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Iraqi Journal of Biotechnology

Contents

Iraqi Journal of Biotechnology

Contents

Iraqi Journal of Biotechnology, 2015, Vol. 14, No. 2 , 1-11

The first report of *Grapevine Algerian latent virus* (GALV) infecting tomato and eggplant in Iraq

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Abstract: A research was initiated to investigate tombusviruses in Iraq. Tomato and eggplant samples collected from fields in Iraq were screened by Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) using tombusvirus specific primers. Sequence analyses confirmed the detection of *Grapevine Algerian latent virus* (GALV) in tomato and eggplant. GALV from Iraq showed maximum (93%) nucleotide identity to the CP region of GALV isolates from Japan (Acc. AY830918). The maximum amino acid identity was (98%) to an isolate from Italy (Acc. AF540885). Neighbor-Joining phylogenetic tree grouped GALV sequences isolated into a single group. Although the tombusvirus sequences from Iraq are clearly representative of the species GALV, their distinct properties (infection of previously unreported hosts, and phylogenetic position) suggest that GALV from Iraq could be a distinct strain.

Keywords: Molecular detection, tombusviruses, phylogeny, plant virus, sequence analyses

التسجيل الاول لفايروس الجزائري الكامن على العنب *Grapevine Algerian latent virus* (GALV) على نباتي الطماطة والباذنجان في العراق

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الخلاصة: اجري هذا البحث بهدف التحري عن الاصابة بالفايروسات العائدة لمجموعة فايروس التقزم الشجيري على الطماطا tombusviruses في عينات من نباتي الطماطة والباذنجان جمعت من الحقول العراقية في بغداد بوساطة تفاعل انزيم البلمرة المتسلسل المعتمد على انزيم النسخ الرجعي (RT-PCR) وباستعمال طقم بوادئ متخصصة بالجنس *Tombusvirus*. اذ اظهرت نتائج المسح الجزيئي اصابتها بمجموعة فايروسات التقزم الشجيري على الطماطا. كما اظهرت نتائج تحليل تسلسل القواعد النيتروجينية للتسلسلات المختبرة اعلى نسبة تطابق نيوكليوتيدي وصلت الى 93% في منطقة الغلاف البروتيني مع الفايروس الجزائري الكامن على العنب عزلة اليابان (Acc. AY830918) كما وصلت اعلى نسبة تطابق حامض اميني الى 98% مع الفايروس نفسه عزلة ايطاليا (Acc. AF540885) . كما جمعت شجرة الاصول الوراثية التسلسلات القاعدية المعزولة من عينات الطماطة والباذنجان مع بعضها بصورة منفصلة ضمن مجموعة التسلسلات القاعدية الخاصة ب(GALV). تشير النتائج الى ان التسلسلات المعزولة قد تعود الى سلالة فريدة النوع اعتماداً على التحليل الجزيئي واصابتها لعوائل نباتية غير مسجلة مسبقاً.

Introdection:

Tomato and eggplant grown in Iraq have a significant economic importance due to their wide consumption (1). They provide a source of direct income as well as employment (2). Tomato and eggplants are grown during the year in both open and protected fields in Iraq. The estimated yield of the solanaceous crops in Iraq were 1059540 and 452050 metric tonnes (MT) for tomato and eggplant, respectively (3). Based on FAO statistics, Iraq ranked the 20th in tomato production among other countries, whereas it ranked the 7th in eggplant production (3). Taxonomically, tomato (*Solanum lycopersicon* L.) and eggplant (*Solanum melongena* L.) belong to the *Solanaceae* family (4). They are infected by many pathogens including several viruses (2). The genus *Tombusvirus*, whose name was abbreviated from the name of its type member *Tomato bushy stunt virus* (TBSV), is the second largest genus (with 17 species) after the genus *Carmovirus* (20 species) within the family *Tombusviridae* (5). The family *Tombusviridae*, whose name was derived from the genus *Tombusvirus*, consists of more than 57 definite and 15 tentative species (6). At least ten of them have been recorded to infect vegetables including tomato and eggplant(7). Members of the genus *Tombusvirus* are transmitted by various ways, including mechanical transmission, fungal transmission, seed and pollen transmission, vegetative propagation and grafting, and through infected soil (6). The tombusvirus particles are icosahedral and ~30-38 nm diameter with T=3 symmetry. Their genome is a single molecule of positive-sense single stranded RNA (ssRNA) of about 4.8 kb in length organized in four ORFs (8). Species demarcation criteria within the genus *Tombusvirus* outlined in the ICTV guidelines are that a distinct tombusvirus species shows less than 87% amino acid similarity in their CP sequence to other members of this genus (9). *Grapevine Algerian latent virus* (GALV: genus *Tombusvirus*; family: *Tombusviridae*) is a member of the genus *Tombusvirus* within the family *Tombusviridae*. It was described for the first time on naturally infected grapevine plants in Italy in 1989 (10). The natural host range of this virus was recorded to be narrow. It is infecting plant species belonging to three families, namely *Chenopodiaceae*, *Solanaceae* and *Vitidaceae*. The virus was shown to infect a wider host range experimentally, namely eggplant, cucumber, common bean and broad bean and cowpea causing local lesion infection (9-11). GALV was reported on a naturally infected nipple-fruit *Solanum mammosum*, an ornamental solanaceous plant in

Japan and a range of other ornamental plants in different geographical regions (9,10). The virus has not been reported to infect vegetables or potato naturally. GALV is mechanically transmissible and transmitted through contaminated soils and has also been detected in river waters (9-11). GALV transmission through seed or by a fungal vector has to date not been confirmed (10).

Materials and methods

Leaf samples were collected separately from symptomatic tomato and eggplant plants from fields in the Abu Ghraib district of Baghdad province in 2008. Leaf samples were dried by calcium chloride in plastic bags at 4 ºC for two weeks, and shipped to NRI at Greenwich University, The UK. Total nucleic acids were extracted from dried leaf samples using an adapted CTAB protocol (12, 13) cDNA synthesis was performed using ImProm-IITM Reverse transcription system (Promega, UK) and the primer CIR2 (9,15). Polymerase chain reaction was performed using Red Hot *Taq* DNA polymerase (Thermo Scientific Inc., UK) following the manufacturer's instructions. The genus specific primers CIR1(5'-GACTCCGCCGTAGCTTGACC) and CIR2(5'-GGTTTATTGACTTGTTCGTATT CAG-3') were used to screen samples as dicribed by (9, 14). Two µl of cDNA was mixed with PCR reaction then the final volume was adjusted to 25 µl using SDW. The following PCR cycle for CIR1/CIR2 primer set was used (9,14): A pre-denaturation cycle for 3 min at 94 ºC, 35 amplification cycles (denaturation for 30s at 94 ºC, annealing for 45s at 60 ºC and extension for 2 min at 72 ºC), with a final extension cycle of 10 min at 72 ºC. PCR products were analyzed using (1% w/v) agarose gel electrophoresis according to standard protocol (15). The gel was stained in 0.5 µg/ml ethidium bromide solution, visualized and photo captured using SYNGENE G: Box photo gel image and analysis system (Synoptics group, UK). DNA fragments of expected size were recovered from the gel by QIAquick gel extraction kit (Qiagen, UK) according to the manufacturer's protocol. Purified DNA was ligated into pGEM®-T easy using the vector system produced by Promega, UK, according to the manufacturer's protocol. The recombinant plasmids were transformed into JM109 high efficiency competent cell (Promega, USA), and the size of inserts in recombinant colonies confirmed by PCR using T7/SP6 primers. Selected clones were sequenced (Source Bioscience UK Limited, UK). Sequence data obtained were analyzed using MEGA5 software (16). Neighbour-Joining

phylogenetic trees were constructed using sequences isolated in this study and equivalent GenBank sequences. Bootstrap test was performed based on scores above 90% and 70% cut-off value to support nt an aa tree topology respectively. The accession codes of sequences generated were (JQ042281-JQ042290).

Results and Discussion:

In this study, tombusviruses were investigated in tomato and eggplant samples collected from fields in Baghdad-Iraq. Leaf mottling, malformation, fruit blisters and malformation symptoms, observed on eggplant resembled those induced by *Tomato bushy stunt virus* (TBSV) or *Eggplant mottled crinkle virus* (EMCV) (17,18). Tomato plants shown no characteristic symptoms, although they were grown nearby the diseased eggplants. As both TBSV and EMCV are members of the genus *Tombusvirus*, CIR1/CIR2 primer set was used to screen the symptomatic eggplant samples collected (9,14). This tombusvirus specific primer set has been designed from the highly conserved motifs in CP gene (9,14). RT-PCR, using A diagnostic ~1.2 kb PCR product amplified by CIR1/CIR2 indicated detection of tombusviruses in tested samples (Figure 1). Sequence analyses confirmed that the 1.2 kb fragments amplified were the coat protein (CP) of *Grapevine Algerian latent virus* when compared to equivalent GenBank sequences. All Iraqi sequences isolated shared 93-94% to GALV infecting nipple fruit *Solanum mammosum* in Japan (Acc. AY830918) and *Gypsophila paniculata* L., in The Netherlands (Acc. AY500880) (Table 1). Deduced amino acid sequences of Iraq GALV sequences showed the 97-98% maximum identity to CP region of Apulia from Italy (Acc. AF540885) (Table 1). Sequence comparison showed unexpected results when sequences obtained from tomato and eggplant samples showed homology to GALV rather than TBSV or EMCV . These two viruses have been reported to infect eggplant naturally (17,18). GALV has been reported to infect eggplant experimentally inducing local lesion infection on inoculated leaves without systemic infection (10,11). Besides, no report of tomato infection by GALV worldwide, naturally or experimentally (10,11). Phylogenetic analyses based on nucleotide (nt) and amino acid (aa) sequences grouped all sequences with the GALV clade (Figure 2A&B). Both Neighbor-Joining phylogenetic trees diverged GALV sequences isolated into separated group supported by 100% and 94% bootstrap values for nt and aa based trees respectively (Figure 2 A&B). The new host range, sequence data

and phylogenetic divergence, suggest GALV detected may be a new strain. However, the means of GALV infectivity to eggplant and tomato is still unknown. The possible route of infection could be through root system by contaminated soil or irrigation water, as GALV is efficiently transmitted by these means (19). Further infectivity tests, therefore, are required to confirm tomato and eggplant could be systemically infected by GALV through roots.

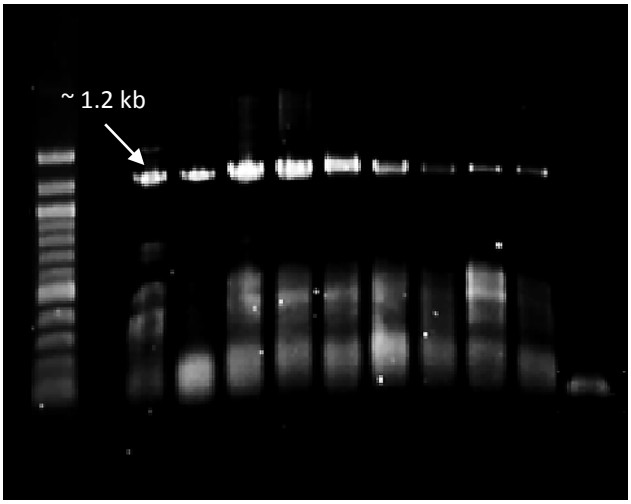


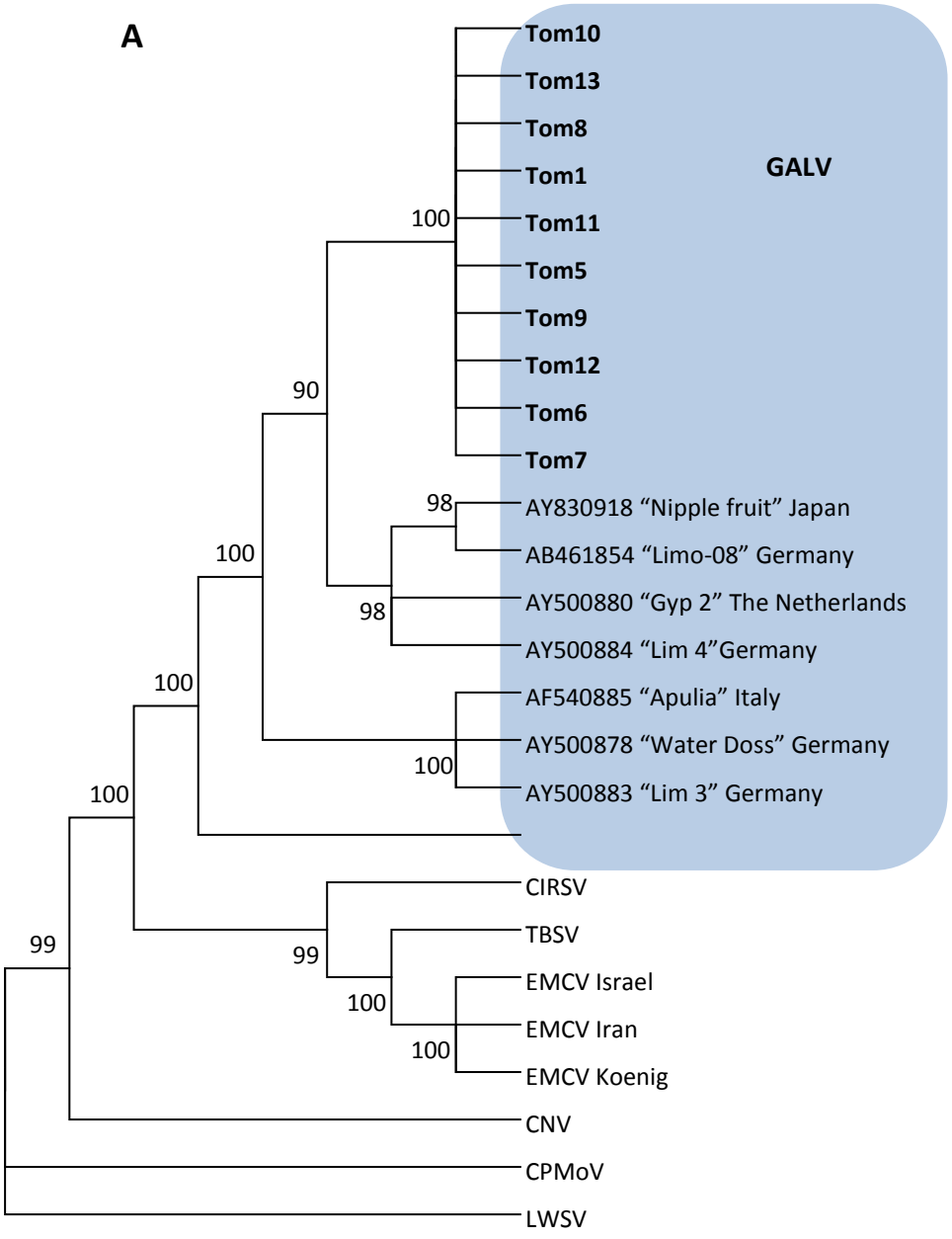
Figure 1: Amplification of tombusviruses genome by RT-PCR using CIR1/CIR2 primers.

Gel electrophoresis profile shows ~1.2 kb DNA fragment amplified from tested samples by CIR1/CIR2 tombusvirus specific primers. Lanes 1-8: tomato samples, 9: eggplant sample, W: water control and L: 100 bp DNA marker (New England Biolabs, UK).

Table 1: Comparison of GALV CP sequences

	Isolate/virus	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1	**Tom1**		**100**	**100**	**99**	**100**	**99**	**100**	**100**	**100**	**100**	**94**	**93**	**92**	**93**	**91**	**91**	**91**	**85**	**37**	**37**	**14**	**36**	**37**	**35**
2	**Tom5**	100		**99**	**99**	**100**	**99**	**100**	**100**	**100**	**100**	**94**	**93**	**92**	**93**	**91**	**91**	**91**	**85**	**37**	**37**	**15**	**36**	**37**	**35**
3	**Tom6**	100	99		**99**	**100**	**99**	**100**	**100**	**100**	**100**	**94**	**94**	**92**	**93**	**91**	**91**	**91**	**85**	**38**	**37**	**14**	**36**	**37**	**35**
4	**Tom7**	100	100	100		**99**	**98**	**99**	**99**	**99**	**99**	**94**	**93**	**92**	**93**	**91**	**91**	**91**	**86**	**38**	**39**	**15**	**37**	**38**	**36**
5	**Tom8**	100	100	100	100		**99**	**100**	**100**	**100**	**100**	**94**	**93**	**92**	**93**	**91**	**91**	**91**	**85**	**37**	**37**	**14**	**36**	**37**	**35**
6	**Tom9**	98	98	98	98	98		**99**	**99**	**99**	**99**	**93**	**92**	**91**	**92**	**90**	**90**	**90**	**84**	**36**	**35**	**14**	**35**	**36**	**34**
7	**Tom10**	100	100	100	100	100	98		**100**	**100**	**100**	**94**	**93**	**92**	**93**	**91**	**91**	**91**	**85**	**37**	**37**	**14**	**36**	**37**	**35**
8	**Tom11**	100	99	99	100	100	98	100		**100**	**100**	**94**	**93**	**92**	**93**	**91**	**91**	**91**	**85**	**37**	**37**	**14**	**36**	**37**	**35**
9	**Tom12**	100	99	100	100	100	98	100	99		**100**	**94**	**93**	**92**	**93**	**91**	**91**	**91**	**85**	**37**	**37**	**15**	**36**	**37**	**35**
10	**Tom13**	100	100	100	100	100	98	100	100	100		**94**	**93**	**92**	**93**	**91**	**91**	**91**	**85**	**37**	**37**	**14**	**36**	**37**	**35**
11	Japan	96	96	96	96	96	95	96	96	96	96		**97**	**95**	**99**	**93**	**93**	**93**	**86**	**37**	**37**	**14**	**35**	**36**	**33**
12	GYP2	97	97	97	97	97	95	97	97	97	97	98		**96**	**96**	**93**	**92**	**93**	**86**	**37**	**37**	**14**	**36**	**37**	**35**
13	Lim 4	96	96	96	96	96	95	96	96	96	96	96	97		**95**	**91**	**91**	**91**	**85**	**37**	**36**	**14**	**36**	**37**	**35**
14	Limo 08	97	96	96	97	97	95	97	97	96	97	100	99	97		**92**	**92**	**92**	**86**	**38**	**38**	**14**	**34**	**36**	**33**
15	Water Doss	97	97	97	97	97	95	97	97	97	97	96	97	95	97		**100**	**100**	**86**	**40**	**38**	**14**	**37**	**38**	**36**
16	Lim 3	97	96	96	97	97	95	97	96	96	97	96	97	95	96	100		**100**	**86**	**39**	**37**	**14**	**37**	**38**	**36**
17	Apulia	98	97	97	98	98	96	98	97	97	98	97	98	96	97	100	99		**86**	**40**	**37**	**14**	**37**	**38**	**36**
18	Schunter River	95	94	94	95	95	93	95	95	94	95	95	95	94	95	95	95	96		**37**	**38**	**15**	**39**	**41**	**39**
19	TBSV	57	57	57	57	57	56	57	57	57	57	57	57	57	58	58	58	58	57		**52**	**7**	**66**	**67**	**66**
20	CIRSV	64	64	63	64	64	62	64	64	63	64	63	63	63	63	64	64	64	64	69		**10**	**47**	**48**	**47**
21	CNV	43	44	43	43	43	43	43	43	43	43	45	44	44	44	43	43	43	46	44	45		**7**	**8**	**7**
22	EMCV Iran	60	60	60	60	60	59	60	60	60	60	60	60	59	59	60	60	60	60	75	68	42		**99**	**97**
23	EMCV Koenig	60	60	60	60	60	59	60	60	60	60	60	60	59	59	60	60	60	60	77	69	42	98		**98**
24	EMCV Israel	59	59	59	59	59	58	59	59	59	59	58	58	58	58	59	59	59	59	75	69	42	96	97	

Nucleotide identity (upper right) and deduced amino acid similarity (lower left) CP sequences of *Grapevine Algerian latent virus* sequences isolated from Iraqi samples (bold letters) with corresponding sequences from GenBank compared to nt sequences identity percent (in bold numbers). Evolutionary divergence conducted by pairwise comparison and calculated by p-Distance method from (MEGA5) (Tamura *et al*., 2011).


A
Tom10
Tom13
Tom8
Tom1
Tom11
Tom5
Tom9
Tom12
Tom6
Tom7
GALV
AY830918 “Nipple fruit” Japan
AB461854 “Limo-08” Germany
AY500880 “Gyp 2” The Netherlands
AY500884 “Lim 4”Germany
AF540885 “Apulia” Italy
AY500878 “Water Doss” Germany
AY500883 “Lim 3” Germany
CIRSV
TBSV
EMCV Israel
EMCV Iran
EMCV Koenig
CNV
CPMoV
LWSV
100
90
98
100
98
100
100
100
99
99
100
100

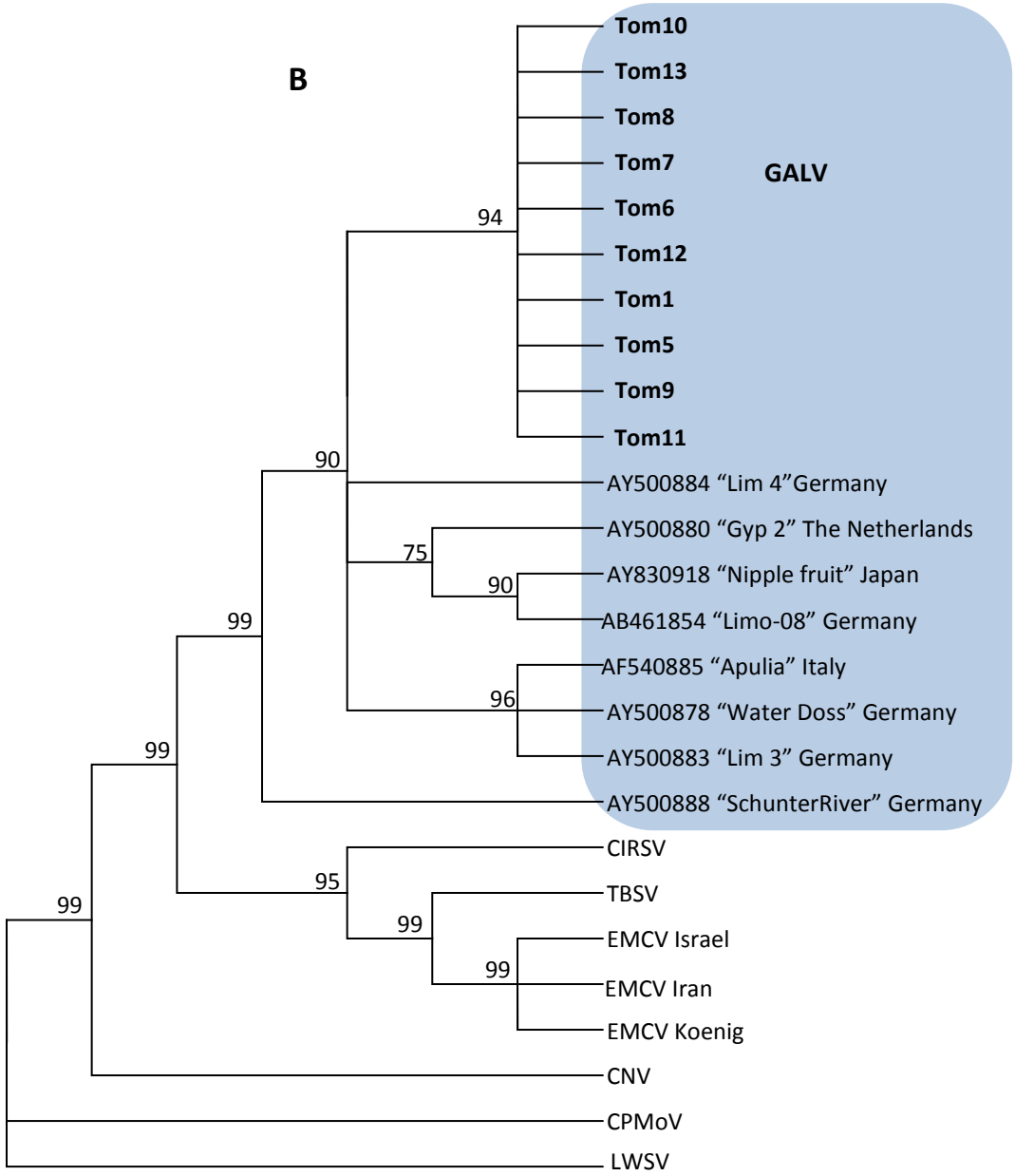


Figure 2: Phylogenetic tree of *Grapevine Algerian latent virus*

Neighbor-Joining phylogenetic analysis of CP nucleotide sequences (A) and CP amino acid sequences (B) of GALV isolated from eggplant and tomato (bold letters) and GALV GenBank sequences referred to as (GenBank acc. No. “isolate name” geographical location). TBSV: *Tomato bushy stunt virus* (TBSV: genus *Tombusvirus*; family: *Tombusviridae*), CIRSV: *Carnation Italian ring spot virus* (CIRSV: genus *Tombusvirus*; family: *Tombusviridae*), EMCV: *Eggplant mottled crinkle virus* (EMCV: genus *Tombusvirus*; family: *Tombusviridae*), CNV: *Cucumber necrosis virus* (CNV: genus *Tombusvirus*; family: *Tombusviridae*), CPMoV: *Cowpea mottle virus* (CPMoV: genus *Carmovirus*; family: *Tombusviridae*) & LWSV: *Leek white stripe virus* (LWSV: genus *Necrovirus*; family: *Tombusviridae*).

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Cytotoxicity and Apoptosis Effect of Purified Arginine Deiminase(ADI) Originating From *Enterococcus Faecium M1* on Rhabdomyosarcoma(RD) Cancer Cell Line and Rat Embryo Fibroblast(REF) normal cell line

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Abstract: Arginine deiminase isolated from a higher productive localy isolated strain *Enterococcus faecium* M1 is avery potent and effective enzyme when used as a cancer theraputic agent. The cytotoxic activity of ADI on RD cancer cell line and REF normal cell linefor (24, 48 and 72h) was examined, inhibition rate increased with raising of ADI concentration and incubation period for RD cell line. The significant effect produced by ADI with (10 to 100ng) concentrations, IC50 and IC90 were (24 and 55ng/ml) after 72h of incubation.ADI showed a slight cytotoxic effect on REF cell line (at high concentrations) and reduced with increase of incubation period and decrease of ADI level and the cytotoxicity disappeared when (24and 55 ng/ml) concentrations of enzyme were used. When ADI was used to investigate its ability to produce mitochondrial apoptosis effect on RD and REF cell lines using concentrations that produced significant cytotoxic effects on RD cell line, the results revealed that the main reason of cell cytotoxicity was the induction of apoptosis process by ADI enzyme and they were compatible to the results of cytotoxicity test. In this study we found that the stability and activity of this enzyme were potentiates the cytotoxic effects of ADI on RD cell line and exerted the strongest antiproliferative effects on their cells. By conclusion ADI has a significant intrinsic mitochondrial apoptosis effect on RD cancer cell line but it was safe for REF normal cell line.

Keywords: Arginine deiminase, *Enterococcusfaecium*, Rhabdomyosarcoma, Rat Embryo Fibroblast.

دراسة التأثير السمي وحث الموت المبرمج لأنزيم أرجنين دي إمينيز المنقى من العزله *Enterococcus Faecium M1* على الخط الخلوي السرطاني Rhabdomyosarcoma والخط الخلوي الطبيعي Rat Embryo Fibroblast

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الخلاصة: يعتبرأنزيم أرجنين دي إمينيز المنقى من العزله الأعلى أنتاجا*Enterococcus Faecium*M1 من أنشط وأقوى الأنزيمات المستخدمه لعلاج السرطان. تم قياس التأثير السمي للأنزيم على الخط الخلوي السرطاني Rhabdomyosarcomaوالخط الخلوي الطبيعي Rat Embryo Fibroblast لثلاث فترات (24 ،48 ،72) ساعة. أظهرت النتائج ازدياد التأثير السمي بزيادة تركيز الإنزيم وفترة الحضن مع الخط السرطاني (RD) حيث تراوحت أهم التراكيز المؤثره بين (10-100) نانوغرام/مل. كان منتصف التركيز المثبط الأقصى للأنزيم50% هو24 نانوغرام/مل ألا أن التركيز 55 نانوغرام/مل أدى ألى تثبيط 90% من الخلايا بعد 72ساعه من الحضن مع الأنزيم. أما عند استخدام الخط الخلوي غير السرطاني (REF) كان التأثير السمي للإنزيم قليلا فقط عند التراكيز العالية منه وقد قل التأثير بزيادة فترة الحضن وتقليل كمية الإنزيم حيث أنعدم التأثير السمي عند استخدام التراكيز24و55 نانو غرام/مل بعد 72 ساعه من الحضن.تم التحري عن قابلية أنزيم أرجنين دي إمينيز لحث الموت المبرمج للخلايا السرطانية المتواسط بالمايتوكوندريا باستخدام بعض التراكيز المهمة والمؤثرة التي استخدمت في فحص السمية للخلايا فأن النتائج أظهرت بان السبب الرئيسي لسمية الخلايا كان الحث على خطوات الموت المبرمج باستخدام التراكيز المختلفة للإنزيم والتي كانت مقاربة لنتائج فحص سمية الخلايا. لاحظنا في هذه الدراسه أن ثبات الأنزيم ونشاطه المتميزفي الظروف البيئيه المختلفه أدى الى تقوية التأثيرالسام له على الخط السرطاني (RD). نستنتج من هذه الدراسه بأن التأثير السام للأنزيم يعود الى حث الموت المبرمج للخلايا السرطانيه والمتواسط بالمايتوكوندريا لكن تأثيره كان امنا مع الخط الخلوي الطبيعي (REF).

