis of meningitis/sepsis and it is about ten times higher in patients with pneumonia. Patients with meningitis and sepsis usually have a much higher bacterial load, as demonstrated by Real Time PCR , so that cultural methods, though less sensitive, can detect infection; patients with meningitis/sepsis usually have a rapid progression of the disease, becoming severe in a few hours, so it is less probable that they have received prolonged antibiotic therapy before hospital admission (13).

A non cultural identification can be due to several factors mainly previous antimicrobial therapy and including for CSF, the time lapse between the lumbar puncture and the smear of the samples. CSF requires immediate processing, otherwise the agents will die (14).

This last point is relevant to hospitals because it is frequent that samples are collected but not immediately processed. Also antibiotics were taken by patients before lumbar puncture in (124) cases (40.92 %) this may led to false-negative CSF culture results .

Identification based on morphology is often not possible. Culture results may be available only after 24 hr to 72 hr. Moreover, culture results may be false negative when fastidious or culture-resistant bacteria are involved or when patient samples are obtained after antimicrobial therapy has started (15).

In order to strengthening of *S.pneumoniae* identification based on the previous reasons, molecular assays could be done with many advantages including more sensitive , more specific and short time needed for confirmed diagnosis of the etiological agents of bacterial meningitis .

Although conventional approaches to identification are still valid and useful, rapid methods and rapid reporting truly became an inevitable goal for clinical microbiology laboratories. Rapid diagnostics can greatly impact patient care, direct the use of appropriate antimicrobial therapies, and reduce hospitalization and overall medical costs (16).

The failure of PCR in these CSF samples possibly is due to the presence of a substance inhibitory for the reaction. Although less likely, another possible explanation for this result would be inadequate conditions of collection and transport. The presence of PCR inhibitory factors could be greatly reduced when bacterial DNA is isolated upon receipt and it also concentrates the DNA present in the sample (17).

Knowing that culture techniques are sensitive to the presence of antibiotics and that PCR analysis is not , I would predict that among

patients whose cultures were negative but whose PCR results were positive there would be a concentration of individuals with evidence of antibiotics (18).

Their findings support my results by finding patients by (124) cases (40.92 %) who taken antibiotics before lumbar puncture resulting of negative culture of the etiological agents of bacterial meningitis .

Actually, it is well known that culture may often give false-negative results because of small sample volume, previous antibiotic therapy (19), or unsatisfactory conditions of sample transport and storage which can impair the viability of pathogens. In all the cases where culture growth is not obtained, also serotyping cannot be performed neither with Quellung reaction nor with molecular methods applied on isolates (20).

The amplification curve of isolates of *S. pneumoniae* showed positive amplification of *lyt A* gene as shown in Figure (3).

One of the main advantages of Real Time PCR is the ability to produce results within a clinically useful period. The turnaround time for the assay is less than 4 hr. It is also possible that the quantitative bacterial load could impact on site of care decisions (outpatient, inpatient, intensive care, etc.). Consistent with this hypothesis, the patient with the highest bacterial load died. However, a much larger cohort of patients will be needed to assess the real clinical utility of the quantitative bacterial load (21).

In accord with (21) that Real Time PCR is more sensitive than culture or end-point PCR for diagnosis of pneumococcal infection . As for serotyping they demonstrated that Multiplex Sequential-PCR can be used directly on whole blood samples for *S. pneumoniae* serotyping so giving results where cultural methods fail (20).

References

1. Crook, D.W. ; Brueggemann ,A. B. ; Sleeman, K. L. and Peto ,T. A. (2004) Pneumococcal carriage. In: " The Pneumococcus". Tuomanen ,E. ; Mitchell ,T.J. ; Morrison ,D.A. and Spratt ,B.G. editors.. Washington, D.C.: ASM Press. p.:136-147.
2. Henriques-Normark ,B. and Tuomanen, E. I. (2013). "The Pneumococcus: epidemiology, microbiology, and pathogenesis". Cold Spring Harb Perspect Med. 3:a010215.
3. Julia E Clark .(2015) Determining the microbiological cause of a chest infection.Review. Paediatric Infectious Diseases, Infection Management and Prevention Service, Royal Children’s Hospital. Australia .

4. López ,R. and García, E. (2004). Recent trends on the molecular biology of pneumococcal capsules, lytic enzymes, and bacteriophage. FEMS Microbiol Rev. 28:553-580.

5. Zhang ,Z. ; Long ,Q.X. ; Xie ,J. (2012). Roles of peptidoglycan recognition protein (PGRP) in immunity and implications for novel anti-infective measures. Crit. Rev. Eukar Gene Expr. ,22: 259-268.

6. Sourav, S. ; Patricia, A. ; Sharma, S. ; Kanungo, R. ; Jayachandran, S. and Prashanth , K. (2010) Detection of pneumolysin and autolysin genes among antibiotic resistant *S.pneumoniae* in invasive infections. Indian J. Med. Microbiol.,28(1): 39-4.

7. Messmer,T.O. ; Sampson,J.S. ; Stinson, A. ; Wong, B. ; Carlone,G.M. and Facklam, R.R. (2004) Comparsion of four polymerase chain reaction assays for specificity in the identification of *S.pneumoniae* .Dia. Microbiol. Infect. Dis.,(49):249-254.

8. Pospiech ,A. and Neumann,B. (1995) A versatile quick-prep of genomic DNA from Gram positive bacteria. Technical tips. TIG June.,11(6): 217-218.

9. Greiner ,O. ; Day , P.J. ; Bosshard, P.P. ; Imeri, F. ; Altwegg, M. and Nadal, D. (2001) Quantitative detection of *S.pneumoniae* in nasopharyngeal secretions by Real-Time PCR .J. Clin. Microbiol. ,39(9):3129-3134.

10. Carvalho ,M.D. ; Tondella, M.L. ; McCaustland, K. ; Weidlich, L. ; McGee, L. ; Mayer ,L.W. ; Steigerwalt, A. ; Whaley ,M. ; Facklam, R.R. ; Fields, B. ; Carlone, G. ; Ades, E.W. ; Dagan,R. and Sampson, J. (2007) Evaluation and improvement of Real- Time PCR assays targeting *lyt A*, *ply* ,and *psa A* Genes for detection of pneumococcal DNA. J. Clin. Microbiol. ,45(8): 2460-2466.

11. Tzanakaki, G. and Mastrantonio, P. (2007) Aetiology of bacterial meningitis and resistance to antibiotics of causative pathogens in Europe and in the Mediterranean region. Int. J. Antimicrob Agents, 29**:** 621–629.

12. Albrich, W. C. ; Madhi, A. ; Adrian, P. C. ; van Niekerk, N. ; Mareletsi,T. ; Cutland, C. ; Wong, M. ; Khoosal, M.; Karstaedt,A. ; Zhao, P. Deatly, A. ; Sidhu, M. Jansen, K. and Klugman, K. (2012) Use of a Rapid Test of Pneumococcal Colonization Density to Diagnose Pneumococcal Pneumonia. CID:54: 601-609**.**

13. Azzari, C. ; Moriondo, M. ; Indolfi, G. ; Massai, C. ; Becciolini, L. ; de Martino, M. and Resti, M. (2008) Molecular detection methods and serotyping performed directly on clinical samples improve diagnostic sensitivity and reveal increased incidence of invasive disease by Streptococcus pneumoniae in Italian children. J. Med. Microbiol. : 57, 1205–1212

14. Mandell, A. ; Bennett, F. and Dolin,S. (2005) "Principles and Practice of Infectious Diseases". 6th ed. Churchill Livingstone, Elsevier ,Amsterdam.

15. Sultlana , S. ; Vindo Kumar , C.S. ; Rathod, V. ; Basavarajappa, K.G. and Kalappanavar, K.N. (2010) Utility of Real Time PCR in the rapid diagnosis of pyogenic meningitis . Int. J. Biotech. Biochem. ,6 (2): 175-186.

16. Aygan , A. (2006) Nucleic acid extraction from clinical specimens for PCR applications. Turk. J. Biol. , 30:107-120.

17. Clarke ,S.C. and Edwards, G.F. (2003) Guidance for requests and interpretation of services provided by the Scottish Meningococcus and Pneumococcus Reference Laboratory. SCIEH Weekly Report ,37: 1-6.

18. Scott ,J.A. ; Marston, E.L. ; Hall, A.J. and Marsh, K. (2003) Diagnosis of pneumococcal pneumonia by *psa A* analysis of lung aspirates from adult patients in Kenya. J. Clin. Microbiol. 41 (6):2554- 2559.

19. Resti, M. ; Micheli, A. ; Moriondo, M. ; Becciolini, L. and Cortimiglia, M. (2009) Comparison of the effect of antibiotic treatment on the possibility to diagnose invasive pneumococcal disease by cultural or molecular methods: a prospective observational study of children with proven pneumococcal infection. Clin. Therapeutics, 31: 1266–1273.

20. Azzari, C. ; Moriondo, M. ; Indolfi, G. ; Cortimiglia, M. ; Canessa, C. ; Becciolini, L. ; Lippi, F. ; de Martino, M. and Resti, M. (2010) Real time PCR is more sensitive than multiplex PCR for diagnosis and serotyping in children with culture negative pneumococcal invasive disease . PLoS ONE, 5 (2): e9282.

21. Kee, C. ; Palladino, S. ; Kay, I. ; Pryce, T.M. ; Murray, R. ; Rello, J. ; Gallego, M. ; Lujan, M. ; Munzo-Almargo, C. and Waterer, G.W. (2008) Feasibility of real-time PCR in whole blood to identify *S. pneumoniae* in patients with community-acquired pneumonia. Dia. Micro. Infec. Dis. ,61: 72-75.

Iraqi Journal of Biotechnology, 2015, Vol. 14, No. 2 , 353-361

Evaluation of some bacteriological aspects and Leukotriene B4 Levels as immunological marker In Pneumonia Iraqi Patients

Karam R. Aljarah*, Dr. Rasmiya A. Abu-resha

Deparetment of Biology, Collage of Science, University of Baghdad

Abstract: From may 2013 to Feburey 2014, 120 sputum and Aspiration samples of patients with pneumonia disease were collected from different hospitals in Baghdad included: Al Yarmouk Teaching Hospital, Hospital Nursing Home sector and Baghdad Teaching Hospital. All samples were diagnosed by biochemical tests , Api 20 E and Api20 strep. Systems. The results was found to be 28 isolates (23.3%) belong to *K. pneumoniae* , 26 isolates (21.7 %) belong to *S. pneumonia* while 66 isolates (55%) belong to causes : *E.coli, Pseudomonas spp., Moraxella catrrhalis, S. pyogenes, Monilia and S. aureus*. 55 blood samples from pneumonia patients and 30 blood samples from healthy individuals (control) were collected in this study, 41(74.55%) with acute pneumonia and 14(25.45%) with chronic infection. Leukotriene B4 levels were measured in pneumonia patients sera and control and showed high level in patients compared with control (36.00 ± 3,82 vs. 25.96 ± 4.44 pg / ml , respectively) with a significant difference (P≤0.05).

Keywords: Leukotriene B4 (LTB4), Pneumonia dise**ase**

تحديد بعض الجوانب البكتريولوجية ومستويات لويكوتيرن B4 كعامل مناعي في المرضى المصابين بذات الرئة

كرم رياض الجراح*، رسمية عبد ابو ريشة

قسم علوم الحياة ، كليه العلوم، جامعه بغداد ،بغداد ،العراق

الخلاصة: جمعت 120 عينة قشع من اشخاص مصابين بمرض ذات الرئه للمدة من ايار 2013 الى شباط 2014 من ثلاثة مستشفيات في مدينة بغداد وشملت مستشفى اليرموك التعليمي، مستشفى دار التمريض الخاص ، مستشفى بغداد التعليمي . شخصت العينات باستخدام الفحوص البايوكيميائية ،نظام Api 20 E و نظام Api strep.20 E . وجد ان 28 (23,3%) عزلة تعود للنوع *K. pneumoniae* و 26 (21,6%) عزله تعود للنوع *S.pneumonia* بينما شخصت 66 (55%) عزله من مجموع العزلات كأجناس اخرى من البكتريا السالبة لملون غرام مثل *E.coli* ،*Pseudomonas sp* ، *Moraxella catrrhalis* والموجبه ملون غرام مثل ،*S.pyogens* ، *S.aureus* . والفطريات *Monilia* . جمعت 55 عينة دم من الاشخاص المصابين بذات الرئه و 30 من الاصحاء (السيطره). حيث اوضحت الدراسه ان 41 (74.55 %) اصابه كانت حاده واكثر عددا من الحالات المزمنه 14(25.45%). وكما قيست مستويات الليوكوترين بي 4 (LTB4) في مصل الاشخاص المصابين بمرض ذات الرئه وكان مستواها 36 ± 3,82 بيكو غرام / مل ومقارنتها بالسيطره 25.96± 4.44 بيكو غرام /مل وكانت المستويات مرتفعه معنويا (P≤0.05) لدى المرضى.

Introduction

Pneumonia is the leading cause of childhood morbidity and mortality worldwide. According to the estimates of the World Health Organization(WHO), pneumonia accounts for almost one-fifth of overall childhood mortality [**1]**. Although the introduction of penicillin in the 1940s resulted in a significant reduction in pneumonia mortality in developed countries, the morbidity caused by childhood pneumonia has remained substantial in the developed world [**2,3]**Seum l vels were significantly higher in patients with pneumococcal pneumonia, the decrease in levels of cytokines was independently influenced by the start of corticosteroid therapy [**4]**. Neutrophils are major specific target for IL-8 action. Many pathophysiological actions of IL-8 depend on activation of neutrophils [**5]**. To obtain further insight into the pathogenesis of Acute respiratory distress syndrome (ARDS), Interleukin 8 role was examined in the recruitment of PMN into the lung and PMN activation in this setting. IL-8, a 72-amino-acid peptide, has chemoattractant activity and can induce PMN activation and degredation [**6** LTB4 induces neutrophil adherence to endothelial cells, promotes chemotaxis, stimulates the generation and release of oxidants, and increases 5-LO activation in neutrophils, resulting in enhanced LTB4 synthesis [**7**,**8]**.LTB4 concentrations are enhanced in bronchoalveolarlage (BAL) fluid and chronic obstructive pulmonary disease (COPD) patients [**9]**.LTB4 and its metabolites, due to a “priming” effect on neutrophils, plays an important role in the development two inflammation mediators of polymorphonuclear-neutrophils -(PMN-) induced lung injury[**10]**.*S. pneumoniae* inhibits the complement pathway in several ways. As a gram-positive bacterium, the pneumococcus is resistant to the bactericidal and lytic activities of complement[**11]**. Pneumococcal surface protein A (PspA) prevents the deposition of C3b onto the surface of pneumococci [12]

Material and Methods*:*

Bacterial isolation

120 sputum and aspiration specimems were collected from pneumonia patients and streaked directly on MacConky agar and blood agar incubated at 37ºC for 24 hr. The large , pink, mucoid colonies were selected and subcultured on another MacConky agar to obtain isolated colonies also β-hemolysis colony in blood agar were selected.

Identification of isolates:

The identification of the isolates included morphological characteristics and biochemical tests which carried out depending on Bergey's Manual of Systematic Bacteriology, 2nd edition **(13).**

Specimen's collection

Fifty five blood specimens from pneumonia patients and 30 blood specimens from healthy persons (control) were collected; 2ml of blood were added to sterilized containers then centrifuged at 3000 r.p.m for 10 minutes to obtain serum.

Leukotriene B4 (LTB4) assay write kit name, company ,country were used to masseur LTB4 levels in patients and control by ELISA technique

1. A volume of 100µL of EIA buffer was added to Non-specific binding wells and 50ML of EIA buffer was added to Standard wells.
2. A volume of 50µL of standard was added started from S8 to S1 .
3. A volume of 50µL of sample was added per well (2 dilution 1/10,1/200).
4. A volume of 50µL of Leukotriene B4 Ach E tracer was added to each well except Total activity (TA) and black (Blk) wells.
5. A volume of 50µL of Leukotriene B4 EIA Antiserum was added to each well except (TA),Non-specific binding(NSB) and Blank wells
6. Incubation the plate overnight at 4 C˚ and cover each plate with plastic film.
7. wiped the bottom of the plate with a clean tissue to remove fingerprints, dirt, etc.
8. Removed the plate cover bening careful to keep Ellman's Reagent from splashing on the cover.
9. Read the plate at a wavelength between 405-420 nm. The absorbance may be checked periodically unital the Standard wells have a minimum of 0.3 A.(blank subtr acted). The plate should be read when the absorbance of the Bo wells in the range of 0.3-1.0 A.U (blank subtracted) ,If the absorbance of the wells exceeds 1.5, wash the plate, add fresh Ellman's Reagent and let it develop again.

Statistical analysis

The Statistical Analysis System-SAS (2010) was used to effect of different factors in study parameters. Chi-square test was used to significant compare between percentage &Least significant difference –LSD test was used to significant compare between means in this study.

Results and discussion:

Isolation of Bacteria

From 120 sputum specimen collected from pneumonia patients ,the genus *Klebsiella* Formed 28(23.3%) isolates identified as *K. pneumoniae* while the genus *Streptococcus* Formed 26(21.6 %) isolates identified as *S.pneumoniae*, However 55% out of all specimens represented other bacteria and fungi which identified as : *E.coli* , *Pseudomonas sp, Moraxella catrrhalis, S.aureus, S.pyogens, monilia*as, *as* shown in figure 1.

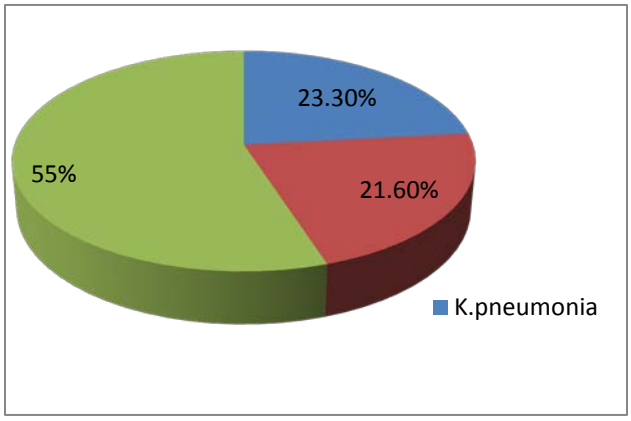


Figure (1): percentages *of bacterial isolates*

In pneumonia patients previous studies, *Klebsiella* spp. formed 54.16% of total clinical isolates while 79.12% was identified as *K. pneumonia* [14]. *neumoniae* was clearly the most common causative agent detected, found in 18 of 34children (53 %) with alveolar pneumonia [15].*S. aureus* has been detected only in 1 % of children with pneumonia in the developed countries [16].In a prospective study of 559 patients hospitalized with pneumonia, patients with a gram-negative bacterial etiology had a significantly higher mortality than those without gram negative bacteria (32% vs 9%) [17]

History of Patients:

- From blood 55 specimens were collected from pneumonia patients, 41 specimenc collected from patients suffering with acute pneumonia infection (74.55%) ,

while 14 (25.45%) specimen collected from patients suffering with chronic pneumonia infection, as shown in figure 2, the percentage of acute pneumonia infection was more than chronic infection in this study with statistical significant difference (P< 0.05). because of most bacterial causative agent of pneumonia are extracellular that cause acute infection , and most chronic infection caused by intracellular agent. Thus, acute pneumonia patients more than chronic patients .

Acute respiratory infections (ARIs) are the most common causes of both illness and mortality in children under five years , who average three to six episodes of ARIs annually regardless of where they live or what their economic situation is[18]Compared 134 patients with Acute respiratory distress syndrome(ARDs) with 744 patients with chronic respiratory distress syndrome (COPDs) and found that non fermenting, gram-negative bacteria caused significantly more cases of pneumonia among patients with ARDS[**19]**.

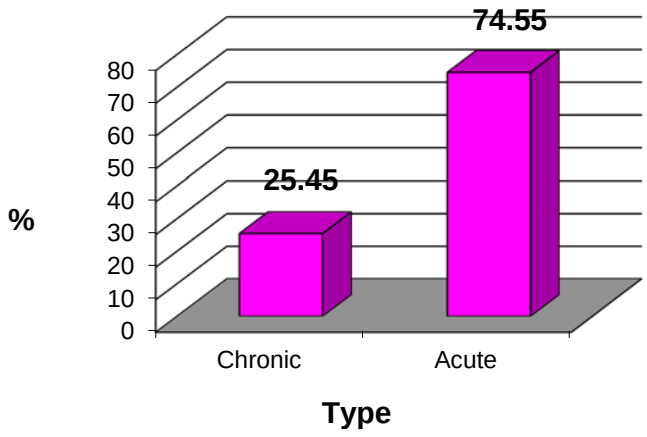


Figure (2): Percentage distrbution of specimen types according to types of disease

Leukotriene B4 (LTB4) Level:

The mean ± SE serum LTB4 concentration in patients groups was generally higher than control groups (36.00 ± 3.82, 25.96 ± 4.44 pg/ml), respectively with statistical significant differences (P < 0.05) as shown in figure 3. In 12 patients who agreed to participate further in the

study the tests were repeated after 2 months when in a stable condition and LTB4 levels were found to decrease further from 10.6 pg/ml to 8.5 pg/ml ($p<0.005$) [**20]**. Patients with exacerbations of chronic obstructive pulmonary distress (COPD) have increased concentrations of LTB4 in the exhaled condensate which return to normal during the recovery period. [**21].**and may be released from macrophages and epithelial cells as well as from activated neutrophils[**22]**. The increase in LTB4 may contribute to the increase in neutrophil influx during an Mexacerbation[**24]**.

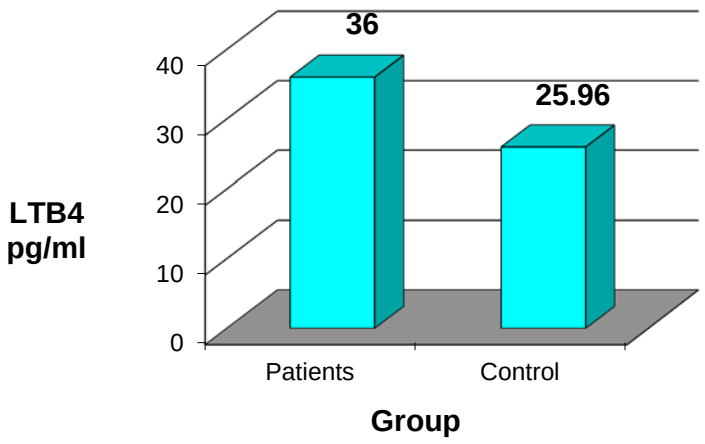


Figure 3 :LTB4 level in pneumonia patients and control

LTB4 levels are increased in the sputum of patients with bacterial exacerbations of COPD and fall rapidly after antibiotic treatment.(23). In another study, which included larger numbers of patients with exacerbations of COPD, the LTB4 level was increased but fell after treatment and remained stable for 56 days[**24]**.Serum levels of LTB4 were determined in 100 patients on admission and for five consecutive days (daily). Twenty healthy volunteers served as control. Thirty patients developed PC (pneumonia, respiratory failure, acute lung injury (ALI), ARDS and pulmonary embolism) and 70 had no PC (ØPC). LTB4-levels in the PC-group (127.8 pg/mL) were significantly higher compared to the ØPC-group on admission (95.6 pg/mL) or control-group (58.4 pg/mL). LTB4 contiously declined to basal levels from day 1 to

5 without differences between the groups.LTB4 was not influenced by overall or chest injury severity, age, gender or massive transfusion. Patients with PC received mechanical ventilation for a significantly longer period of time, and had prolonged intensive care unit and overall hospital stay[**25]**

References

1. Williams, B.G.; Gouws,E.; Bochi-pinto ,C.; Bryce, J.; Dye, C.(2002). Estimates of worldwide distribution of child deaths from acute respiratory infections. Lancet Infect, Dis ;2:25-32
2. 2-Yorita, K.L.; Holman, R.C.; Sejvar .J.J; Steiner, C.A.; Schonberger, L,B.(2008). Infectious disease hospitalizations among infants in the United States. Pediatrics.;121:244-52.
3. Marsh, D R.; Gilroy, K E.; Weerdt Van de R.; Wansi, E.;Shamim Qazi. 2008. Community case management of pneumonia: at a tipping point?, Bulletin of the World Health Organization, Vol. 86, 381–389
4. Endeman, H.; Meijuis, S.C.A.; kers, R.J.; Van-valzen-blad,H.; Van morsel, C.H.; Grutters, J.C.; Biesma, D.H.(2011). Systemic cytokines response in patients with community-acquired pneumonia. EurRespir. J; 37:1431-1438.
5. Birgit Auner; Emanuel, V.; Geiger; Dirk Henrich; Mark Lehnert; IngoMarzi; and Borna Relja.(2012) Circulating Leukotriene B4 Identifies Respiratory Complications after Trauma. Hindawi Publishing Corporation Mediators of Inflammation .
6. Baggiolini, M. A.; Walz, and Kunkel, S.L. (1989). Neutrophilactivatingm peptide-1/interleukin-8, a novel cytokine that activates neutrophils. J. Clin. Invest. 84:1045-1049.
7. Peters-Golden, M.; and Henderson, W.R.(2007). Leukotriene .New England . Jouranl of medicine ; 357: pp 1798-1854.
8. Hicks, A.; Monkarsh, S.P.; Hoffman, A.F.; and Goodnow, R.(2007). “Leukotriene B4 receptor antagonists as therapeutics for inflammatory disease: preclinical and clinical developments,” *Expert Opinion on Investigational Drugs*, vol. 16, no. 12, pp. 1909–1920
9. Antczak, A.; Piotrowski,W.; Marczak, J.; Ciebiada, M.; Gorski, P.; and Barnes, P.J.(2011). “Correlation between eicosanoids in bronchoalveolar lavage fluid and in exhaled breath condensate,” *Disease Markers*, vol. 30, no. 5, pp. 213–220.

10. Eun, J.C.; Moore, E.E.; Banerjee, A.; et al., "Leukotriene b4 and its metabolites prime the neutrophil oxidase and induce proinflammatory activation of human pulmonary microvascular endothelial cells," *Shock*, 2010.

11. Kemper, C.A.; and S. C. Deresinki, S.C. (1994). The immunology of pneumococcal pneumonia, p. 29–49. *In* H. Chmel, M. Bendinelli, and H. Friedman (ed.), Pulmonary infections and immunity. Plenum Press, New York, N.Y.

12. Ren, B.; Szalai, A.J.; Hollingshead, S.K.; and Briles, D.E. (2004). Effects of PspA and antibodies to PspA on activation and deposition of complement on the pneumococcal surface. Infect. Immun. 72:114–122.

13. Farmer, J.J.; Boatwright, K.D. and Janda, J.M. (2007). Enterobacteriaceae*In* : Murray, P.R.; Baron, E.J. ; Jorgensen, J.H.; Landry, M.L.; and Pfaller, M.A. (eds.), Manual of Clinical Microbiology, 9th ed., vol. 1. ASM Press, Washington, DC.

14. Omar-Zahid, L. A. (2009). Extraction and Purification of the Klebocin from Clinically Isolated *Klebsiella*and Studying Its Biochemical and Biological Characteristics. Msc. thesis. College of Science. University of Baghdad.

15. Assal, S.D.A. (2010). A study on inhibitory effect of local Lactobacilli filtrate on formation of Klebsiella pneumoniae biofilm. Msc. thesis. College of Science. The University of Baghdad .

16. Tsolia, M.N.; Psarras, S.; Bossios, A.; Audi, H.; Paldanius, M. Gourgiotis, D.; etal.(2004). Eitology of community[acquired pneumonia in hosptilized school[agwchildren:evidence for hogh prevalence of viral infectious. Clin Infect Dis ;39:361-6.

17. Vuori-Holopainen, E.; Salo, E.; Saxen, H.; Hedman, K.; Hyypiä, T.; Lahdenperä, R.; et al.(2002) Etiological diagnosis of childhood pneumonia by use of transthoracic needle aspiration and modern microbiological methods. Clin Infect Dis.;34:583 90.

18. Don, M.; Valent, F.; Korppi ,M. Fallet, E.; De Candia, A.; Fasoli, L.; etal.(2007). Efficacy of serum procalicitonin in evaluating severity of community-acquired pneumonia in childhood. Scand J Infect Dis;39:129-37.

19. Arancibia, F.; Bauer, T.T; Ewig ,S.; et al.(2002). Community-acquired pneumonia due to gram-negative bacteria and Pseudomonas aeruginosa. *Arch Intern Med*;162:1849-1858. (Prospective observational study, 60Patients).

20. Monoto, A.S.; and Ulman, B.M.(1974). Acute respiratory illness in a American community: The tecumesh study. Journal of the American Medical Association ;277:164-69.

21. Markowicz, P. ; Wolff, M.; Djedaini, k.; etal.(2000). Muttcellatarproipective study of ventilator-associated pneumonia during acute respiratory distress syndrome :incideuce prognosis , and risk factor Am J RespirCrit Care Med ;161:1942-8.

22. Hicks, A.; Monkarsh, S.P.; Hoffman, A.F.; and Goodnow, R. (2007). "Leukotriene B4 receptor antagonists as therapeutics for inflammatory.

23. Hubbard, R.C.; Fells, G.; Gadek, J.; et al.(1991). Neutrophil accumulation in the lung in alpha1-antitrypsin deficiency. Spontaneous release of leukotriene B4 by macrophages. J ClinInvest ;88:891–725.

24. Aaron, S.D.; Angel, J.B.; Lunau, M.; et al.(2001). Granulocyte inflammatory markers and airway infection during acute exacerbation of chronic.

25. Gompertz, S. O.; Brien, C.O.; Bayley, D.; et al.(2001). Changes in bronchial inflammation during acute exacerbations of chronic bronchitis. EurRespirJ ;17:1112–9.

26. Birgit, A.; Emanuel, V. G.; Dirk ,H.; Mark, L.; Ingo, M.; and Borna, R.(2011) Circulating Leukotriene B4 Identifies Respiratory Complications after Trauma. Received 22 August 2011; Accepted 9 December.

Iraqi Journal of Biotechnology, 2015, Vol. 14, No. 2 , 362-373

Evaluation antimicrobial activity of *Annona muricata* against bacteria

Majid S. Jabir [1], Zainab J. Taqi [2], Ahmed J. Jasim[3], Ali H. Saadoon[4], Buthena A. Hassoon[5], Raghad KH. Maieh[6]

University of technology/ School of Applied science/ Department of biotechnology

Abstract: *Annona muricata* have been used as traditional medicine for chemoprevention and treatment of chronic inflammatory diseases although the evidence supporting their functions is still poor. The present study was carried out to investigation of chemical composition as well evaluate antimicrobial activities from *Annona muricata peel*. Methanol 80% extract of *Annona muricata*peel was tested for antimicrobial activity against Gram positive and Gram negative bacterial strains. The results showed that the extract has a significant antimicrobial activity; it inhibited significantly the growth of ***Staphylococcus aureus***, ***Streptococcus pyogenes***, ***E coli***, *and* ***Serratiamarcescens*** *suggesting* that it can be used in the treatment of bacterial infections, and it showed no effect on the other bacterial strain ***Pseudomonas areuginosa***. Phytochemical analysis of the extract showed the presence of Carbohydrates, Terpenes, Tannins and Flavonoids. These results of this study may help to discover new chemical classes of natural antimicrobial antioxidant substances.

Key words: Annona muricata, Antimicrobial, Antioxidant, Peel extract.

تقيم الفعالية المضادة للاحياء المجهرية لنبات فاكهة القشطة ضد البكتريا

ماجد صخي جابر ، زينب تقي ، احمد جاسم، علي سعدون، بثينة حسون و رغد

قسم علوم الحياة - كلية العلوم التطبيقية - الجامعة التكنولوجية

الخلاصة: استخدم نبات فاكهة القشطة بصورة تقليدية في الطب كعلاج كيميائي وفي علاج الالتهابات المزمنة على الرغم من الادلة التي تشير الى أهمية الطبية لاتزال فقيرة وغير مدروسة بشكل مفصل. صممت الدراسة الحالية للتحري عن المحتوى الكيميائي وكذلك الفعالية المضادة للاحياء المجهرية لقشور نبات فاكهة القشطة. اختبر المستخلص الكحولي للاميثانول بتركيز 80% ضد البكتريا الموجبة والسالبة لصبغة كرام. اظهرت النتائج ان المستخلص يثبط نمو بكتريا المكورات العنقودية الذهبية وبكتريا المسبحيات وكذلك يثبط نمو بكتريا القولون وبكتريا السرشيا مرسسنس ولم يظهر فعالية مضادة لبكتريا الزوائف الزنجارية . التحليل الكيمائي للمستخلص اظهر وجود مركبات كيمائية مثل الكاربوهيدرات والتربينات والتانينات وكذلك الفلوفونات. نتائج الدراسة الحالية ربما تساعد في اكتشاف انواع كيمائية جديدة تعمل كمضادات للاحياء المجهرية وكعوامل مضادة للاكسدة.

Introduction

Medical plants are the oldest known healthcare agent. Their importance is still growing although it varies depending on the ethnological, medical and historical background of each place. It's important for biological research such as pharmacological and drug development, not only when plant constituents are used directly as therapeutic agents, but also when they are used as basic materials for the synthesis of drugs or as a models for pharmacologically active compounds (1). Annonaceae family is one of the large families showing so many characteristic features such as antitumor, antioxidant and antimicrobial activities. The previous studies on Annona species demonstrated that this family is a potent source of a wide variety of secondary metabolites belonging to several categories (2). The effect of organic component of aqueous extracts studied from the defatted seeds of *Annona squamosa* on different tumors cell lines and reported that the two extracts from *A. squamosa* seeds induced apoptotic features like formation of apoptotic bodies, DNA fragmentation and phosphotidyl serine externalization by Annexin-V staining in MCF-7 breast carcinoma and K-562 erythroleukemic cells (3). Previous studies have demonstrated that the leaf, bark, root, stem, and fruit seed extracts of *Annona muricata* are anti-bacterial (4), antifungal (5) and anti-malarial (6). *Annona muricata* leaves extract were also found to possess antioxidant (7) and molluscicidal properties (8). Recently, it has also been reported to exhibit anti-inflammatory (9) and analgesic effects (10). Annonaceous acetogenins, from *Annona muricata* were found to be a promising new anti-tumor and anticancer agent in numerous *in vitro* studies (11). These acetogenins demonstrated to be selectively toxic against various types of the cancerous cells without harming healthy cells (12).