Introduction

Cancer still takes millions of lives every year around the world, Recently in Iraq there is a terrible number of unpublished cancer cases. The world health organization (WHO) estimated that if unchecked, annual global cancer deaths could be rise to 15 million by 2020 (1, 2). The current conventional cancer treatment options for localized tumors and advanced disease are typically associated with risks and side effects (3). The discovery of anticancer drugs remains a highly challenging endeavor and cancer a hard-to-cure disease (4). l-Arginine deiminase enzyme (ADI; EC 3.5.3.6) catalyzes the irreversible hydrolysis of arginine to citrulline and ammonia. This enzyme participates in arginine metabolism which is widely expressed in bacteria including *Enterococcus* that the production of this enzyme is used as routinely test to identify many species of this genus (5). This enzyme that hydrolyze arginine to generate energy in many parasitic microorganisms has a potent anticancer activities and can halt growth of solid tumors (6). The

restriction of arginine inhibits the growth of metastatic tumours by the depletion of extracellular arginine using arginine deiminase enzyme (7). Controlling the cell cycle and apoptosis has been considered a promising target for cancer chemoprevention agent due to ADI potential activity in inhibiting blood vessel growth (anti-angiogenesis) and cell division in laboratory tests (8, 9). The goal of this project was designed to study the cytotoxicity and apoptosis effect of arginine deiminase enzyme purified from the higher productive *Enterococcus faecium* M1 isolation on RD cancer cell line and (REF)normal cell line, because There is no study about the effect of this enzyme on any cancer cell line in Iraq.

Materials and methods

Cytotoxic activity of arginine deiminase on REF and RD cell lines

The effect of purified arginine deiminase(ADI) enzyme on REF and RD cell lines was determined. In the first step, five concentrations (200- 1000 ng/ml) of ADI were used and compared with controls. In the second and third step lower concentrations(10 to 100 ng/ml) and (2-8ng/ml) were used.

Purified arginine deiminase enzyme: this enzyme was obtained from (10).

Tissue culture cell line media (for cytotoxicity assay): Rosswell Park Memorial Institute -1640 culture medium with or without Fetal calf serum for REF cell line

Minimum Essential Media: with or without Fetal calf serum for RD cell line (11).

Cell lines used in the study: Rhabdomyosarcoma (RD) cell line:it was kindly provided by ICCMGR at passage75- 77 of RD cell line were used throughout this study and MEM medium with 10% fetal calf serum was used in maintaining the cells.

Rat Embryo Fibroblast (REF) cell line, It was kindly supplied by ICCMGR at Passage 63.

In vitro cytotoxicity assay: Preparation and Maintenance of the cell lines has been done according to(11)**.**

Viable Cell Counting of control cell lines contained more than 95% cell viability of a confluent monolayer and It was performed according to (12).

Cytotoxicity assay: Cytotoxicity effect of various concentration of arginine deiminase enzyme on proliferation of the adherent cells in 96-well microtiter plate has been performed according to (11) method as follows: The purified arginine deiminase was sterilized by filtration throughout 0.22 µm Nalgene Millipore membrane filter

and diluted (when the cytotoxicity test was done) with serum free medium in a manner of concentrations. Cytotoxic effect of arginine deiminase on cell lines was evaluated by crystal violet stain. A set of five concentrations (1000, 800, 600, 400 and 200 nanogram/ml) , then another set of tenth concentrations from (100 to10 ng/ml) and the last four concentrations (8, 6, 4 and 2 ng/ml) of arginine deiminase enzyme, were concerned, the exposure time assay were (24, 48 and 72 hours) for each concentration under aseptic conditions, the remaining steps of Cytotoxicity assay were completed according to (11). The percentages of Inhibitory Rate (IR) were calculated (13) according to the equation as below:

$IR\% = \frac{C-T}{C} \times 100$ IR%: The percentage of inhibition rate C: The absorbance(optical density at 492nm) of control. T: The absorbance (optical density 492nm) of the test of each concentration. The optical densities (O.D) at wave length 492 nm of 3 cell lines after 24, 48 and 72 hour exposure to all concentrations of (ADI) enzyme were compared to those of their controls (ADI-free treated groups). The change in O.D was referred as the percentages of Inhibitory Rate (IR) were calculated (13) according to the last equation .

Apoptotic effect on cell-lines

The principle of this assay depends on the disruption of the mitochondrial transmembrane potential, which is one of the earliest intracellular events that occur following the induction of apoptosis. The dye of Mitocapture reagent kit will be concentrated in the mitochondria of healthy cells, thereby creating red fluorescent region within the cell, while dispersed in apoptotic cells; these cells will not have red aggregates in the mitochondria, rather the entire cell will appear green. The assay can be carried out according to (14) as follows:-Four-tenth ml of cell suspension (1×10^6cells/ml) with medium was added in each chamber of tissue culture 8-chamber slide. The chambers were sealed and incubated at 37˚C until the confluent monolayer was formed. The medium was withdrawn and discarded, then o.4ml of ADI enzyme selected concentrations (table 4) were added, leaving appropriate control chambers that were treated with SFM, the slide was sealed and incubated at 37˚C for 24,48 and 72hr for each concentration. The medium was aspirated and discarded, then 0.4ml of diluted mitocapture reagent, A2299-92A (diluted immediately prior to use by mixing 1µl mitocapture to 1ml pre-warmed incubation buffer A2299-92B, for each assay, the solution was

vortexed and centrifuged for 1min at 13000 RPM and carefully transferred the supernatant), the slide was sealed and incubated at 37˚C for 15-20min.

Dye reagent was aspirated and the chambers were removed from the slide, then the slide was examined under fluorescent microscope. The number of healthy cells (red fluorescent region), and apoptotic cells (green region) were counted in five fields and the mean of them were calculated then the percent of apoptosis was calculated from the following equation:-

Apoptosis % = (No. of apoptotic cells/Total No. of cells) × 100

The final apoptosis % of treated groups = Apoptosis % of treated groups - Apoptosis % of their controls.

Statistical Analysis

The Statistical Analysis System (15) was used to determine the effect of different factors (Concentration and Time) on inhibition rate of different cell lines. Least significant difference –LSD test was used to significant compare between the means of this study

Results and Discussion

Effect of high ADI concentrations on cell lines.

The results showed a variable effect of treatments on the cell lines proliferation among the three periods of incubation,.

Table(1) showed all treated groups IR% of REF cell line after began with high level then gradually decreased whereas vice versa about control groups which were began with low IR% level then increasing occurred gradually according to time, these results indicated that arginine was decreased in culture medium during the first time of incubation in treated normal REF cell line by arginine deiminase enzyme which lead to starvation of some cells to this amino acid then to cell cycle arrest and inhibited the proliferation of them, but during the time the cells could synthesize the needed amount of arginine by induction the production of two enzymes argininosuccinate synthetase and argininosuccinate lyase because their expression, localization and regulation differs significantly depending on the tissue specific needs for arginine, thus the arrested cells were retained their ability in proliferation and decreasing the I.R. during the time, the best and significant con. of ADI was 200ng/ml after 72h incubation which revealed a slight cytotoxicity effect on the viability of (REF) normal cell line. About control groups the I.R. enhanced with time due to accumulation of metabolites and deprivation of nutrients in cell line culture medium.

Table1: Cytotoxic effect(Inhibition Rate%) of ADI high concentrations on REF and RD cell lines after different times of incubation

Cytotoxic effect (Inhibition Rate%)						
	REF			RD		
Inc. time	24 hrs	48hrs	72hrs	24hrs	48hrs	72hrs
Control range	0-0.8	1.6-2	2.7-3.3	0-0.5	2.1-2.7	3.1-3.4
Test ADI con. ng/ml						
200	18.2	9.0	6	81	90.5	94
400	17.7	9.6	6.7	80	91.8	94.7
600	19.7	10.2	6.2	87	93.2	94.5
800	20.3	11	7.8	90	95	96
1000	22.3	11.6	8	92.7	96.9	96.3

Statistical analysis of differences (Mann-Whitney test) between each concentration and its control group showed non-significant difference $P \leq 0.05$ in comparison to control but they were significant (LSD) value between different ADI concentrations and times.

These data described the lowest IR to REF cell line produced by ADI after 72h of incubation which confirmed the ability to produce arginine increased with the time of incubation lead to gradually diminish of the cytotoxicity effect to this normal cell line and exit the cells from stationary stage then proliferation of them. (16) found that arginine deprived normal cells will have become quiescent but soon recover on restitution of the missing nutrient, whereas tumor cells in cycle can be hit by low doses of cycle-dependent cytotoxic drugs. Arginine is required by all tissues in human and other mammalian bodies for protein synthesis, and by some tissues for specialized needs.

2. When RD cell line exposed to ADI it exhibited a significant toxic effect started from the concentration 200ng/ml till the concentration of 1µgm/ml. Statistical analysis of differences (Mann-Whitney test) between each concentration and its control group showed that all concentrations of ADI revealed significant differences at $P \leq 0.05$ in comparison with control and LSD value between concentrations and times.

The results explains that IR% values were between (81.2 - 92.7%) at (200- 1000 ng/ml respectively) of enzyme after 24h of incubation, this results indicates that the sensitivity level of RD cell line to ADI may be due to higher requirement of arginine to produce important metabolites for growth and proliferation of RD cell line depending on control group which had very low inhibition rate values(table 1). The effect of ADI on RD cell line after 48hr and 72 hr. of exposure was more toxic than 24 h exposure with high level of inhibition rate for all concentrations, the IC90 of ADI was 200ng after 48hr of incubation with RD cell lin. The cytotoxicity effect of ADI high concentrations for cancer cell line may be partly due to pH effectiveness of NH_3 produced as a product of enzyme activity during the time and due to accumulation of cell line metabolic products with the time which increased the pH of culture media, because the color converted from orange(pH 7.2 was a suitable neutral value for cell growth) to pink alkaline not suitable value (in the presence of phenol red indicator) for the activity of enzymes and proteins used during cellular growth and proliferation, while this effect was diminished as concentration dropped. In the other hand this effect was not observed with REF cell line, may be this normal cell line can tolerate the low difference of pH value, the optimum pH for cell growth varies among different cell strains (17).

These results describes that arginine decreases with the time leading to induce more dead cell ratios and this probability raised with time, that explain this enzyme was very toxic to RD cell with these efficient concentrations. Many studies reported that arginine deiminase has cytotoxic effect at low concentrations toward many cell lines because ADI induces G0/G1-phase arrest then sub-G1 accumulation (3,18,19).

Effect of low ADI concentrations on REF and RD cell lines

The high toxic effect of the previous concentrations lead to use lower ADI concentrations (10-100 ng/ml) to determine the lower significant con. of enzyme which will be toxic for RD but safe for REF cell line.

1. Table (2) showed all treated groups IR% of REF cell line, it revealed a slight effect on the viability of normal (REF) cell line. The IR% of the first three concentrations (10- 30 ng/ml) were 0% and other seven concentrations(40-100ng) had very low cytotoxic effect which will be diminished during the time, whereas vice versa about control groups which were began with low IR% level then increasing occurred gradually according to time.

Table 2:Cytotoxic effect(Inhibition Rate%) of ADI lower concentrations on REF and RD cell lines after different times of incubation

Cytotoxic effect (Inhibition Rate%)						
	REF			RD		
Inc. time	24 hrs	48hrs	72hrs	24hrs	48hrs	72hrs
Control range	0-0.6	1.1-2.4	2.9-3.5	0-0.7	2-2.8	3-3.6
Test ADI con. ng/ml						
10	0	0	0	18.5	20	34
20	0	0	0	24.1	31.5	41
30	0	1.8	0	22.6	43.2	57
40	2.1	1.6	0	31.8	56	70.3
50	4.4	4.1	0	40.6	72.3	88
60	3.2	3.7	1.1	47.2	69.5	91.2
70	5	4.9	3.4	54.2	77.2	90.7
80	5.7	5	2.5	65.9	73.8	92.3
90	6.1	5.3	4.8	55.4	82	91.8
100	6.8	5.5	5	61.7	86.8	93.1

These results indicated that the enzyme had a very little cytotoxic effect on REF normal cell line with those ten concentrations (which were less than the effect of first five higher concentrations (200-1000ng/ml). The results confirmed the ability of REF normal cell line to synthesize arginine by ASS and ASL enzymes induced during the time. Statistical analysis showed no significant differences between each concentration and its control group showed non-significant difference at $P>0.05$ in comparison with control but they were significant (LSD) value between different ADI concentrations and times.

2. The cytotoxic effect of ADI against RD cell line after 24h incubation with ten concentrations(10-100ng) is described in table (2), the IC50 of ADI was 62 ng/ml after 24h of incubation. After 48h incubation the IR were augmented, the IC50 was 33ng/ml and IR significantly expanded with increasing the

concentrations and reached to 86.8% at 100ng ADI. That prove this cell line was very sensitive to deprivation of enzyme.

When the incubation time increased to 72h, the cytotoxic effect of the ten concentrations significantly raised to higher cytotoxic effects, These data indicate that the IC50 of enzyme is 24ng/ml and the IC90 for ADI enzyme is 55ng/ml after 72h. of incubation with significant differences at $P \leq 0.05$ in comparison with control and LSD value between concentrations and times. The results identified the robust cytotoxic ability of ADI enzyme to inhibit the proliferation of RD cell line especially after 72h of incubation, but in the same time it hadn't any cytotoxicity to normal REF cell line with the same concentrations, the IR was nearly 0% at 55ng/ml after 72h of incubation(Table2). In other words, the enzyme will be safe to REF normal cell line but in the same time it has very cytotoxic effect toward RD cell line with this amount of enzyme, which mean RD couldn't express(ASS) enzyme the rate-limiting enzyme for the biosynthesis of arginine from citrulline. ASS-negative cancer cells require arginine from extracellular sources for growth and survival, thus the absence of arginine in the presence of ADI in culture media components lead to suffering the cells from starvation then to death. some authors(20) found that melanoma and hepatocellular carcinoma (HCC) are auxotrophic for arginine, because they do not express(ASS) enzyme thus they die because of arginine starvation; where as normal cells which express ASS were able to survive.

Effect of very low concentrations (2-8 ng/ml) of purified arginine deiminase on REF and RD cell lines.

In order to know the cytotoxicity of ADI at lower concentrations on the proliferation of three studied cell lines, four amounts were used (2- 8 ng/ml).

1. Table(3) showed all treated groups IR% of REF cell line, the IR values were 0% with all enzyme concentrations.

2. In table (3) results indicate that the cytotoxicity of RD cell line was gradually deprived with decreasing of ADI con. and during the time, which mean that the enzyme had a concentration and time depending effect, Non- significant differences at $P \leq 0.05$ between inhibition rates in comparison with control but they were significant LSD values between concentrations and times.

Inhibition rate reached to 29% when 8ng/ml of enzyme used, but it was not sufficient to inhibit RD cell line like higher concentration (55ng/ml) which was the best amount of ADI enzyme to inhibit about 90% of RD cell line.

Table3: Cytotoxic effect(Inhibition Rate%) of very low concentrations of ADI on REF and RD cell lines after different times of incubation

	(Cytotoxic effect) Inhibition Rate%					
	REF			RD		
Inc. time	24 hrs	48hrs	72hrs	24hrs	48hrs	72hrs
Control range	0-0.3	1.5-2.2	2.7-3.2	0-0.2	1.9-2.5	2.8-3
ADI con. ng/ml						
2	0	0	0	I.6	6.5	9
4	0	0	0	3.2	11.4	12.3
6	0	0	0	8.8	18.1	19
8	0	0	0	12.3	20.2	29

From the results of three groups of concentrations used it can be concluded that all concentrations of arginine deiminase possess a cytotoxic effect toward the cancer cell lines but the severity of cytotoxicity was varied between enzyme quantities and two cell lines.

Apoptotic effect of arginine deiminase on cell-lines

The significant concentrations of ADI caused inhibitory effect on cell proliferation, particularly in RD tumor cell line and in normal REF cell line were chosen to investigate their ability to cause apoptotic effect (table 4).The apoptotic cells (green regions) and healthy cells (red regions) of both ADI-treated and untreated (control) cells have been illustrated and computed under the fluorescent microscope using cationic fluorescence dye (mitocapture reagent). The percentage of apoptosis was calculated as a mean of five fields for every test which was clearly showed apoptosis induction in treated groups in comparison to their controls. Arginine deiminase treatment caused significant induction of apoptosis in RD cell lines. IC50 and IC90 and other important enzyme quantities that produced high percentage of inhibition rate for cancer cell lines were chosen here. The results of apoptosis test were compared with the results of cytotoxic test especially in both RD and REF cell lines.

Table (4): The apoptosis ratio of cell lines induced by significant concentrations of ADI enzyme during incubation times.

ADI concentration (Nanogram/ml)	RD	REF
24	54% during 72h (2)	0% during 72h (3)
33	51% during 48h (5)	0% during 48h
37	-	0% during 72h
55	94% during 72h (6)	0% during 72h (7)
100	-	2% during 72h (9)
200	100% during 48h (8)	4% during 72h (10)
400	-	5% during 72h
600	-	5% during 48h

The results in this study showed the ability of ADI enzyme to induce (mitochondrial) apoptosis in RD tumor cell line, the number of apoptotic cells were ADI dose and time dependent which are directly proportional to each other. The apoptosis ratio in RD cell line (table 4) and figures (2, 5, 6 and 8) showed that the main reason of cell cytotoxicity was inducing the apoptosis process by different concentrations of ADI enzyme with high significant differences at $P \leq 0.05$ in comparison to control. Therefore, apoptotic effect of ADI to cancer cell lines may be due to, its ability to cause deprivation of arginine, growth factors and other stimulatory survival signals leading to cause the production of anti apoptotic members of the Bcl-2 family (21) and survival facter deprivation then activate the Intrinsic apoptotic pathway. The apoptotic effect increased with increasing incubation time and ADI concentration, this indicates that the enzyme is potent with low amounts in death program of RD cell line, but it was safe with REF cell line when the same concentrations were used as described in table (4) and in figures(3, 7, 9, and 10) with non-significant differences at $P \leq 0.05$ in comparison to control.(22) found that the killing of cancer cell lines by arginine deprivation is also selective because deprived normal cells will have become quiescent but soon recover on restitution of the missing nutrient, whereas tumor

cells in cycle can be hit by low doses of cycle-dependent cytotoxic drugs. (23) found that the purified methionine γ- lyase has a potent inhibitory activity against cancer cells especially RD cell line because apoptotic activity showed that RD had high level of cytochrome C in comparison with ANG cell line.In order to compare the effect of ADI the same concentrations were used with REF(normal cell line). The results described in table (4) and in figures: (9 and 10) showed that the ADI enzyme had very low cytotoxicity which increased with increasing the amount of enzyme. When 400 and 600ng/ml of ADI used, the apoptosis ratio of REF cell line was 5% during 72h of incubation, this may be due to inability of these cells to recover 100% of cellsin the presence of high amount of ADI. Many researchers were described the tumor cells (in the presence of ADI enzyme)in many cases as: 1- Lost the ability to make arginine from citrulline (24, 25). 2- Stay in cycle instead of moving out of it into G1 or G0 (26). 3- Die within 3-4 days in many cases, probably as a result of trying to cycle when insufficiently resourced, and without any further intervention (22, 26).

4- Because they stay in cycle, they continue to be suitable targets for cell cycle-dependent cytotoxic agents (27), where as normal cells become quiescent and relatively resistant. 5- As long as arginine is reduced to the micromolar level, many cancer cells will die, while normal cells recover from quiescence when enzyme is removed (22). (3) stated that ADI triggers apoptosis pathway by increasing the expressions of p53 and p27Kip1, and decreasing the expressions of cyclic D1, c-myc and Bcl-xL and sub-G1 accumulation, DNA condensation and DNA fragmentation, of SNU-1 cells (Stomach adenocarcenoma cells) by acting as chemotherapeutic agent.

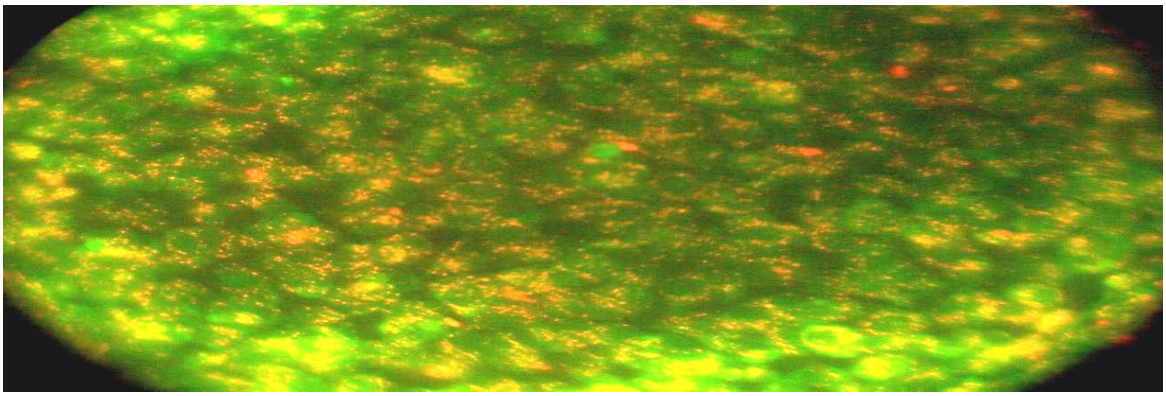

Figure 1 : RD (untreated) cell line after 72h of incubation showed attached and healthy cells (cytoplasmic red regions) Magnification power: 400X, using cationic fluorescence dye

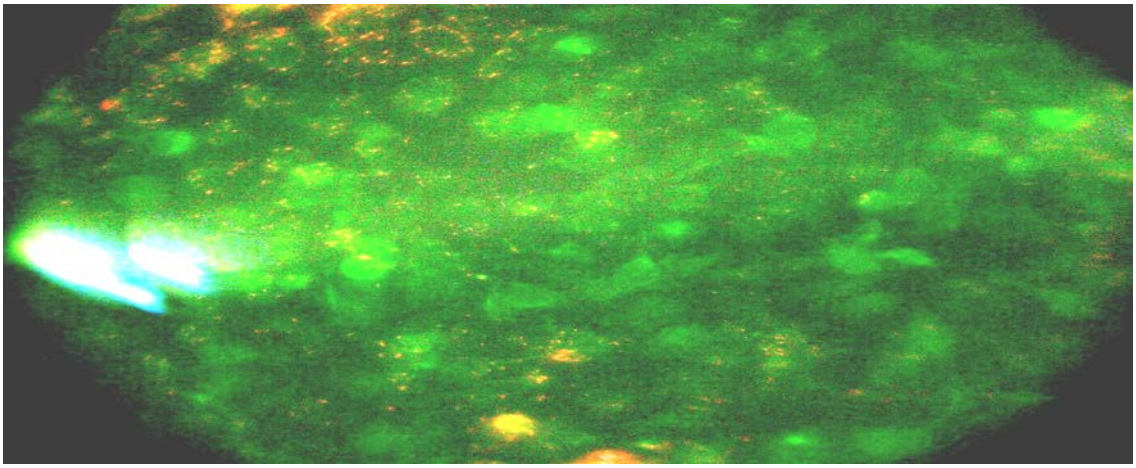

Figure2 : RD cell line displayed about 54% apoptosis (green regions) treated with 24ng/mlof ADI after 72h incubation showed dispersed and cell death with their exudes. (Magnification power: 400X)

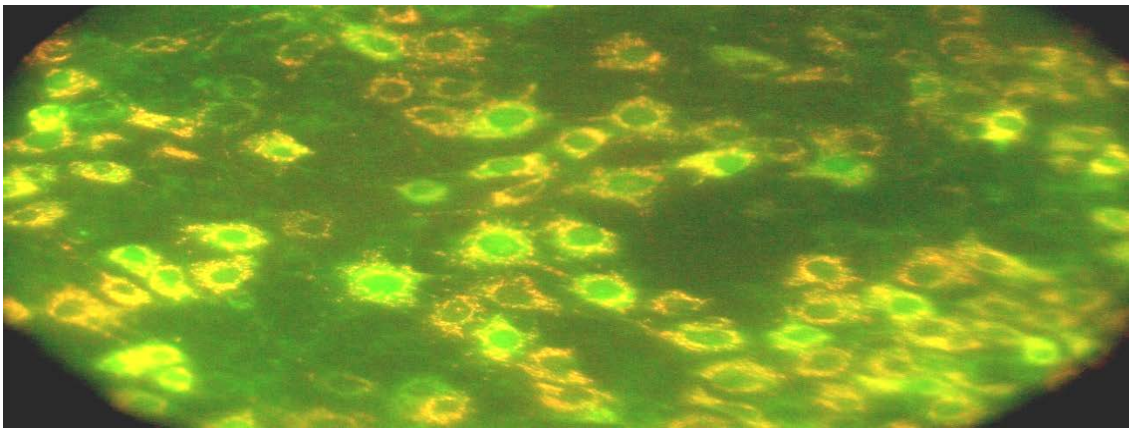

Figure 3 : Shows 0% apoptosis of REF cell line treated with 24ng/ml after 72h of incubation presented healthy cells (with red cytoplasmic regions). (Magnification power: 400X)

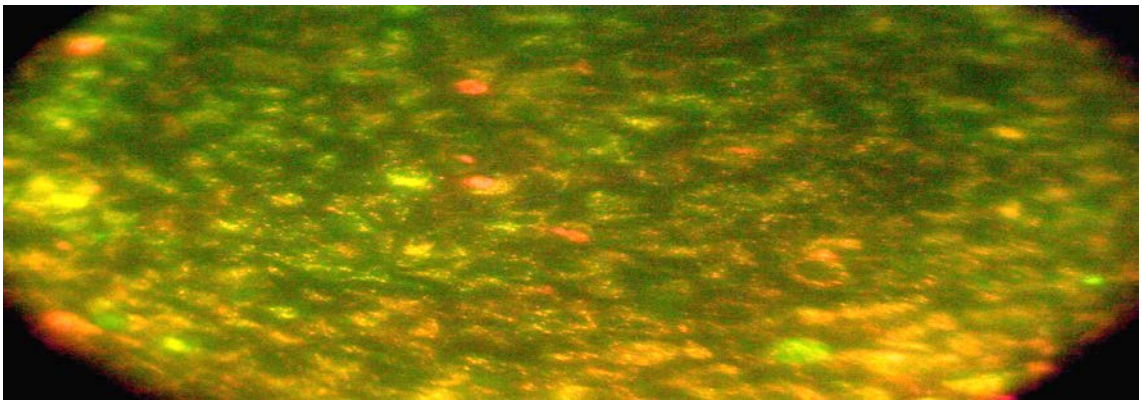

Figure 4 : RD (untreated) cell line after 48h of incubation showed attached and healthy cells (cytoplasmic red regions) Magnification power: 400X, using cationic fluorescence dye.

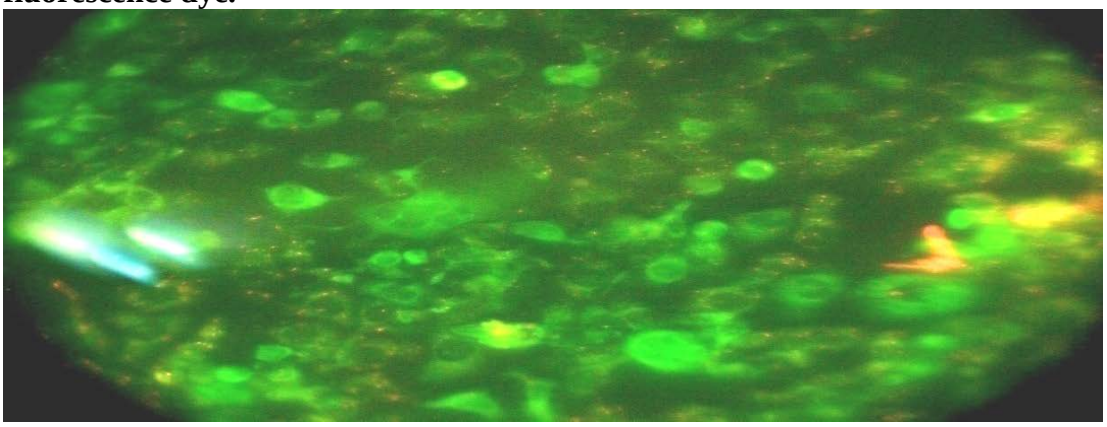

Figure 5 : Represented(51%) apoptosis of RD cell line after 48h of incubation with 33ng of ADI displayed disperesed (green cytoplasmic regins) cells with losing its natural forms.