Hence, the present study has been undertaken to investigate the antimicrobial and antioxidant activity of the peel of Annona muricata using in different *In-vitro* and *In-vivo* model.

Materials and methods

Preparation of Annona muricata peel Extract

Annona muricata peels were collected in Iraq during January and February 2015. The peels were cleaned before dried at room temperature. Dried peels were ground into powder form. 300gm of dried powder of plant was then subjected to extraction with 80% ethanol (300ml) by using a soxhlet apparatus. The crude extract obtained was concentrated using rotary evaporator under reduced

pressure and dissolved in 1% acetone in to different concentration (50, 100, 250 mg/kg) (13).

Antimicrobial activity

Microorganisms

The test microorganisms included the following Gram-positive bacteria: *Staphylococcus aureus* and *Streptococcus pyogenes*. Gram-negative bacteria: *Escherichia coli, Serratia marcescens*, and *Pseudomonas areuginosa*. These micro-organisms were diagnosed in Department of biotechnology, university of Technology.

Disc-diffusion assay

Antimicrobial activity test was done according to the protocol described by (14) with slight modification. Briefly, a loopful of the microorganisms working stocks was enriched on a tube containing 20 ml of Mueller-Hinton broth (LAB,Cat.No. lab 39. UK) for bacteria. Then incubated at 37 °C for (18-24) h. The overnight cultures were used for the antimicrobial activity of the *Annona muricata* peel extract which is used in this study and the optical density was adjusted at 0.5 McFarland turbidity standards with a DENSIMAT (Biomérieux) which are used as a reference to adjust the turbidity of the number of bacteria will be within a given range to standardize microbial testing. The inoculum of the respective bacteria streaked on to Mueller-Hinton MH agar (Sigma,Cat.No. 70191. USA) plates using a sterile swab. Sterile filter discs (diameter 6 mm) (Whatman Paper No. 1Cat.No. Q/FML61-2004, England) were impregnated with *Annona muricata* peel extract in different concentration (62.5, 125, 250, and 500 mg/ml) placed on the appropriate agar medium (LAB,Cat.No. lab 108170. UK), D.W. (20 µl/disc) were used as negative reference standards to determine the sensitivity of one strain/isolate in each microbial species tested. The antimicrobial activity was determined by using Mueller–Hinton agar plates as described by (15). After incubation at 37 °C for (18-24) h, the diameter of the inhibition zone was measured (16). The diameter of the zones of inhibition around each of the discs was taken as measure of the antimicrobial activity. Each experiment was carried out in triplicate and the mean diameter of the inhibition zone was recorded.

Bacterial cultures

E. coli and S. aureus were cultured in LB broth (Invitrogen. Cat. No.12780-052, USA) to mid-log phase (OD 0.4-0.6) immediately prior to use. The bacteria were then centrifuged at 3500 rpm for 15 minutes at 4˚C, the pellet was washed twice in sterile Phosphate buffer saline and then resuspended in the same Pen Strep -free media as

the cells being infected to a concentration of approximately 1× 10^6cfu/µl=(OD600/0.4)*1.8ml(17).

In-vivo model experiment

Mice were maintained according to Institutional and National (UK Home Office) guidelines. C57/BL6 mice were obtained from Harlan UK. Male C57/BL6 mice aged (7-9) weeks, (20-24 g) weight were used in this study. Mice were randomly divided into to 3 groups (3 mice per group). The control groups were injected intraperitoneally with sterile PBS. Experimental groups were injected intraperitoneally with *E.coli*, *Staphylococcus aureus* (10^6 CFU) alone or with Annona muricata peel extract 3mg/kg. Seven hrs after infection, mice were sacrificed and peritoneal fluid collected for analysis as indicated (18).

Antimicrobial activity of Annona muricata in liquid media

We studied the effect of *Annona muricata* peel extract on bacterial growth in liquid media in the presence and absence of *Annona muricata* peel extract at different concentration (250, 500mg/ml). *E. coli* and *S.aureus* were cultured in nutrient broth (HIMEDIA. Cat.No.M002-500G, India), for 24 hr. 10µl from bacterial strains were taken from nutrient broth each 3 hr. at (0, 3, 6, 9, and 12 hr) were cultured on nutrient agar (LAB, Cat. No. LAB8, UK) plates. The plates containing the test microorganisms were incubated at 37˚C For 24 hr. then we counted CFU/ml (17).

Enzymatic antioxidant assay

Estimation of catalase activity

The procedure described by manufacture (Gentaur, Cat.No. ECAT-100, Belgium). Briefly, Prepare the sample of *Annona muricata* peel extract in different concentration (100, 200, 300, 400, and 500µg/ml). Transfer 10µl from each concentration and 10µl from positive control and 10µl from sample blank into 96 well plate. Then add 90µl of the 50µM substrate to these wells to initiate the catalase reaction. Incubate for 30 min at room temperature. Add 100µl detection reagent and reading the optical density at 570 nm. The change in color is directly proportional to the catalase activity in the sample.

Statistical analysis

Values are expressed as the mean ± SD. Two-tailed unpaired Student's *t*-test, and ANOVA-One way were used to compare means. Analyses were processed using GraphPad Prism software for Windows (version 5.0, GraphPad Software, Inc., San Diego, CA) (17, 18).

Results and discussion

The chemical composition of Annona muricata peel extract was investigated by using Evans protocol (19). The results showed presence of different chemicals such as Alkaloid, Saponins, tannins, flavonoids, triterpenes, and steroid. Our results agree with results of (20, 21), their results shows that the chemical composition of *Annona muricata* peel extract are Alkaloid, Saponins, tannins, flavonoids, triterpenes, and steroid.

Table1: Chemicals composition of *Annona muricata* peels extract.

Chemicals composition	Reagents	Results
Alkaloids	Mayer's Reagent	White precipitate
Saponins	$HgCl_2$	White precipitate
Flavonoids	Equal value of 50% KOH+ 50% Ethanol	Yellow precipitate
Turbines	Chloroform + Acetic acid + Sulfuric acid	Brown ring
Steroids	Chloroform + Acetic acid + Sulfuric acid	Deep Blue ring

The peel extract was screened for antibacterial activity against Gram's positive bacteria *Staphylococcus aureus, and Streptococcus pyogenes*. The peel extract showed antimicrobial activity against these bacteria in different concentration (Figure1). The same results demonstrated against Gram negative bacteria *E. coli*, and *S. marcescens*. While, the peel extract showed no antimicrobial activity against *P. areuginosa* (Figure 2). Neither 250 mg/ml nor 500mg/ml of Annona muricata peel extract had a significant effect on the growth of *E. coli* and *S. aureus* in culture broth (Figure 3).

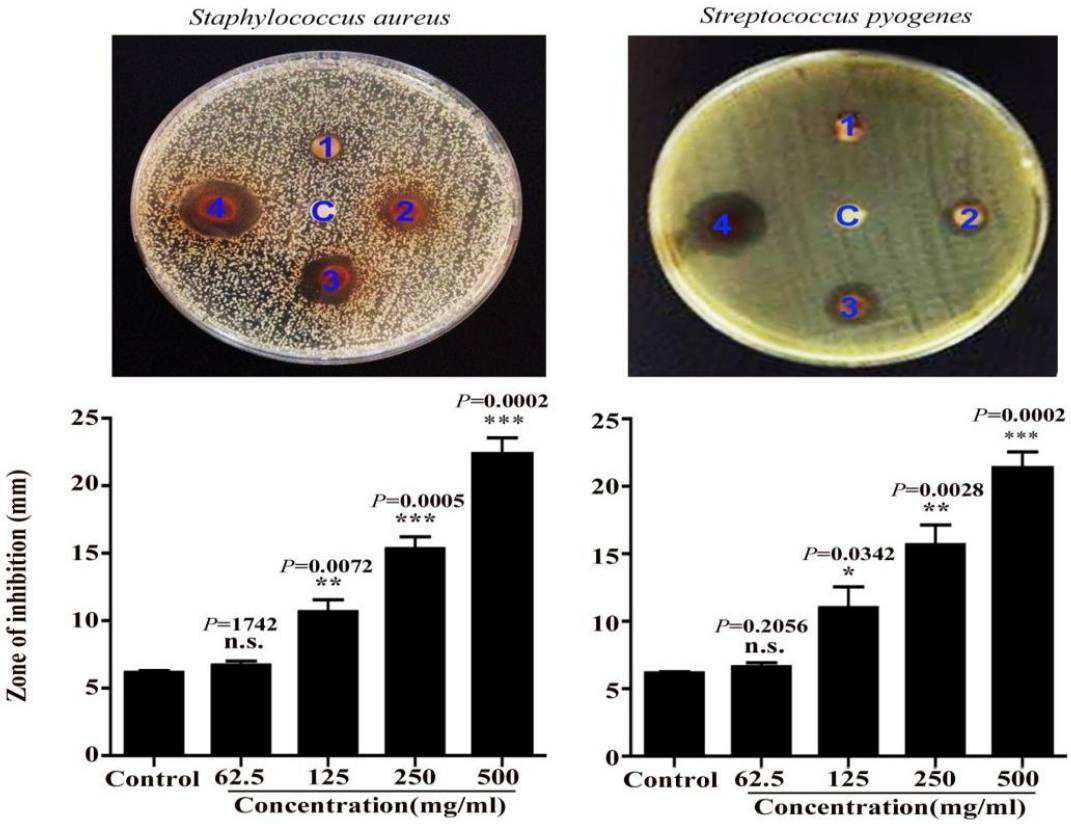


Figure: 1. Antimicrobial activity of *Annona muricata* peels extract against Gram positive bacteria. C= Control (D.W.), 1= 62.5mg/ml, 2=125mg/ml, 3=250mg/ml, 4=500mg/ml of peel extract. Asterisks indicate statistically different from control (D.W). Columns are mean of triplicate determination; error bar are SEM. * *P* <0.05, ** P<0.01, *** P<0.001.

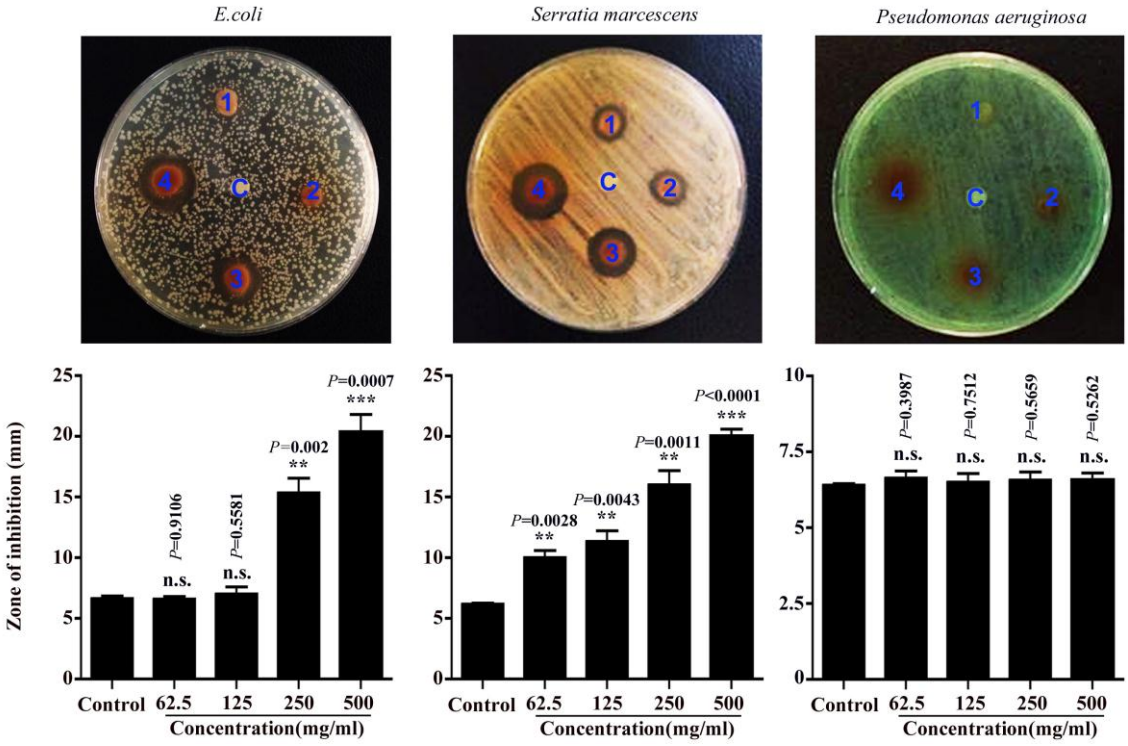


Figure: 2. Antimicrobial activity of *Annona muricata* peels extract against Gram negative bacteria. C= Control (D.W.), 1= 62.5mg/ml, 2=125mg/ml, 3=250mg/ml, 4=500mg/ml of peel extract. Asterisks indicate statistically different from control (D.W). Columns are mean of triplicate determination; error bar are SEM. * *P* <0.05, ** P<0.01, *** P<0.001.

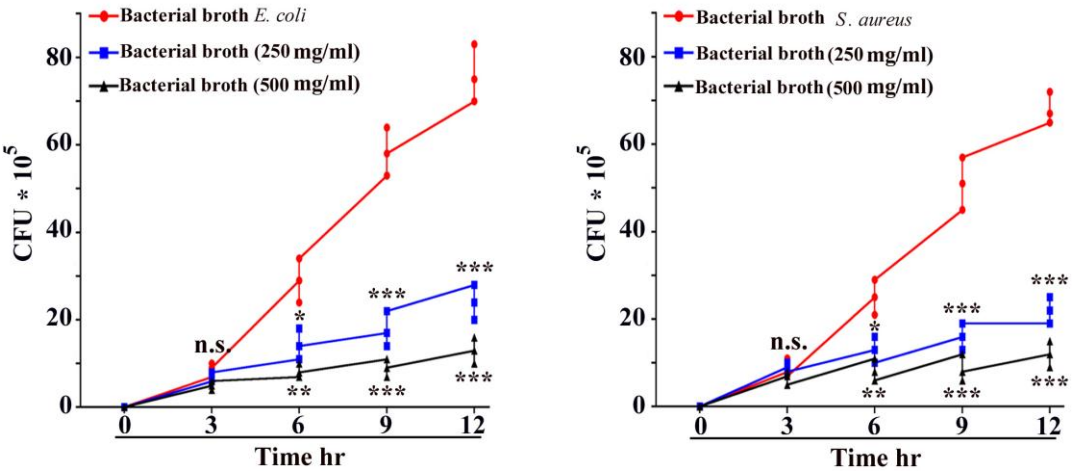


Figure3: Effect of *Annona muricata* peels extract on bacterial growth. Asterisks indicate statistically different from bacterial broth without peel extract. * *P* <0.05, ** P<0.01, *** P<0.001.

Next, we tested the effect of Annona muricata peel extract in an *In-vivo* model of *E.coli*, and *S. aureus* infection in mice. Animals were infected with the microbes intraperitoneally for 7 hrs, then mice were sacrificed and peritoneal fluid collected for analysis as indicated. We measured the numbers of viable bacteria recovered from the peritoneal cavity following infection (Figure 4). This showed that the peel extract reduced the numbers of bacteria.

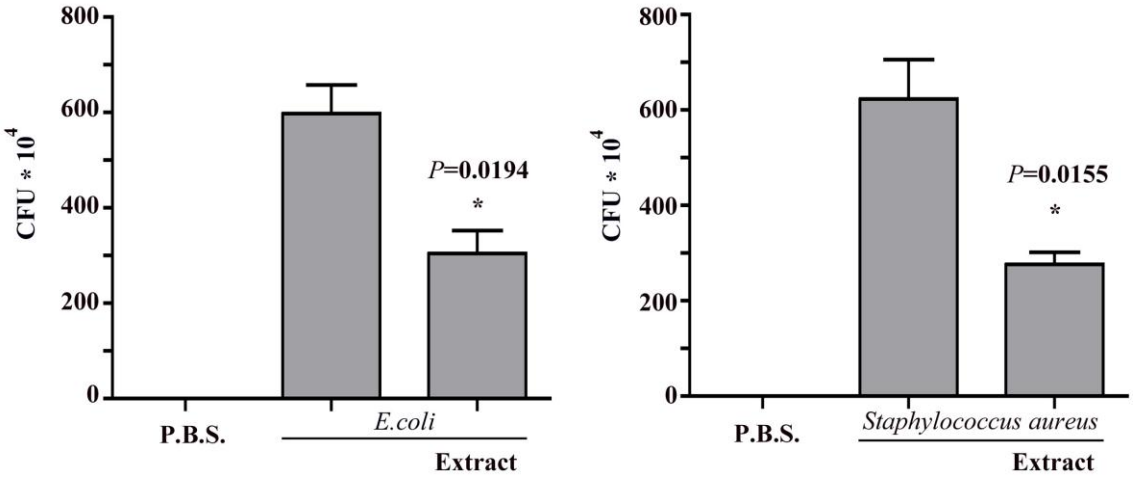


Figure 4: *Annona muricata* peel extract contributes to bacterial killing *In-vivo* bacterial infection. Bacterial colonies count per ml of recovered fluid from the peritoneal cavity with treatment as shown. Asterisks indicate statistically different from bacterial infection without peel extract. Columns are mean of triplicate determination; error bar are SEM. * *P* <0.05.