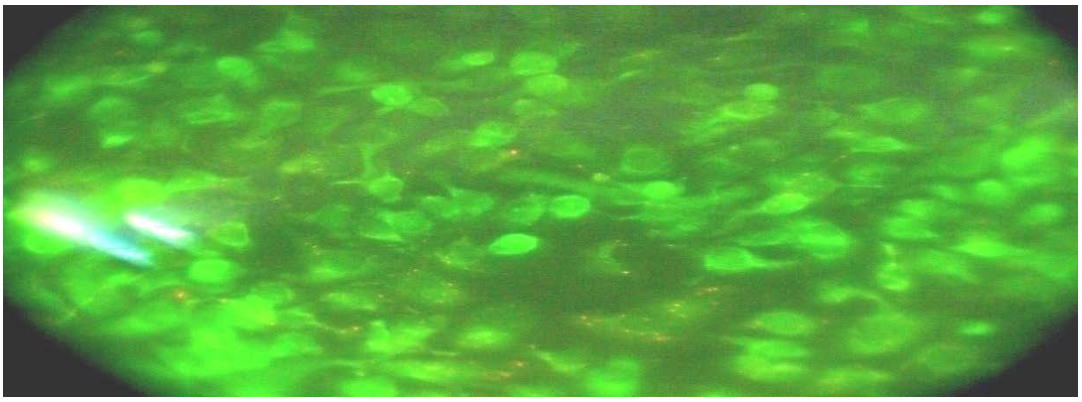

Figure 6 : Shows 94% apoptosisof RD cell line treated with 55 ng/mlof ADI after 72h of incubation presented dettached dead (cytoplasm shrinking) green cells. Magnification power(M.p.): 400X.

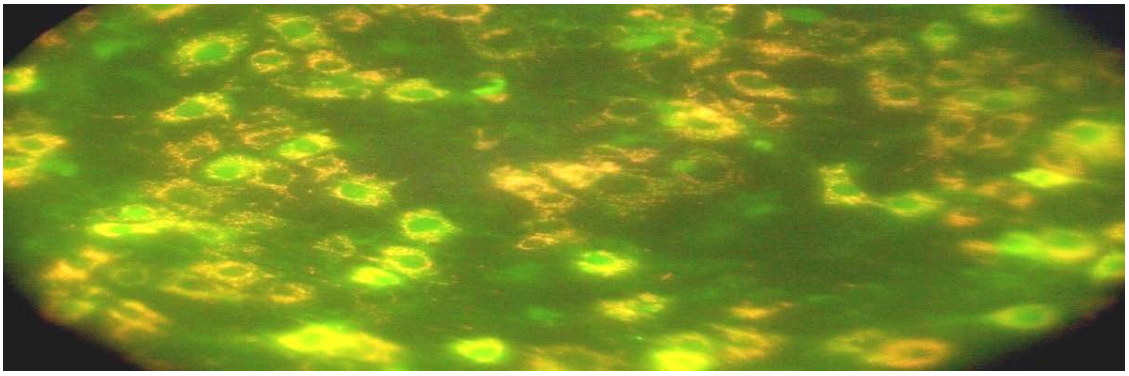

Figure 7 : Apoptosis 0% of REF normal cell line treated with 55 ng/mlof ADI after 72h of incubation at expressed red cytoplasmic healthy cells (M.p. power:400X)

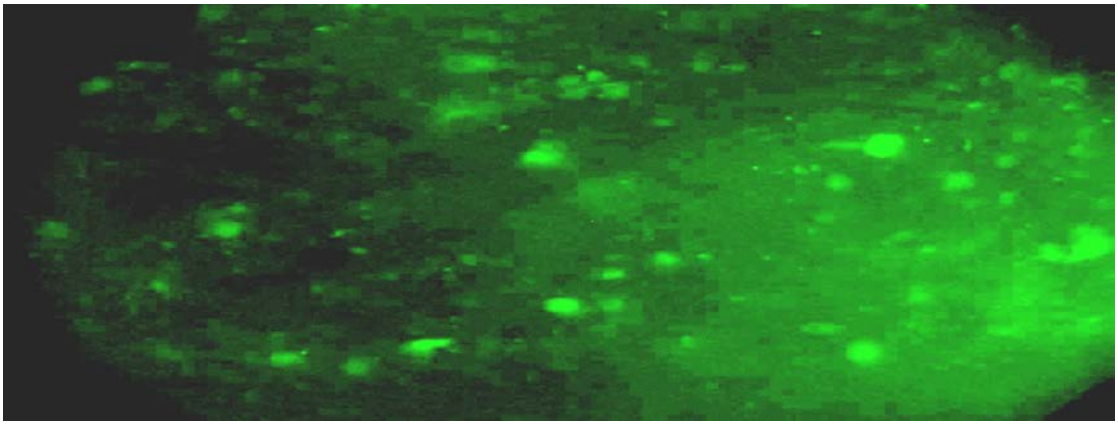

Figure 8 : Late event of (100%) apoptosis for RD cell line after 48h of incubation with 200ng/ml of ADI enzyme presented debris of lytic cells and their exudes. (M.p. power:400X) using cationic fluorescence dye

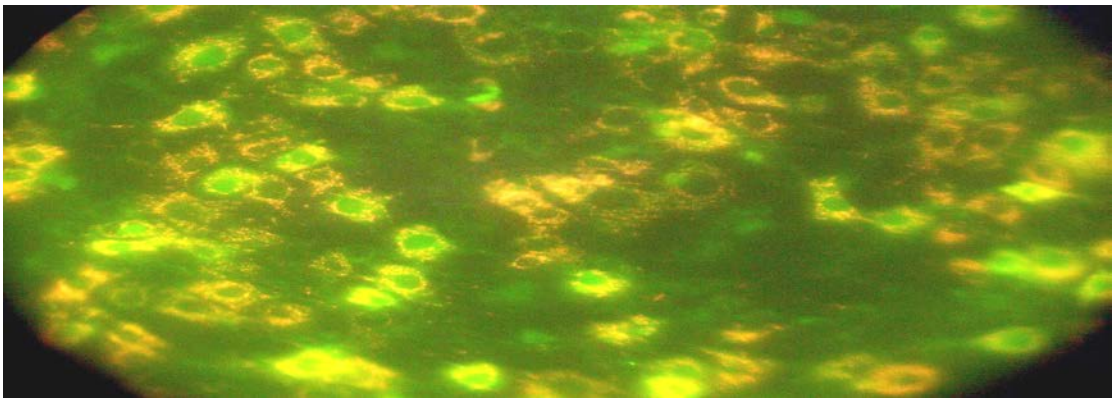

Figure 9 : Apoptosis (2%)of REF cell line after adding 100ng of ADI enzyme during 72h of incubation presented mostly healthy red cytoplasmic cells with a small number of dead shrinking green cells. (M.p. power:400X) using cationic fluorescence dye

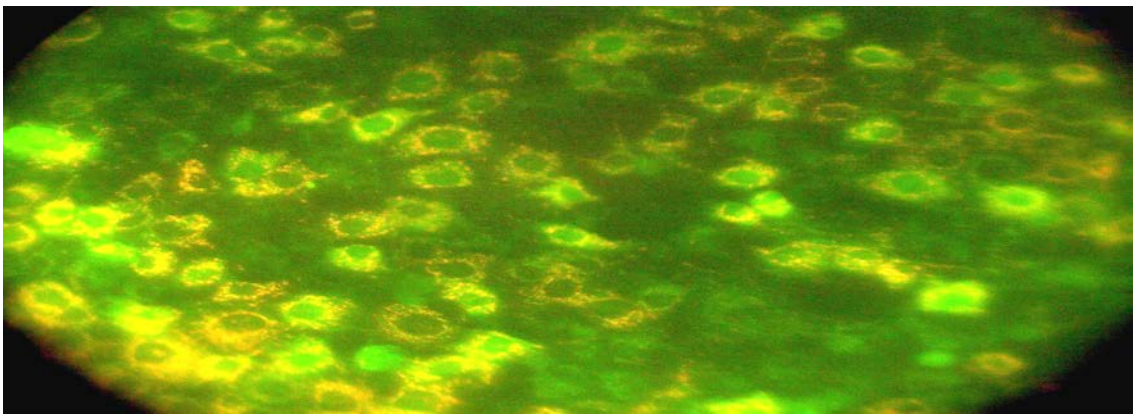

Figure 10 : Shows 4% apoptosis of REF normal cell line after adding 200 ng ADI for 72h incubation presented the large number of red cytoplasmic healthy cells and few shrinking dead cytoplasmic green cells. (M.p. power:400X)

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Seroprevalence of Hepatitis C Virus in Type 2 Diabetic Patients in Relation with Interleukin 10 in Kirkuk Province

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Abstract:

Objectives: The study aimed to evaluate the seroprevalence of HCV in type 2 diabetic patients and its relation with serum IL-10.

Methods: A cross-sectional study was carried out in Kirkuk city from 15th of December 2012 to 15th of June 2013. The study included 391 diabetic patients whose ages were between 22-81 years old. The control group including 288 non diabetic individuals who were apparently didn't have any chronic diseases and their ages were between 21-81 years old. These individuals were attended to Kirkuk General Hospital and Primary Health Care Centers of Kirkuk First Health Care Sector. The patients and control group were examined for the presence of antibodies against HCV by using of ELISA technique. **Results:** The study showed that the rate of HCV infection in diabetic patients was 6.65% and (0.34% in non-diabetic control group. The study also showed that 64.16% of HCV infected diabetic patients have elevated level of serum IL-10. **Conclusions**: from the current study we concluded that there was a significant relation between HCV infection and type 2 diabetes, increased risk of HCV infection with increasing of age. Surgical procedures were very important to establish HCV infection in the society. Level of IL-10 was elevated in diabetic patients infected with HCV.

الانتشار المصلي لفيروس التهاب الكبد نوع C في مرضى السكري- النوع الثاني وعلاقته بمستوى Interleukin 10 في محافظة كركوك

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الخلاصة:

الأهداف: تهدف الدراسة الى تقدير نسبة الانتشار المصلي لفيروس التهاب الكبد نوع C في مع مرضى داء السكري-النوع الثاني وعلاقته بـ IL-10 في العراق

الطريقة: لقد أجريت الدراسة في مدينة كركوك للفترة من 15 كانون الاول 2012م ولغاية 15 حزيران 2013 م. شملت الدراسة 391 مريضاً بالسكري وكانت أعمارهم من 22 الى 81 سنة، وشملت مجموعة السيطرة 288 شخصاً من غير المصابين بالسكري ولا يشكون من اية امراض مزمنة والذين كانت اعمارهم 21-81 سنة. كل المشمولين بالدراسة كانوا قد راجعوا مستشفى كركوك العام وقطاع الرعاية الصحية الاولية الاول في كركوك-جمهورية العراق. لقد اجري لكل المرضى فحص التلازن المناعي الانزيمي ELISA للتحري عن الاجسام المضادة تجاه فيروس التهاب الكبد نوع C.

النتائج: أظهرت الدراسة أن نسبة انتشار فايروس التهاب الكبد نوع C في مرضى السكري هي 6,65% و 0,34% في مجموعة السيطرة ممن هم غير مصابين بالسكري. وكذلك اظهرت الدراسة ان 64,16% من المصابين بالفيروس كان لديهم مستوى IL-10 مرتفعاً.

الاستنتاج: من هذه الدراسة نستنتج ان هنالك علاقة قوية بين التهاب الكبد الفيروسي نوع C وداء السكر، وأن مستوى IL-10 يزداد في مرضى السكري المصابين بالتهاب الكبد الفيروسي نوع C .

Introduction:

Hepatitis C virus (HCV) is a single stranded RNA virus classified within hepacivirus genus of the Flaviviridae family. It is enveloped in a lipid bilayer in which two or more envelope proteins (E) are anchored. The envelope surrounds the nucleocapsid, which is composed of multiple copies of a small basic protein (core or C), and contains the RNA genome.[1] A major characteristic of hepatitis C-infection is its tendency to establish chronic liver disease, such as cirrhosis, and eventually hepatocellular carcinoma (HCC).[2] Parenteral exposure to the hepatitis C virus is the most efficient means of transmission. The risk of chronic HCV infection is high, 80-100% of patients remain HCV positive after acute infection.[3]

Hepatitis C virus causes asymptomatic chronic hepatitis in up to 85% of those infected.[4] Hepatitis C infection has also been strongly linked to several extra-hepatic manifestations, based on early clinical observation. Type II diabetes mellitus (DM) was suggested to be one of the potential extra hepatic manifestation of HCV infection.[3] It is now clear that hepatitis C conveys a risk for developing DM, in particular type 2. Moreover, several studies have found a high prevalence of anti-HCV antibodies among patients with diabetes, especially those with type 2 DM.[5] The liver is a key organ in intermediary metabolism and plays a pivotal role in the pathogenesis of insulin resistance. Hence, glucose intolerance is common in patients with liver cirrhosis of whatever etiology, and around 20% of cirrhotic patients have overt diabetes.[6]

Type 2 Diabetes (T2D) is a common complication of all liver diseases, independently of the etiology, especially at the advanced stage.[5] Although individuals may develop insulin resistance (IR) independently of HCV, a considerable amount of clinical and experimental data suggest that HCV contributes to its pathogenesis. This aspect is important, because IR seems not only to accelerate the course of chronic hepatitis C, but also to influence the response to antiviral therapy. [7]

In this study we aim at evaluating the seroprevalence and relation of HCV with type 2 diabetic patients.

Methods:

The present study was carried out between December 2012 and May 2013 in Kirkuk City-Iraq was presented in Kirkuk General Hospital.

Patients:

Peripheral venous blood (5 ml) was aspirated from 391 diabetic

patient (22-81 years old) and 288 non diabetes control group (22-81) who presented in Kirkuk General Hospital. Types 1 and 2 diabetes were defined on the basis of a history of therapy with oral hypoglycemic agents or insulin at the date of inclusion. Patients older than 40 years of age, and treated by oral hypoglycemic agents were considered to have type 2 diabetes. [17] A control group of non-diabetic patients were recruited from the same center at the same time. The HCV status was not known for any of the recruited diabetic and control groups at the time of patient visit and blood collection. [7]

Procedures:

Serum was separated to determine the presence of HCV-specific antibodies by Enzyme Linked ImmunoSorbent Assay (ELISA) (CTK Biotech inc. USA), which was used according to the manufacturer's instructions. The optical density (OD) values were determined at 450nm by an ELISA reader. Testing was performed strictly according to the manufacturer's instructions. The results were then interpreted on the basis of antibodies as seropositive or seronegative, then results submitted to determine the relation of the HCV antibodies positive and negative with liver function tests, GPT(Alanine Aminotransferase ,ALT) using ALT biochemical kit (RANDOX UK), GOT (Aspartate Aminotransferase , AST) using AST biochemical kit (RANDOX UK), and Alkaline Phosphatase using (Alkaline Phosphatase ,Biomerieux , France) and study the relation of HCV infection with the value of IL-10 by using of (Omnikine Co. USA) for estimating of human IL-10 serum samples of all diabetic patients enrolled in the study.

Results:

A total of 391 type II diabetic patients and 288 non diabetic individuals (control group) were examined, their age ranged between 21-81 years old were investigated for seroprevalence of HCV antibodies by ELISA.

Seroprevalenc of HCV in type 2 diabetic patients: The rate of HCV infection in diabetic patients (6.65%) was higher than that in non-diabetic individuals (0.34%). The result was highly significant (Table 1).

Distribution of HCV seropositive according to sex and age: The high rate of HCV infected individuals (7.53%) were males while 6.12% were females (Table 2). Regarding distribution of HCV seropositive according to the age groups, the highest rate of HCV seropositive (9.01%) occurred in the age group 52-61 years old followed by 7.24% of group 42-51 years. However, no one of diabetic patients within the

age group 22-31 years old had HCV infection (Table 3).

Relation of HCV infection with surgical operation: Regarding the relation between HCV infection and transmission patterns, the highest rate of HCV seropositive (65.38%) occurred in patients who were previously exposed to surgical operations and the lowest rate (34.62%) in un-exposed patients. The result was highly significant (Table 4).

Relation of HCV infection with liver function tests: Table 5 shows that the highest rate (80.77%) of diabetic patients with HCV infection had elevated level of ALT, while 59.18% of diabetic patients without HCV infection have elevated level of ALT. The result was highly significant. Table 6 shows that the highest rate (65.38%) of diabetic patients with HCV infection had elevated level of AST, while the highest rate (53.15%) of diabetic patients without HCV infection had normal AST level. The result was significant. The current study revealed that in diabetic patients, the alkaline phosphatase elevated with a higher rate (69.23%) in HCV-infected patients than those without HCV-infection (51%), while only 34.38% of non-diabetics had an elevated level of serum alkaline phosphatase.. (Table 7).

Relation of HCV infection with serum IL-10: In relation of HCV infection with IL10, the study showed that 46.16% of HCV seropositive diabetic patients had increased IL-10 level comparing with 24.93% of HCV negative patients and 22.57% of non-diabetic control group. The result was significant.. (Table 8).

Table 1: Frequency of HCV antibodies in diabetic patients and control group by ELISA.

HCV antibodies	Diabetics		Control	
	No.	%	No.	%
Positive	26	6.65	1	0.34
Negative	365	93.35	287	99.66
Total	391	100	288	100
X^2 = 17.252 p= 0.0003 P < 0.01 Highly Significant(HS)				

Table 2: Relation of HCV seropositive to sex of diabetic patients.

Sex	Diabetic group		
	Total No.	HCV +ve (No.=26)	
		No.	%
Male	146	11	7.53
Female	245	15	6.12
X^2 = 1.61	**p= 0.607**	**P > 0.05**	**Not Significant(NS)**

Table 3: Distribution of HCV seropositive according to age groups of diabetic patients.

Age groups (Years)	Diabetic group		
	Total No.	HCV +ve	
		No.	%
22-31	7	0	0
32-41	31	2	6.45
42-51	69	5	7.24
52-61	122	11	9.01
62-71	70	5	7.14
72-81	92	3	3.26
X^2 = 2.564	**p= 0.81**	**P > 0.05**	**Not Significant(NS)**

Table 4: Frequency of anti-HCV antibodies among diabetic patients in relation to Surgical operation.

Exposure	Surgical operation			
	HCV+ve		HCV-ve	
	No.	%	No.	%
Exposed	**17**	**65.38**	**135**	**36.99**
Unexposed	**9**	**34.62**	**230**	**63.01**
Total	**26**	**100**	**365**	**100**
X^2= 8.237	**p= 0.004**	**P < 0.01**	**Highly Significant(HS)**	

Table 5: Relation of ALT level with the study groups.

ALT level	Study groups					
	Diabetics				Non diabetics	
	HCV +ve		HCV -ve			
	No.	%	No.	%	No.	%
Normal	5	19.23	149	40.82	165	57.29
Increased	21	80.77	216	59.18	123	42.71
Total	26	100	365	100	288	100
X^2 = 25.88 p= 0.0001 P < 0.01 Highly Significant(HS)						

ALT: Alanine Aminotransferase

Normal range: (ALT: up to 12 U/L)

Table 6: Relation of AST level with the study groups.

AST level	Study groups					
	Diabetics				Non diabetics	
	HCV +ve		HCV -ve			
	No.	%	No.	%	No.	%
Normal	9	34.62	194	53.15	166	57.64
Increased	17	65.38	171	46.85	122	42.36
Total	26	100	365	100	288	100
X^2 = 5.999 p= 0.049 0.01≤ P ≤ 0.05 Significant(S)						

AST: Aspartate Aminotransferase

Normal range: (AST: up to 12 U/L).

Table 7: Relation of alkaline phosphatase level with the study groups.

Alkaline phosphatase	Study groups					
	Diabetics				Non diabetics	
	HCV +ve		HCV -ve			
	No.	%	No.	%	No.	%
Normal	8	30.77	179	49	189	65.62
Increased	18	69.23	186	51	99	34.38
Total	26	100	365	100	288	100
X^2 = 3.247 p= 0.072 P > 0.05 Not Significant(NS)						

Normal range: (alkaline phosphatase: 21- 92 U/L).

Table 8: Relation of HCV infection with IL-10 level in the study groups.

IL-10 Level	Study groups					
	Diabetic groups				Non diabetics	
	HCV positive		HCV negative			
	No.	%	No.	%	No.	%
Normal	14	53.84	274	75.07	223	77.43
Elevated	12	46.16	91	24.93	65	22.57
Total	26	100	365	100	288	100
$X^2 = 7.139$ P= 0.028 0.01≤ P ≤ 0.05 Significant(S)						

IL-10: Interleukin-10

Normal Range: 1.3-37.4 pg/ml.

Discussion:

Hepatitis C Virus infection affects not only the liver but the extra hepatic tissues as well and may combine with many unrelated diseases and morbid conditions. A number of extra hepatic manifestations have been recognized including diabetes mellitus. [8]

In this study HCV infection was relatively common among diabetic patients by using of ELISA for detecting and estimating of anti HCV antibodies among them and among control (non-diabetic individuals) with the aid of liver function tests and IL-10. In the present study, the frequency of HCV infection among diabetic patients was 6.65% by using ELISA technique (as shown in Table 1), the study showed a highly significant result of HCV seropositive in diabetic patients in comparing with 0.34% in control group ($P < 0.01$).

Results of the current study are in agreement with some studies conducted earlier in other countries. A study done in Kuwait showed that the rate of HCV was 7% in diabetics vs. 1% in healthy controls[9], similarly, in a Turkey, HCV was found in 7.5% of diabetic patients vs. 0.1% in control group.[10] In Italian study seroprevalence rate of HCV was 7.6% in diabetic patients.[11] In Taiwan, HCV was found in 6.8% of diabetic patients.[12] The differences of HCV seroprevalence in those studies compared to current study could be explained by a difference in the demographic data between the populations. Differences in source of controls, case definition, sample size and underlying target population may explain much of this observed variability among studies. Various reasons have been adduced for the increased occurrence of HCV infection in DM patients. These are frequent parenteral injections, extra-hepatic manifestations of HCV infection which includes DM, and

the fact that patients with liver diseases are known to have a higher prevalence of glucose intolerance. A higher prevalence of DM in HCV related liver cirrhosis in comparison with cirrhosis secondary to other causes has also been postulated.[13]

The present study showed that 7.53% of diabetic males were HCV seropositive and 6.12% diabetic females were HCV positive and the there was no significant relation between them, whereas the overall females rate of total patients involved in this study were 62.66% (345 female of 391diabetic patients).

Several studies didn't support a significant relation between sexes concerning HCV infection.[14-16]

The current study was disagreed with several studies. Abass, *et al* [17], Al-Khazraji *et al* [18] recorded a significant association between HCV infection and sex. The explanation for these variations may be attributed to the difference in sample size, type of patients involved in addition to the different time of blood collection. Moreover diabetes as a disease affects both the male and female gender. The sex distribution of the diabetic patients in this study showed that women attend hospitals more than men. This corroborates an earlier that there were more women diabetics than men. Some of the men who were diabetic might have refused to show up at the clinic which may be due to that they consider it as a time killing exercise.[19] The proportion of worldwide deaths attributable to diabetes mellitus is estimated to be higher in females than in males, with 1.5 million and 1.4 million deaths respectively.[20]

The present study showed that highest rate of HCV seropositive diabetic patients were within the age group 52-61 years old followed by 42-51 years old (9.01% and 7.24% respectively) and no HCV seropositive occurred in group 22-31. There was no significant relation between HCV infection and age. Similar results were obtained by several studies. Mehta, *et al*[21] showed that the highest rate of HCV infection occurred in the age group 50 -54 years old. The high seropositivity recorded in older group may be due to more parenteral exposures as compared to younger people and thus greater chances of transmission of infection.[22]

The present study showed that 65.38% of HCV seropositive diabetic patients were previously exposed to surgical operation and the result was statistically highly significant. Al-Mashhadani, *et al* [23] proved a correlation of hepatitis C infection with surgical procedure and blood transfusion in health care workers. Habib, *et al* [24] found that surgical practice such as suture was

associated with 32.3% of HCV positive and much higher in dental practice as it reached 62.2%.

Regarding the association between HCV infection and liver function tests, the present study showed that 80.77% of HCV seropositive diabetic patients had an increased level of ALT comparing with 59.18% of HCV seronegative and 42.71% of non-diabetic individuals with a highly significant relation between them (Table 5), 65.38% of HCV seropositive diabetic patients had an increased AST level with a highly significant relation (Table 6) and 69.23% of HCV seropositive diabetic patients had an increased level of alkaline phosphatase with no significant relation (Table 7)

The current study was in agreement with Ali, *et al*[25] who showed that serum ALT levels were found to be raised in 55.5% of HCV seronegative cases. Mehta, *et al*[21] also reported an elevated levels of transaminases in diabetes mellitus. Furthermore, Ni, *et al*[26] showed that serum ALT level was raised in 73.7% of the positive cases as compared to the 18.5% of the seronegative patients.

The elevated level of liver enzymes concerned with HCV seropositive offered more liver inflammation and that chronic hepatitis C is associated with a wide variation in ALT, from normal ALT to persistent elevation of ALT, although studies have shown that patients with persistently normal ALT usually have slower progression and lower prevalence of cirrhosis.[27] However, some cases remain asymptomatic with normal levels of ALT after HCV infection and detection of the infection in these cases may occur only through screening, such as with an anti-HCV antibody test. The ALT level usually increases in hepatitis, but it is normal in approximately 20-30 % of HCV carriers.[28] Previous studies have shown that elevated ALT levels predict an increased rate of HCV associated HCC in a community-based population and that serial measurements to identify persistent ALT abnormality may be useful in determining the HCC risk.[29]

Interleukin-10 is a cytokine which play key roles in the regulation of cellular immune response in HCV infection.[30] In the present study, 46.16% of HCV seropositive diabetic patients showed an increased level of IL-10 comparing with 24.93% of HCV seronegative and 22.57% of non-diabetic control group, there was a significant relation between HCV infection and elevated level of IL-10,(Table 8). The study was in agreement with Paladino, *et al* [31] who noticed that IL-10 level was increased in patients with HCV

infection. Moreover, Liu[32] showed that serum from chronic HCV patients has a significantly higher level of IL-10 as compared with serum from healthy individuals.

The present study disagreed with Bozkaya, *et al* [33] who noticed that the number of patients with elevated IL-10 was not different as compared to controls when all patients were analyzed together. The anti- inflammatory cytokine IL-10, is known to exert a protective role in hepatic damage caused by viruses, alcohol and autoimmunity. Its main biological function seems to be the limitation and termination of inflammatory responses and the regulation of differentiation and proliferation of several immune cells.[34] The high levels of IL-10 present in chronic HCV infection have been suggested as responsible for the poor antiviral cellular immune responses found in HCV patients.[35]

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Iraqi Journal of Biotechnology, 2015, Vol. 14, No. 2 , 42-51

New selective media for the isolation and acid production screening of concrete fouling microbes

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Abstract: Microorganisms fouling concrete infrastructures are gaining more attention for their role in concrete deterioration or remediation. There is an urgent need to design a new media for the extremophiles isolation with as limited nutrients as possible and high selectivity. Collected samples were inoculated on Cement extract agar (CEA) supplemented with Nutrient agar or Potato dextrose agar or Heterotrophic plate count agar. Three different pH (5 ،7 and9) were used for microbial screening in addition to pH 12.5 for acid production screening using cement extract solution (CES). A total of 266 isolates were successfully isolated. Bacteria appeared the most abundant (75%), 9% are Actinomycetes . Only 39.1% were able to produce organic acid(s). Most of the acid producers were Bacteria and molds 48% and 43% respectively .The new invented media were highly selective in microbial isolation and acid production screening and are highly recommended in related researches.

Key words: selective media, concrete, fouling, acid, screening

وسط زرعي جديد لعزل و غربلة انتاج الحوامض للميكروبات الملوثة للأسطح الخرسانية

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الخلاصة: هناك إهتمام متزايد بالميكربات الملوثة لأسطح المنشآت الخرسانية وذلك لدورها في إتلاف أو تحسين مواصفات الخرسانة. هناك حاجة ملحة لتصميم أوساط زرعية جديدة لعزل هذه الميكروبات، التي تعيش في بيئة متطرفة، بوجود أقل كمية من المواد المغذية و ذات إنتقائية عالية. تم زرع العينات على وسط أكر مستخلص السمنت مدعم بالأكر المغذي او بأكر دكستروس البطاطا او بأكر عد متباينة التغذية. تم إستخدام ثلاث درجات حموضة (5 و 7 و 9) لغربلة الميكروبات بالأظافة الى رقم هيدوجيني 12.5 لغربلة إنتاج الحوامض بإستخدام محلول مستخلص السمنت (CES). تم عزل 266 عزلة حيث ظهرت البكتريا بنسبة عالية (75%)، 9% منها بكتريا خيطية. فقط 39.1% من عزلات كانت قادرة على إنتاج ألاحماض العضوية معظمها من البكتريا و الاعفان 48% و 43% على التوالي. أظهرت الأوساط الزرعية الجديدة إنتقائية عالية في عزل الميكروبات و غربلة إنتاج الأحماض و يوصى بإستخدامها في الأبحاث ذات العلاقة.

Introduction

Concrete as a cementitious material is an extreme habitat in both pH (12-13) and availability of organic nutrients, which create a very harsh environment for microbes to survive. Microorganisms that foul concrete surfaces can be deleterious on concrete microstructure, durability and aesthetic appeal [1, 2]. Most of the destructive processes are either chemical or direct and indirect physical actions [3, 4]. The biological processes represent 30% of the total concrete destructive factors [5, 6]. Heterotrophic bacteria and fungi are the most inhabitants of concrete surfaces and are implicated for the concrete destructive action [7, 8]. They produce metabolites, which are chemically aggressive to building materials especially concrete mainly organic and mineral acids. [9 - 12]. Biofilms formation might be more aggressive by generating local high concentration of destructive materials [13] or physically by fungi hyphae penetration of the concrete surface. [14, 15].