In this study, we used several concentrations ranging from 100-500 µg/ml of the Annona muricata peel extract were tested for their antioxidant activity in vitro model. It was observed that free radicals were scavenged by the test components in the concentration manner (Figure5). Antioxidant was measured the activity of catalase. The enzyme extract was added to the reaction mixture containing H_2O_2 and phosphate buffer (pH 7.0) and the OD change was measured at 570 nm. The activity of the catalase enzyme is expressed in the terms of µ mole of H_2O_2 consumed/ min/ µg protein (22). Our results agree with the results of (23). Enzymic antioxidants play an important role in cellular defense against reactive oxygen species (ROS) (24).

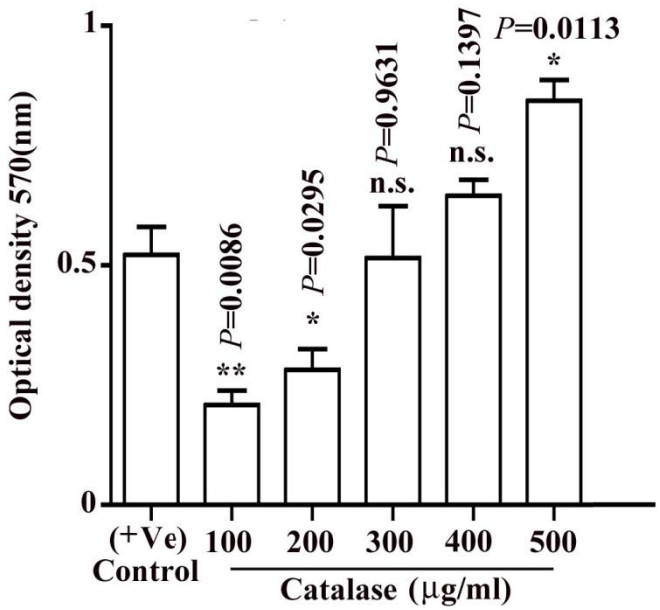


Figure 5: Level of antioxidant of catalase enzyme in peel extract of *Annona muricata*. Asterisks indicate statistically different from positive control without peel extract. Columns are mean of triplicate determination; error bar are SEM. * *P* <0.05. ***P*<0.01.

Antibacterial effects of aqueous and ethanolic extracts of *Annona muricata* were examined against Gram positive and negative bacteria such as *S. aureus*, *V. cholerae* and *E. coli*. The results demonstrated that the bioactivity of water-based soursop extracts against *S. aureus* and *V. cholerae* may be related to the chemical structure of the active substances. In an investigation of the bactericidal properties of eight species of annonaceae, it was confirmed the ability of trachylobanoic acid to inhibit *S. aureus*. Annonaceae contain other bioactive substances, including a range of acetogenins with a wide spectrum of action, including antibiotic effects. Structurally, Annonaceous acetogenins are series of C-35/C-37 natural products derived from C-32/C-34 fatty acids and combined with a 2-propanol unit (25). *Annona muricata* extract of leaves exhibited a broad spectrum of activity against a panel of bacteria such as *B. subtilis*, *S. aureus*, *K. pneumonia*, and *P. vulgaris* which are responsible for common bacterial diseases like pneumonia, diarrhoea, UTIs and skin infections (26).

Finally, from our results it can be concluded that *Annona muricata* peel extract plays an important role as antimicrobial and antioxidant activity due to the presence of

bioactive chemical compounds such as flavonoids, triterpenes, and others. Our results suggest that the peel extract could serve as potential source of bioactive compounds. Further research is needed in which the extract could possibly be exploited for pharmaceutical use.

References

1. Biba V. S.; Amily A.; Sangeetha S.; and Remani P. (2014). Anticancer, antioxidant, and antimicrobial activity of Annonaceae family. World journal of pharmacy and pharmaceutical science, 3(3):1595-1604.

2. 2-Pardhasaradhi, B.V.; Madhurima R. V.; Mubarak, A., A.; Leela, K., A.; and Ashok, K.(2005). Differential cytotoxic effects of *Annona squamosa* seedd extracts on human tumour cell lines: Role of reactive oxygen species and glutathione. J. Biosci., 30: 237- 244.

3. Oberlies, N.H.; Chang, C.J. and McLaughlin, J.L.(1997). Structure-activity relationships of diverse Annonaceous acetogenins against multidrug resistant human mammary adenocarcinoma (MCF-7/Adr) cells. J. Med. Chem., 40(3): 2102-2106.

4. Sundarrao, K.; Burrows, I. and Kuduk, M. (1993). Preliminary screening of anti-bacterial and anti-tumor activities of papuan new guinean active medicinal plants. Pharmaceutical Biol., 31:3-6.

5. Heinrich, M.; Kuhnt, M. and Wright, C.W. (1992). Parasitological and microbiological evaluation of mixe Indian medicinal plants (Mexico). J. Ethnopharmacol, 36: 81-5.

6. Antoun, M.; Gerena, L. and Milhus, W. (1993). Screening of the flora of puerto rico for potential antimalarial bioactives. Int. J. Pharmacol., 31(2): 255-8.

7. Baskar, R.; Rajeswari, V. and Kumar, T. (2007). *In vitro* antioxidant studies in leaves of annona species. Indian J. Exp. Biol., 4(3): 480-485.

8. Luna, J.; Carvalho, J.and Lima, M. (2006). Acetogenins in *annona muricata* L. (annonaceae) leaves are potent molluscicides. Nat. Prod. Res., 20: 253-257.

9. De Sousa, O.V; Vieira, G.D. and De Pinho, J.J. (2010). Antinociceptive and anti-inflammatory activities of the ethanol extract of *annona muricata* L. Leaves in animal models. Int. J. Mol. Sci., 1: 2067-2078.

10. Roslida, A.H., Tay, C.E. and Zuraini, A. (2010). Anti-inflammatory and anti-nociceptive activities of the ethanolic extract of *annona muricata* leaf. J. Nat. Remedies, 10: 97-104.

11. Zhao, J.; Wang, J. and Chen, Y. (1999). Anti-tumor-promoting activity of a polyphenolic fraction isolates from grape seeds in the mouse skin two stage initiation-promotion protocol and identification of procyanidin B5-30-gallate as the most effective antioxidant constituent. Carcinogenesis, 20 (3):1737-1745.

12. Zeng, L. R.; Wu, F.E. and Oberlies, N.H. et al (1996). Five new monotetrahydrofuran ring acetogenins from the leaves of *annona muricata*. J. Nat. Prod., 59: 1035-1042.

13. Sulaiman, H.; Roslida, A.; Fezah, O.; Tan, K.; Tor, Y. and Tan, C.(2012). Chemopreventive Potential of *Annona Muricata* L Leaves on Chemically-Induced Skin Papillomagenesis in Mice. Asian Pacific J. Cancer Prev., 13:2533-2539.

14. Vuddhakul, V.; Bhoopong, P.; Hayeebilana, F.; and Subhadhirasakul, S.(2007) Inhibitory activity of Thai condiments on pandemic strain of Vibrio parahaemolyticus. Food Microbiol., 24:413-418.

15. Ottaviani, D.; Bacchiocchi, I.; Masini, L.; Leoni, F.; Carraturo, A.; and Giammarioli, M. (2001). Antimicrobial susceptibility of potentially pathogenic halophilicvibrios isolated from seafood. Int. J. Antimicrob. Ag. , 18:135-140.

16. Cavallo, J.; Chardon, H.; Chidiac, C.; and Choutet P.(2006). Comité de l'antibiogramme de la société Française de Microbiologie, Communiqué,

17. Majid, S.; Neil, D.; Dong Li, Hannah, K.; Panagiotis, T.; Daniel, P.; Alison L.; Lee, H.; Anna, S.; Clare, B.; and Thomas J. (2014). Caspase-1 Cleavage of the TLR Adaptor TRIF Inhibits Autophagy and b-Interferon Production during Pseudomonas aeruginosa Infection. Cell Host & Microbe, 15: 214–227.

18. Majid, S.; Lee, H.; Neil, D., Ihsan, U.; Hannah, K.; Dong, L.; Panagiotis, T.; Alison, L.; Daniel, P.; Anna, S.; Clare, B.and Thomas, J. (2015). Mitochondrial damage contributes to Pseudomonas aeruginosa activation of the inflammasome and is downregulated by autophagy . Autophagy, 11(1): 166-182.

19. Evans, W.C.(1989). *Trease* and *Evan's Pharmacognosy*. 13th ed. Balliere Tindal, London 419-420.

20. Santhoshkumar, M. and Brindha, D.(2015). Evaluation of antioxidant and free radical scavenging activity of *Annona muricata*. European Journal of Experimental Biology, 2015, 5(3):39-45.

21. Abubacker, M. and Deepalakshmi,T.(2012). Antioxidant and antibacterial activity of *(Annona muricata* L.) leaf aqueous extract. International journal of plant sciences, 7 (2):301-306.

22. Luck. H. (1993). Methods of enzymatic analysis, Ed Begmeyer HU, Academic press, New York.

23. Bashar, R.; Rajeswari, V. and Sathish, T.(2007). In-vitro antioxidant studies in leaves of Annona species. Expermental biology, 45:480-485.

24. Ray, G. and Husain, S. (2002). Oxidants, antioxidants and carcinogenesis, Indian Journal of Experimental Biology, 40(11):1213-1232.

25. Vieira, G.; Mourao. J.; Ângelo, A.; Costa, R. and Vieira, R. (2010). Antibacterial effect (in vitro) of Moringa oleifera and Annona muricata against Gram positive and Gram negative bacteria.‖ Rev. Inst. Med. Trop. Sao Paulo, 52(3):129-132.

26. Solomon-Wisdom, G.; Ugoh, S. and Mohammed, B.(2014). Phytochemical Screening and Antimicrobial activities of Annona muricata (L) leaf extract, American Journal of Biological, Chemical and Pharmaceutical Sciences 2(1): 01-07.

Iraqi Journal of Biotechnology, 2015, Vol. 14, No. 2 , 374-390

Evaluation the Physiological Effect of Pregnancy on Some Hematological and Biochemical Parameters for Pregnant Women

Mohammed Abdul-Daim Saleh[1] , Abbas Aboud Farhan [2], Mohammed Hussein Alwan[2]

[1] University of Diyala. College of Science. Biology Department. Iraq.
[2] University of Diyala. College of Education for pure Science. Biology Department. Iraq.

Abstract: The present study was designed to focus on some haematological and biochemical changes accompanied pregnancy. The sample included 120 women who were attending Al-Batool hospital for Gynecology and Children in Baqubah city during the period from September 2014 to May 2015. Women divided into two groups: Pregnant women include 60 pregnant women with apparently healthy pregnancy; their ages range between 17-37 years, Age Mean of them was 26.95 years. Non-pregnant women include 60 women their ages range between 17-37 years. Age Mean of them was 27.13 years, and they considered as control group. The haematological parameters studied were included (total WBC, neutrophils, monocytes, lymphocytes, RBC, Hb, PCV and platelets).The biochemical parameters studied were (ALT, AST, ALP, total bilirubin, BUN, creatinine and glucose). The results showed the following features: A significant($P<0.001$) increase in the levels of (WBC count, neutrophils and monocytes percentage) in the plasma of pregnant women as compared with control group, also significant ($P<0.001$) decrease in the levels of (lymphocytes, Hb, PCV and platelets count) in the plasma of pregnant women as compared with control group, moreover significant ($P<0.05$) decrease in the levels of RBC count in plasma of pregnant women as compared with control group, moreover no significant ($P>0.05$) in the levels of (ALT, AST and total bilirubin) in the serum of pregnant women as compared with control group, addition to significant ($P<0.001$) increase in the levels of ALP in the serum of pregnant women as compared with control group, finally significant ($P<0.001$) decrease in the levels of glucose, BUN and creatinine in the serum of pregnant women as compared with control group.

Key word: Pregnancy, WBC, RBC, ALT, ALP, Urea.

تقيم التاثير الفسيولوجي للحمل على بعض الجوانب الدموية والبايوكيميائية للنساء الحوامل

محمد عبد الدايم صالح[1] ، عباس عبود فرحان[2] ، محمد حسين علوان[2]

[1]جامعة ديالى ، كلية العلوم ، قسم علوم الحياة [2] جامعة ديالى ، كلية التربية للعلوم الصرفة ، قسم علوم الحياة

الخلاصة: اجريت هذه الدراسة بهدف تسليط الضوء على بعض الجوانب الدموية والبايوكيميائية عند النساء الحوامل اللواتي ترددن الى مستشفى البتول للنسائية والاطفال في مدينة بعقوبة للمدة من تشرين الاول2014 الى آيار 2015 حيث تم اختيار 120 امرأة. 60 امرأة منها حامل من الأصحاء ظاهريا تراوحت اعمارهن بين 17-37 سنة بمتوسط عمري 26.95 و60 امرأة غير حامل تراوحت اعمارهن بين 17-37 سنة بمتوسط عمري 27.13 والتي تمثل مجموعة سيطرة (Control). شملت الدراسة المتغيرات الدموية كل من (WBC, neutrophils, monocytes, lymphocytes, RBC, Hb, PCV, platelets) اما المتغيرات البايوكيميائية فشملت تقدير ALT, AST, ALP, total bilirubin, BUN, creatinine, glucose) وقد توصلت الدراسة إلى النتائج الآتية :-
أظهرت النتائج وجود ارتفاع معنوي ($P<0.001$) في مستوى كل من (WBC (neutrophils, monocytes في النساء الحوامل مقارنة مع مجموعة السيطرة. كما وجد انخفاض معنوي ($P<0.001$) في مستوى كل من ((Hb, PCV, platelets في النساء الحوامل مقارنة مع مجموعة السيطرة. فضلا عن ذلك وجد انخفاض معنوي ($P<0.05$) في مستوى كريات الدم الحمراء RBC في النساء الحوامل مقارنة مع مجموعة السيطرة. كما اظهرت النتائج عدم وجود اختلاف معنوي ($P>0.05$) في مستوى كل من (ALT, AST, total bilirubin) في مصل النساء الحوامل مقارنة مع مجموعة السيطرة. وسجل انزيم الفوسفتيزالقاعدي ALP ارتفاعا معنويا ($P<0.001$) في مصل النساء الحوامل مقارنة مع مجموعة السيطرة. ووجد انخفاض معنوي ($P<0.001$) في مستوى كل من اليوريا BUN والكرياتينين creatinine في مصل النساء الحوامل مقارنة مع مجموعة السيطرة. وأخيرا وجد انخفاض معنوي ($P<0.001$) في مستوى الكلوكوز glucose في مصل النساء الحوامل مقارنة مع مجموعة السيطرة.

Introduction

Pregnancy is the state of a female after conception until the birth of the baby (1). Normal pregnancy is characterized by profound changes in almost every organ and system to accommodate the demands of fetoplacental unit (2). Pregnancy is one of the most important periods in human life with hormonal, immunological, vascular, metabolic and psychological changes (3). Pregnancy induces several physiological adaptations to meet the needs of the developing fetus and the health requirements of the mother (Jiang *et al.* , 2012). With 50% of its genetic material derived from its father, successful pregnancy has been considered a biologic example of semiallogeneic graft acceptance, in which the semiallogeneic fetus is protected from immune attack from the mother (4), therefore pregnancy represent a unique immunological period for the mother (5). The hematological profile of an individual to a large extent reflects their general health (6). However, many hematologic problems develop in pregnancy or can be triggered by the pregnant state. Normal physiologic changes during pregnancy can alter hematologic indices during pregnancy and make recognition of pathologic states difficult. These conditions are a significant source of morbidity and mortality during pregnancy that has implications for both the mother and the fetus (7). The alanine aminotransferase (ALT) is an enzyme that catalyzes the transfer of amino groups to form the hepatic metabolite oxaloacetate. It is composed of 496 amino acids, which are encoded by a gene located in the long arm of chromosome 8 (8). ALT is found primarily in the liver and kidney with lesser amount in the heart and skeletal muscles (9). Therefore ALT is more specific for hepatic injury because it is present mainly in the cytosol of the liver cells and in low concentration in other tissues (10). In the case of hepatocellular injury or death, release of ALT from damaged liver cells increases measured ALT activity in the serum (8). Aspartate aminotransferase (AST) is an enzyme found in very high concentrations within highly metabolic tissue, such as the liver cells, heart muscle, skeletal muscle cells, and to a lesser degree in the kidney, pancreas, and RBCs (11). When body tissue or an organ such as the liver or heart is diseased or damaged, additional AST is released into the bloodstream, causing levels of the enzyme to rise. Therefore, the amount of AST and in the blood is directly related to the extent of the tissue damage. After severe damage, AST levels rise 10 to 20 times larger than normal (12). Alkaline phosphatase (ALP) is present in many tissues of the body with

particularly high levels in the liver, bone, placenta, intestine and kidney. These are referred to as isoenzymes. The precise metabolic function of the enzyme is still unknown, but some suggestions made is that it participates in the calcification process in bone synthesis, and is involved in the transfer of glucose and fatty acids across the cell membrane (13). Bilirubin is the end product of hem breakdown. About 80% of bilirubin originates from degradation of erythrocyte hemoglobin in the reticuloendothelial system; the remaining 20% comes from inefficient erythropoiesis in bone marrow and degradation of other heme proteins. Water insoluble, unconjugated bilirubin (UCB) bound to albumin is transported to the liver where it is removed from the plasma (14). Renal function can be evaluated by the determination of urea, creatinine and uric acid levels in serum/plasma (15). Urea is synthesized in the liver, primarily as a by-product of the deamination of amino acids. It's elimination in the urine Represents the major route for nitrogen excretion (16). Creatinine is the breakdown product of creatine phosphate released from skeletal muscle. The daily production of creatine, and subsequently creatinine depends on muscle mass. Creatinine as blood urea nitrogen (BUN), is excreted entirely by the kidneys and therefore is directly proportional to renal excretory function, thus with normal renal excretory function, The serum creatinine level remain constant and normal (11). Glucose is the major energy source for both fetus and placenta. Fetal glucose production is minimal; therefore, it is entirely dependent upon placental supply of glucose from the maternal circulation. Glucose crosses the placenta by facilitated diffusion. Net glucose transfer is therefore highly dependent on maternal-fetal concentration gradients. Placental glucose uptake increases with advancing gestation (17).