Concrete fouling microbes are not always harmful; some microbes (mainly bacteria such as *pseudomonas*) excrete beneficial metabolites mostly low molecular weight organic acids such as acetate, oxalate and citrate, that can be utilized by other microorganisms as a sole carbon and energy source, which lead to increase in both pH and the concentration of dissolved inorganic carbon leading to calcium carbonate precipitation which increase concrete strength [16, 17].

Up to our knoledge, this is the first research using cement extract (CE). All previous researches used commercially available media like nutrient agar (NA) and potato dextrose agar (PDA) for concrete fouling microbes cultivation which is represented as rich media that are not suitable to cultivate environmental microbes live in extreme conditions.

Concrete fouling microbes and its interaction with concrete are gaining more concern and becoming the subject of more research projects. This study aim to use a new media preparation that is economic and highly selective for concrete fouling microbes screening with as minimum nutrients as posssible that mimics the natural habitat.

Materials and methods

Biological sample collection

Concrete surface areas with highly dense biofilm coverage (stain) were selected for sample collection. Aseptic conditions were provided as much as possible. Four labeled swab samples from each location were collected using

readymade sterile Amies transport medium (ATM) wetted cotton swabs. Slight rubbing is necessary in order to collect as much as possible of the covering biofilm. Swabs were kept in a cooling box along the way to the lab. where they refrigerated till the time of use.

Cultivation media composition and preparation

In order to mimic the natural habitat with as low nutrients as possible, the following media were prepared.

Cement extract

One kg of regular Portland cement was added to 2.0L distilled water gradually with magnet stirring for 30 min. at room temperature. The mix was left to sit until the aqueous phase is clearly separated, then filtered through Whatman No.1 filter paper in screw capped bottles. (CE) has been added to the standardized media (Nutrient agar, Potato dextrose agar and Heterotrophic plate count agar).

Media for bacteria, fungi and actinomycetes screening and isolation

The readymade media were provided from HIMEDIA, India. Three different new media [Nutrient cement extract agar (NCEA), Potato-Dextrose cement extract agar (PDCEA) and Heterotrophic Plate Count cement extract Agar (HPCCEA)] with three different pH values (5, 7 and 9) were prepared as follows: Half of the manufacturer recommended amount of each ready-made medium component was added to 900 ml cement extract with continuous stirring and gentle heating. Desired pH were adjusted using 1N HCl. Distilled water was added to reach 1.0L final volume. The mixture was brought to boiling then autoclaved. The pH of the solidified media (in plates) were checked using Extech concrete pH kit (EXTECH instruments, USA).

Media for cyanobacteria screening and isolation

A modified Bristol's medium was prepared in both solid and biphasic forms as in [18] with four different pH values (5, 7, 9 and 12.5), and as the following: The ingredients were dissolved one at a time in a desired amount of CE in order to prepare Bristol's Cement extract solution (BCES). To prepare Bristol's Cement extract agar (BCEA), an amount of 15g/L of agar-agar were added to the BCES. The pH was adjusted to the desired point using 1N HCl. The mixture was brought to boiling then.The pH of the solidified media (in plates) were checked using Extech concrete pH kit (EXTECH instruments, USA).

Biphasic Bristol's Cement Extract Medium (BBCE)

An amount of 50g of standard sand was added to 250ml glass containers then autoclaved for 15min. at 15psi pressure and 121°C. Millipore filter (0.45µm) sterilized BCES of 20ml were added to the warm containers, according to [18].

Sample screening

Every two ATM tubes were mixed in one tube by adding 3.0ml of normal saline in one tube to liquefy the medium and then added on the other under aseptic conditions in order to have whole microbial community collected from different spots in one tube. One tube was used for Cyanobacteria cultivation and the other was used for the other microbes screening.

Cultivation and isolation of concrete fouling bacteria and fungi

The above selective media were streaked with the ATM sample swab and incubated at 20-26°C. The plates were checked daily for colonies appearance for one month period in order to give chance for the slow growing microbes to develop visible growth. Morphologically different colonies were selected immediately then sub-cultured on the same medium. ATM swabs were prepared in duplicates for every pure cultures. The ATM swabs with purified colonies were parafilm sealed and preserved in the fridge for further use.

Cyanobacteria screening

Biphasic media containers were inoculated with the ATM sample swab which was also used to streak the agar plates. The containers and agar plates were incubated at 20-25°C in an illuminated incubator for 2 months. Microscopic investigation for any growth was followed out monthly.

Acid production screening

In order to test the ability of acid production of each pure isolate, Cement extract solution (CES) pH12.5 and cement extract agar (CEA) of three different pH (5, 7 and 9) plates were prepared and as follows: For pH12.5, 3ml portions of CE were Millipore (0.45µm) filtered in sterile screw cap glass tubes. The tubes were inoculated in two groups of triplicates with a loopfull of bacteria or 5mm square of fungal growth and incubated at 20-26°C for 1 month (group one) and 2 month (group two) periods with control tubes (no inoculum). The pH of each tube was measured after each incubation period. For CEA plates, 15g/L of agar were added to the CE with the desired acid-base indicator solution (Table 1). The mixture was brought to boiling then autoclaved. Warm media were poured into sterile Petri

dishes. The pH of the solidified media was checked using Extech concrete pH kit. Each plate was sectioned and inoculated with the representative bacteria streaking or ~5mm square of fungal growth. Plates were incubated at 20-26°C and checked daily for indicator color change for a month period.

Table 1: Acid-base indicators used [19, 20].

Medium / pH	Acid-base indicator	Color Change (pH)
CEA / 5	Bromophenol blue	Blue-purple (4.6) to Yellow (3.0)
CEA / 7	Bromocresol purple	Purple (6.8) to Yellow (5.2)
CEA / 9	Phenol red	Red (8.4) to Yellow (6.8)

Results and discussion

Biological Samples Cultivation and Microbial Screening

The newly invented cultivation media for screening of concrete fouling microbes had proved its efficiency in the selectivity and maintenance of concrete fouling microbes especially by using different pH where 266 isolates were successfully cultured and isolated. Bacteria appeared the most abundant (75%) among the other groups, 9% of which are actinomycetes (Actino.) (Figure 1).

Cyanobacteria have not appeared in the culture either because they are absent in the collected samples since they require high humidity to grow (require long water retention times) or they are uncultivable.

The most majority of microorganisms were isolated on NCEA and PDCEA media and “as expected” these media have been found very efficient for both bacteria and molds isolation respectively.

Most of the isolated molds and non-filamentous Bacteria (NF-Bacteria) were neutrophils while alkaliphilic Actinomycetes were the most abundant (48%). However, 31% of the molds and 35% of the Bacteria have been isolated as alkaliphils (Figure 2).

The biofilm community members have showed variable tendency in growth behavior for both media type and pH which reflects the biofilm complexity.

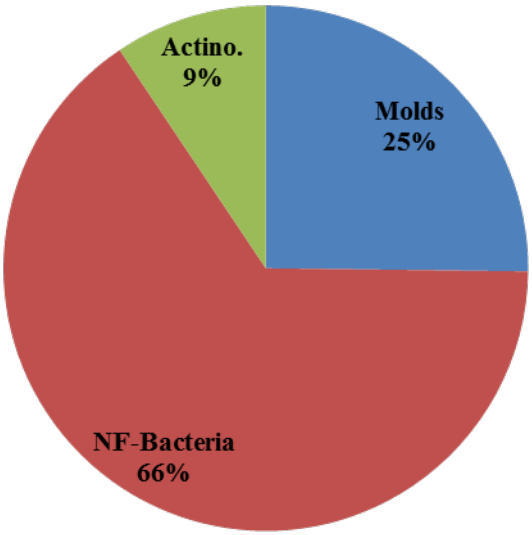

Figure 1: Distribution of the total isolated microbes. Actino.: Actinomycetes.

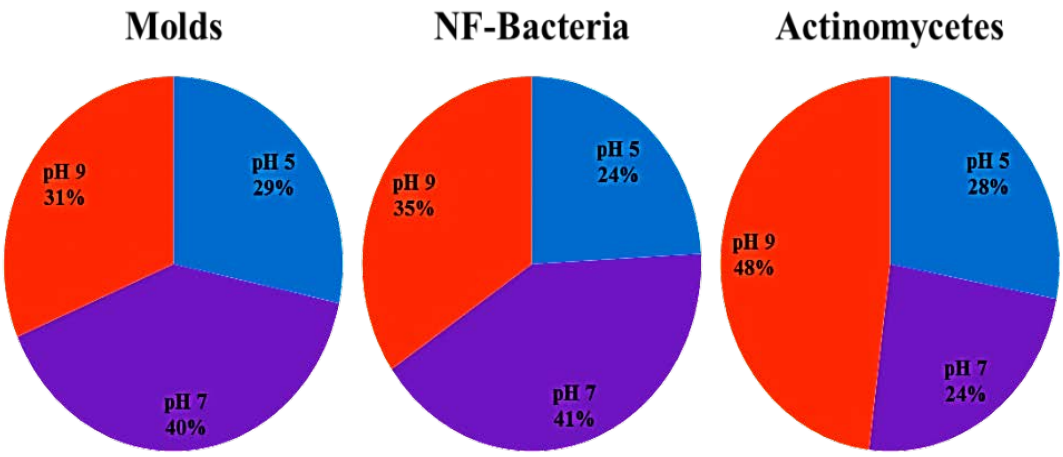

Figure 2: Distribution of the isolated microbes according to pH tendency. NF-Bacteria: Non-filamentous Bacteria.

Microbial Acid Production Screening and Analysis

From the total successfully isolated colonies, only 39.1% were able to produce acids when cultured on CES and CEA with different pH. Most of the acid producers were NF-bacteria and molds 48% and 43% respectively (Figure 3) since they are the major inhabitants of the concrete surface.

It should be mentioned that CES has been used for pH12.5 since the available pH indicators

(phenolphthalein, thymolphthalein, alkaline blue and Nile blue) has been found unstable when used in CEA pH12.5 in addition to their toxicity. Add to that, CES can be incubated for two months easily without risk of media dryness.

Most of the isolates were able to produce organic acid(s) on either CEA pH 7 or pH 9 or both especially basophilic bacteria (Figure 4).

All acid producers were able to produce organic acid(s) since no isolate was able to decrease the media pH to less than five. It is obviously clear that most isolated microorganisms produce organic acids under stress of high pH. However, some others produce organic acids on their optimum pH probably due the stress of nutrients lack in the medium by utilizing the organic materials of the dead cells.

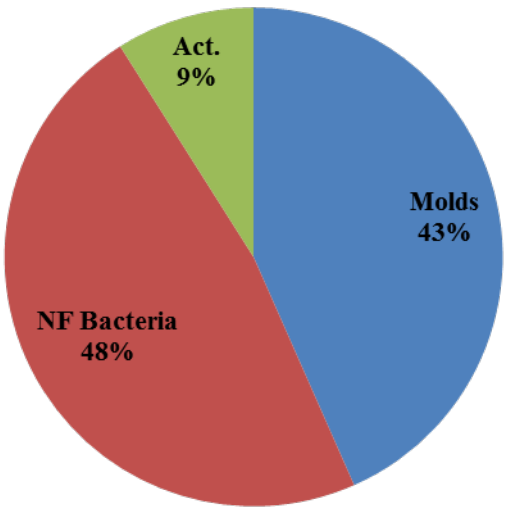

Figure 3: Contribution of each microbial group among the acid producers. Actino.: Actinomycetes. NF-Bacteria: Non-filamentous Bacteria.

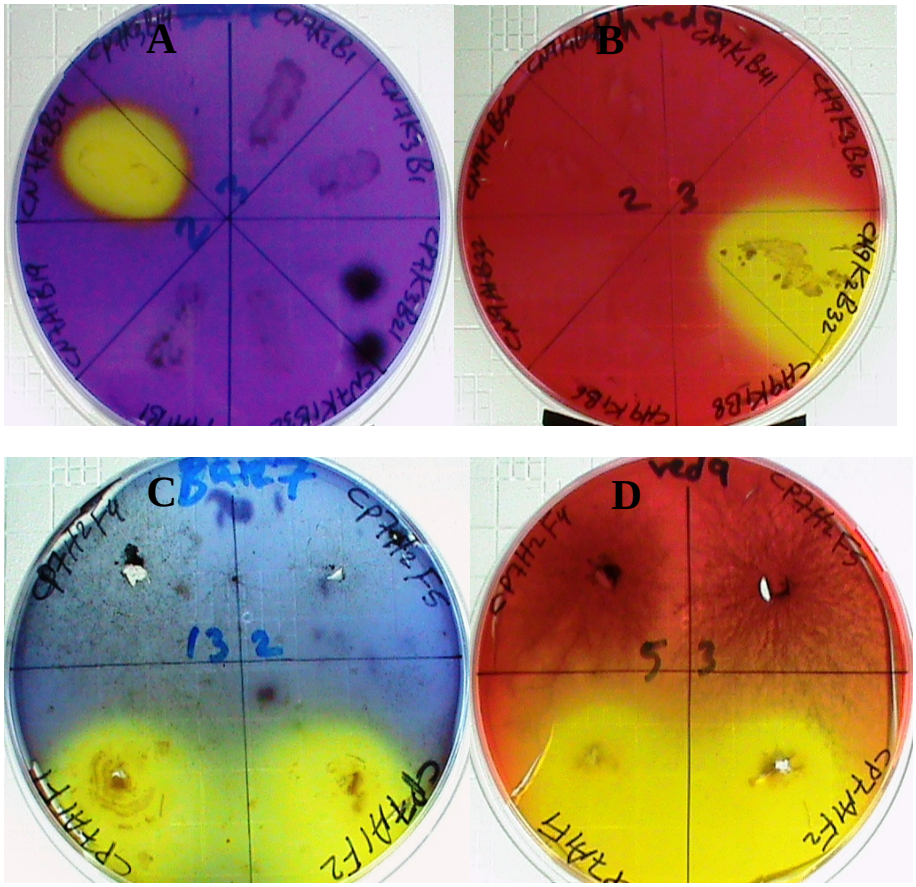

Figure 4: Organic acid production by Bacteria on NCEA. A: pH 7. B: pH 9, and Molds on PDCEA. C: pH 7, D: pH 9, after 2 weeks of incubation.

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Iraqi Journal of Biotechnology, 2015, Vol. 14, No. 2 , 52-62

Determination norovirus genotypes in Baghdad children associated with Acute Gastroenteritis during year 2012-2013

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Abstract: Noroviruses (NoV) have been shown to be an important cause of morbidity and mortality in children worldwide. The disease in most cases occurs with diarrhea and vomiting, affecting mainly children, the elderly and immunocompromised persons. Norovirus being considered the first causative agent of viral gastroenteritis in children under five years old in Baghdad the Capital of Iraq .Two hundred and fifty two fecal samples, negative for pathogenic bacteria and gastrointestinal parasites, were collected from children admitted Baghdad hospitals from May 15, 2012 to May 15, 2013. The presence and genetic diversity of NoV was determined by RT-PCR technique and nucleotide sequencing . Nucleotide sequence and phylogenetic analysis of A and C regions of 60/81 (74.07%) positive samples results found that the appearance of five genotypes: GII.4, GGII.2, GII.17, GII.21, GI.3 .The NVGII.4 Sydney was the most dominant strain with percentage 66.66% Three recombinant genotypes (GII.17/GII.4 Sydney_2012, GII.21/GII.4 Sydney, GII.4 Alberta -2011/GII.4 Sydney-2012) . The results showed a continuous circulation of NoVs in children throughout the one year of study and an extensive diversity of genotypes, highlighting the need for better surveillance of NoVs infection in Iraqi children.

Key words : norovirus ,genotypes, Acute gastroenteritis, capsid protein**.**

تحديد الأنماط الجينية لفيروس النورو في أطفال بغداد المصابين بالتهاب الأمعاء الحاد في العام 2012-2013

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الخلاصة: يعد فيروس النورو أحد أهم أسباب الاعتلال والوفيات بين الأطفال في جميع أنحاء العالم .يظهر المرض في معظم الحالات مع الإسهال والقيء، ويصيب بصورة رئيسية الأطفال وكبار السن وضعاف المناعة .ويأتي الفيروس في مقدمة المسببات الفيروسية لالتهاب الأمعاء الحاد في بغداد.جمعت مائتان واثنان وخمسون عينة من برازالاطفال المترددين على المستشفيات في بغداد والسالبة لفحص البكتيريا الممرضة والطفيليات المعوية للفترة من 15 أيار 2012 ولغاية 15 أيار 2013.وتم التحري عن وجود الفيروس والتباينات الجينية من خلال تقنية تفاعل إنزيم البلمرة التسلسلي العكسي وتتابعات النيوكليوتيدات. من خلال دراسة تتابعات القواعد النتروجينية وتحليل الشجرة الجينية للقطع الجينية Aو C ل 81/60 من العينات الموجبة ومن خلال تحليل النتائج تبين ظهور خمسة أنماط جينية هي , GII.4 GII.2, GII17 ,GII.21, GGI.3وظهر تفوق النمط الجيني NVGII4 Sydney على بقية الأنواع بنسبة 66,66% وظهور ثلاث أنماط جينية هجينة هي(-GII.17/GII.4 Sydney_2012, GII.21/GII.4 Sydney, GII.4 Alberta -2011/GII.4 Sydney 2012).أظهرت نتائج الدراسة استمرار دوران الإصابة بفيروس النورو خلال عام الدراسة والتنوع الواسع للأنماط الجينية.مما يسلط الضوء على ضرورة إجراء مراقبة أفضل لإصابات فيروس النورو في الأطفال العراقيين.

Introduction

Since the development and application of novel sensitive molecular

assays, Noroviruses (NoVs) have been recognized as the leading cause of epidemics of gastroenteritis and an important cause of sporadic gastroenteritis in individuals of all ages in both developed and developing countries(1) .People may remain infectious even after their diarrhea has ended. Infected hosts can shed virus in stool for up to two weeks (2). Viruses cause about 70% of episodes of infectious diarrhea in the pediatric age group(3). NoV is a single positive-strand RNA of 7.7 kb that contains three Open Reading Frames (ORFs)(4) . ORF1 encodes several nonstructural proteins involved in replication of the genome, including RNA-de-pendent RNA polymerase (RdRp), nucleoside triphos-phatases (NTPases), and proteases. ORF2 and ORF3 encode the major capsid protein VP1 and minor capsid protein VP2,respectively(5) .NoV are genetically diverse; 35 different genotypes are now classified within five genogroups (GI-GV) based on their capsid and/or polymerase genes: 14 genetic genotypes in GI; 17 in GII; two in GIII; one in GIV, and one in GV (6). Genetically,

NoVs are grouped by the major capsid protein amino acid sequence. Viruses with less than 14.3%difference are classified as the same strain, those with 14.3 to43.8% difference are classified as the same genotype, and those with 45 to 61.4% difference are classified as the same genogroup (7). Recombination between NoV strains has occurred in nature at high frequency and represents a major driving force of viral evolution. Recombination allows the virus to increase its genetic fitness, to evolve, and to spread in the host population by escaping the host immune response (8).

Materials and Methods

Studying groups: The study involved the collection of 765 stool samples with acute gastroenteritis children under 5 years, and 252 stool samples was chosen from nonbacterial and non-parasite samples for one year from May15,2012- May15,2013.Four Pediatric hospitals were chosen in Baghdad City :-Ebn –Albalady Hospital ,Al- Elwia Hospital , Al-Kademia Hospital and Child Central Hospital

Sample collection: Stool samples were collected from children under 5 years with clinical symptoms of non-bacterial non- parasitic acute gastroenteritis: nausea, vomiting and/or three or more loose stools in 24 hrs. During the acute phase of the infection in sterile plastic water proof container labeled with patient name ,patient number, hospital ,and date of collection. Samples were transported to the laboratory on ice in sealed bag stored at +4ºC in refrigerator until processing. After examination, samples were stored at -20ºC (9).

RNA extraction : 30% (w/v) stool suspensions were made in phosphate-buffered saline (PBS;7.2pH)and centrifugation 8000xg for 10 min .Extractions were performed using 140 µl of supernatant stool suspension with the QIAamp1Viral RNA Mini kit (Qiaen, Germany) according to the manufacturer's instructions. (10). Viral RNA concentration and purity was determined using Nanodrope technique The extracted RNA was dissolved in 60 µl of RNase-free water and stored at -70-C until used.

Genomic amplification for genotyping

For genotyping the primer sets G1SKF:CTGCCCGAATTYGTAA ATGA /G1SKR: CCAACCCARCCATTRTACA and G2SKF:CNTGGGAGGGCGATCG CAA/G2SKR: CCRCCNGCATRHCCRTTRTAC AT were used to amplify the 5' end of the capsid gene (region C in ORF2) for GI and GII, respectively. The primer set JV12Y: ATACCACTATGATGCAGAYTA /JV13I

:TCATCATCACCATAGAAIGAG and 290d GATTACTCCASSTGGGAYTCM AC /289d: TGACGATTTCATCATCMCCRT A, was used to amplify the 3' end of the RdRp gene (region A in ORF1) (11).DNA was generated by QIAGEN One Step RT PCR Kit(Qiagen, Germany) according to the manufacturer's instructions. All amplicons were visualized by electrophoresis (12) Sequencing of PCR product was carried out by Microgen company (USA)in forward and reverse direction ,and primer was used in each sequencing reactions.

Sequencing alignment

Homology search was conducted between the sequence of standard gene BLAST program which is available at the National Center Biotechnology Information (NCBI) online at(http://www.ncbi.nlm.nih.gov) and using BioEdit program ,and Evolutionary analysis were conducted in MEGA 5.2 (13)

Results and discussion

In the present study stool specimens collected from Baghdad children under five years were tested for NoVs by real-time RT-PCR. Of the 251 specimens ,and 81 (32.27%) specimens were positive to NoVs ,data was published previously in (14). NVGII infections were predominant on NVGI infection, representing 74 (88.32 %) of the total NoV infections. The amplicons were visualized by electrophoresis using gel concentration of 2% for best separation of small molecular weight Fig (1,2,3,4) respectively .Analysis of the genetic diversity according to the RdRp and capsid sequence located in region A and C of the NoV strains in our study showed a variety of GII genotypes were identified ,included GI.3(11.66%) ,GII.4(66.66%) ,GII.2 (11.66%), GII.21(5%)and GII.17(5%) (Fig.5,6,7). sequence comparison with archived GII.17 strains from GenBank suggests that the GII.17 genotype identified in Baghdad, differing percentage was 10% from GII.17 strains detected before 2011depending on the C region sequence and these finding is consistent with (15,16).Beginning in 1995, the emergence of novel GII.4 variants caused six pandemics of NoV-associated acute gastroenteritis and most recently the Sydney_2012 variant. After the first detection of the Sydney_2012 variant in March 2012 in Australia, many countries, including Iraq, reported increased levels of NoV activity associated with this novel variant during winter 2012–2013 (17,18,19,20) . But In winter 2014–15,norovirus out-breaks in detected in Maryland ,USA, and Guangdong ,China, increased. Sequence analysis

indicated that 82% of the outbreaks were caused by a norovirus GII.17 variant(15,16).Most of studies refer to the analysis of the recombinants were suggested that the majority of recombination points are located near or within the ORF1/ORF2 overlap (21,22).In the present study, recombinant strains represented an important portion, and 8 of the 81 (9.87 %) NoVs genotyped using both the capsid genes and RdRp corresponded to GII recombinant strains, highlighting the role of recombination in NoV evolution. The GII.17/GII.4 Sydney_2012, GII.21/GII.4 Sydney, GII.4 Alberta -2011/GII.4 Sydney-2012, were detected for the first time in Baghdad. Detection of new NoV recombinant strains shortly after their initial detection in other countries suggests that some recombinant NoV strains can spread widely and rapidly.

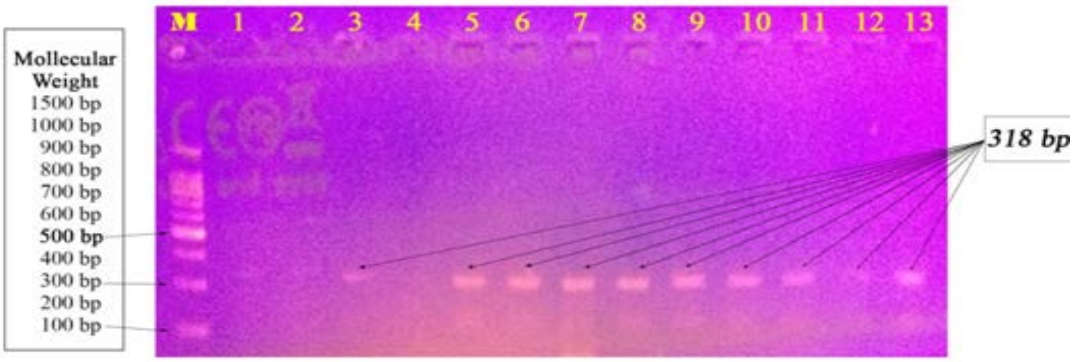

Fig 1. The gel-electrophoresis result of the amplified samples using 2% Agarose and 3vol/cm in TBA buffer. Lane M-100 base pair DNA ladder, Lane 1- 2 negative control. Lane 3-13 Amplicons 318 bp ORF1partial polymerase gene A primer set 290d/289d product .

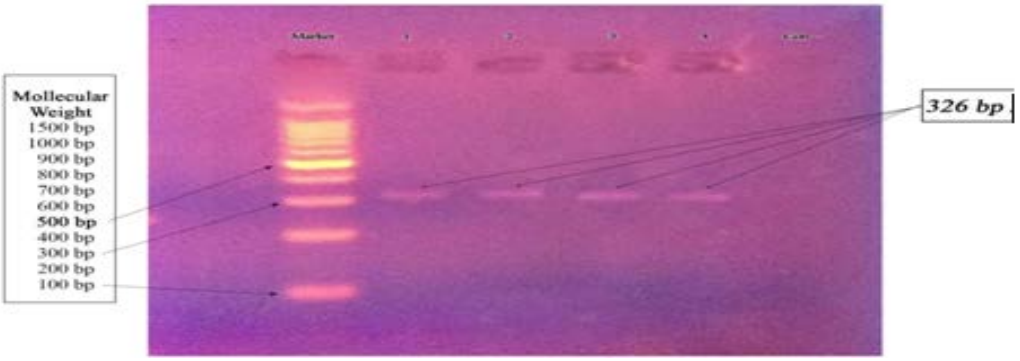

Fig 2. The gel-electrophoresis result of the amplified samples using 2% Agarose and 3vol/cm in TBA buffer. Lane M-100 base pair DNA ladder, Lane 5 negative control. Lane 1-4 Amplicon 326 bp ORF1partial polymerase gene A primer set JV12Y/ JV131product.

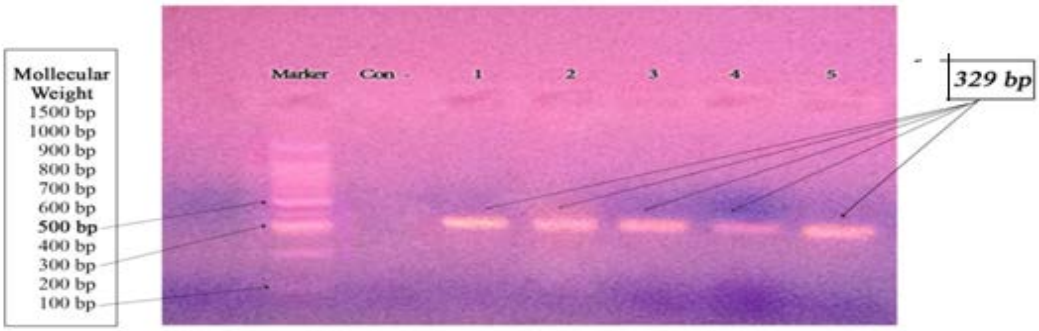

Fig 3. The gel-electrophoresis result of the amplified samples using 2% Agarose and 3vol/cm in TBA buffer. Lane Marker-100 base pair DNA ladder, Lane -con negative control. Lane 2-6 Amplicon 329 bp partial capsid gene C primer set G1SKF/ G1SKR product.

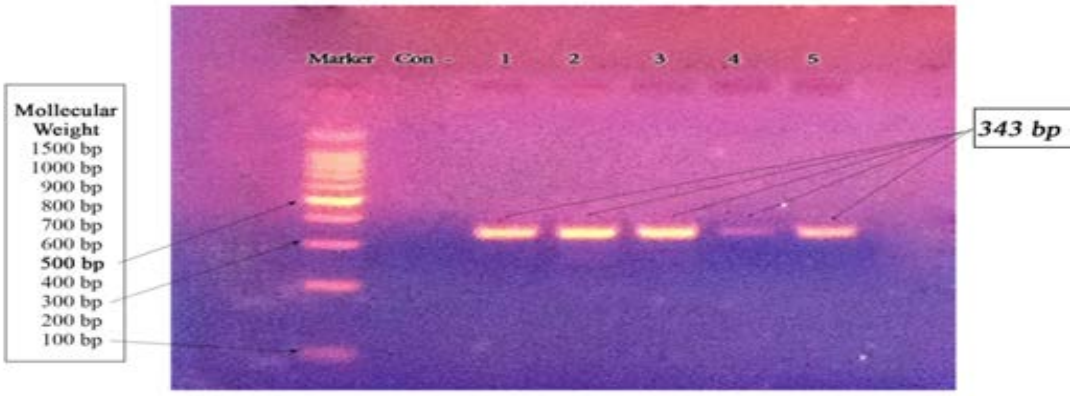

Fig4. The gel-electrophoresis result of the amplified samples using 2% Agarose and 3vol/cm in TBA buffer. Lane Marker-100 base pair DNA ladder, Lane -con negative control. Lane 1-5 Amplicon 343 bp C Junction: ORF1-ORF2 overlap, primer set G2SKF/ G2SKR product.

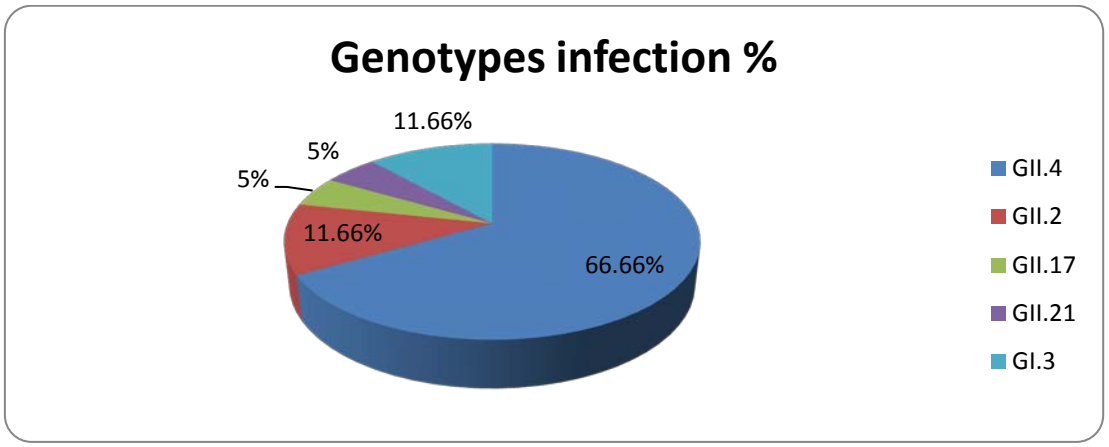

Fig 5. The percentage of different norovirus genotypes

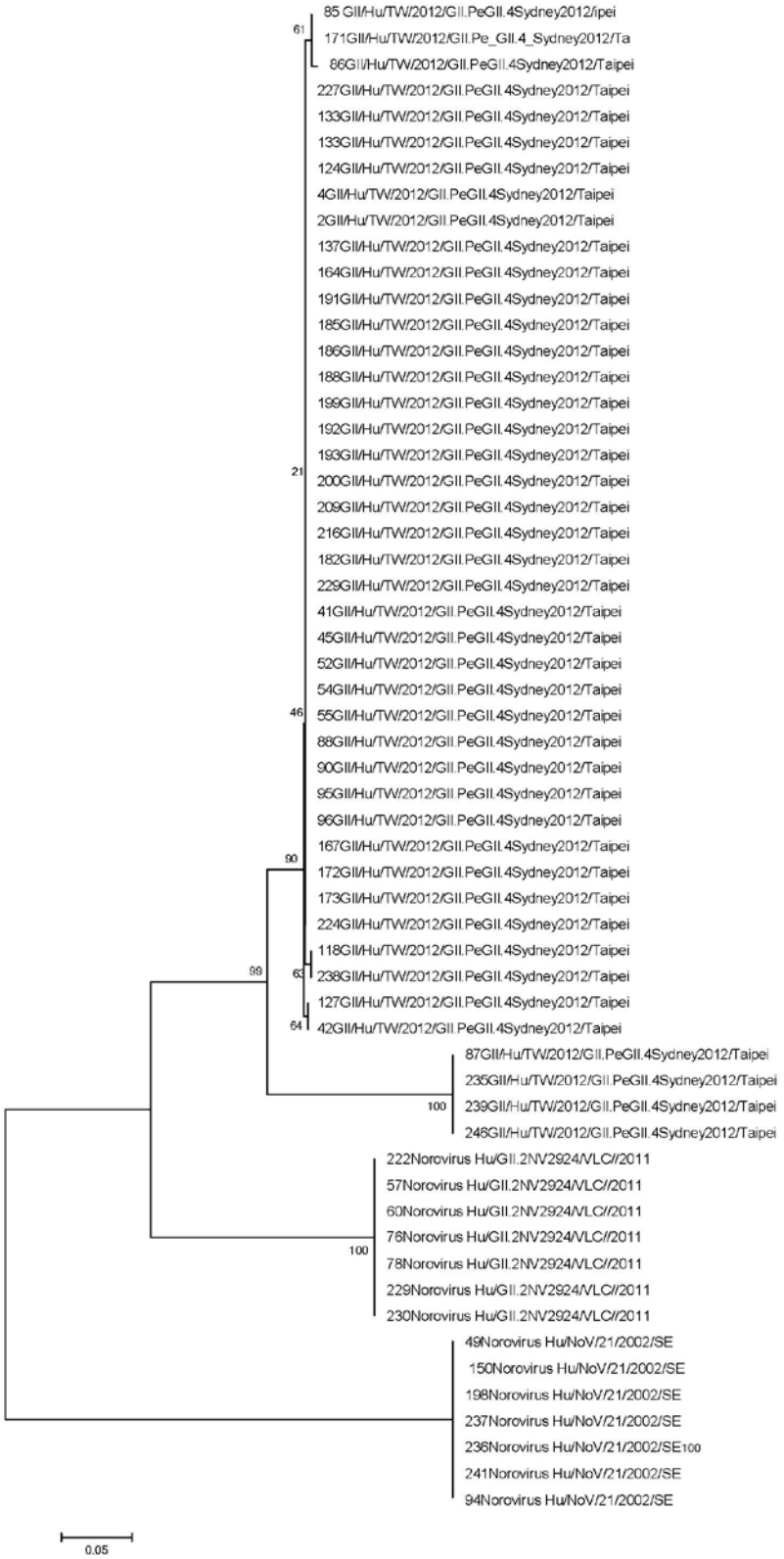

Fig 6.Phylogenetic analyses of the partial RpRd region A of the detected Norovirus genomes

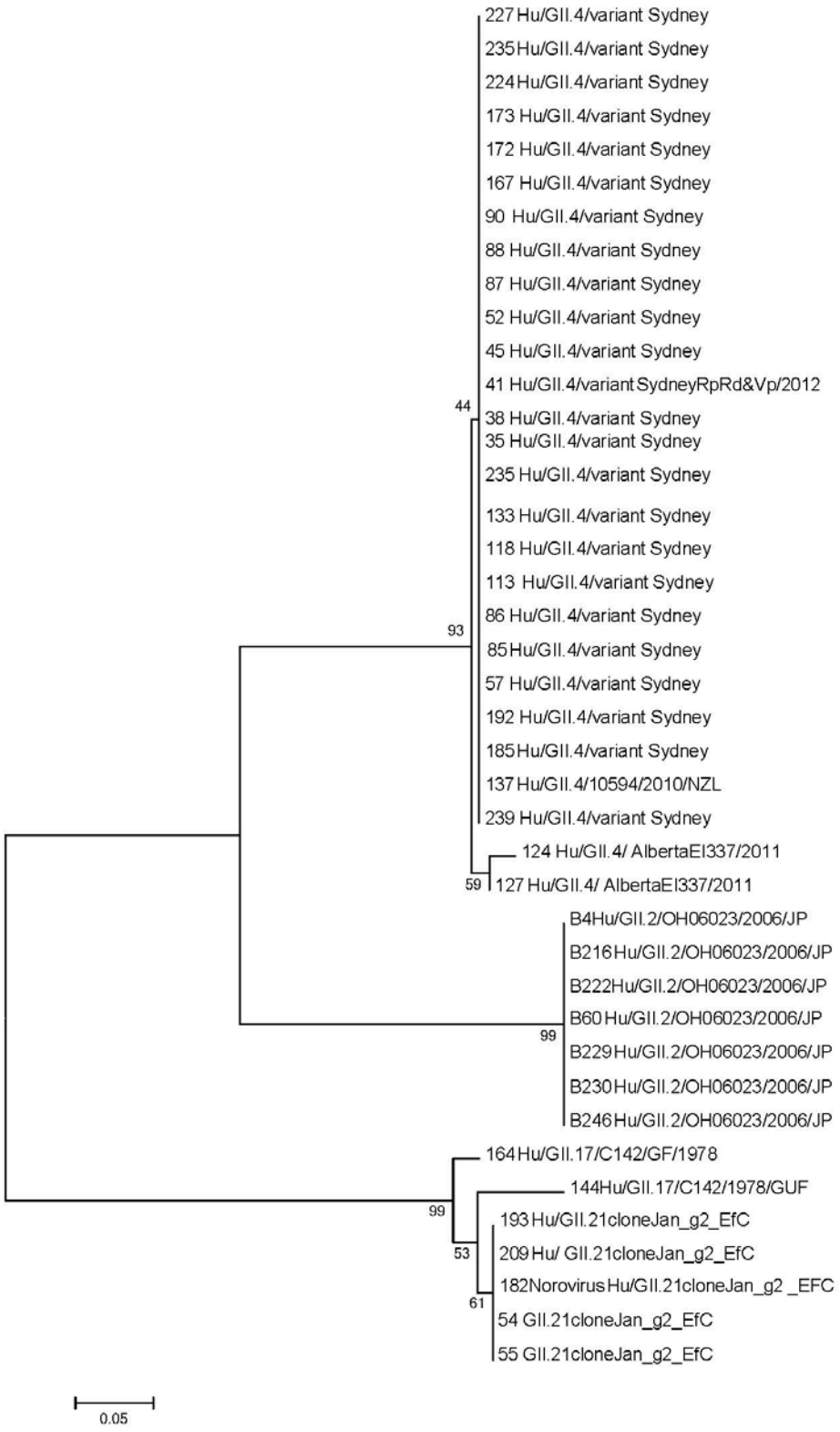

Fig 7. Phylogenetic analyses of the RpRd&Vp1 overlap region C of the detected Norovirus genomes

Conclusion

In our study provides a detailed description of the genetic diversity of NoVs in adults with acute gastroenteritis in Baghdad/Iraq. During the study period, the NoVs circulating in children in Baghdad were predominantly GII.4 Sydney_2012 variants and GII NoV recombinants. Three recombinant genotypes (GII.17/GII.4 Sydney- 2012, GII.21/GII.4 Sydney, GII.4 Alberta -2011/GII.4 Sydney-2012, were identified in this study by phylogenetic. The findings of our study indicate that recombination makes an important contribution to the generation of diversity within No Vs.

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Electrochemical study of the effect of ascorbic acid on redox current peaks of paracetamol in blood sample

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Abstract: A new study was conducted on the paracetamol that are important material in the broad medical applications material. Cyclic voltammetric technique was used to detect redox current peaks of paracetamol in human blood medium with and without present of ascorbic acid. Also, it was studied the paracetamol in normal saline and in KCl solution as supporting electrolyte. The results appear single oxidation potential peak of paracetamol in normal saline as supporting electrolyte at 125mV with anodic current peak at 21uA. It was found new phenomena that paracetamol in normal saline acts antioxidative agent because disappearing of the anodic current peak and appear cathodic current peak at 100mV. Moreover, the present of ascorbic acid (AA) solution in blood medium enhance the cathodic current peak (antioxidative) of the paracetamol.

Keywords: cyclic voltammetry, paracetamol, ascorbic acid, blood sample, GCE.

دراسة الكيمياء الكهربائية لتأثير حامض الاسكوربيك على قمم الاكسدة والاختزال للباراسيتامول في نموذج الدم

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كلية التقنيات الصحية والطبية-بغداد-الجامعة التقنية الوسطى

الخلاصة: تم اجراء دراسة جديدة لمادة الباراسيتامول الواسعة الانتشار في الاستخدامات الطبية. حيث استخدمت تقنية الفولتامتري الحلقي لكشف قمم تيار الاكسدة والاختزال لمادة الباراسيتامول في محيط الدم البشري وذلك بوجود حامض الاسكوربيك وفي حالة عدم وجوده. كذلك تم دراسة الباراسيتامول في محلول النورمال سيلاين وفي محلول الالكتروليتي KCl . لقد اظهرت النتائج بظهور قمة جهدية للاكسدة لمادة الباراسيتامول في النورمال سيلاين عند 125mV مع قمة انودية للتيار عند21uA. وحصلنا على ظاهرة جديدة بان الباراسيتامول في محلول النورمال سيلاين يعمل كعامل مختزل اي عامل مضاد للاكسدة وذلك بسبب اختفاء قمة الاكسدة وظهور قمة كاثودية اي مضادة للاكسدة عند 100mV . بالاضافة الى ان تركيبة الباراسيتامول في محيط الدم بوجود حامض الاسكوربيك الذي يقوم برفع قمة تيار الكاثودية اي يرفع من عامل المضاد للاكسدة للباراسيتامول في الدم.

الكلمات المفتاحية: الفولتامتري الحلقي، الباراسيتامول، حامض الاسكوربيك، نموذج الدم، قطب الكاربون الزجاجي

Introduction

Paracetamol also known as acetaminophen chemically named N-acetyl-p-aminophenol is classified as a mild analgesic. It is commonly used for the relief of headaches and other minor aches and pains and is a major ingredient in numerous cold and flu remedies. [1-3]. A method is proposed for the determination of paracetamol in whole undiluted blood, based on the enzymatic hydrolysis of the drug to p-aminophenol, which is then measured by chronoamperometry at a glassy carbon electrode [4]. The Bi_2O_3 modified electrode was used for determination of paracetamol in human blood plasma samples using 0.1 M KH_2P0_4 solution. The reaction showed signals due to the oxidation of parecetamol [5]. voltammetric study was used on the effect of paracetamol concentration, scan rate, pH, and temperature at a SWCNT/Ni-modified electrode in the determination of paracetamol. The characterization of the SWCNT/Ni/GCE was performed by cyclic voltammetry. Results indicate that electrodes modified with SWCNT and nickel nanoparticles exhibit better electrocatalytic activity towards paracetamol. [6].

Cyclic voltammetry (CV) and chronoamperometry (CA) have been used to sense and determine simultaneously L-ascorbic acid (AA) and acetaminophen at a boron-doped diamond electrode (BDDE) in a Britton-Robinson buffer solution. The anodic CV and CA data were obtained for individual and mixture standard solutions of ascorbic acid and acetaminophen at unmodified BDDE in acidic buffered media [7]. Electrochemical sensor used to detect acetaminophen by electrochemically co-depositing glutamic acid and gold nanoparticles on a single-walled carbon nanotube. Cyclic voltammetry indicated that the electrochemical oxidation of acetaminophen at the modified electrode involved a two-electron, one-proton process and was pH dependent [8].

One of studies was described that the selective electrochemical determination of paracetamol in the presence of important interference with ascorbic acid (AA) using an ultrathin electro-polymerized film of 5-amino-1,3,4-thiadiazole-2-thiol (p-ATT) modified glassy carbon (GC) electrode in 0.20 M phosphate buffer solution (pH 7.20). Bare GC electrode failed to resolve the voltammetric signals of AA and PA in a mixture. On the other hand, the p-ATT modified electrode not only separated the voltammetric signals of AA and paracetamol but also enhanced their peak currents [9].

Zinc oxide (ZnO) microparticles have been mechanically attached on the surface of a glassycarbon (GC)

electrode. The modification of GCE with Zinc oxide was studied the effect on oxidation of paracetamol in 0.1 M KH2PO4 electrolyte solution by cyclic voltammetry (CV). Excellent electrocatalytic activity towards the oxidation of paracetamol was observed. Peak potential was observed to shift slightly to less positive value by about 150 mV and current was significantly enhanced by about 1.1 folds as compared to bare GCE [10].

A chemically - modified electrode has been constructed based on a single walled carbon nanotube/chitosan/room temperature ionic liquid nanocomposite modified glassy carbon electrode. It was demonstrated that this sensor could be used for simultaneous determination of acetaminophen, uric acid and ascorbic acid (AA). The measurements were carried out by application of differential pulse voltammetry, cyclic voltammetry (CV) and chronoamperometry (CA) methods. Electrochemical studies suggested that the modified electrodes provided a synergistic augmentation that can increase current responses by improvement of electron transfers of these compounds on the electrode surface. [11].

Electrochemical behaviors of acetaminophen at a muti-wall carbon nano-tube composite film modified glassy carbon electrode were investigated by cyclic voltammetry, linear sweep voltammetry and chronocoulometry. Compared with that obtained at the unmodified electrode, the peak currents were enhanced significantly, and the oxidation peak shifted towards more negative potential with the reduction peak separation turned narrow, and suggested that the reversibility was improved greatly [12,13].

Paracetamol is involved in a large proportion of accidental pediatric exposures and deliberate self-poisoning cases, although subsequent hepatic failure and death are both uncommon outcomes [14].

In this work, paracetamol compound was studied by electrochemical analysis to finding the redox current peaks properties of paracetamol in blood medium in present with AA.

Experimental

Reagent and chemicals

Paracetamol as standard solution (10mg/ml) from Bristol-Myers Squibb (Anagni, Italy), Normal saline (0.9%NaCl) from Iranian company, KCl (pure powder) from SCRC (China), the blood samples was used from healthy human. Other chemicals and solvents were of annular grade and used as received from the manufacturer. Deionized water was

used for the preparation of aqueous solutions. All solutions used in the cell of cyclic voltammetry were deaerated with oxygen free nitrogen gas for 10-15 min prior to making the measurement. All experiments were carried out at the room temperature of the laboratory.

Instrumentation

EZstat series (potentiostat / glvanostat) NuVant Systems Inc. pioneering electrochemical technologies USA. Electrochemical workstations of Bioanalytical system with potetiostate driven by electroanalytical measuring softwares was connected to personal computer to perform Cyclic Voltammetry (CV), an Ag/AgCl (3M NaCl) and Platinum wire (1 mm diameter) was used as a reference and counter electrode respectively. The working electrode used in this study was glassy carbon electrode (GCE).

Results and Discussion

Effect paracetamol in normal saline

The cyclic voltammograms of 0.1 mM paracetamol(10 mg/ml) in normal saline (0.9% NaCl) using GCE, Fig 1 shows one of oxidation potential and current peak at 150mV and 21uA respectively. Also, the reduction potential peak appears at 50 mV.

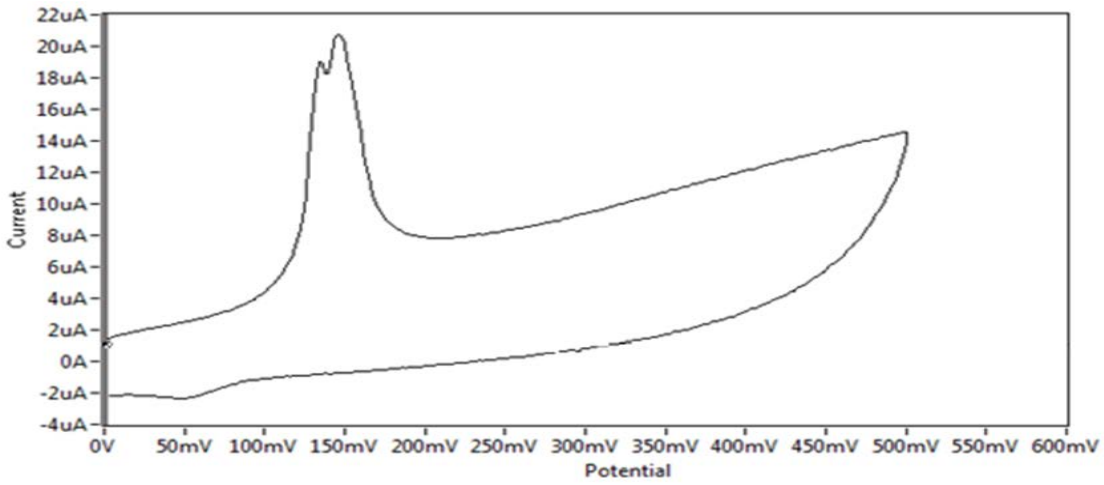

Figure 1 Voltammograms of the oxidation current peak of 1 mM paracetamol (10 mg/ml) in normal saline (0.9% NaCl) (using GCE, 100mVs^{-1} versus Ag/AgCl).

Effect paracetamol in blood sample

Fig.2 illustrated the cyclic voltammogram of 10 mM paracetamol (10 mg/ml) in mixing of normal saline (0.9% NaCl) and blood sample(healthy human sample) using GCE as working

electrode. It showed that the effecting of blood medium on the redox current peak of paracetamol. It was appeared that the cathodic potential peak at 100 mV with disappearing anodic potential peak which mention that the paracetamol has antioxidative properties in blood medium. So, it can be used paracetamol as safety medicine without side effect.

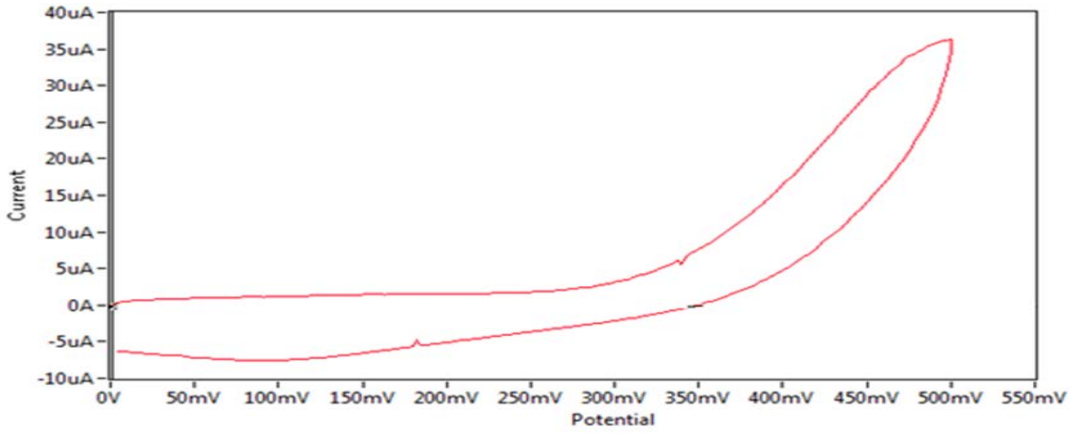

Figure 2 Voltammograms of 10 mM paracetamol (10 mg/ml) in normal saline (0.9% NaCl) and blood sample (using GCE, 100mVs-1 versus Ag/AgCl).

Affecting of ascorbic acid on paracetamol in blood sample

Ascorbic acid has highly electrochemical affecting on the cathodic potential peak (at 100 mV) of paracetamol in blood medium by

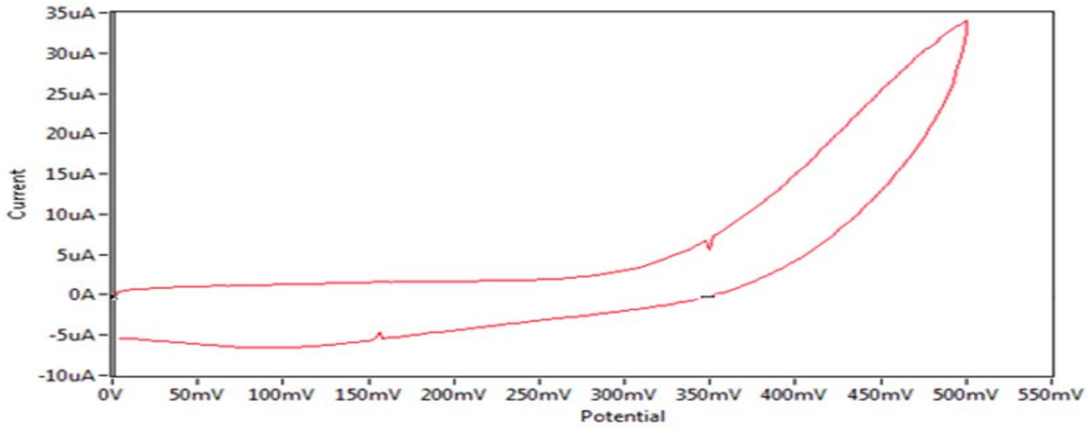

Figure 3 Voltammograms of 10 mM paracetamol (10 mg/ml) with 5 mM AA in normal saline (0.9% NaCl) and blood sample (using GCE, 100mVs-1 versus Ag/AgCl).

Enhancement the reduction current peak as show in figure 3. So, AA acts as electrocatalyst of paracetamol for human body in blood medium and used as antioxidative ragent because disappearing of oxidation current peak from the voltammogram of paracetamol in blood medium in present of AA.

Effect KCl electrolyte on the redox current peaks of paracetamol

Figure 4 shows the effective of AA on the redox current peaks of paracetamol in KCl electrolyte and blood medium. It was found that disappearing of the redox reaction of the paracetamol in the blood medium except of redox peaks of AA.

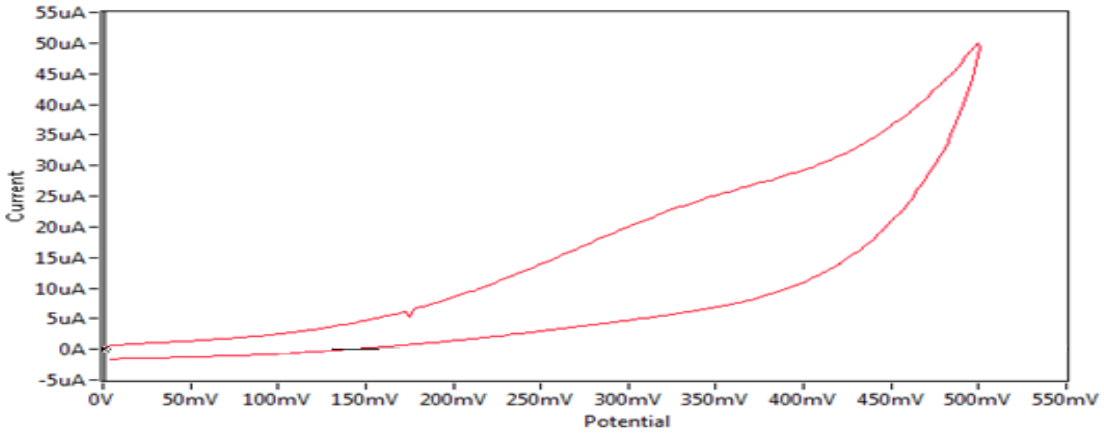

Figure 4 . Voltammograms of 10 mM paracetamol (10 mg/ml) with 10 mM AA in 0.1M KCl and blood sample (using GCE, 100mVs-1 versus Ag/AgCl).

Conclusion

Highly sensitive and selective electrochemical determination of paracetamol in the presence of important interference with AA using GCE in blood sample was reported. It was found that the redox current peaks response of paracetamol was improved significantly and the oxidation peak was disappeared in present of AA in blood medium. The enhanced of cathodic current peak of paracetamol mainly came from the AA solution in blood medium. As a result, the using of paracetamol in medicine with AA was successfully employed for the voltammetric determination in electrochemical analysis as electrocatalyst. Its advantages, such as simple, sensitive, rapid and accurate, were demonstrated by the determination of paracetamol in the pharmaceutical samples with good result.

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Extraction, Purification and Characterization Of Polyohenoloxidase From Broccoli *(Brassica oleracea Var)* .

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Abstract: The activity of polyphenoloxidase (PPO) in broccoli was evaluated using spectrophotometric method. The enzyme was extracted from the broccoli stem with 0.1 M phosphate buffer solution pH (7. 0). The activity of PPO was determined using catechol as a substrate. The effects of the concentration enzyme extract, substrate concentration, pH and temperature were investigated. The highest activity of ppo at 2.5 mg /ml concentration enzyme .The highest activity of PPO was obtained when using catechol concentration of 100 Mm . The optimum pH was 5.0 for PPO. The optimum temperature for PPO was 50°C. These optimum conditions were used to determine the enzyme activity in broccoli sample. Polyphenoloxidase (PPO) enzyme was purified from a soluble extract of broccoli stems. The PPO was purified by using Ion exchange chromatography was purified showed a specific activity 285.71 U/mg ,18.81 times and with a 42.45% yield. Then PPO was purified by gel filtration chromatography, increased the specific activity to 1444.4 units/mg ,95.18 timesand with a yield 44.16%. Optimum activity and stability were at pH 6 .0 and 6.0 respectively. Opt. temperature and stability were 50 , 60°C respectively.

Keywords: Broccoli ; Polyphenoloxidase ; optimization; purification.