The aims of the present study were to estimate changes in some haematological and biochemical indicators in apparently healthy pregnant women compared with apparently healthy non-pregnant women (control).

Materials and Methods

The study included 120 women were divided into two groups: First group included 60 pregnant women with apparently healthy pregnancy; their age range (17-37) years with mean age 26.95±5.58 years. Second group included 60 non-pregnant women, their age range (17-37) years with mean age 27.13± 6.14 years, and they considered as control group. Samples were collected from those individuals

only if they were not having a history of a chronic or acute illness.

From each woman, 5 ml of venous blood was collected from a suitable vein. Blood sample divided into two parts, 2 ml of fresh blood was put in sterile tube contains ethylene diamine tetra acetic acid (EDTA) to estimate the haematological parameters, and 3ml was transferred to sterile plain gel tube and let to clot and then centrifuged at 5000 r.p.m. for 10 minutes at room temperature to separate the serum and estimate the biochemical parameters. The haematological parameters (total WBC count, neutrophils, monocytes, lymphocytes, RBC, Hb, PCV and platelets) were makes with automated method by ABX Pentra DX 120 (Horiba Company) as an automatic laboratory analysis system. This automotive test system characterized by speed and exactness, whereas it can estimate blood cells numbers as electric pulses. Biochemical tests (ALT, AST, ALP, total bilirubin, BUN, creatinine and glucose) were makes with automated method by COBAS INTEGRA 400 plus system as an automatic laboratory analysis system by using commercial test kits (COBAS INTEGRA systems).

Statistical Analysis

Data Analysis was computer aided. Statistical analysis was done using SPSS (Statistical Package of Social Science) version 20 computer software. Frequency distribution and percentage for selected variable were done. The independent t-test was used and P-Value (less than 0.05, 0.001) was considered as the level of significance (18).

Results

Table (1) shows that there were no significant ($p > 0.05$) differences in the mean age between the studied groups. The mean age of pregnant women was 26.95±5.58 years compared with the mean age of non-pregnant women (control) was 27.13± 6.14 years.

Table (1): The mean of ages in pregnant and control groups (mean ± SD).

Studied Groups	No.	Age (Years)		P-Value
		Range	Mean ± SD	
Pregnant women	60	17-37	26.95 ± 5.58	> 0.05
Control	60	17-37	27.13 ± 6.14	

Haematological parameters for both pregnant women and control group are shown in table (2). There was significant ($p<0.001$) increase in the mean value of (WBC, neutrophils and monocytes) in pregnant women (7.43±1.61 X 10^9/L, 69.82±5.44 % and 5.82±1.49 % respectively) when compared with the mean of control group (6.13±1.04 X 10^9/L, 59.33±5.90 % and 4.99±0.93 % respectively). Also table (2) shows a significant ($p<0.001$) decrease in the mean of (lymphocytes, Hb, PCV and platelets) in pregnant women (20.70±4.83 % , 11.57±1.32 gm/dl , 36.32±3.60 % and 229.23±59.60 x10^9/L respectively) when compared with the mean of control group (30.95±5.19 % , 12.97±0.96 gm/dl , 40.52±2.94 % and 273.43±49.94 x10^9/L respectively). Furthermore there were a significant ($p<0.05$) decrease in the mean of RBC in pregnant women (4.41±0.39 x10^{12}/L) when compared with the mean of control group (4.97±0.41 x10^{12}/L).

Table (2): Haematological parameters in Pregnant and control groups(mean ± SD).

Parameter	Pregnant (mean ± SD)	Control (mean ± SD)	P-value
WBC (X 10^9/L)	7.43±1.61	6.13±1.04	< 0.001
Neutrophils (%)	69.82±5.44	59.33±5.90	< 0.001
Monocytes (%)	5.82±1.49	4.99±0.93	< 0.001
Lymphocytes (%)	20.70±4.83	30.95±5.19	< 0.001
RBC x10^{12}/L	4.41±0.39	4.97±0.41	< 0.05
Hb(gm/dl)	11.57±1.32	12.97±0.96	< 0.001
PCV %	36.32±3.60	40.52±2.94	< 0.001
Platelets (X 10^9/L)	229.23±59.60	273.43±49.94	< 0.001

The biochemical parameters for both pregnant women and control groups are shown in table (3). There were non-significant ($p>0.05$) differences in the mean value of serum ALT, AST activity and total bilirubin levels in pregnant women (13.02±14.51 U/L, 16.98±9.47 U/L and 5.98±3.26 µmol/L respectively) when compared with the mean value

of control group (13.82±7.64 U/L, 15.95±5.47 U/L and 6.23±3.06 µmol/L respectively). Also table (3) explained a significant ($p<0.001$) increase in the mean value of ALP in pregnant women (154.12±57.11 U/L) when compared with the mean value of control group (62.92±15.31 U/L). Moreover there is a significant ($p<0.001$) decrease in the mean value of serum BUN, creatinine and glucose levels in pregnant women (2.32±0.78 mmol/L , 42.07±9.96 mmol/L and 4.55±0.68 mmol/L respectively) when compared with the mean value of control group (3.47±0.97 mmol/L, 52.97±11.14 mmol/L and 4.93±0.52 mmol/L respectively).

Table (3): Biochemical parameters in pregnant women and control groups (mean±SD).

Parameter	Pregnant (mean ± SD)	Control (mean ± SD)	P-value
ALT (U/L)	13.02±14.51	13.82±7.64	> 0.05
AST (U/L)	16.98±9.47	15.95±5.47	> 0.05
ALP (U/L)	154.12±57.11	62.92±15.31	< 0.001
Total bilirubin (µmol/L)	5.98±3.26	6.23±3.06	> 0.05
BUN (mmol/L)	2.32±0.78	3.47±0.97	< 0.001
Creatinine (mmol/L)	42.07±9.96	52.97±11.14	< 0.001
Glucose (mmol/L)	4.55±0.68	4.93±0.52	< 0.001

Discussion

White blood cells count is used as a clinical marker of innate immune function, the increase in total WBC was due to an increase in the number of circulating neutrophils, granulocytes, and monocytes (19). Alteration in total and differential count of leucocytes, which may indicate the physiological compensation of the body's defense mechanism through nonspecific immunity, exerted by neutrophils the migratory phagocytes and other leukocytes like monocytes and eosinophil in different trimesters of pregnancy, this alteration in innate immunity represent a tries to compensate at least partly, the weakened specific immunity of the mother's body (20).

Increase WBC (Leucocytosis) occurring during pregnancy in spite of hemodilution is due to the physiologic stress induced by the pregnant state and because increased inflammatory response during normal pregnancy, which can be as a consequence of selective immune tolerance, immunosuppression and immunomodulation of fetus (21). The stress probably stimulate the release of certain factors called leucocytosis inducing factor (LIF) and colony stimulating factors (CSF) which are known to increase haemopoietic activities and blood cells mobilization into circulation (22). Monocytes arise from precursors in the bone marrow and comprise about 5–10% of the circulating blood leukocytes. They circulate in the blood for a few days before migrating into tissues to become macrophages or dendritic cells. They have important functions in homeostasis, tissue repair, and inflammation. Not only count of monocytes increase but monocytes are functionally changed in pregnant women (19).

Neutrophils are the major type of leucocytes on differential counts. This is likely due to impaired neutrophilic apoptosis in pregnancy. The neutrophil cytoplasm shows toxic granulation, neutrophil chemotaxis and phagocytic activity are depressed, especially due to inhibitory factors present in the serum of a pregnant female (23). In the advanced stage of gestation, there is an endogenous adrenaline release which induces the greater mobilization of neutrophils in the circulation resulting in an increase in total leucocyte count (24). Leukocyte and neutrophil count increased significantly on day 1 but start decreasing until fifth day postpartum when the value returns back to normal (21).This important finding should always be kept in mind to avoid the unnecessary use of antibiotic in the postpartum period.

Decrease in lymphocytes can be illustrated through that, it has been reported that stimulated by oestrogen the adrenal cortex produces increasing levels of total and free plasma cortisol and other corticosteroids from 12 weeks to term. It decreased the circulating lymphocyte count, size of lymph node and thymus by inhibiting lymphocyte mitotic activity (20,25). Decrease in lymphocytes percentage and increase of WBC, neutrophils and monocytes in pregnant women in this study are in agreement with (2,6,20)**.**

Nutritional deficiencies, metabolic disorder and changes during gestation can be detected by analysis and monitoring of blood and other body fluids (26). Blood volume begins to increase as early as 6 weeks of gestation and increases to more than 50% of the

prepregnant state. The change in plasma volume during pregnancy is attributed to increased plasma renin activity and reduced atrial natriuretic peptide levels (26).

Changes in RBC, Hb and PCV during pregnancy can explanation as rise in plasma volume more as compared to increase in the red cell mass, plasma volume increases 25%–80% between the sixth and twenty-fourth week of gestation. However, the increase in RBC mass has been found to be approximately 30% between the twelfth and thirty-sixth week of gestation when iron and folate are supplemented (27). Hemoglobin (Hb), hematocrit (HCT) will reflect a normal physiologic anemia of pregnancy. The HCT will appear to fall as the volume increases more than the packed cell count (PCV). Increase in RBC mass due to erythropoietin concentration that increased during pregnancy, this increased probably derived from placental prolactin (28). Erythropoietin effect bone marrow becomes increasingly active and produces extra red blood cells to go with the excess fluid volume (29). The net result being a dip in hemoglobin concentration Thus, there is “physiologic anemia of pregnancy”. The decline in hemoglobin is typically by 1–2 g/dL by the late second trimester and stabilizes thereafter in the third trimester. Women who take iron supplements have less pronounced changes in hemoglobin, as they increase their red cell mass in a more proportionate manner than those not on hematinic supplements (23). Furthermore progressive decline in Hb concentration may be due to an increased demand for iron as pregnancy progresses. More iron is required to meet the expansion of maternal Hb mass and the needs of fetal growth (27).

The haematocrit value (the percentage of red blood cells relative to plasma volume) in non-pregnant women ranges from 38 to 45%. However, in pregnant women because of hemodilution normal values can be much lower, e.g. 34% in single and 30% in twin or multiple pregnancy even with normal stores of iron, folic acid and vitamin B12. This lower range simply reflects "the physiologic hemodilution of pregnancy" and does not indicate a decrease in oxygen carrying capacity or true anaemia (30). Similar results of RBC, Hb and PCV were obtained by (2,6,31)

Decrease in platelet count during pregnancy due to hemodilution and partly due to increased platelet activation and accelerated clearance. This is termed as ‘‘gestational thrombocytopenia.’’ (23). Also increased consumption of platelets as well as decreased life span in the uteroplacental circulation has been suggested to be

the explanation of the reduction in the number of circulating platelets during pregnancy (21). Similar result of platelets was recorded by (2,6).

Table (3) illustrated simple decrease (no significantly) in ALT activity in pregnant women. Also this study illustrated simple increase (no significantly) in AST activity in pregnant women. Thus results in this study indicated that during pregnancy the liver enzymes (AST, ALT) were not clinically affected. This study is in line with other studies, whereas (32) found no significant different in ALT and AST in all trimester of pregnancy compared with non-pregnant women. (33) recorded significantly higher ($P \leq 0.05$) in ALT activity only in third trimester and no significant difference in first and second trimester, while serum AST activity did not give significant differences between pregnant and non-pregnant women. (34) found ALT and AST activity was slightly but significantly increased in third trimester compared to non-pregnant, while no significant increase in ALT and AST activity during first and second trimester compared to non-pregnant women.

Elevate ALP activity in the serum in pregnancy are due to placental ALP (heat stable ALP) entering the maternal blood especially during the second and third trimesters. Serum total alkaline phosphatase (TALP) begins to rise at the fourth (4th) month of gestation. This elevation is a reflection of placental ALP entering the maternal blood, since human trophoblastic cells are rich in ALP. Measurement of serum ALP of placental origin is, therefore, of particular interest in the investigation of placental insufficiency. In addition ALP from other sources other than placenta such as liver and bone; with the highest contribution coming from the placenta (13). As gestation progresses, the concentration of PALP rises till term and this can be caused by the detachment of ALP from the membrane into the maternal circulation. In normal pregnant women it rises to a level 2-3 times higher than that of non-pregnant women and do possess a long half-life (seven days postpartum) (35).

Increase ALP in normal pregnant women associated with it functions, it has a growth factor – like effect on human fetus and mouse embryo fibroblast, transfer of maternal IgG to fetus, destined for the growth and development of the fetus (36), and have an important role in fetus musculature via transfer of phosphate (26). So PALP is used as useful indicator for placental function. In pregnant women, decreased serum level of PALP may be associated with delay uterine

growth (9), premature rupture of membrane and premature labour (36). This study is in line with other studies, that includes (9,33,34) found significantly higher in ALP activity in second and third trimester pregnant women compared to non-pregnant women.

Table (3) illustrated simple decrease (no significantly) in total bilirubin in pregnant women. Pregnancy is characterized by dynamic changes in multiple body systems resulting in increased basal oxygen consumption and in changes in energy substrate use by different organs including the feto-placental unit. The human placenta influences maternal homeostasis, it is rich in mitochondria and when fully developed it consumes about 1% of the basal metabolic rate of the pregnant woman (37).

A phenomenon called oxidative stress is occurred when free radicals and oxidants produced in excess, a deleterious process that can seriously alter the cell membranes and other structures such as proteins, lipids, lipoproteins, and deoxyribonucleic acid (DNA). Biological free radicals are thus highly unstable molecules that have electrons available to react with various organic substrates such as lipids, proteins, DNA. The roles of antioxidants are to neutralize the excess of free radicals, to protect the cells against their toxic effects and to contribute to disease prevention (38). Oxidative stress can cause DNA damage, which in turn can lead to aberrant gene expression and apoptosis. For example, leukocyte DNA damage was approximately two times greater among third trimester pregnant women than non-pregnant control women. This elevation in DNA damage among pregnant women may arise from increases in oxidative stress particularly as pregnancy advances (4).

Thus, decrease in bilirubin due to action of bilirubin as an antioxidant and its role in remove different free radicals that produced by pregnancy. Bilirubin provides potent protection against lipid peroxidation. Therefore bilirubin may be the most abundant endogenous antioxidant in mammalian tissues (39). Also hemodilution could at least partly be responsible for the decrease in bilirubin concentration because albumin is the protein that transports bilirubin (34). This study is in line with other studies, whereas Hussein (40) found similar result of our study, (9) recorded significantly lower ($P \leq 0.05$) in total bilirubin only in third trimester and no significant difference in first and second trimester. (34) found significantly lower in total bilirubin in second and third trimester and no significant difference in first trimester, While Ahmed *et al.* (41)

found significantly lower in total bilirubin in pregnant women compared to non-pregnant women.

The decrease in serum urea of pregnant women although additional nitrogen waste products consequent to the enlarging uterus, placenta, and fetus, might be due to hydration, a rise in glomerular filtration rate (GFR), increased anabolic rate and demand of the developing foetus on the protein of pregnant mothers (42).

Creatinine is a muscle metabolite excreted by the kidney in the urine. When formed, creatinine diffuses passively into the blood stream where it is removed by the glomerular filtration action of the kidney, thus the level of creatinine in the bloodstream is reasonably constant (11). The statistical significant ($p<0.001$) decrease in the levels of serum creatinine in pregnant women may be due to increase in GFR which occurs during pregnancy. The increase in GFR results in an increase in the clearance rate of creatinine, while results a decrease in creatinine level in the serum (43). The increased clearance is thought to be necessary to handle the increased production from placenta and fetus (44). So most pregnant patients who have serum creatinine at the upper limit of normal, defined by laboratory tests for non-pregnant individuals, should be viewed with marked suspicion of renal impairment. Serum creatinine is probably the most widely used indirect measure of GFR and one of the means of assessing kidney function. In several renal failures, the plasma concentration of creatinine is raised. Creatinine is freely filtered, so the serum creatinine levels depend on the GFR (45), while GFR increases in normal pregnancy, so the serum concentration of creatinine decreases.

This study is in line with other studies, whereas (32) found significant decrease in BUN and creatinine in all trimester of pregnancy compared with non-pregnant women. (15) found significant ($p<0.05$) decrease in the levels of serum creatinine in all trimester of pregnancy and they observed progressive but not significant ($p>0.05$) reduction in the level of serum urea from the first to the third trimester of pregnancy.