استخلاص تنقية وتوصيف انزيم البولي فينول اوكسديز من البروكلي

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الخلاصة: استخلص الانزيم من سيقان البروكلي باضافة حجم معين من دارىء فوسفات الصوديوم بتركيز 0.1 مولار برقم هيدروجيني 7.0 وقدرت فعالية انزيم البولي فينول اوكسديز من البروكولي بطريقة المطياف الضوئي . قدرت الظروف المثلى لفعالية الانزيم باستخدام تركيز الانزيم ،تركيزالمادة الاساس (الكاتيكول) ، أضافة للرقم الهيدروجيني ودرجة الحرارة . سجلت اعلى فعالية للبولي فينول اوكسديز عند تركيز 2.5 ملغم/ مل ، اما تركيز المادة الاساس سجلت اعلى فعالية عند تركيز 100 ملي مولار. وسجلت اعلى فعالية لانزيم البولي فينول اوكسديز عند الرقم الهيدروجيني 6.0 و لوحظت اعلى فعالية للانزيم عند درجة الحرارة 40 °م. نقي البولي فينول اوكسديز من مستخلص سيقان البروكلي ،باستخدام تقنية التبادل الايوني وسجل اعلى فعالية كانت بفعالية نوعية 285.71 وحدة /ملغم وعدد مرات التنقية 18.81 و حصيلة 42.45 % وبعدها نقي انزيم البولي فينول أوكسديز بالترشيح الهلامي وارتفعت الفعالية النوعية الى 1444.4 وحدة /ملغم وعدد مرات التنقية لها 95.18 وحصيلة 44.16 % ،. وكانت اعلى فعالية عند الرقم الهيدروجيني الامثل والثبات عند القيم 6.0 و6.0 على التوالي. وسجلت اعلى فعالية عند درجة الحرارة المثلى والثبات عند الدرجات 50، 60 °م على التوالي .

Introduction

Many vegetables and fruits become discoloured during storage or processing, an action mediated by the enzyme polyphenol oxidase (PPO) [1]. PPO (EC 1.14.18.1) is a copper-containing enzyme that is widespread in plants, and synthesised early in tissue development and stored in chloroplasts [2]. The enzyme is a copper protein widely distributed in a multitude of organisms, from bacteria to mammals [3] .Enzymatic browning is the main function of PPO in fruits and vegetables but is often undesirable and responsible for unpleasant sensory qualities as well as losses in nutrient quality [4]. When cell membrane integrity is disrupted, phenolic substrates encounter the enzyme and are converted to o-quinones in a two-step process of hydroxylation of monophenols to diphenols (monophenolase activity), followed by the oxidation of diphenols to o-quinones (diphenolase activity). These highly reactive quinones polymerize with other quinones, amino acids and proteins to produce coloured compounds, and nutrient quality and attractiveness is reduced. PPO from different plant tissues shows different substrate specificities and degrees of inhibition. Therefore, characterisation of the enzyme could enable the development of more effective methods for controlling browning in plants and plant products. Guaiacol is a common hydrogen donor substrate traditionally used to check the adequacy of the thermal treatment. PPO from different plant tissues shows different substrate specificities and degrees of inhibition. Therefore, characterisation of the enzyme could enable the development of more effective methods for controlling browning in plants and plant products, Substrate and temperature effects were also studied. One unusual characteristic of this enzyme is its ability to exist in an inactive or latent state [5].

Materials and methods

Materials: Fresh broccoli *(Brassica oleracea var.)* was obtained from a local market and washed with distilled water. Broccoli stems and florets were separated. Only the stems were used for polyphenoloxidase extraction due to their relatively higher activity of polyphenoloxidase as compared to the floret [6]. Fresh prepared samples were frozen and stored at -20 °C until used.

Enzyme Extraction: Broccoli stems were removed from frozen storage and homogenized at 4 °C for 1h. using phosphate buffer 0.1M, pH 7.0, in a ratio of 1: 2 (grams of broccoli per milliliter of buffer). The extract was centrifuged, and the

supernatant was used for further purification.

Enzyme assays

PPO activity was determined using a spectrophotometric method based on an initial rate of increase in absorbance at 410 nm [7] . 1.95 mL of 0.1 M Phosphate buffer solution pH (7 .0) , 1 mL of 100 mM catechol as a substrate and 50 µL of the enzyme extract with pipetted into a test tube and mixed thoroughly. Then the mixture was rapidly transferred to a 1-cm path length cuvette. The absorbance at 410 nm was recorded continuously at 25°C for 5 min using ultraviolet-visible spectrophotometer, Spain.. One unit of enzyme activity was defined as the amount of enzyme that causes an increase 0.001 of absorbance per min.

$$\textbf{PPO activity (unit/ml)} = \frac{\triangle A_{410nm}}{0.001 \times 0.05 \times RM}$$

0.05 = volume of enzyme

RM= reaction mixture (3 ml)

Protein concentration was measured according to Bradford method [8] .

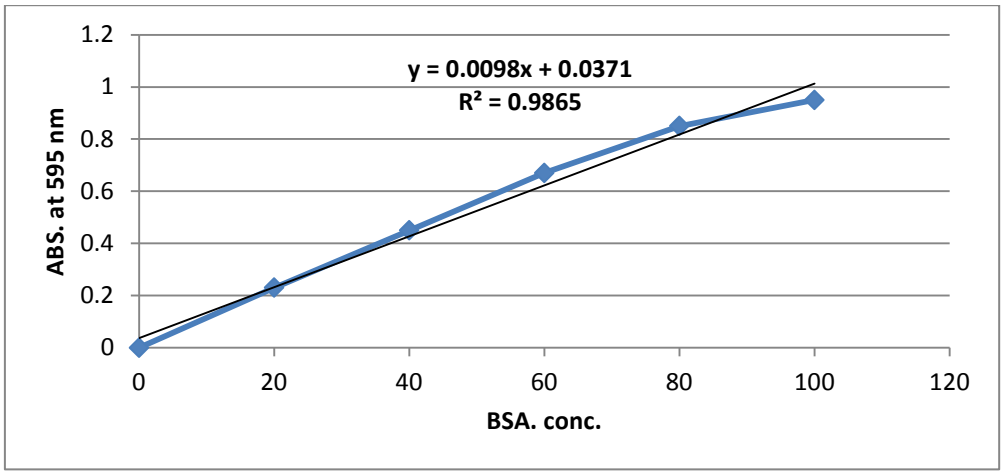

Figure.1: Bovine serum albumin standard curve using Bradford method.

Effect of amounts of enzyme extract on enzyme activity

The activity of PPO as a function of amounts of enzyme extract was investigated. PPO activity was assayed at various amounts of the enzyme extract from (1 , 1.5, 2, 2.5 ,3, 3.5) mg/ml by

mixing with 2 mL of 100 mM catechol, and 1 mL of 0.1 M phosphate buffer pH (7.0) [9] .

Effect of substrate concentration on enzyme activity

PPO activity was performed using the substrate concentrations (40, 60 ,80, 100, 120) mM , PPO activity was observed by using the mixture containing 50 µL of the enzyme extract,1 mL of 100 mM catechol and1.95 mL 0.1 M phosphate buffer pH (7.0) at a selected volume.The enzyme activity was measured in a quartz cuvette of 3 mL volume [9] .

Effect of pH on enzyme activity

The activity of PPO was determined at pH values of (4, 5, 6, 7 , 8 ,9) using 0.1 M citrate buffer (pH 3- 5) and phosphate buffer (pH 6 - 8). The optimum pH for PPO was obtained using catechol as substrate in these buffers. The effect of pH on PPO activity was observed by using the reaction mixture containing 1 mL of 100 mM catechol, 1.95 mL of 0.1 M buffer solution and 50 µL of the enzyme extract [9] .

Effect of temperature on enzyme activity

PPO activity was determined at(20, 30, 40, 50, 60 , 70°C). The substrate and buffer solutions were incubated for 5 min at various temperatures from 20 to 70°C before adding of the enzyme extract. Spectrophotometric measurement for 5 min was carried out 1 mL of 100 mM catechol, 1.95 mL of 0.1 M phosphate buffer pH 7.0 and 50 µL of the enzyme extract [9] .

Protein Precipitation. Precipitation of protein was carried out using ammonium sulfat$(NH_4)_2SO_4$, first with 50% saturation and centrifugation, and then the saturation level was increased to 90% followed by centrifugation. After 1 h, the precipitated proteins for each stage were separated by centrifugation at 10000 rpm for 30 min. The precipitate was redissolved in a in 0.05 M Tris-HCl, pH 7.8 and dialyzed at 4ºC against the same buffer for 24 h with 4 changes of the buffer during dialysis, and used in the purification steps [10].

Anion Exchange Chromatography. A 3 × 50 cm column packed to a height of 31 cm with DEAE-Cellulose (Sigma Chemical) was equilibrated with 0.05 M Tris-HCl buffer, pH 7.8. Broccoli extract was loaded onto the column and washed with the equilibrating buffer using a 86 mL/h flow rate. The retained protein was eluted at the same flow rate using a linear 1 L gradient of 0.0 - 0.5 M NaCl in the above buffer. Fractions of 6.5 mL were collected, the absorbance was read at 280 nm, and PPO activity was measured [10].

Gel Filtration Chromatography. Pooled fractions from the DEAE-Cellulose column that were eluted using a linear salt gradient. Each sample was loaded onto a 2 × 75 cm column packed with Sephacryl S-300 and equilibrated with 0.1 M sodium phosphate, pH 7.0. Elution of the protein was carried out at 22 mL/h flow rate with the equilibrating buffer. Fractions of 5 mL were collected.

Concentration sample by dialysis. Fractions from the DEAE- Cellulose column eluted during washing with equilibrating buffer that showed PPO activity were combined. These fractions were concentrated by dialyzed against 0.04 M sodium phosphate buffer, pH 7.8.

Activity of PPO in Different pHs and Temperatures Activity of purified PPO was measured in pHs (3 -9) using the substrate 100Mm catechol in these buffers 0.05 M buffers of sodium acetate for pH ranging from 3.5 - 6.5 and Tris-base for pH ranging from 7-9. and the activity was measured, Activity of PPO in different temperatures (30 - 70°C) was estimated as the enzyme assay [7].

Stability of PPO in Different pHs and Temperatures Determination of pH stability of PPO was incubated for 4 h at 37°C, and then enzyme activity was measured as [7]. For measuring of thermal stability of PPO enzyme . phosphate buffer (50 mM, pH: 7.8) was incubated for 30 min in different temperatures, then proportion of remained activity was compared with the initial activity. Enzymeassay was performed as [7].

Results

Optimization conditions for enzyme activity measurements PPO is oxidative enzymes which catalyze the oxidation of phenolic substrates mainly due to enzymatic browning [11] . The substrate oxidation was found to be dependent on the amounts of the enzyme extract. (Figure 2) the enzyme PPO concentration range assayed (1 , 1.5 , 2 , 2.5 ,3) mg/ml, The highest activity was 65 U/ml at 2.5 mg/ml concentration enzyme.

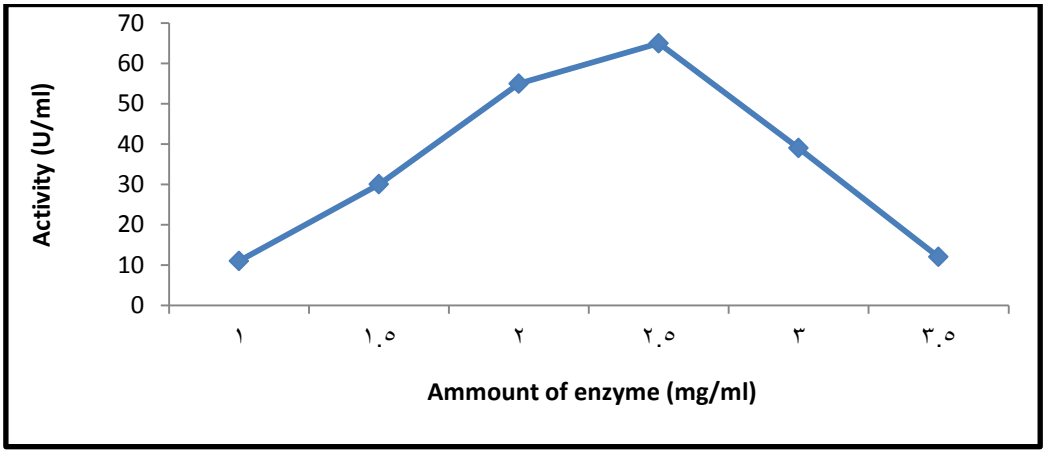

Figure (2) . Effect of amounts of the PPOenzyme

Using different amounts of the substrate (Figure 3). As expected, an increase in the substrate concentration resulted in an increase in pigment formation. The rate of which stayed practically constant at saturating catechol concentration. Therefore, the concentration of 100 mM catechol was routinely chosen because at higher concentrations of the substrate did not significantly affect the formation of the O-quinone intermediate.

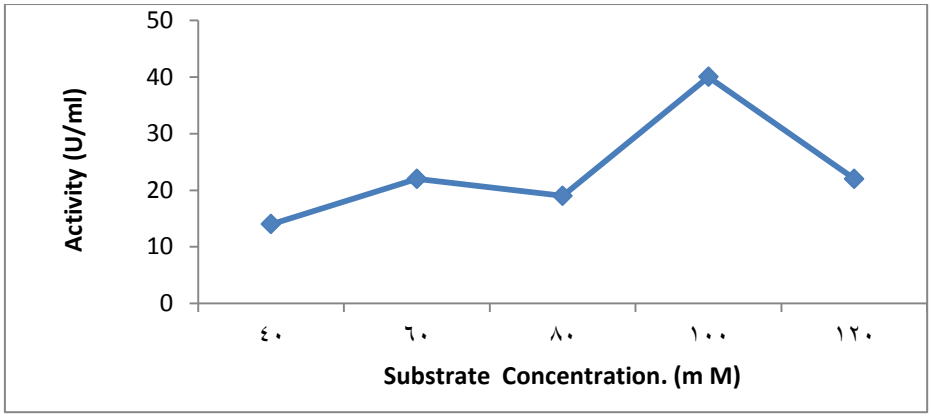

Figure (3) . Effect of catechol concentration on the PPO activity .

The activity of PPO was measured at different pH values using catechol as substrate. As shown in (Figure 4) the optimum pH 6.0 of enzyme PPO was obtained. It is known that the optimum pH for any enzymes depends on plant materials and substrate in the activity assay. In general, most plants show maximum enzyme activity at or near neutral pH. Different optimum pH values

for both enzymes obtained from various sources and substrates used have been reported.The optimum pH values are 6.8 and 5.5 for butter lettuce PPO using 4-methycatechol and catechol as substrates, respectively [12] .

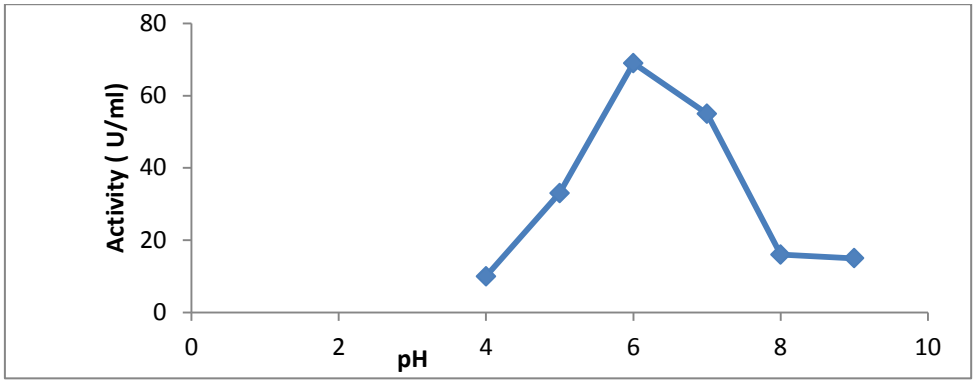

Figure (4) . Effect of pH on PPO activity.

The optimum temperature for enzyme activity usually depends on experimental conditions. Generally, the reaction rate decreases because of thermal denaturation when the temperature is increased. This situation is similar for most enzymes. Temperature dependence in the enzyme activities is presented in (Figure 5). It was found that the highest activity of PPO was obtained at 50°C. PPO showed the highest activity at 30°C, and its activity decreased slightly between 40 and 70°C, and then decreased probably due to denaturation of the enzyme at higher temperatures. From previous studied, the temperature at which PPO showed the highest activity was in the range of 25-30°C, and then decreased at temperature above 40°C [13] .

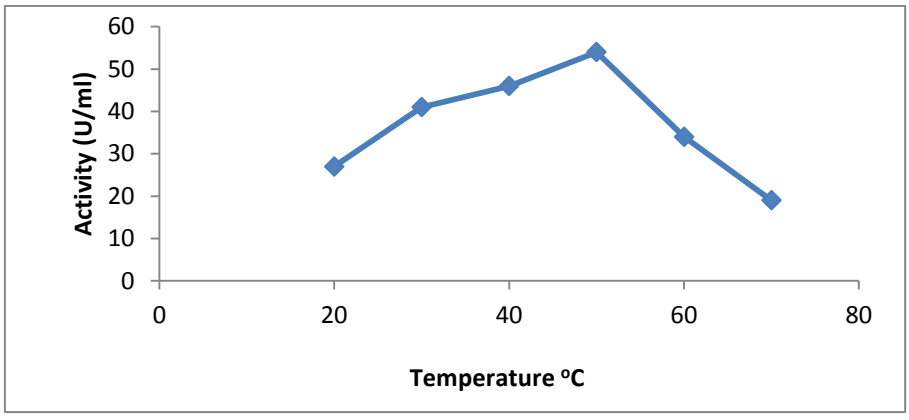

Figure (5). Effect of temperature on PPO activity.

Purification of PPO A summary of the purification procedure and specific information on the degree of purification obtained at each step appears in (Figure 6) and (Table 1). Ammonium sulfate precipitation helped to improve PPO purification and concentrate the crude extract. The specific activity and purification-fold following ammonium sulfate treatment were twice those of the crude extract. After anion exchange chromatography (AEC), PPO was distributed into two peaks, the first of which was eluted during the washing step and the second eluted with the salt gradient (Figure 6).

DEAE-cellulose chromatography was mostly used for PPO purification [14]. Two fractions of PPO activity were eluted from DEAE-cellulose column (Figure .6), the purified fraction was showed a specific activity 285.71U/mg and 18.81 times with a 42.45 % yield. Other investigators were used DEAE-cellulose get 14.08 [14] and 9.7 [15] times of purification. This relatively of increase in specific activity may be associated with the large amount of absorbing materials eluted along with the enzyme. The fractions eluted by the salt gradient were pooled, concentrated, and then further purified by gel filtration chromatography. Gel filtration chromatography separated out some contaminating materials and increased the specific activity to 1444.4 U/mg and 44.16 % with 95.18 time (Figure 7) .

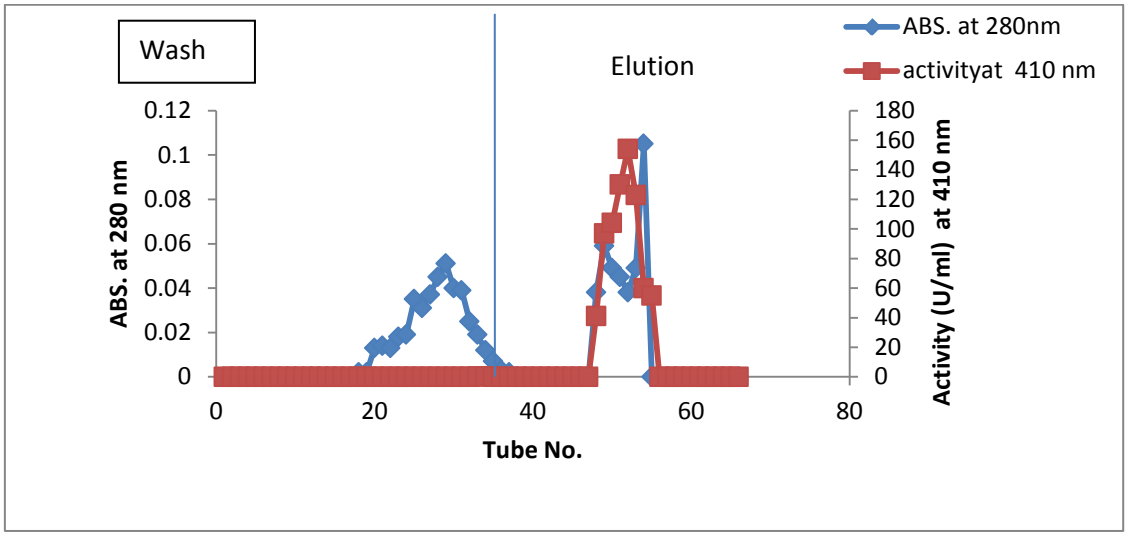

Figure (6): Ion exchange chromatography for polyphenol oxidase extracted from broccoli stems DEAE-cellulose column (3 X 31 cm) equilibrated and washed with 5 mM tris buffer (pH: 7.8) and eluted with [5 mM tris buffer (PH 7.8) buffer and 0 – 0.5 M Nacl gradient], at a flow rate (6.5 ml/4.5 min).

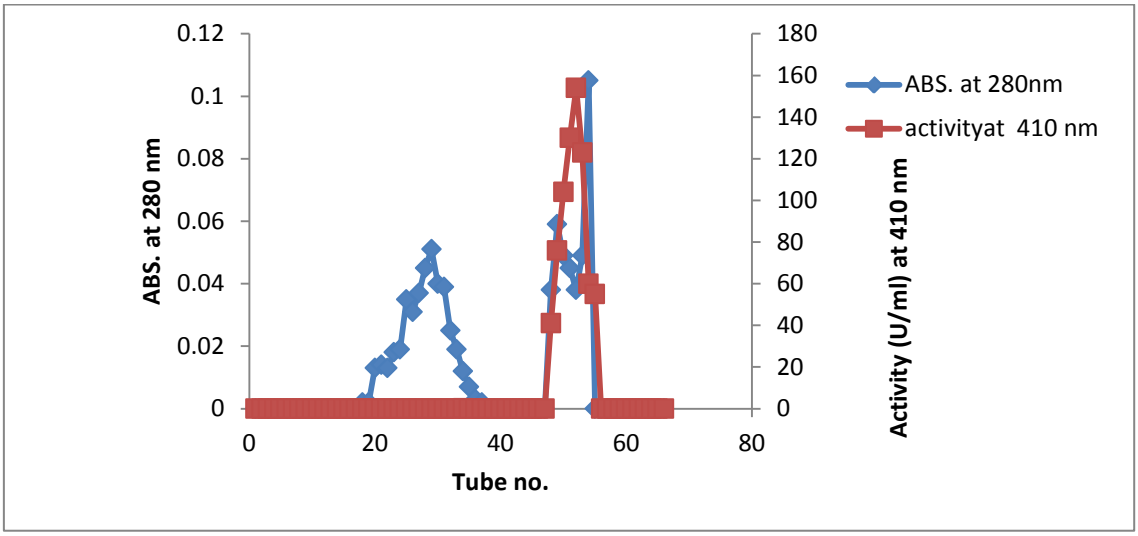

Figure (7): Gel filtration(2 × 75 cm) column packed with Sephacryl S-300 and equilibrated with 0.1 M sodium phosphate,(pH 7.0) Elution of the protein was carried out at 22 mL/h flow rate with the equilibrating buffer. Fractions of 4.6 mL were collected .

Table 1. Fractionation protocol of broccoli *Brassica (oleracea capitata L.*)

Purification stepes	Vol. (ml)	Activity (U/ml)	Protein (mg/ml)	Specific activity (U/mg)	Total activity (U)	Recovery (%)	Purification fold
Crude extract	125	47.10	3.1	15.19	5888	100	1
Ammonium precipitation	45	. 76.12	1.64	46.41	3425.4	58.18	3.14
dialysis	14	93.38	0.87	107.3	1307.32	22.20	7.15
Ion exchange	25	100	0.35	285.71	2500	42.45	18.81
Gel - filteration	20	130	0.09	1444.44	2600	44.16	95.18

Optimum pH for PPO activity with catechol as substrates was 6.0 (Figure 8). As the pH increased from 3 to 9, the enzyme activity increased, with maximal activity occurring at pH 6.0. Differences in optimum pH for PPO with distinct substrates have been reported for the enzyme from various sources [16, 17 , 13]. However, pH optima for PPO activity in presence of catechol and pyrogallol in wild pear is the same.

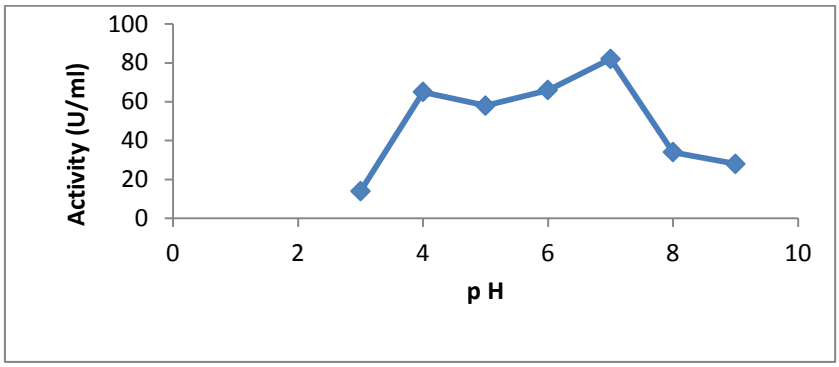

Figure (8). Effect of pH on activity of PPO from broccoli

Optimum Temperature. The optimum temperture was 50ºC Other reported values include 25 °C for grape PPO [18] and 30 °C for banana PPO [19]. The optimum temperature obtained in this study is 40°C for catechol and pyrogallol at pH 5, 45°C for catechol at pH 7 and 55°C for pyrogallol at pH 7 that are dependent on the substrate and pH.

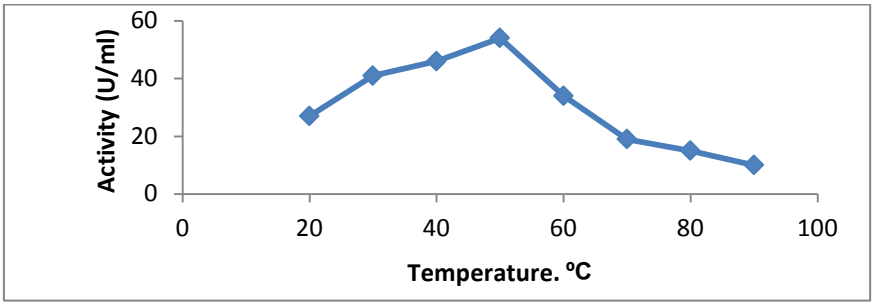

Figure (9). Effect of different temperature on the of PPO .

Also shows pH stability of PPO in 0.05 M buffers with pHs between 3 - 9.After four hours of incubation of PPO in mentioned pHs at 37°C, the activity was assayed. The maximum stability for PPO was 83 .22% after four hours is in pH 6.0 .

PPO activity in different temperatures was measured by incubating of enzyme in temperatures ranging from 30 - 90°C (Figure .10). The best temperature for highest activity of PPO at 50°C was 88.8 U/ml .

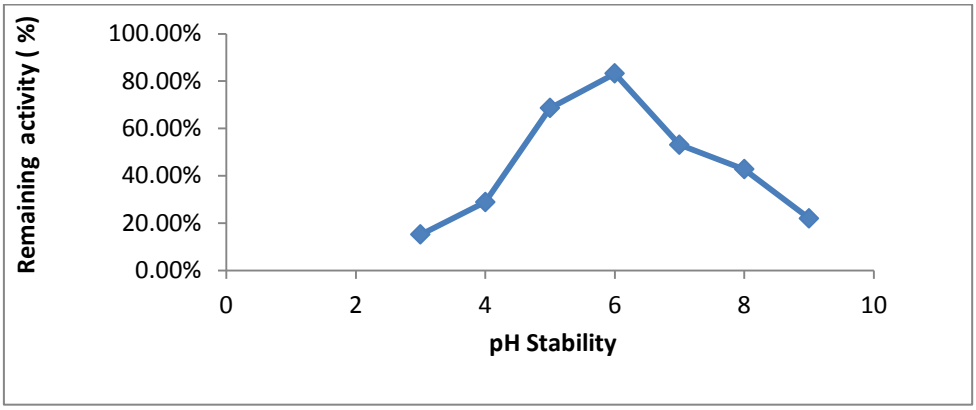

Figure (10) . Effect of different Temp.on the activity of PPO

Thermal stability of PPO at temperatures ranging from 30 - 90°C after 30 min was also measured. As it is shown in (Figure 14) , PPO keeps more than 77% of its activity at 60°C, but in higher temperatures it loses most of its activity.