Changes in carbohydrate metabolism occur during pregnancy to support the fetus and to prepare maternal organs for delivery and lactation. Although insulin sensitivity deteriorates in normal pregnancy, most women are able to maintain normoglycemia (46). Increased fasting blood glucose in pregnant women could indicate danger signs which pose a threat to both the woman and the foetus since glucose is an important substrate for

metabolism. A high increase in blood glucose during pregnancy could lead to gestational diabetes which is characterized by difficulty during delivery, abnormal foetal weight, adolescent obesity, and neonatal hypoglycaemia (47).

During pregnancy, insulin resistance is the natural physiological state (48). This state which appears to be due, in part, to circulating levels of inflammatory mediators (49). Also placental lactogen (PL) promotes insulin resistance. As gestation progresses to term, the mother becomes increasingly insulin resistant. The purpose behind this phenomenon is to limit maternal glucose uptake in order to provide adequate substrates for the growing fetus, as it requires 80% of its available energy to come from glucose (50). On the other hand the development of increasing insulin resistance during pregnancy is compensated for by a simultaneous increase in insulin secretion (51). Whereas in early pregnancy, increasing oestrogen and progesterone levels, which lead to pancreatic B-cell hypertrophy and insulin excretion (48).

Decreases in fasting glucose concentration can be explained as a result of increases in plasma volume and facilitated transport of glucose from mother to fetus (50). Also decreased glucose reabsorption from the renal tubules during pregnancy may be playing a significant role in the contribution to the fall in glucose levels of maternal serum. Furthermore an inverse relationship between cortisol hormone and plasma glucose level since cortisol hormone is a stress hormone, expected to be accompany by increase metabolism with consequence usage of glucose (52). This result is in agreement with (52), but not agreement with (41,47).

References

1- Gacek, C. M. (2009). Conceiving “pregnancy”. Fam. Res. Can. Ins. 9 (1): 1-18.

2- Ifukor, P. C. ; Jacobs, J. ; Ifukor, R. N. and Ewrhe, O. L. (2013). Changes in haematological indices in normal pregnancy. Physiology J.: 1-4.

3- Akkoca, A. N. ; Ozdemir, T. Z. ; Kurt, R. ; Sen, B. B. ; Yengil, E. ; Karatepe, C. ; Karapınar, O. S. and Ozer, C. (2014). The physiological changes in pregnancy and their distribution according to trimester. J.Gyn. Obs. 2 (6): 86-90.

4- Jiang, X. ; Bar, H. Y. ; Yan, J. ; West, A. A. ; Perry, C. A. ; Malysheva, O. V. ; Devapatla, S. ; Pressman, E. ; Vermeylen, F. M. ; Wells, M. T. and Caudill, M. A. (2012). Pregnancy induces

transcriptional activation of the peripheral innate immune system and increases oxidative DNA damage among healthy third trimester pregnant women. Plos One, 7 (11): 1-10.

5- Oliveira, L. J. ; Barreto, R. S. ; Perecin, F. ; Mansouri-Attia, N. ; Pereira, F. T. and Meirelles, F. V. (2012). Modulation of maternal immune system during pregnancy in the cow. Rep. Dom. Anim. 47 (4): 384-393.

6- Ifeanyi, O. E. ; Ndubuisi, O. T. ; Leticia, E. O. and Uche, E. C. (2014). Haematological profile of pregnant women in Umuahia, Abia State, Nigeria. Int. J. Curr. Microbiol. App. Sci. 3 (1): 713-718.

7- Townsley, D. M. (2013). Hematologic complications of pregnancy. Sem. Hem. 50 (3): 222-231.

8- Kim, W. R. ; Flamm, S. L. ; Di Bisceglie , A. M. and Bodenheimer , H. C. (2008). Serum activity of alanine aminotransferase (ALT) as an indicator of health and disease. Hepatology J. 47 (4): 1363-1370.

9- Al-Hamdani, A. H. and Al-Doski, F. S. (2012). Evaluation of some liver function tests during different gestational period. Raf. J. Sc. 23 (1): 44-51.

10- Hall, P. and Cash, J. (2012). What is the Real Function of the Liver 'Function' Tests?. Ulster Med. J. 81 (1): 30-36.

11- Pagana, K. D. and Pagana, T. J. (2010). Manual of diagnostic and laboratory tests (4th ed.).El Sevier, London, pp: 125, 204.

12- Huang, X. J. ; Choi, Y. K. ; Im, H. S. ; Yarimaga, O. ; Yoon, E. and Kim, H. (2006). Aspartate aminotransferase (AST/GOT) and alanine aminotransferase (ALT/GPT) detection techniques. Sensors, 6: 756-782.

13- Aliyu, I. S. ; Randawa, A. J. ; Isah, H. S. and Afonja, O. A. (2013). Pattern of serum total alkaline phosphatase activity in different stages of normal third trimester pregnancy in Zaria, Northern Nigeria. Ann. Nig. Med. Jan-Jun. 7 (1): 28-31.

14- Sticova, E. and Jirsa, M. (2013). New insights in bilirubin metabolism and their clinical implications. Wor. J. Gas. 19 (38): 6398-6407.

15- Patricia, O. O. ; Christiana, B. A. and Raphael, O. J. (2013). Evaluation of changes in renal functions of pregnant women attending antenatal clinic in Vom Plateau State, North-Central Nigeria. Arch. App. Sc. Res. 5 (4): 111-116.

16- Marshall, W. J. and Bangert, S. K. (2008). Clinical chemistry. (6th ed.). Mosby Elsevier, Edinburgh, pp: 74.

17- Lager, S. and Powell, T. L. (2012). Regulation of nutrient transport across the placenta. J. Pregnancy, 179827: 1-14.

18- Nici, A. D. (2004). Statistical analysis in medical research (2nd ed.). Appleton and Lange, Norwalk, Connecticut, pp: 21-30.

19- Faas, M. M. ; Spaans, F and Vos, P. D. (2014). Monocytes and macrophages in pregnancy and pre-eclampsia. Fron. Imm. Inf. 5 (298): 1-11.

20- Pramanik, T. ; Ghosh , A. ; Tuladhar, H. and Pradhan, P. (2005). Variation of total and differential count leukocytes and increment in the number of young neutrophils in different trimesters of pregnancy. Pak. J. Med. Sci. 21 (1): 44-46.

21- Kaur, S. ; Khan, S. and Nigam, A. (2014). Hematological profile and Pregnancy : a review. Int. J. Adv. Med. 1 (2): 68-70.

22- Waziri, M. A. ; Ribadu, A. U. and Sivachelvan, N. (2010). Changes in the serum proteins, hematological and some serum biochemical profifi les in the gestation period in the Sahel goats. Vet. Arh. 80: 215-224.

23- Chandra, S. ; Tripathi, A. K. ; Mishra, S. ; Amzarul, M. and Vaish, A. K. (2012). Physiological changes in hematological parameters during pregnancy. Ind. J. Hem. Bl. Tran. 28 (3): 144-146.

24- Roy, S. ; Roy , M. and Mishra, S. (2010). Hematological and biochemical profile during gestation period in Sahiwal cows. Vet. Wor. 3 (1): 26-28.

25- Chng, W. J. ; Tan, G. B. and Kuperan, P. (2004). Establishment of adult peripheral blood lymphocyte subset reference range for an Asian population by single-platform flow cytometry: influence of age, sex, and race and comparison with other published studies. Clin. Diag. Lab. Imm. 11: 168-173.

26- Abdulkareem, T. A. (2013). Some hematological and blood biochemical profile of Iraqi riverine buffaloes (Bubalus bubalis) during different gestation periods. J. Buf. Sc. 2 (2): 78-84.

27- Akinbami, A. A. ; Ajibola, S. O. ; Rabiu, K. A. ; Adewunmi, A. A. ; Dosunmu, A. O. and Adediran, A. (2013). Hematological profile of normal pregnant women in Lagos, Nigeria. Int. J. Wom. Heal. 5 (1): 227-232.

28- Satue, K. ; Hernández, A and Muñoz, A. (2012). Physiological factors in the interpretation of equine hematological profile. In: Hematology-science and practice. Lawrie , C. (Edt). In Tech, Shanghai, pp: 581.

29- Mansoor, A. J. (2010). Some parameters of anemia during pregnancy of women in Tikrit teaching hospital. Tik. J. Pur Sc. 15 (1): 180-183.

30- Shah, A. R. ; Pate, N. D. and Shah, M. H. (2012). Hematological parameters in anaemic pregnant women attending the antenatal clinic of rural teaching hospital. Inn. J. Med. Heal. Sc. 2 (5): 70-73.

31- Ali, N. K. and Ali, I. S. (2013). Prevalence of anemia among pregnant women attending primary health care center in kirkuk city. Kir. Un. J. Sc. St. 8 (3): 12-25.

32- Al-Tawil, S. R. (2013). Biochemical and hematological profile of normal pregnant women in Gaza Governorate, Gaza strip. MS. C. Thesis, Coll. Faculty of Science, Univ. The Islamic University-Gaza pp: 42-49.

33- Salman, M. I. (2009). Changes in liver functions tests during pregnancy. J. Al-An. Uni. Pu. Sc. 3 (1).

34- Gohel, M. G. ; Joshi, A. G. ; Anand, J. S. ; Makadia, J. S. and Kamariya, C. P. (2013). Int. J. Rep. Con. Obs. Gyn. 2 (4): 616-620.

35- Rajagambeeram, R. ; Raghavan, S. A. ; Ghosh, S. ; Basu, S. ; Ramasamy, R. and Murugaiyan, S. B. (2014). Diagnostic utility of heat stable alkaline phosphatase in hypertensive disorders of pregnancy. J. Clin. Diag. Res. 8 (11): 10-13.

36- Mangal, A. ; Shrivastava, P. ; Gaur, U. ; Jain, A. ; Goyal, U. and Rath, G. (2005). Histochemical analysis of placental alkaline phosphatase in hypertensive disorders complicating pregnancy. J. Anat. Soc. Ind. 54 (2): 1-9.

37- Mehde, A. A. ; Ali, K. F. and Mehdi, W. A. (2013). Study the Effect of Folic Acid as A supplement on Selected Oxidative Stress and Biochemical Parameters in First Trimester of Pregnancy. Ira. J. Pha. Sci. 22 (1): 50-55.

38- Abdul-Barry, J. ; Al-Rubai, S. A. and Qasim, Q. A. (2011). Study of oxidant-antioxidant status in recurrent spontaneous abortion. Thi-Qar Med. J. 5 (1): 35-46.

39- Baranano, D. E. ; Raom, C. D. and Snyder, S. H. (2002). Biliverdin reductase:A major physiologic cytoprotectant. Pr. Nat. Ac. Sc. 99 (25): 16093-16098.

40- Hussein, Z. G. (2012). Study of liver and kidney functions in non-pregnant, pregnant and preeclamptic women. J. Bag. Sc. 9 (2): 277- 284.

41- Ahmed, T. Y. ; Al-Helaly, L. A. and Mla-Alw, F. Y. (2011). Follow-up oxidative stress in pregnant women. Al-Raf. Sc. J. 22 (4): 88-106.

42- Mustafa, R. ; Ahmed, S. ; Gupta, A. and Venuto, R. C. (2012). A Comprehensive review of hypertension in pregnancy. J. Pregnancy, 105918: 1-19.

43- Hussein, W. and Lafayette, R. A. (2014). Renal function in normal and disordered pregnancy. Cur. Opin. Neph. Hyp. 23 (1): 46-53.

44- Cheung, K. L. and Lafayette, R. A. (2013). Renal physiology of pregnancy. Adv. Chr. Kid. Dis. 20 (3): 209-214.

45- Traynor, J. ; Mactier, R. ; Geddes, C. C. and Fox, J. G. (2006). How to measure renal function in clinical practice. B M J 333: 733-737.

46- Ley, S. H. ; Hanley, A. J. ; Retnakaran, R. ; Sermer, M. ; Zinman, B. and O'Connor, D. L. (2011). Effect of macronutrient intake during the second trimester on glucose metabolism later in pregnancy. Am. J. Clin. Nutr. 94: 1232 1240.

47- Ekhator, C. N. and Ebomoyi, M. I. (2012). Blood glucose and serum lipid profiles during pregnancy. Afr. J. Diab. Med. 20 (1): 16-19.

48- Yanamandra, N. and Chandraharan, E. (2012). Anatomical and physiological changes in pregnancy and their implications in clinical practice. Obs. Int. Em. : 1-10.

49- Lowe, L. P. ; Metzger, B. E. ; Lowe, W. L. ; Dyer, A. R. ; McDade, T. W. and McIntyre, H. D. (2010). Inflammatory mediators and glucose in pregnancy: Results from a subset of the hyperglycemia and adverse pregnancy outcome (HAPO) study. J. Clin. End. Met. 95 (12): 5427-5434.

50- Greiner, L. E. (2011). Markers of maternal metabolism and maternal glucose responsiveness following supplementation with docosahexaenoic acid. Ms. C. Thesis, Coll. Allied health sciences, Univ. Cincinnati, pp: 3-5.

51- Suhonen, L. (2009). Glycemic control in diabetic pregnancies: Effects on fetal and maternal outcome. Ph. D. Thesis, Coll. Obstetrics and Gynecology, Univ. Helsinki, Finland, pp: 14.

52- Akinloye, O. ; Obikoya, O. M. ; Jegede, A. I. ; Oparinde, D. P. and Arowojolu, A. O. (2013). Cortisol plays central role in biochemical changes during pregnancy. Int. J. Med. Biom. Res. 2 (1): 3-12.

Iraqi Journal of Biotechnology, 2015, Vol. 14, No. 2 , 391-404

Effect of NB-UVB phototherapy on total protein, albumin and globulins in sera of vitiligo patients

Yasser A.H. Al-Issa[1], Hathama R. Hasan[2], Hayder R. Al-Hamamy[3]

[1, 2]Chemistry Department, College of Science, Baghdad University, Baghdad, Iraq.

[3]Chairman of the Scientific Council of Dermatology, Iraqi Board of Medical Specialization, Baghdad, Iraq.

Abstract: Vitiligo is a relatively common dermatologic finding observed since ancient times. The cause of this disease is unclear yet. Narrow-band ultraviolet B (NB-UVB) is an emerging, promising phototherapy for vitiligo. Total protein content of serum is made up of a large number of individual proteins, and diagnostic information can be obtained by the evaluation of changes in the total serum protein concentrations or in different protein fractions. Because the total proteins represent the sum of albumin and globulins, it is more important to know which protein fraction is altered. A total of 48 patients with vitiligo and 47 control individuals were the participants of the present study. Vitiligo patients received NB-UVB treatment for three days weekly. The concentration of serum protein, albumin and globulin were measured for control group as well as vitiligo patients group before and after treatment. Polyacrylamide gel electrophoresis technique was used to detect the changes in the protein profile according to the gender. There were no significant differences in serum protein, albumin, globulin concentration and [albumin] / [globulin] ratio between the control and vitiligo patients groups. While when these parameters were measured to look out the effect of NB-UVB phototherapy treatment on them, the results showed a significant differences in the concentration of total serum protein ($p=0.002$) and albumin ($p=0.027$). Meanwhile no significant differences was observed in serum globulin concentration and [albumin] / [globulin] ratio upon using this type of treatment. On the basis of current study results, NB-UVB phototherapy seems to affect protein amount and profile. The most observed effect was on albumin's level. The present study results support the role played by UV light in increasing the oxidative stress in the blood by forming many types of cytotoxic free radicals.

Keywords: Vitiligo, NB-UVB treatment, protein, albumin, globulin.

تأثير المعالجة الضوئية فوق البنفسجية ضيقة النطاق على البروتين الكلي والزلال والجلوبيولينات في أمصال مرضى البهاق

م. د. ياسر عبد الحسين آل عيسى[1] ، أ.د. حذامة رزوقي حسن[2] ، أ.د. حيدر رؤوف الحمامي[3]

[1، 2] قسم الكيمياء، كلية العلوم، جامعة بغداد، بغداد، العراق.
[3] رئيس المجلس العلمي للأمراض الجلدية، المجلس العراقي للاختصاصات الطبية، بغداد، العراق.

الخلاصة: البهاق مرض جلدي شائع نسبيا لوحظ منذ العصور القديمة. سبب هذا المرض غير واضح حتى الآن. وتعتبر معالجة هذا المرض بالأشعة فوق البنفسجية ضيقة النطاق من المعالجات الضوئية الواعدة. يتكون مصل الدم من عدد كبير من البروتينات، ويمكن الحصول على المعلومات المتعلقة بتشخيص الامراض بتقييم التغيرات في تركيز بروتين المصل الكلي أو من خلال التغيرات في بعض البروتينات. نظراً لأن مجموع البروتينات تمثل مجموع الزلال والجلوبيولينات، أصبح من المهم أكثر التعرف على اي جزء من البروتين المتأثرة. بلغ إجمالي المشاركين في هذه الدراسة 48 مريضا بالبهاق و47 من عينات السيطرة. تلقى مرضى البهاق العلاج الضوئي بالأشعة فوق البنفسجية ضيقة النطاق لمدة ثلاثة أيام أسبوعيا. تم قياس تركيز المصل البروتين والزلال والجلوبيولينات لمجموعة عينات السيطرة، فضلا عن مجموعة مرضى البهاق قبل وبعد العلاج. استخدمت تقنية الترحيل الكهربائي الهلامي للكشف عن التغييرات حسب نوع الجنس. لم تلاحظ هناك فروق معنوية في تراكيز البروتين والزلال والجلوبيولينات ونسبة [الزلال] /[الجلوبيولينات] في مصول مجموعات مرضى البهاق وعينات السيطرة. بينما عندما تم قياس هذه التراكيز بعد العلاج الضوئي بالأشعة فوق البنفسجية ضيقة النطاق، أظهرت النتائج فروقات معنوية في تركيز بروتين المصل الإجمالي ($p = 0.002$) والزلال ($p = 0.027$). ولم تلاحظ فروقات معنوية في تركيز الجلوبيولينات ونسبة [الزلال]/[الجلوبيولينات] عند استخدام هذا النوع من العلاج. استناداً إلى نتائج الدراسة الحالية، يبدو أن المعالجة الضوئية بالأشعة فوق البنفسجية ضيقة النطاق تؤثر على كمية البروتين محتواه خصوصاً على مستوى الزلال. تدعم نتائج الدراسة الحالية الدور الذي تلعبه الأشعة فوق البنفسجية في زيادة الأكسدة في الدم عن طريق تشكيل أنواع عديدة من الجذور الحرة السامة للخلايا.