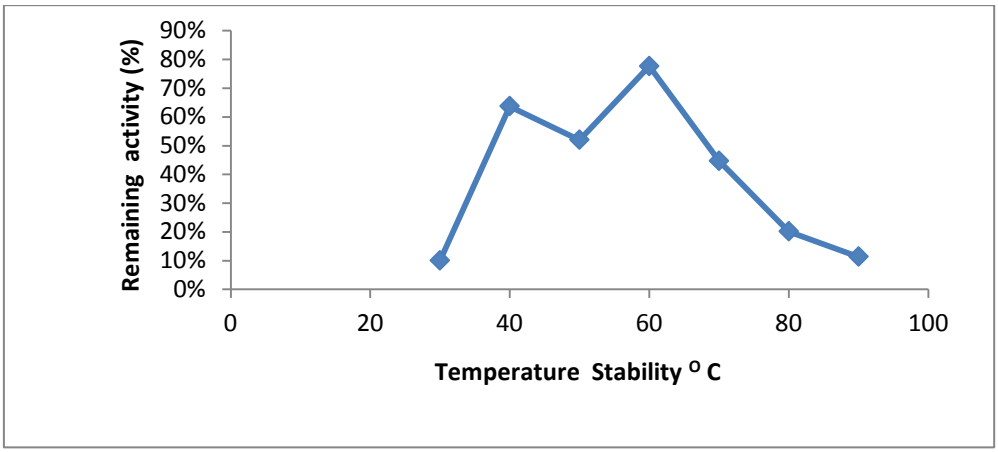

Figure (11) . Effect of different temperatures on the thermal stability of PPO

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Association among family history and some microbial infectious (*Helicobacter pylori IgG and Hepatitis B and C Virus) as Risk Factors for Atherosclerosis in Iraqi Patients*

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Abstract: Certain bacterial and viral infectious agents may play a role in the activation of inflammation in atherosclerosis lesions. Epidemiological studies indicate that infectious agents may predispose patients to atherosclerosis as Infections have been associated with an increased risk of this disease. Moreover, a positive antibody status has been detected against some infectious organisms associated with atherosclerotic rupture. Infectious agents found in human atheroma, which may directly cause or accelerate atherosclerosis , include many pathogens but the present study focused on *Helicobacter pylori*, hepatitis B virus surface antigen and C. In order to evaluate the possible association between *H. pylori*, HBV, and HCV infections and the risk of atherosclerosis. Biochemical markers and acute inflammatory factors that may be involved in atherosclerosis disease were investigated in relation to microbial infections and atheroma formation in Iraqi patients.

The present study shows a significant increase in *H. pylori IgG* antibody concentrations in the sera of the patients (2.941±1.350) [U/L] compared to the controls(1.962±0.873) [U/L] and thus provides evidence that *H. pylori* infection is a risk factor for atherosclerosis. Furthermore patients with positive family history of atherosclerosis were significantly more likely to be positive for *H. pylori IgG* antibodies 86.3%. While hepatitis B virus infection is not associated with atherosclerosis in our Iraqi patients, there was a significant positive correlation between HBV infection and both the levels of the inflammatory protien ceruloplasmin and family history of atherosclerosis indicating that the HBV association needs further study . No subject was found to be positive for anti-HCV antibodies.

Key words : Hepatitis , H.pylori IgG, Family history & atherosclerosis.

العلاقة بين التاريخ العائلي والاصابة ببعض الاحياء المجهرية (الاجسام المضادة نوع جي لبكتريا Helicobacter pylori) سي ونوع بي والتهاب الكبد الفايروسي كعوامل خطورة لمرض تصلب الشرايين في مرضى عراقيين

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الخلاصة: بعض الانواع المحددة من البكتريا والفايروسات تؤدي دورا مؤثرا في تنشيط العمليات الالتهابية لصفائح تصلب الشرايين. الدراسات الوبائية اشارت الى ان العدوى باصابات معينة لدى مرضى تصلب الشرايين ترتبط بتحفيز العمليات الالتهابية وزيادة خطورة المرض، بالاضافة الى ذلك شخصت حالات موجبة لوجود الاجسام الضدية لبعض الاحياء المجهرية الممرضة ترتبط بنيسهل تمزيق الصفائح التصلبية ويسرع الاصابة بمضاعفات تصلب الشرايين وقد شخص العديد منها لكن الدراسة الحالية ركزت على البعض مثل بكتريا وفايروس التهاب الكبد نوع بي ونوع سي لغرض تقييم العلاقة المحتملة بينهم كعوامل خطورة لمرض تصلب الشرايين .كذلك درست الدلائل الكيميائية وبعض عوامل الالتهاب الحاد والتي يمكن ان ترتبط مع العدوى الميكروبية وتكون الصفائح الدهنية لدى المرضى العراقيين.

اظهرت الدراسة الحالية زيادة معنوية في الاجسام المضادة نوع جي للبكتريا المدروسة مقارنة بالسيطرة مما يوكد الى دورها في تطور المرض واعتبارها كعامل خطورة لمرض تصلب الشرايين وقد اضهر المرضى ذوي التاريخ العائلي الموجب للاصابة بتصلب الشرايين اظهروا اصابة موجبة بالبكتريا86.3% . كذلك سجلت الدراسة الحالية ارتباطا معنويا موجبا بين الاصابة بالتهاب الكبد الفايروسي نوع بي لدى مرضى تصلب الشرايين ومستوى البروتين الالتهابي السيريلوبلازمين وكذلك مع التاريخ العائلي الموجب للمرض ولم تسجل اصابة بالتهاب الكبد الفايروسي نوع سي .

Introduction

Atherosclerosis is one of the most common disease, atherosclerotic lesion development is mostly confined to regions of arterial curvature and branch points, which are exposed to disturbed blood flow causing cardiovascular complications. Atherosclerosis is characterized by local inflammation that includes an inflammatory component (1). Activated inflammatory cells and mediators can influence the development progression of atherosclerosis (2, 1). The inflammation can activated by possible role of certain infectious agents , since some pathogens have been identified in atherosclerosis plaques or remnants of them are present in atherosclerotic plaque (3), moreover a positive antibody status detected against some infectious organisms which it is associated with atherosclerotic diseases, while other Epidemiological studies indicate that infectious agents may predispose patients to atherosclerosis or may be Infections have been associated with an increased risk of atherosclerosis. (4) Furthermore Immunity-related

injury by infectious may precipitate vascular inflammation with increased acute phase protein like C-reactive protein , Ceruloplasmin and albumin(5).

Pathogens may directly cause or accelerate atherosclerosis include chlamydia pneumoniae, cytomegalovirus, herpes simplex virus and helicobacter pylori. Periodontal pathogens have also been found in human atheroma's (3). Recently, a report investigated a potential role for viral inflammation by different Hepatitis virus A, B and C , in the atherosclerotic process (5).Another research work suggested that direct effect of chronic HCV infection raised the progress of cardiovascular disease as any other atherosclerosis risk factors in Egyptian Patients (6).

Two previous studies suggest hepatitis B and C virus infection may be independent risk factors for carotid atherosclerosis (7-8). A recent study reported that seropositivity for the hepatitis C virus (HCV) has a positive association with carotid atherosclerosis (9). Previous results have not been confirmed by other study, which even produced contradictory results with hepatitis virus infections being protective against atherosclerosis (10). On the other hand, other researchers have failed to demonstrate any link between infection and atherosclerotic disease (11-4).

The aim of this study was to evaluate the possible association between H. pylori, HBV, and HCV infections and the risk of atherosclerosis. Family history, Biochemical markers and acute phase proteins that may be involved in atherosclerosis disease were investigated in relation to microbial infections and atheroma formation in Iraqi patients, in order to find any in prospect link among them.

Materials and methods

For each one of immunological tests used specific ELISA kit. Immune enzymatic assay for serum human IgG antibodies to H. pylori by ELISA was provided by Bio-RAD company, France. Bioelisa HCV:third generation enzyme-linked immunosorbent assay for the detection of antibodies to HCV in human serum (Biokit , Spain), Bioelisa HBs Antigene : enzyme-linked immunosorbent assay for the detection of HBS Ag in human serum (Biokit , Spain). For each one of chemical tests used specific zymatic and colorimetric methods in the sera of patients and controls, by Using kits from Randox company, UK. Serum Ceruloplasmin (Cp) level estimated by signal radial immunodiffusion (SRID) plates for accurate quantitative determination of proteins in human serum

(Biomaghreb-Tunisia). Albumin level was determined by dye-binding technique, which using bromo-cresol sulphonphthalin (bromocresgreen, BCG). (Randox, UK.)

Results

The present study includes 410 subjects, 320 diagnosed with atherosclerosis and 90 healthy individuals. Thirteen of the patients were positive for HBs Ag, indicating that they were hepatitis B virus (HBV) carriers, but there was no significant differences between patients and control for HBs Ag. No subject was found to be positive for anti-HCV antibodies. There were significant differences between patients and healthy controls in the level of *H.pylori IgG* . The mean concentrations of GOT ,GPT ,Cholesterol ,Triglyceride , total serum protein and ceruloplasmin showed significant increases relative to controls (table 1). There were non significant increases in the concentration of albumin , HDL, HBs Ag.There were no individual with positive result of antibodies to HCV (anti-HCV).

Table(1) Comparison of study parameters between patients group and control group.

Parameters level	Control (No.=90) Mean ± SD	Patients (No.=320) Mean ± SD	t. test value
HBs Ag [ng/ml]	0.142 ± 0.042	0.214 ± 0.104	0.191 NS
HCV	Null	Null	-
H.pyloriIgG [U/L]	1.962±0.873	2.941±1.350	0.121*
GOT [g/dL]	9.93 ± 0.46	14.35 ± 0.62	2.355*
GPT [g/dl]	6.47 ± 0.43	10.34 ± 0.46	1.783*
Alb [g/dl]	4.563±0.631	4.541± 0.309	NS
Cholesterol [g/dl]	4.73 ± 0.19	5.32 ± 0.13	0.533*
Triglyceride [g/dl]	0.95 ± 0.05	1.61 ± 0.06	0.237*
HDL[g/dl]	1.09 ± 0.28	1.27 ± 0.27	0.193 NS
TSP[g/dl]	8.16 ± 0.83	7.01 ± 0.46	2.728*
Ceruloplasmin [g/dl]	3.166±0.997	4.107±0.465 *	1.001 *

P≤ (0.05)* , NS: non-significan

Table (2) shows the association among study parameters. HBs antigens were positively correlated with GOT,GPT,TSP and Ceruloplasmin. Also the positive correlation between H.pylori IgG and Ceruloplasmin was.

Table (2) The correlation among study characteristic's for patients

Join characteristics	R	P
HCV & HBS	-0.04	NS
***H.pylori IgG* & HBS**	0.026	NS
GOT & HBS	0.239	*
GPT & HBS	0.378	*
Alb & HBS	-0.161	NS
Cholesterol & HBS	- 0.088	NS
Triglyceride &HBS	-0.050	NS
HDL & HBS	- 0.025	NS
TSP & HBS	0.375	*
Cp. & HBS	0.095	*
Cp.& *H.pylori IgG*	0.112	*

P≤ (0.05)* , NS: non-significan

The comparison between study groups according to the family history of atherosclerosis with hepatitis B infectious is show in Table 3

Table (3) Compare between study groups according to the Family history of presence HBS

Groups	No.	*H.pylori* IgG G antibodies level			
		Presence		Absence	
		No.	%	No.	%
Patients with positively family history	204	176	86.30	28	3.70
Patients with negatively family history	116	84	72.40	32	27.60
Healthy control	90	18	20	72	80

X^2=98.88 df=1 p<<0.005 S (between patients and control for family history

Table (4) Level of H.pylori IgG in control group and in patients classified by family history of artherosclosis.

Study groups	No.	Family history			
		Positive		Negative	
		No.	%	No.	%
Patient group	320	258	80.6	62	19.4
Control group	90	23	25.6	67	74.4

X^2=89.01 df=1 . p<<0.005 (S) (total comparison).

X^2=9.22 df=1 P<0.005 (S) (between patients with positively family history of Atherosclerosis and patients with negatively family history)

Discussion

The present study indicated that family history is associated with the development of artherosclerosis and patients regardless of family history of artherosclerosis recorded a significant higher positive frequency of *H.pylori IgG* antibodies compared to controls . These results support the earlier suggestion that *H.pylori* is trigger for atherosclerosis (1). In addition there is an increasing (but not statistically significant) -level of HBs Ag in patients sera when compared to controls . Both results are in agreement with previous reports showing that viral and bacterial infections contribute to the pathogenesis of atherosclerosis (2)

The absence of correlations between HBs Ag and classical risk factors of atherosclerosis which such as Albumin, Cholesterol, Triglyceride and HDL was unexpected (table 2), and may suggest that HBs Ag acts as an independent factor. There were positive significant correlations among HBs Ag and the *H.pylori*, acute face proteins, ceruloplasmin, total serum protiens, these results agree with previous report that have been demonstrated an elevated levels of acute face proteins in the sera of atherosclerosis patients (4), and may be related with pathogenesis of atherosclerosis (7). GOT and GPT levels were significantly increased in patients compared to controls, observations may be explained by the fact that liver dysfunction in the majority of the HBs Ag-positive subjects was minor.

The increased level of ceruloplasmin is associated with increased levels of oxidative stress (5). Cp. is an acute face protein related to immuno-system activity. In present data. This factor showed significant positive correlation with both HBs Ag and *H.pylori IgG*. so

HBV may colonize in the vascular tissues (7,8), leading to vascular damage (4), along with a significant increase in *H.pylori IgG l*evels which facilitateing the developing of other factors like atherogenesis because antibodies of any pathogens have the ability to block the import of modified Lipoproteins via macrophage scavenger receptors (2,19). Thus they became as a synergistic effect between different pathogenic factors and the serum antibody titers (14). Moreover because the obligate intracellular pathogens having a latent state may contribute to the atherogenic process where resistance is observed in the cellular or by humeral response (1)], Cp can act as a mark for the activation of the inflammatory responses, that play a role in the progression and destabilization of atherosclerotic plaques (3).

No individuals (neither in the patient group nor in the control group) were positive for HCV, which indicates there is no association between atherosclerosis and hepatitis C infection, which disagree with a previous report considerd chronic HCV infection independent risk factor (6).

Furthermore present data agree with previous finding that the prevalence of disease among Asians not be much different from that Caucasians in spite of the high rate of *H.pylori* infection for both races(20) this high ratio relates to family history, as 86.3% of our patients with high levels of *H.pylori* IgG also have a positive family history for Atherosclerosis. The presence can not be predicted by measuring serum antibodies, further studies must conduct to obtain more clarification about the relationship among different factors in positively patients with HBs Ag, *H.pylori* and others factors. Moreover, follow-up studies are needed to establish the association among study parameters and progressive of disease, especially ceruloplasmin and other factors that showed a positive correlation with positive HBsAg patients.

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Iraqi Journal of Biotechnology, 2015, Vol. 14, No. 2 , 93-106

Bio treatment of tannery waste water by use some species of fungi isolated from local soils

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Abstract: The current study deal with two aspect as the following isolated fungi from soil samples and try use some fungal species to remove and reduce some pollutants in industrial wastewater .The study was isolated and diagnosed 74 isolation of 7 species 5 genus where the 25 isolation belonging to the fungus *Aspergillus niger* and 14 isolation of the fungus *Aspergillus terreus* and 12 isolation of the fungus *Aspergillus flavus* and 10 isolates of the fungus *Penicillium expansum* and 5 isolation belonging to the fungus *Alternaria alternate* and 6 isolates belonging to the fungus *Fusarium* sp.and 2 isolation of *Bipolaris* sp. This was choice two species (*Aspergillus terreus* and *Fusarium* sp.) In treatment of wastewater to tanning industry by removal and reducing the pH, color, Total Hardness, nitrite and nitrate, the biomass was study before and after treated also.

The results were showed that the mix fungal *Aspergillus terreus* and *Fusarium* sp , it was more effective in the removal of the top ten compared to the use of each species separately for treatment total hardness pretreatment from 4500 mg / L to 300 mg / L after the addition of the mixture was innate in the using of fungus *Aspergillus terreus* or fungus *Fusarium* sp. The results also showed that the mix fungal *Aspergillus terreus* and *Fusarium* sp. More efficient in removing pollutants compared to the use of two fungi separately. As was the removal of pollutants ratio (nitrite and nitrate) the existence of innate mix more as compared to the use of two fungi individually.

The results showed the possibility of removing some pollutants using some species of fungi through biodegradation and did not need this technology to high cost.

Keywords: Bioremediation, Industrial waste water, fungi, water pollution, Biodegradation.

المعالجة الإحيائية لمياه فضلات الدباغة باستخدام بعض أنواع الفطريات المعزولة من ترب محلية

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الخلاصة: يهتم البحث الحالي بجانبين هما عزل فطريات من عينات تربة محلية وتجربة استعمال هذه الانواع في ازالة او اختزال التلوث بمياه الفضلات الصناعية . تضمنت الدراسة عزل وتشخيص (74) عزلة تعود (7) انواع و(5)اجناس ، منها (25) عزلة للنوع *Aspergillus niger* و(14) عزلة للنوع A.terreus و (12) عزلة للنوع *A.flavus* و(10)عزلة تعود للنوع *Penicillium expansum* و(5) عزلة تعود للنوع *Alternaria alternata* و (6) عزلة للنوع .*Fusarium* sp (2) عزلة للنوع .*Bipolaris* sp .

تم اختبار نوعين من الفطريات المعزولة في اعلاه هما *Aspergillus terreus* و.*Fusarium* sp لمعالجة وازالة بعض الملوثات او تحسين خصائص مياه الفضلات الصناعية لمعامل الدباغة او الجلود مثل الاس الهيدروجيني واللون والعسرة الكلية والنتريت والنترات اضافة الى دراسة الكتلة الحية قبل وبعد المعالجة بشكل مفرد او خليط من انواع الفطريات المستخدمة .

اظهرت النتائج خليط الفطريات المستخدمة كان اكثر كفاءة في عملية الازالة مقارنة بأستخدام كل نوع على حدة ، اذ لوحظ انخفاض تراكيز الخصائص المدروسة بشكل كبير مثل العسرة الكلية من (4500 مل/لتر) الى (300 مل/لتر) بعد اضافة خليط الفطريات ، كما ان النتائج اظهرت ان استخدام المزيج كان افضل من خليط الفطريات من استخدام كلا نوعي الفطريات المستخدمة بشكل منفصل ويكون ذلك واضحاً ايضاً في اختزال تراكيز النترات والنتريت في المياه الصناعية اظهرت المعالجة النتائج امكانية ازالة بعض انواع الملوثات بأستخدام انواع من الفطريات من خلال التحطيم الحيوي والذي لايتطلب تقنية عالية الكلفة.

كلمات مفتاحية : معالجة حيوية ،مياه الفضلات الصناعية ،فطريات،تلوث مياه ،تحطيم حيوي.

Introduction

The tanning industry is an important sector in the economy of many countries. The result of the industry large quantities of wastewater with ammonium, sulphates, acids, dyes, and organic materials, given that biggest part of the organic compounds are resistant to conventional chemical and biological treatments, and waste disposal in natural water leads to increased environmental pollution and health risks and that the treatment of this type of waste water is a very complicated process because of the diversity in industrial wastewater chemical products and different concentrations(1-4).

Fungal treatment is a form of biological treatment, used to get rid of fungus or adjust the ratio of pollutants in the environment. And to raise the effectiveness of microbial and enzyme activity, where the mycelium by reducing

toxins in the soil or water rates. For some types of fungi ability to absorb heavy metals and concentration in the body of fungi(5) .That one of the main roles of fungi in the ecosystem Healthily, where mycelium secretes enzymes and acids as well as outside the cell that break down lignin compound and also cellulose, the two blocs main in the construction of the plant fibers. Those two compounds are an organic composite materials made up the chains of carbon and hydrogen also, similar in composition with many organic pollutants. It is also the essence of fungal treatment in determining the appropriate and correct fungal species to target a specific pollutant. That certain breeds have proved successful in cracking and decomposition of nerve gases and Article siren(6,7).

One of the ways used to remove colored liquid waste re-direct recycling(8), blood clots(9), flocculation(10), chemical precipitation (8,11), ion exchange (12), adsorption (13), biological treatment (14,15,16) and electrochemical treatment (17,18), and membrane separation (19,20,21), thermal and other technologies (7,19). The physical chemistry processes, such as coagulation / flocculation, adsorption and membrane separation, was the most widely used to remove colored liquid waste. However, these treatments do not solve the problem because of the transfer of pollutants from one stage to another. However, the biological treatment, and microorganisms decomposition of organic contaminants and used as a source for the production of Carbone metabolic energy to keep life(22). Use of fungi is economical and eco-friendly technique for the fine tuning of waste water treatment(23).

This research deals with two axes isolate fungal types of local soils and the use of fungi isolated(*Aspergillus terreus* , *Fusarium sp*.) in industrial waste water treatment.

Material and Methods

Isolation purification of fungi from soil

This method is used to get the largest number of fungal genera and species in soil samples where scattering 5 g of soil on solid circles in the dish 9 cm diameter by repeating after the dishes were incubated at a temperature of 28 c° for 7 days(24) **.** Purification process has fungus after the incubation period ranging from five to seven days by taking part of the edge of the fungi colonies by piercing Cork and distributed to the circles culture dishes and incubated at a temperature of 28c° until the degree of growth (25).

Treatment of sterilized wastewater with single fungal isolate

The sterile waste water distribution in conical flasks one liter capacity by 570 ml of water per vial and each transaction add 30 ml of the vaccine for primary fungi *Aspergillus terreus*, *Fusarium* sp. Both individually and in three replicates for each of them, and then put the bottles in the incubator at a temperature of 28c˚. The tests were conducted in the first, third, fifth and seventh day for physical and chemical tests of water (26,27) .

Treatment of sterilized wastewater with a mixture of fungal isolate

The sterile waste water distribution in flask cone one liter capacity by 570 ml of water per vial and then added to 30 ml of a combination vaccine innate equal sizes each individually and as follows *Aspergillusterreus* with *Fusarium*sp. After that mixing the two types of vaccine innate previously mentioned and added by 10 ml of each vaccine and put this transaction in the incubator at a temperature of 28c˚ and then taken from the farm for each of the first, third, fifth and seventh day for physical and chemical tests(26,27) .

Physical and Chemical Factors

1- pH

PH was measured for samples using pH meter after calibration with standard solutions of pH(4,7,9) .

2-Color and The percentage of removal account

Absorbance was measured for a sample of industrial water before and after treatment fungal device v1100 Digital spectrophotometer after pass the sample to a centrifuge and the speed of 3000 r / 10 minutes (28) .

The removal percentage is calculated using the equation:-

The percentage of removal=(the concentration of pollutants before treatment - the concentration of pollutants before treatment/ the concentration of pollutants before treatment *100 (26).

3-Total Hardness

The total hardness was estimated in water by usingNa_2 - EDTA titration Method ,Then take 10 ml of the sample and mitigation with distilled water to 50 ml and burette with the standard solution 0.01M after the addition of one ml of the solution organizer and dye Eriochrome Black T (EBT) and Expressed in units of Mg/L (29) .

4-Nitrate and Nitrite

Use the cadmium column to measure the effective nitrate dissolved by passing 50 ml sample of the candidate, as is the reduction of nitrate to nitrite which is added after his 1 ml of ammonium chloride Center and across from the output unit mg/l(30) .nitrite ws measured adding 1 ml of slvanal amaid for a period of one to eight minutes, then add 1 ml of Naphthalene - ethylene Di amine Di Hydrochloride to 50 ml of the candidate and measured the optical absorption of the sample along the 543 nm(28).

5-Biomass

Biomass account in the last day of the experiment and that the nomination of the sample by the nomination and put it in a glass cup known weight papers and then placed in the oven at a temperature 85c˚ (31) .

Results and Discussions

The results showed that *Aspergillus* more visible races at isolating fungi from the soil because it has a variety of enzymes that help him to throw his living on the residue in the soil

During this research were are isolate and diagnose 80 isolation belonging to 7 species under 5 genus and *Aspergillusniger* was the most frequency by 33.78% While the least frequency *Bipolaris* sp. by 2.70 % and species *(Aspergillus terreus , Fusarium* sp.)were selected fungal least risk to human health in treatment contaminated water as shown in the Table (1).

Table(1) showing the number isolates for each type of fungi isolated from soil and percentage of frequency

Fungi	Isolate No.	Frequency (%)
Aspergillus niger	25	33.78
Asp.flavus	14	18.91
Asp.terreus	12	16.21
Penecillium expansum	10	13.51
Fusarium sp.	6	8.10
Alternaria alternate	5	6.75
Bipolaris sp.	2	2.70
Total isolates	74	100

The fungal ability to remove color from colored industrial waste because of the characteristics of cellular grandparents harm and is composed of chitin-chitosan In addition to their ability to secrete enzymes that break down the middle, which is composed of carbon, nitrogen and nutrients such as calcium, magnesium, zinc, potassium and pH (5).

The results showed the test mixture of *Aspergillus terreus* with *Fusarium* sp. more effected for decrease total hardness 300 mg/l (table 2) than when we used single fungi *Aspergillus terreus* about 500 mg/l or *Fusarium* sp. about 3200 mg/l (Table 3,4) total hardness represent a sign of the presence of some dissolved solids in the water (32) ,and industrial waste that poses to the water cause rising total hardness due to the presence of additional ions such as calcium ions and magnesium(33).

pH was one of the most important environmental factors that effect on the growth of fungi and the process of the production of enzymes ,filamentous fungi was growth in acidic media and this culture media including nitrogenous sources and carbohydrates , the decline in PH shows that the fungus produced salts of organic acids then degraded by enzymes produced by the fungus called manganese peroxidase enzyme(27,34,35,22,).the table 2 shows the PH and Acidic were changed from 8.3, 5 to 6.93 , 0.1 Respectively , But when using *Aspergillus terreus* the PH and Acidic were changed from 8.3,4.5 to 5.3,0.2 Respectively in the seventh day, The nitrogen source.

(nitrate , nitrite, ammonium) is essential to the process of fungal growth, especially the production of enzymes that most fungi are used as a source of ammonia nitrogen and accompanied by low pH in the culture media (36) .

Nitrate ,Nitrite was changed from 1.53 ,1.2 to 0.228 ,0.210 Respectively (table 2) while when used *Aspergillus terreus* , nitrate ,nitrite also was changed from 1.79 ,1.54 to 0.306,0.215 Respectively (table 3) And when using *Fusarium* sp.the nitrate and nitrite also was changed from 1.93 ,1.65 to 0.263, 0.250 Respectively in the seventh day (table 4).

Table(2) showing some chemical Factors for industrial waste water treatment and sterile by *Aspergillus terreus,Fusarium sp.*

Chemical Factors	Before treatment	After treatment			
		First day	Third day	Fifith day	Seventh day
Total Hardness mg/L	4500	2300	2150	600	300
pH	8.6	7.5	7.1	6.5	4.5
Acidic	6	3.4	1.3	0.9	0.1
Nitrate	400	1.53	0.42	0.306	0.228
Nitrite	150	1.2	0.35	0.2	0.210

Table (3)showing some chemical tests for industrial waste water treatment and sterile by *Aspergillus terreus*

Chemical Factors	Before treatment	After treatment			
		First day	Third day	Fifith day	Seventh day
Total Hardness mg/L	4500	4200	3700	3500	3200
pH	8.6	8.3	7.9	7.21	6.93
Acidic	6	5	4.2	0.7	0.1
Nitrate	400	1.93	1.5	0.952	0.263
Nitrite	150	1.65	1.32	0.3	0.250

Table(4)showing some chemical tests for industrial waste water treatment and sterile by *Fusarium* sp.

Chemical Factors	Before treatment	After treatment			
		First day	Third day	Fifth day	Seventh day
Total Hardness mg/L	4500	9300	1100	700	500
pH	8.6	8.3	7.5	6.8	5.3
Acidic	6	4.5	2.5	1.3	0.2
Nitrate	400	1.79	0.934	0.802	0.306
Nitrite	150	1.54	0.72	0.69	0.215

It was the highest rate for the removal of nitrate and nitrite in last day for treatment about 89.623%, 96.359% Respectively when use mixture fungal(table 5 and figure1,2, 3,4)While the percentage of removal of nitrate and nitrite when using *Aspergillus terreus* in last day of treatment about 88.38%, 95.468% Respectively (table 6 and figure1,2, 3,4)While the lowest percentage for the removal of nitrates and nitrites about 86.09%, 93.392 % Respectively when using *Fusarium* sp. (table7 and figure1,2,3,4).