Introduction

Total protein content of serum is made up of a large number of individual proteins, and diagnostic information can be obtained by the evaluation of changes in the total serum protein concentrations or in different protein fractions (1).

Because the total proteins represent the sum of albumin and globulins, it is more important to know which protein fraction is altered. Albumin is one of oldest known proteins of plasma (2). Normal concentration of albumin is 3.5–5.0 gm/dl, which makes it the most abundant protein in plasma with a wide variety of physiological functions. Human albumin represents 50% of the normal individual's plasma protein (3). Due to its low molecular weight (67 kDa), albumin contributes to osmotic pressure maintenance of plasma, compared with other plasma globulins (4), and also because of its weak isoelectric point, this protein has a global negative charge at physiological pH (5). Its structure allows it to bind and transport diverse metabolites such as metal ions, fatty acids, bilirubin, and drugs (6). It acts as a free radical scavenger and is able to bind toxic substances (e.g. free fatty acids). A large proportion of total serum antioxidant properties can be attributed to albumin(7). Hundreds

of serum proteins found in globulin fraction including carrier proteins, enzymes, complement, and immunoglobulins. Many of them are synthesized in the liver while the immunoglobulins are synthesized by plasma cells. Electrophoresis can divide globulins into four groups α1, α2, β, and γ, depending on their migratory pattern between the anode and the cathode. Increases in the globulin fraction usually result from an increase in immunoglobulins, but there can be an increase in other proteins in pathologic states that have characteristic electrophoretic patterns (8).

Vitiligo is an epidemic dermatologic finding observed since ancient times (9). It is an acquired skin disorder, characterized by white macula of variable shapes and sizes, with a tendency to increase in size with time(10). About 0.38%-2.9% of the world population suffer from this disease, and its distribution changes depending to the region studied (11,12). Several studies concerned with the vitiligo treatment consider narrow band ultraviolet B (NB-UVB) as an active and safe phototherapy for treatment vitiligo (13-16).This was supported by a previous study results in our laboratory which showed this type of treatment has no effect on thyroid function(17).

The current study test whether this disease ,as well as, this type of treatment has any effect on serum proteins and it's different fractions .In addition ,it focus on the role played by UV treatment and albumin in the oxidative stress that has been previously reported by others to be present in vitiligo patients(18,19).

Materials and methods

The present study inculde 48 vitiligo patients and 47 healthy control. The cases of vitiligo were selected from Dermatology Department in Baghdad Teaching Hospital including 27 males and 21 females with the mean ages of 29.7 and 27 years respectively. A careful history and examination ruled out any other associated disorder and use of any drug(s). Blood samples of the patients was drawn twice before and after 20 session of phototherapy with NB-UVB (after this period of time a response was observed in the treated patients). Treatment with NB-UVB was usually administered three days per a week but never on two consecutive days, for an average of 7 weeks. Usually, a standard starting dose of 200 (mJ/cm^2) was used with increments 20%per treatment depending on skin photo type. The blood sample was drawn once from control subjects.

Total protein concentration of all samples was determined using Biuret method (20), and bovine serum albumin (BSA) as standard. Serum albumin was determined by dye-binding method (21) using kit

manufactured by Randox. The differences between total serum protein and serum albumin represent the concentration of serum globulin. Total protein, albumin and globulin concentrations of serum were expressed in gm/dl.

In order to identify the differences in proteins and glycoproteins that are released into the serum of male and female patients & control groups, polyacrylamide gel electrophoresis was carried out the gel was stained with coomassie brilliant blue R-250 for protein and with Schiff's reagent for glycoprotein.

Statistic program IBM SPSS version 20 was used in the statistical calculations. The z-test (independent samples) was used for analysis the differences between the control group and patient group before treatment, while the t-test (paired samples) was used for the analysis the differences between the patients before and after treatment with NB-UVB. A value of the $p<0.05$ was used for considering the significant differences.

Results

Throughout this study total serum protein, albumin, and globulin concentrations were measured as explained in material and method section, as well as the ratio of [albumin] / [globulin] was calculated in sera of both healthy control group and vitiligo patients groups before treatment and the results are presented in table 1. It is clear from the results that there are no significant differences in serum protein, albumin, globulin concentration and [albumin] / [globulin] ratio between the control and vitiligo patients groups.

Table (1): Comparison of protein, albumin, and globulin concentrations levels and [Albumin] / [Globulin] ratio between the healthy control group and vitiligo

	Groups	N	Mean ± SD	P value
S. Protein (gm/dl)	control	47	8.232 ± 0.732	0.575
	Patient	48	8.142 ± 0.838	
S. Albumin (gm/dl)	control	47	4.749 ± 0.369	0.739
	Patient	48	4.777 ± 0.435	
S. Globulin (gm/dl)	control	47	3.483 ± 0.625	0.44
	Patient	48	3.365 ± 0.850	
[Alb]/[Glo] ratio	control	47	1.403 ± 0.244	0.086
	Patient	48	1.553 ± 0.601	
*** P value is significant at the 0.05 level (2-tailed).** *** N= number of cases.**				

In order to look if there is any variation in the above parameters according to the gender, a comparison between these levels in males and females were done.

It can be observed from the table 2 that no significant differences were found between males and females for the healthy group and vitiligo patients group when the comparison of the above mentioned parameters was done.

Table (2): Comparison of protein, albumin, and globulin concentrations and [Albumin] / [Globulin] ratio between the healthy control group and vitiligo patients group for both gender.

		Groups	N	Mean ±SD	P value
Male	S. Protein (gm/dl)	control	18	8.397 ± 0.791	0.206
		Patient	27	8.078 ± 0.851	
	S. Albumin (gm/dl)	control	18	4.815 ± 0.390	0.905
		Patient	27	4.800 ± 0.423	
	S. Globulin (gm/dl)	control	18	3.582 ± 0.730	0.186
		Patient	27	3.278 ± 0.764	
	[Alb]/[Glo] ratio	control	18	1.392 ± 0.272	0.136
		Patient	27	1.557 ± 0.455	
Female	S. Protein (gm/dl)	control	29	8.130 ± 0.687	0.673
		Patient	21	8.224 ± 0.835	
	S. Albumin (gm/dl)	control	29	4.708 ± 0.357	0.751
		Patient	21	4.747 ± 0.460	
	S. Globulin (gm/dl)	control	29	3.421 ± 0.554	0.811
		Patient	21	3.477 ± 0.956	
	[Alb]/[Glo] ratio	control	29	1.409 ± 0.230	0.424
		Patient	21	1.549 ± 0.761	
*** P value is significant at the 0.05 level (2-tailed).** *** N= number of cases.**					

Valuable diagnostic information can be obtained using electrophoresis to fractionate serum proteins and to determine the concentrations of the major protein fractions (Burtis et al., 1994). Since computer programs are available now that digitally analyze the pictures of the gels for comparing the differences in the patterns between the groups, J-image program was used in the present study to look for such differences.

In order to identify the differences in proteins that are released into the serum of male and female patients & control groups, polyacrylamide gel electrophoresis

was carried out. After the electrophoresis was complete, the gels were stained with coomassie brilliant blue R-250 to detect such difference in the protein profile (figure 1 and 2).

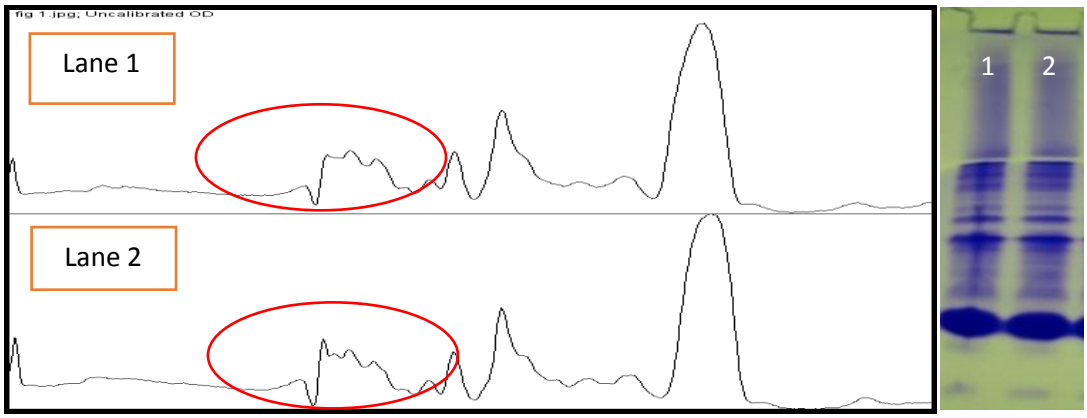


Figure (1): Serum protein electrophoresis pattern using Tris-glycine buffer, pH 8.9 as electrode buffer. Electrophoresis was carried out for 3 hours at 4ºC using a constant current of 40mA and a voltage of 20V/cm. The gel was stained by coomassie brilliant blue R-250 stain: lane [1] control (male); lane [2] vitiligo patients (male).

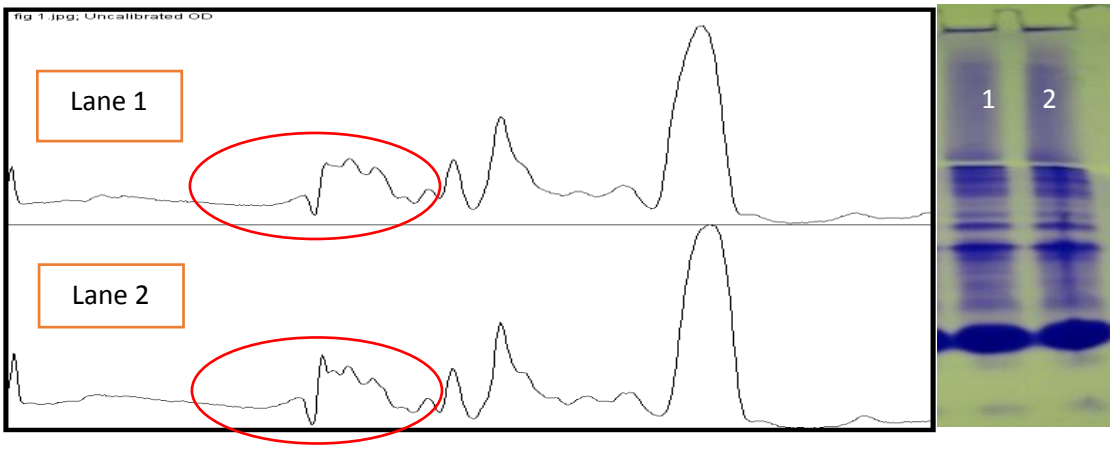


Figure (2): Serum protein electrophoresis pattern using Tris-glycine buffer, pH 8.9 as electrode buffer. Electrophoresis was carried out for 3 hours at 4ºC using a constant current of 40mA and a voltage of 20V/cm. the gel was stained by coomassie brilliant blue R-250 stain: lane [1] control (female); lane [2] vitiligo patients (female).

It is obvious from the comparison of the proteins profile (figure 1), that the proteins of all groups were separated into several bands with some differences in the globulin regions for males. While no differences in protein pattern of females specimen was observed

(figure 2) and glycoproteins (figure 4). The results in (figure 3) reveal that the differences appeared in protein electrophoresis patterns are in glycoproteins as Schiff's stain of the gel showed.

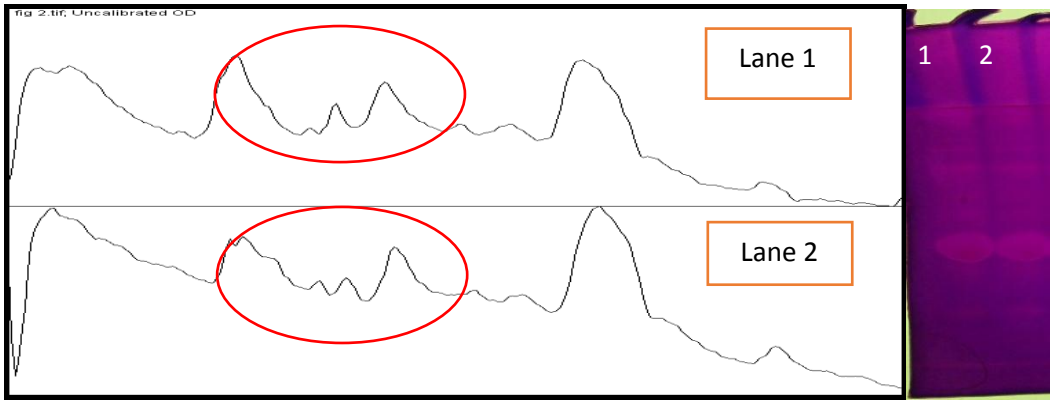


Figure (3): Sera glycoprotein electrophoresis pattern using Tris-glycine buffer, pH 8.9 as electrode buffer. Electrophoresis was carried out for 3 hours at 4ºC using a constant current of 40mA and a voltage of 20V/cm. the gel was stained by basic fusion stain: lane [1] control (male); lane [2] vitiligo patient (male).

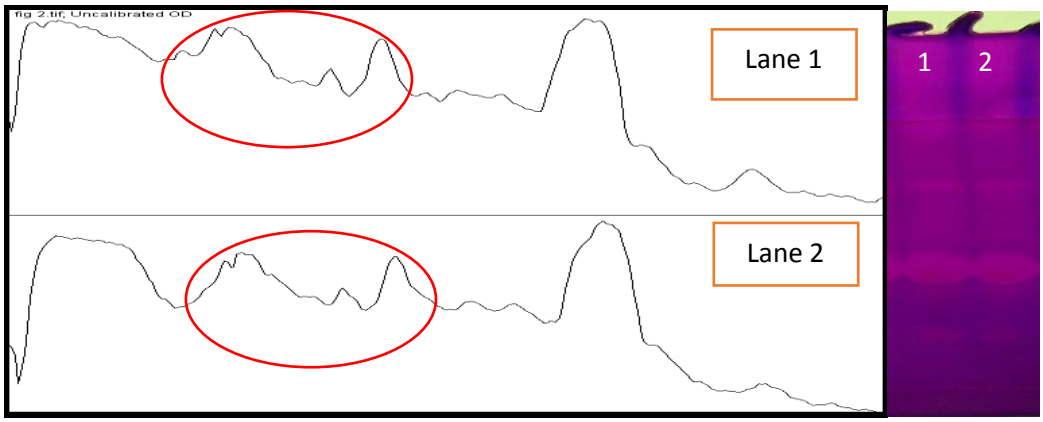


Figure (4): Sera glycoprotein electrophoresis pattern using Tris-glycine buffer, pH 8.9 as electrode buffer. Electrophoresis was carried out for 3 hours at 4ºC using a constant current of 40mA and a voltage of 20V/cm. the gel was stained by basic fusion stain: lane [1] control (female); lane [2] vitiligo patient (female).

The great advantage of electrophoresis compared with the quantitation of specific proteins is the overview it provides. The electrophoretic pattern can give information about the relative increases and decreases within the protein population, as well as information about the homogeneity of a fraction (22).

The electro-zymograms in figures 1 and 2 which represents

proteins and figures 3, and 4 which represents glycoproteins electrophoresis patterns of separation, such separation is based on difference in the net charge as well as in the molecular weights. As it is obvious from these figures, the differences are clear in these peaks between the males and females groups. Also there are differences in protein peaks among the indicated studied groups between control and patients in male specimen. These different proteins seems to be glycoprotein since they stained with Schiff base (figure 3 and 4).

Because of the unknown pathogenesis of vitiligo, many treatment strategies have been devised. Some of these treatments were developed to restore the functional integrity of the epidermis and melanocytes by reactivating residual melanocytes. While the others suppressed the immune reaction (23).

When the concentration of total protein, albumin, globulin and [albumin] / [globulin] ratio were compared between patients before and after NB-UVB phototherapy treatment, the results show a highly significant difference (pair 1) in the concentration of total serum protein ($p<0.01$). Pair 2 show a significant difference in the concentration of serum albumin between vitiligo patients before and after phototherapy treatment ($p<0.05$), while no significant differences was observed between these groups for serum globulin concentration (pair 3) and [albumin] / [globulin] ratio (pair 4) at ($p>0.05$) as illustrated in table 3.

Table (3): Comparison of protein, albumin, globulin concentrations and [Albumin] / [Globulin] ratio for vitiligo patients before and after NB-UVB phototherapy.

		N	Mean ± SD	P value
Pair 1	S. Protein before treatment (gm/dl)	31	8.125 ± 0.787	0.002*
	S. Protein after the treatment (gm/dl)	31	8.671 ± 0.724	
Pair 2	S. Albumin before treatment (gm/dl)	31	4.751 ± 0.388	0.027*
	S. Albumin after the treatment (gm/dl)	31	5.002 ± 0.501	
Pair 3	S. Globulin before treatment (gm/dl)	31	3.374 ± 0.780	0.088
	S. Globulin after the treatment (gm/dl)	31	3.669 ± 0.849	
Pair 4	Alb/Glo ratio before treatment	31	1.499 ± 0.444	0.948
	Alb/Glo ratio after treatment	31	1.491 ± 0.627	
*** P value is significant at the 0.05 level (2-tailed).** *** N= number of cases.**				

Polyacrylamide gel electrophoresis of serum proteins and glycoproteins for males and females was done to detect any

changes in proteins profile after treatment of vitiligo patients with NB-UVB phototherapy (figures 5, 6, 7, and 8). It is clear from these figures that few changes in protein profile migrations and intensities of the bands are present between males and females. These differences are more obvious in glycoprotein profile (the red circulated area) in figures 7 and 8 in female and male groups before and after treatment.

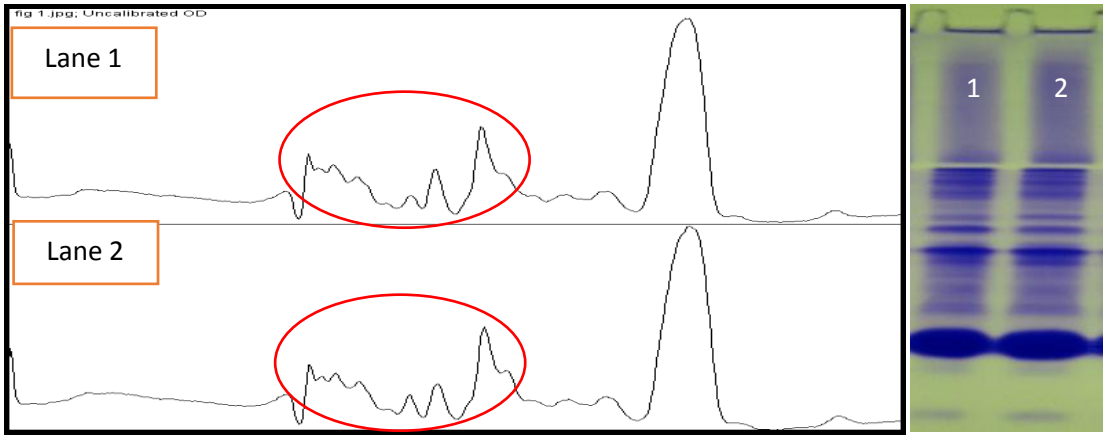


Figure (5): Serum protein electrophoresis pattern using Tris-glycine buffer, pH 8.9 as electrode buffer. Electrophoresis was carried out for 3 hours at 4ºC using a constant current of 40mA and a voltage of 20V/cm. the gel was stained by coomassie brilliant blue R-250 stain: lane [1] Vitiligo patients before treatment (male); lane [2] vitiligo patients after treatment (male).

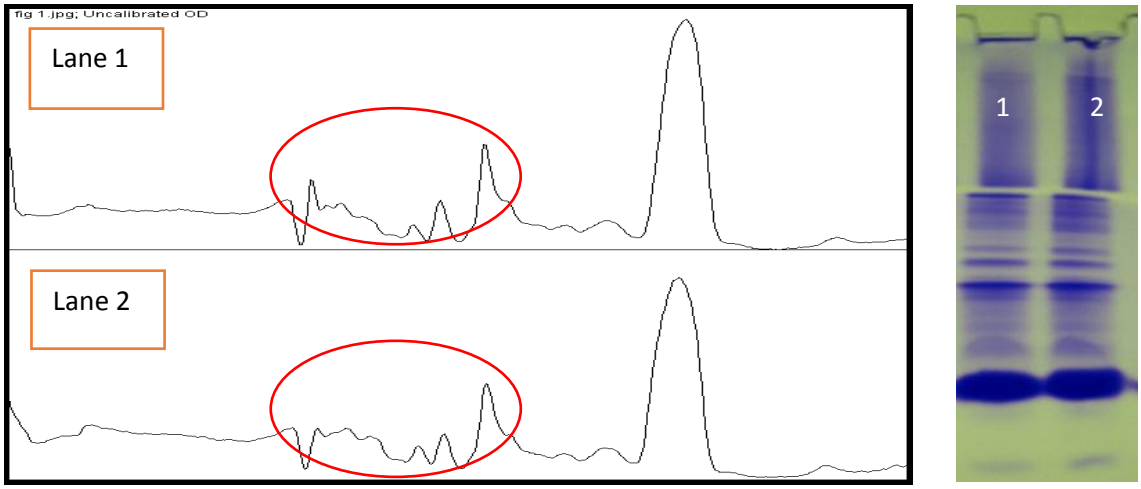


Figure (6): Serum protein electrophoresis pattern using Tris-glycine buffer, pH 8.9 as electrode buffer. Electrophoresis was carried out for 3 hours at 4ºC using a constant current of 40mA and a voltage of 20V/cm. the gel was stained by coomassie brilliant blue R-250 stain: lane [1] Vitiligo patients before treatment (female); lane [2] vitiligo patients after treatment (female).

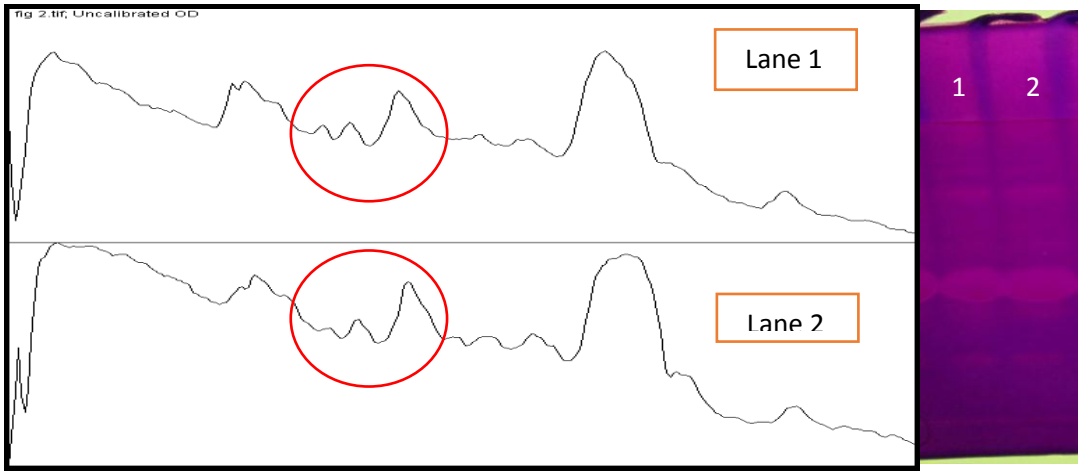


Figure (7): Serum glycoproteins electrophoresis pattern using Tris-glycine buffer, pH 8.9 as electrode buffer. Electrophoresis was carried out for 3 hours at 4ºC using a constant current of 40mA and a voltage of 20V/cm. the gel was stained by basic fusion stain: lane [1] vitiligo patients before treatment (male); lane [2] vitiligo patient after treatment (male).

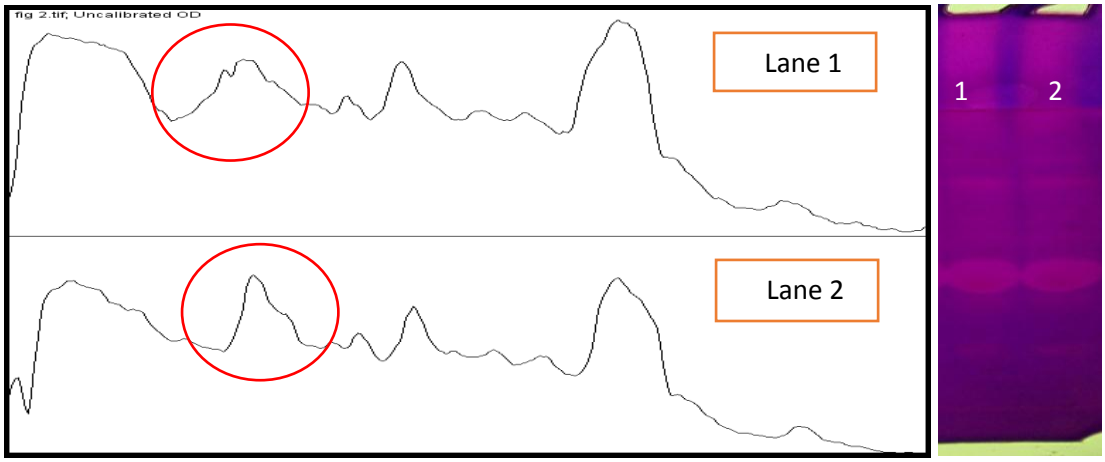


Figure (8): Serum glycoproteins electrophoresis pattern using Tris-glycine buffer, pH 8.9 as electrode buffer. Electrophoresis was carried out for 3 hours at 4ºC using a constant current of 40mA and a voltage of 20V/cm. the gel was stained by basic fusion stain: lane [1] vitiligo patients before treatment (female); lane [2] vitiligo patient after treatment (female).

Discussion

Oxidative stress is considered to be one of the possible pathogenic events in melanocyte loss (24,25). Antioxidant can be defined as "any substance that delays, prevents or removes oxidative damage to a target molecule" (26). Albumin has been considered not only as an

antioxidant, but as the major circulating antioxidant in plasma known to be exposed to continuous oxidative stress. No significant differences in albumin concentration was found when the comparison was done between the control group and the vitiligo patients group. This result reflects that the albumin already present in the serum, in addition to the other antioxidants mechanisms exist there, are capable to cope with the increasing levels of free radicals in sera of vitiligo patients& scavenge them without the need to produce an excess amount of albumin (27,28).

On the other hand, it is clear from the results in table 3 a significant increase in sera total protein and albumin concentration occur in the patients group after they received NB-UVB phototherapy treatment. Such increase can be explained on the basis of the role of UV light in generating free radicals, which necessitate the presence & requirement of more antioxidants to scavenge such radicals. Albumin, as it is known, is considered as the main extracellular molecule responsible for maintenance of the plasma redox state(29,30).This protein exerts specific antioxidant functions due to its multiple ligand-binding capacities & free radical-trapping properties, which are both closely related to its structure(29,30). The structural stress that induced by reactive species has been reported to impair the antioxidant capacity of albumin and the entire albumin molecular structure is highly involved in the antioxidant response(31). Therefore the body needs to synthesize more albumin so that it can carry on its different physiological functions.

It can be concluded from the results of this study that this disease has no effect on proteins concentration but NB-UVB phototherapy seems to have an effect on protein amount and profile. The most observed effect was on albumin's level. The present study results support the role played by UV light in increasing the oxidative stress in the blood by forming many types of cytotoxic free radicals.

References

1. David Eckersall, P. (2008) In Bruss, J. J. K. W. H. L. (ed.), Clinical Biochemistry of Domestic Animals (Sixth Edition). Academic Press, San Diego, pp. 117-155.
2. Stein, D.A. (2010) Protein aggregation. Nova Science Publishers, Hauppauge, N.Y.
3. Evans, T.W. (2002) Review article: albumin as a drug—biological effects of albumin unrelated to oncotic pressure. Alimentary Pharmacology & Therapeutics, 16, 6-11.

4. Scatchard, G., Batchelder, A.C. and Brown, A. (1944) Chemical, Clinical, and Immunological Studies on the Products of Human Plasma Fractionation. Vi. The Osmotic Pressure of Plasma and of Serum Albumin. J Clin Invest, 23, 458-464.

5. Figge, J., Rossing, T.H. and Fencl, V. (1991) The role of serum proteins in acid-base equilibria. J Lab Clin Med, 117, 453-467.

6. Wood, M. (1986) Plasma drug binding: implications for anesthesiologists. Anesth Analg, 65, 786-804.

7. Roche, M., Rondeau, P., Singh, N.R., Tarnus, E. and Bourdon, E. (2008) The antioxidant properties of serum albumin. FEBS Letters, 582, 1783-1787.

8. Busher, J.T. (1990) In Walker, H. K., Hall, W. D. and Hurst, J. W. (eds.), Clinical Methods: The History, Physical, and Laboratory Examinations. Butterworth Publishers, a division of Reed Publishing, Boston.

9. Bellet, J.S. and Prose, N.S. (2005) Vitiligo in children: a review of classification, hypotheses of pathogenesis and treatment. Anais Brasileiros de Dermatologia, 80, 631-636.

10. Steiner, D., Bedin, V., Moraes, M.B., Villas, R.T. and Steiner, T. (2004) Vitiligo. Anais Brasileiros de Dermatologia, 79, 335-351.

11. Laberge, G., Mailloux, C.M., Gowan, K., Holland, P., Bennett, D.C., Fain, P.R. and Spritz, R.A. (2005) Early disease onset and increased risk of other autoimmune diseases in familial generalized vitiligo. Pigment cell research / sponsored by the European Society for Pigment Cell Research and the International Pigment Cell Society, 18, 300-305.

12. Birlea, S.A., Fain, P.R. and Spritz, R.A. (2008) A Romanian population isolate with high frequency of vitiligo and associated autoimmune diseases. Arch Dermatol, 144, 310-316.

13. Kist, J.M. and Van Voorhees, A.S. (2005) Narrowband Ultraviolet B Therapy for Psoriasis and Other Skin Disorders. Advances in Dermatology, 21, 235-250.

14. Scherschun, L., Kim, J.J. and Lim, H.W. (2001) Narrow-band ultraviolet B is a useful and well-tolerated treatment for vitiligo. Journal of the American Academy of Dermatology, 44, 999-1003.

15. Kanwar, A.J., Dogra, S., Parsad, D. and Kumar, B. (2005) Narrow-band UVB for the treatment of vitiligo: an emerging effective and well-tolerated therapy. International journal of dermatology, 44, 57-60.

16. Hercogova, J., Buggiani, G., Prignano, F. and Lotti, T. (2007) A rational approach to the treatment of vitiligo and other hypomelanoses. Dermatologic clinics, 25, 383-392, ix.

17. Al-Issa, Y.A.H., Al-Hamamy, H.R. and Hasan, H.R. (2015) Evaluation the effect of NB–UVB phototherapy on thyroid function in sera of vitiligo patients. Iraqi National Journal of Chemistry, 15, 97-102.

18. Karsli, N., Akcali, C., Ozgoztasi, O., Kirtak, N. and Inaloz, S. (2014) Role of oxidative stress in the pathogenesis of vitiligo with special emphasis on the antioxidant action of narrowband ultraviolet B phototherapy. The Journal of international medical research, 42, 799-805.

19. Sitar, M.E., Aydin, S. and Cakatay, U. (2013) Human serum albumin and its relation with oxidative stress. Clinical laboratory, 59, 945-952.

20. Janairo, G., Linley Sy, M., Yap, L., Llanos-Lazaro, N. and Robles, J. (2011) Determination of the Sensitivity Range of Biuret Test for Undergraduate Biochemistry Experiments. e-Journal of Science & Technology, 6, 77-83.

21. Doumas, B.T., Ard Watson, W. and Biggs, H.G. (1971) Albumin standards and the measurement of serum albumin with bromcresol green. Clinica Chimica Acta, 31, 87-96.

22. Bishop, M.L., Fody, E.P. and Schoeff, L.E. (2013) Clinical Chemistry: Principles, Techniques, and Correlations. Wolters Kluwer Health.

23. Nina, S. and Sharon, K. (2011) Combination therapy enhances effect of NB-UVB for treatment of vitiligo. A systematic review. Erasmus Journal of Medicine 1, 43-46.

24. Schallreuter, K.U. (1999) Successful treatment of oxidative stress in vitiligo. Skin pharmacology and applied skin physiology, 12, 132-138.

25. Maresca, V., Roccella, M., Roccella, F., Camera, E., Del Porto, G., Passi, S., Grammatico, P. and Picardo, M. (1997) Increased sensitivity to peroxidative agents as a possible pathogenic factor of melanocyte damage in vitiligo. The Journal of investigative dermatology, 109, 310-313.

26. Halliwell, B. and Gutteridge, J. (2007) Free Radicals in Biology and Medicine. 4 ed. Clarendon Press, Oxford.

27. Soriani, M., Pietraforte, D. and MinettI, M. (1994) Antioxidant potential of anaerobic human plasma: role of serum albumin and thiols as scavengers of carbon radicals. Archives of biochemistry and biophysics, 312, 180-188.

28. Halliwell, B. (1996) Antioxidants in human health and disease. Annu Rev Nutr, 16, 33-50.

29. Guidet, B. (2009) In Richard C, Teboul JL and JL, V. (eds.), Réanimation. Elsevier, pp. 343–356.

30. Oettl, K. and Stauber, R.E. (2007) Physiological and pathological changes in the redox state of human serum albumin critically influence its binding properties. British Journal of Pharmacology, 151, 580-590.

31. Medina-Navarro, R., Corona-Candelas, I., Barajas-González, S., Díaz-Flores, M. and Durán-Reyes, G. (2014) Albumin antioxidant response to stress in diabetic nephropathy progression. PLoS One, 0.1371/journal.pone.0106490.