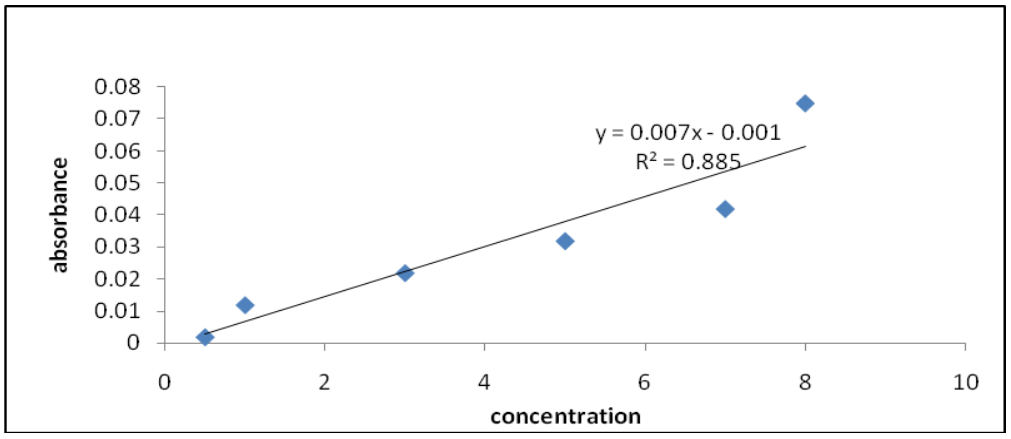

Fig.1 showing stander curve of nitrate

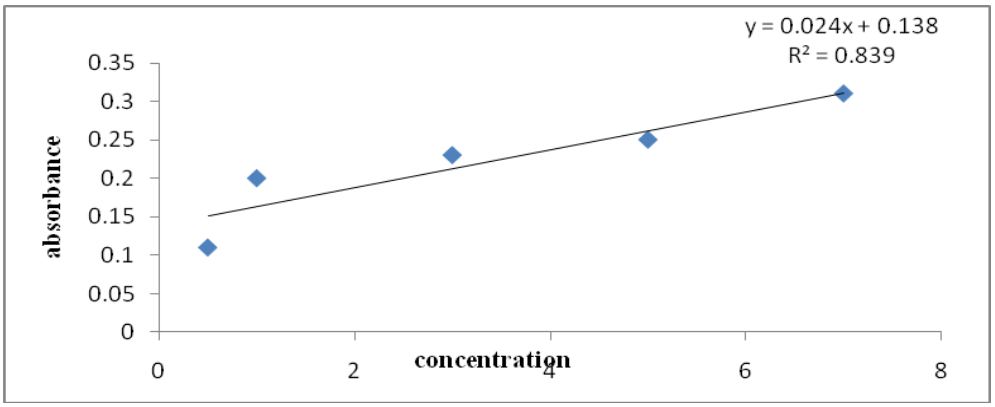

Fig.2 showing stander curve of nitrite

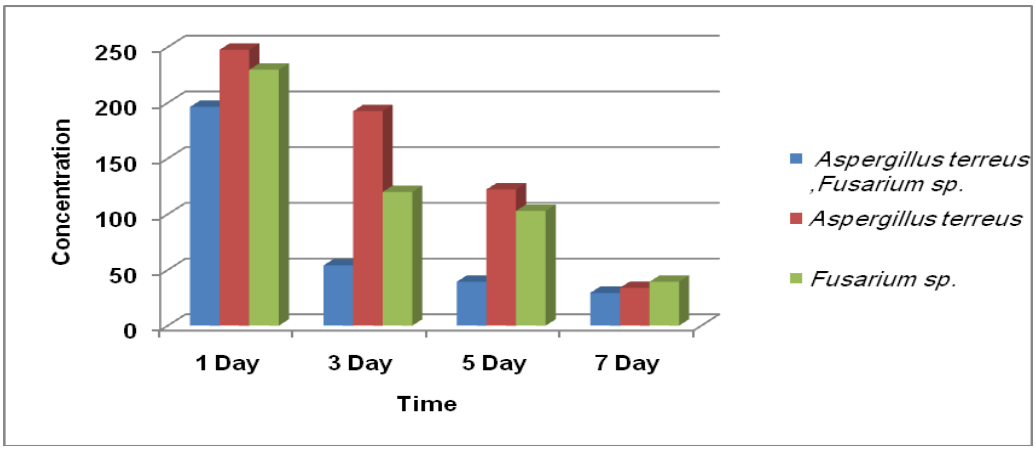

Fig.3 showing concentration of nitrate when used fungi for treatment waste water

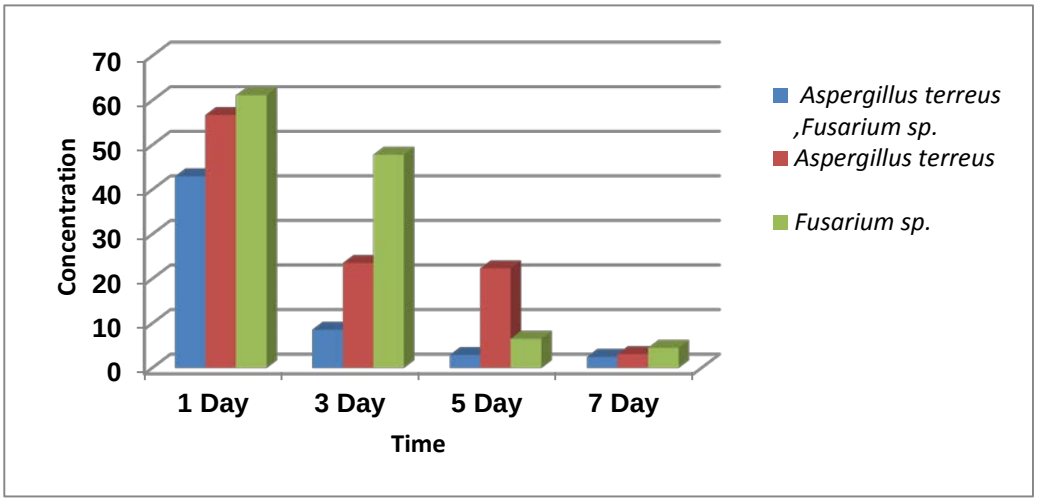

Fig.4 showing concentration of nitrite when used fungi for treatment waste water

Table(5) showing the percentage of removal of nitrate and nitrite by using mix fungal *Aspergillus terreus* with *Fusarium* sp.

Exposure time days	nitrate		nitrite	
	Residual concentration mg/l	Removal efficiency %	Residual concentration mg/l	Removal efficiency %
1	196.297	30.651	42.971	37.039
3	53.987	80.927	8.558	87.46
5	39.371	86.09	2.89	95.765
7	29.371	89.623	2.485	96.359

Table(6) showing the percentage of removal of nitrate and nitrite by using *Aspergillus terreus*

Exposure time days	nitrate		nitrite	
	Residual concentration mg/l	Removal efficiency %	Residual concentration mg/l	Removal efficiency %
1	247.576	12.535	56.736	16.871
3	192.448	32.011	23.538	65.512
5	122.192	56.831	22.323	67.292
7	33.858	88.38	3.093	95.468

Table(7) showing the percentage of removal of nitrate and nitrite by using *Fusarium* sp.

Exposure time days	nitrate		nitrite	
	Residual concentration mg/l	Removal efficiency %	Residual concentration mg/l	Removal efficiency %
1	229.628	18.875	61.19	10.345
3	102.961	63.625	47.829	29.921
5	119.884	57.646	6.534	90.426
7	39.371	86.09	4.51	93.392

The results showed in this work that fungal mix has the largest critical mass in comparison with the use of each fungus alone in biological treatment reaching biomass after treatment 8.7 gm/l (table 8) ,The fungal ability to exploit food sources such as Carbon, Nitrogen,Calcium, Magnesium in the polluted water and thus increase the biomass of fungi(37,5,38).

Table(8) showing The biomass of fungi before and after treatment

Fungi	Biomass before treatment gm/l	Biomass after treatment gm/l
***Aspergillus terreus* with *Fusarium* sp.**	6.3	8.7
Aspergillus terreus	5.9	6.7
***Fusarium* sp.**	1.5	2.4

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Measurement of Some Biochemical Markers in Sera of Women Infected with Uterus Cancer and Screening on Some Accompained Microorganisms

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Abstract :

Objective : The research had targed to measure some biochemical markers in sera of women infected with uterus cancer as well as , isolate and diagnose accompanied microorganisms which may be the major cause of cancer in women .

Materials and Method: Fifty endometrium scrappings and fifty blood specimens from patients with cancer of uterus in Educational Baghdad hospital and Tumores Hospital were collected during the period from 1/8/2014 to 14/1/2015, these scrapping had cultured aerobically and aerobically with the presence of 5–10% Co_2 , and the direct examination to isolate the yeasts and parasites as well as,the cytological test (pap smear test)for these specimens ,all these microorganisms isolated and diagnosed by using a group of microscopic and biochemical tests , as well as, the sensitivity of isolated bacteria against eight types of antibiotics had screened ,therewhere, the sera had separated from specimens of blood and screened on some biochemical markers such as acid phosphatase , alkaline phospatase enzymes and estrogen hormone and all these results had compared with control group which consist of 10 healthy women.

Results : The results of this study had showed presence of abnormal tumerous cells in scrapping sample ,also isolated of different types of microorganisms such as bacteria , yeasts and parasites with high percentages and the sensitivity of isolated bacteria against tested antibiotics with different percentages, as well as, showed the increasing of the activity of acid phosphatase,alkaline phosphatase enzymes and estrogen hormone at propability level $P \leq 0.05$.

Recommendation : The study had been suggested to treat any infection in genitourinary tracts , even it was simple , also the study had confirmed to use the antibiotics that had bacteria were Sensitive to it ,and this study had also considered as a first step to other future studies to detect the affect and role of bacteria and other microorganisms to cause cancer by using recent and advanced immunomolecular tests .

Key words : Cancer, Infection , Causes of Cancer .

قياس بعض المؤشرات الكيموحيوية في مصول النساء المصابات بسرطان الرحم والتحري عن بعض الأحياء المجهرية المرافقة

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الخلاصة :

الهدف : يهدف البحث الى قياس بعض المؤشرات الكيموحيوية في مصول النساء المصابات بسرطان الرحم والتحري عن بعض الأحياء المجهرية المرافقة والتي قد تكون المسبب الرئيسي للسرطان عند النساء .

المواد وطرائق العمل : جمعت (50) مسحة scrappings من بطانة الرحم و(50) عينة دم من النساء التي يعانين من حالة سرطان الرحم والمراجعات لمستشفى بغداد التعليمي ومستشفى الأورام للفترة من 2014/8/1 ولغاية 2015/1/14 ، زرعت هذه المسحات هوائياً وهوائياً بوجود 5 – 10% Co_2 ، كما أجري لها الفحص المباشر للتحري عن الخمائر والطفيليات فضلاً عن إجراء الفحص الخلوي pap test للتحري عن وجود الخلايا غير الطبيعية السرطانية، عزلت وشخصت جميع الأحياء المجهرية بأستخدام عدد من الفحوصات المجهرية والبايوكيميائية ، كما تم التحري عن حساسية البكتريا المعزولة لثمانية مضادات حياتية ،أما عن عينات الدم فقد فصل المصل منها وتم التحري عن بعض المؤشرات الكيموحيوية مثل إنزيمي الفوسفاتيزالحامضي و القاعدي وهرمون الإستروجين وأجريت مقارنة جميع النتائج مع مجموعة السيطرة والتي شملت 10 نساء سليمات .

النتائج : أظهرت نتائج هذه الدراسة وجود خلايا غير طبيعية ورمية من المسحات المأخوذة من المريضات كما عزلت أنواعاً مختلفة من الأحياء المجهرية منها البكتريا والخمائر والطفيليات وبنسب عالية جداً كما أظهرت النتائج حساسية البكتريا المعزولة للمضادات الحياتية المستخدمة في الدراسة وبنسب مختلفة ،فضلاً عن وجود زيادة ملحوظة في فعالية إنزيمي الفوسفاتيز الحامضي و القاعدي وتركيزهرمون الإستروجين عند مستوى إحتمالية $P\leq 0.05$.

التوصيات : إقترحت الدراسة ضرورة معالجة الإصابات التي تحدث في المسالك البولية والتناسلية حتى وإن كانت بسيطة ، كما أكدت الدراسة على إستخدام المضادات الحياتية المستخدمة في الدراسة والتي أظهرت البكتريا حساسيتها لها ، وتعد هذه الدراسة خطوة أولى لدراسات اخرى مستقبلية لتحديد تأثير ودور البكتريا وغيرها من الأحياء المجهرية في إحداث السرطان بإستخدام إختبارات مناعية جزيئية متقدمة وحديثة .

الكلمات المفتاحية: السرطان ، إلتهابات ، مسببات السرطان .

Introduction :

Cancer is a disease of the cell cycle , in that the cell cycle is out of control and cellular reproduction occurs repeatedly without end , cancers are classified according to their location: carcinomas are cancers of epithelial tissue that lines organs , sarcomas are cancers arising in muscular or connective tissue especially bone and cartilage and leukemia which are cancers of the blood [1]. A high rates of cell division makes a tissue susceptible to cancer because it increases the chances of a mutation that causes a cell to divide uncontrollably or to ignore apoptosis signals [2] . Cancer of uterus is considered as one of the important diseases occur in women , resulting about of 16.96% of death rate [3] , where the uterus is one of essential part in female reproductive system which also includes the ovaries , the oviducts and vagina [4] . There are many risk factors that increase of uterus cancer which are : age , family history , viruses and

bacteria , lifestyle , contact with harmful substances , early onset of menstruation, prolonged exposure to estrogen , late menopause and never been pregnant , as well as , obesity , smoking , high blood pressure and type 2 diabetes also associated with increased risk [5,6]. Endometrial cancer is usually detectable by pelvic examination, transvaginal ultrasound , biopsy for hysteroscopy , x-ray and blood tests [7] , while it is treated surgically , commonly by hysterectomy , radiation treatment , dilation and curettage and chemotherapy may used in addition to surgery , when the tumor is detected at an early stage , about 96% of patients are alive and may be treated successfully[8]. Approximately18%of cancer related to infectiousdiseases, this proportion varies in different regions of the world from a high of 25% in Africa to less than 10% in the developed world [9,10] . Viruses are the usual infectious agents that cause cancer, but *Mycobacterium*, some other bacteria and parasites also have an effect to cause it [11,12,13,14] .

Materials and Method:

Specimens Collections:

During the period from 1/8/2014 to 14/1/2015 ,fifty endometrium scrapping from patients with cancer of uterus in Educational Baghdad Hospital and Tumores Hospital were collected before eradication of uterus (hysterectomy), these swabs had transported to the laboratory to make the following tests [15,16] :

1. Pap test had done to detect the abnormal tumerous cells in specimens.
2. The scrappings had cultured on blood and MacConkey agar , then incubated aerobically at 37^{o} C for 24 hours .
3. The same scrappings also had cultured on chocolate agar and incubated aerobically with the presence of 5-10% Co_2 at 37^{o} C for 24 hours .
4. Direct examination of scrappings which had done by making a smear from them on a clean slide after putting a drop of saline,then putting the cover slipe and examined microscopically , then the results had recorded .

Also, fifty blood specimens had been collected from same patients and the sera had been separated to detect the concentrations of some biochemical markers such as alkaline phospatase ,acid phosphatase enzymes and estrogen hormone ,these tests had done by using specific kits of Biomeriex company [17] ,then all the results had read and recorded and compared with control group which consist of 10 endometrium scrappings and 10 blood specimens obtained from healthy women,and the obtained results had been analyzed statistically by using applied

program(SPSS) while the significant differences between the averages of biochemical markers had obtained by using Duncan test at propability level $P \leq 0.05$ [18].

The Isolation of bacteria :

The aerobically isolated bacteria hadbeenpurified by ABCD streaking and cultured on nutrient agar slants , and chocolate slants for bacteria isolatedaerobicallywith the presence of 5– 10% Co_2 , these purifications inorder to diagnose the bacteria and do other diagnostic tests [19] .

The Diagnosis of bacteria :

The all isolated bacteria had diagnosed by the microscopic examination with Gram stain and by the other biochemical tests which were done according to [20].

Sensitivity Testing to Antibiotics :

The sensitivity of isolated bacteria against eight types of antibiotics had tested, these antibiotics were : Ciprofloxacin , Penicillin , Amoxicillin , Gentamycin , Cefotaxime , Cefixime , Metronidazole, and Augmentin, the test was done by preparing of suspension from bacteria under study and the turbidity of it had compared with the third tube of Macferland, so the aerobically isolated bacteria had been cultured by swabbing on Muller-hinton agar , than the antibiotics discs had sterilly distributed and the plates incubated at 37^oC for 18 – 20 hours , while the bacteria which isolated with the presence of 5 – 10 % Co_2 had swabbed on chocolate agar plates , also the antibiotics discs had sterilly distributed , then the plates had incubated with 5–10 % Co_2 for 18 - 20 hours and finally the results had read and recorded according to [21] .

Results and Discussion :

In this study ,the results of pap smear test showed that all specimens had presented an inflammatory changes in endometrium cells as well as ,presence of abnormal cells with abnormal nuclear chromatin patterns and variations of shape and size and these results commite with [22] ,also the results of culturing had showed the isolation of many microorganisms from the endometrium scrapping of patients with cancer of uterus such as bacteria , yeasts and protozoa , and these results agreed with [23,24] . Also the results had confirmed the isolation of bacteria aerobically and with the presence of 5 -10 % Co_2 , the bacteria which isolated aerobically such as *D. pneumoniae* which was isolated in highly percentage(37.5%),then *S.pyogenes*, *K.pneumoniae*, *P.vulgaris* and *P.aeruginosa* which were isolated with percentages 30% , 20% , 7.5% and 5% respectively ,while the bacteria which isolated aerobically with the presence of 5 – 10% Co_2 which were : *M. catarrhalis* , *G. vaginalis* , *H. ducreyii* and *N.*

gonorrhoeae with percentages 45.7% , 31.43% , 14.3% and 8.57% respectively , also the study had showed the isolation of yeasts such as *C.albicans* and other *Candida ssp*. with percentages 53.33% and 26.67% respectively , as well as , the results confirmed the isolation of *T.vaginalis* with percentage (20%) , all these results also confirmed there were many types of microorganisms accompanied with cancer of uterus , this reffered either these microorganisms were the etiological agents of uterus cancer directly because they isolated with highly percentages and may be because the presence of microorganisms inside the tissues of uterus led to activate the abnormal divisions of cell for a result of interaction between the surface antigens of these microorganisms and the tissue , or indirectly the accumulations of primary and secondary metabolites of these microorganisms such as alcohols , free radicals , nitrous compound and organic acids after inflammation led to cause the cancer of uterus which act as a carcinogenic agent and activate the abnormal divisions of cells [25,26,27] and the tables(1, 2, 3 and 4) demonstrated thes results.

Table (1) The results of diagnostic tests for the isolated bacteria in this study

The name of bacterium	The result of diagnostic test													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Diplococcus pneumonae	diplococci	+	-	-	+	+	+ alpha hemolytic	-	+	-	-	+	-	-
Streptococcus pyogenes	streptococci	+	-	-	-	-	+ beta hemolytic	-	+	-	-	+	-	-
Klebsiella pneumonae	bacilli	-	-	-	+	+	+ no hemolysis	-	+	+	-	+	+	+
Proteus vulgaris	coccobacilli	-	+	-	+	+	+ beta hemolytic	-	+	+	+	+	-	+
Pseudomonas aeroginosa	bacilli	-	-	+	+	+	+ beta hemolytic	+	+	+	+	+	-	-
Moraxella catarrhalis	cocci	-	-	-	-	+	-	-	+	-	-	-	-	+
Gardenerella vaginalis	pleomorphic	-	-	-	+	+	-	-	-	+	-	+	+	+
Haemophilus ducreyii	pleomorphic	-	-	+	-	+	-	-	-	-	-	+	+	+
Neisseria gonorrrhoeae	diplococci	-	-	-	-	+	-	+	+	-	-	+	+	+

+ /positive result , - /negative result , 1= cell shape , 2= reaction with Gram stain , 3= indol test , 4= methyle red ,
5= voges proskauer , 6= citrate utilization , 7= growth on blood agar , 8= oxidase , 9= catalase , 10= urease , 11= motility ,
12= glucose fermentation , 13= lactose fermentation , 14= sucrose fermentation

Table (2) The types and percentages of bacteria isolated aerobically

The name of bacterium	The number of isolates (the percentage)
Diplococcus pneumonae	15(37.5)
Streptococcus pyogenes	12(30)
Klebsiella pneumonae	8(20)
Proteus vulgaris	3(7.5)
Pseudomonas aeroginosa	2(5)
Total number	40*(100)

***= The total number of samples were (50) while the positive samples were(40).**

Table (3) The types of bacteria isolated aerobically with 5 – 10 % and the percentages of isolation

The name of bacterium	The number of isolates (the percentage)
Moraxella catarrhalis	16(45.7)
Gardenerella vaginalis	11(31.43)
Haemophilus ducreyii	5(14.3)
Neisseria gonorrrhoeae	3(8.57)
Total number	35*(100)

***= The total number of samples were (50) while the positive samples were(35).**

Table (4) The types of microorganisms isolated in direct examination and the percentages of isolation

The name of microorganism	The number of isolates (the percentage)
Candida albicans	8(53.33)
Candida ssp.	4(26.67)
Trichomonas vaginalis	3(20)
The total number	15*(100)

***= The total number of samples were (50) while the positive samples were(15).**

As well as, the obtained results of biochemical markers showed the increasing of the enzyme activity of acid phosphatase and alkaline phosphatase enzymes and the concentration of estrogen hormone at propability level P≤ 0.05 than the normal averges of them which may be between(2.5-11.7 U/L), (30-85 U/L) and (43-214 pg /ml) respectively,also there were the significant differences between the tested specimens and control groups where the activity of acid phosphatase and alkaline phosphatase enzymes in tested specimens were 25 U/L and 121U/L respectively while the activity of these enzymes in control groups were 8.3 U/L and 63.21 U/L respectively , also the results showed there were the significant differences between the tested specimens and control groups in the concentration of estrogen hormone where the concentrationthis hormone in control groups was 55.62 pg/ml while in tested specimens was 502 pg/ml,all these results reffered to increase the concentrations of tested biochemical markers than the normal averages as a results of uterus cancer and these results consistent with [28, 29],and the table (5) demonstrated these results

.

Table(5)The average ± standared error for the concentrations of Acid phosphatase, Alkaline phosphatase and Estrogen in the sera of women infected with uterus cancer in this study.

Group of study	Concentrations of biochemical markers		
	Acid phosphatase U/L	Alkaline phosphatase U/L	Estrogen pg/ml
Control (healthy women)	$(8.3\pm 0.35)^{a*}$	$(63.21 \pm 2.9)^{c*}$	$(55.62 \pm 2.61)^{e*}$
Patients with Uterus Cancer	$(25 \pm 1.18)^{b*}$	$(121 \pm 5.69)^{d*}$	$(502 \pm 23.63)^{f*}$

***/ The different leters in the same column reffered to significant differences at P≤ 0.05.**

On the other hand , the results of antibiotics sensitivity test of isolated bacteria that there were many of isolated bacteria were sensitive to Ciprofloxacin , Cefotaxime , Cefixime , Metronidazole and Augmentin with percentage between (80% to 100%), these results reffered to ability of using these antibiotics for treating the genitourinary tract infections to avoid developing these infections to cancer [30] and the table (6)demonstrated these results.

Table (6)The results of antibiotics sensitivity test of isolated bacteria in this study.

The name of bacterium	The number of sensitive isolates for the antibiotic (percentage)							
	1	2	3	4	5	6	7	8
Diplococcus pneumonae	0(0.0)	14(93.3)	15(100)	15(100)	13(86.6)	10(66.6)	13(86.6)	15(100)
Streptococcus pyogenes	2(16.6)	10(83.3)	12(100)	12(100)	10(83.3)	5(41.6)	11(91.6)	12(100)
Klebsiella pneumonae	0(0.0)	8(100)	7(87.5)	8(100)	7(87.5)	4(50)	7(87.5)	8(100)
Proteus vulgaris	0(0.0)	3(100)	3(100)	3(100)	3(100)	2(66.6)	2(66.6)	3(100)
Pseudomonas aeroginosa	0(0.0)	2(100)	2(100)	2(100)	2(100)	1(50)	1(50)	2(100)
Moraxella catarrhalis	8(50)	15(93.75)	16(100)	15(93.75)	16(100)	10(62.5)	8(50)	15(93.75)
Gardenerella vaginalis	5(45.4)	11(100)	10(90.9)	10(90.9)	11(100)	5(45.4)	9(81.8)	10(90.9)
Haemophilus ducreyii	3(60)	5(100)	5(100)	4(80)	5(100)	4(80)	4(80)	4(80)
Neisseria gonorrrhoeae	3(100)	3(100)	3(100)	3(100)	2(66.6)	2(66.6)	3(100)	3(100)

1= Penicillin 10 IU/disc , 2= Augmentin 5µ/disc , 3= Ciprofloxacin 1 µ/disc , 4= Cefotaxime 30 µ/disc , 5= Metronidazole 50 µ/disc , 6= Amoxicillin 10 µ/disc , 7= Gentamycin10 µ/disc , 8= Cefixime 5 µ/disc

Recommendation :

The present study had been suggested to treat any infection in genitourinary tracts especially in uterus , even it was simple, to avoid the complications which occur after infection and recurrent of infection which may be led to cancer at a long time and eradication of uterus , also , the study had recommended to use the antibiotics: Ciprofloxacin , Cefotaxime , Cefixime , Metronidazole and Augmentin to treat these infections of genital and urinary tracts , as well as , to use Nystatin and Metronidazole to treat the infections caused by yeasts and protozoa respectively.This study was a first step for another future studies to detect the affect and role of bacteria and other microorganisms to cause uterus cancer by using recent and advanced immunomolecular tests.

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Detection and subgrouping of *Enterobacter cloacae* in Iraqi child patients with UTI

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Abstract: A urinary tract infection (UTI) is an infection involving the kidneys, ureters, bladder, or urethra are caused by many bacterial and fungal pathogens. In many studies, *Enterobacter cloacae* was reported to cause (UTI) in human. In this work the prevalence and grouping of *E. cloacae* was determined in patients suffering from UTI. The study was conducted in AL-Kadhumia hospital for children / Baghdad; through period April- June 2014. Urine samples were collected from 76 patients with clinical symptoms of UTI and cultured for further biochemical and microbiological identification of *E. cloacae*. Genotyping and grouping of the pathogen were performed based on PCR amplification of *chuA, yjaA* and *TspE4C2* genes. Out of 76s patient's urine samples, 16 (21%) have *E. cloacae* infection. The isolated bacteria were shown resistance to pipercillin, cefotaxim, ampicillin and sensitive to Gentamycin, ciprofloxacin, chlormphicol, impene, aztreonam and ticracillin clavulunic acid antibiotics. The most prevalence group is B (50%) and subgroup (B2) 50% of group B. Groups A and D present with different ratios. Determination the prevalence and genotypes of the *E. cloacae* will enhance diagnosis, treatment and developing health control programs in our country.

Key words: *Enterobacter cloacae*, UTI, Diagnosis, PCR

الكشف وتحديد المجاميع الفرعية لبكتريا الامعائية الزرقية في الاطفال العراقيين المصابين بالتهاب المسالك البولية

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الخلاصة :التهاب المسالك البولية تتضمن اصابات الكليتين والمثانة او الحالب تسببها البكتريا او الفطريات المرضية . في العديد من الدراسات ، تعد بكتريا الامعائية الزرقية واحدة من اهم مسببات التهاب المسالك البولية في الانسان . في هذه الدراسة تم تحديد نسب الإصابة والمجاميع الفرعية لبكتريا الامعائية الزرقية في المرضى الاطفال العراقيين الذين يعانون من التهاب المسالك البولية . اجريت هذه الدراسة في مستشفى الاطفال في الكاظمية \ بغداد للفترة من نيسان – حزيران في عام 2014 ، حيث جمعت عينات ادرار67 من الاطفال بعمر 1-4 سنوات لديهم اعراض التهابات مجاري بوليه ، تم زرع هذه العينات على اوساط زرعيه لغرض عزل العامل المسبب ثم اجريت كافه الاختبارات الكيمياحيوية لتأكيد العزل . تم تحديد النمط الوراثي والمجاميع الفرعية باعتماد تقنيه البلمرة المتسلسل باستخدام ثلاث (جينات)معلمات .chaA,yjaA,TspE4E2 . اظهرت النتائج ان 16(21%)عزله موجبه لعزله بكتريا الامعائية الزرقية ، اظهرت هذه العزلات مقاومه لكل من البيبراسيلين والامبيسلين ، وحساسيه لكل من جنتاميسين وسبروفلوكسيسين، وكلورومفينيكول ، الامبيين والازيرونيم واخيرا لحامض الكلفيوليين ، اما تحديد المجاميع اظهر ان نسبه مجاميع B (50%) المجاميع الفرعية B2 (50%) اما مجاميع Aو D فقد ظهرت بنسب مختلفة .

ان تحديد نسب الاصابة والنمط الوراثي لبكتريا الامعائية الزرقية يساعد على تطوير الأنظمة الصحية للسيطرة على الإصابة بهذه البكتريا في العراق من خلال ايجاد وسائل التشخيص السريع والمبكر لهذه البكتريا .

Introduction

Urinary tract infection (UTI) is the most common disease both in community and hospitalized patients. About 10% of individuals will suffer from UTI in their lifespan (1). *Enterobacter* species, particularly *Enterobacter cloacae* and *Enterobacter aerogenes*, are important nosocomial pathogens responsible for various infections including UTI infection (2). Three genes were used for phylogenetic genotyping of *E. coli*, *chuA* gene, required for hem transport in enterohemrrhagic O157:H7 E. coli [3,4)], *yjaA* gene, encodes an uncharacterized protein and is a known housekeeping gene in *E. coli* K-12, but its function has not yet been determined and TspE4C2 gene, an anonymous DNA fragment that has been recently identified as part of a putative lipase esterase gene (3,5). Phylogenetic analysis has shown that *E. coli* strains can be assigned to one of the main phylogenetic groups (A, B1, B2, and D) (6). In Iraq one study was conducted for the phylogenetic analysis of *Enterobacter* spp. isolated from urine of patients with cystitis in Babylon province (7). No study conducted for the molecular grouping of *Enterobacter cloacae* in Iraq. The aim of this work is to investigate the prevalence of *Enterobactercloacae* in UTI patients in a hospital in Baghdad city.

Materials and methods

Samples:

In total, (67) patients with clinical symptoms of UTI referred Kadhmiya pediatrich hospital, Iraq, were investigated. There were 50 females and 17 males, with an age range of 1-4 years. Midstream urine of the patients was collected in sterile containers and immediately transferred to the laboratory. (8)

Bacterial cultures:

Urine sample were cultured on blood agar, nutrient agar and MacConkey agar for isolation of the microbial agents of UTI. All bacterial isolated were identified by biochemical and ApiE20 test (8,9).

Antimicrobial susceptibility testing:

Antimicrobial susceptibility testing was done on Mueller- Hinton agar (Merck, Germany) using disk diffusion (Kirby Bauer's) method (10). This method was done according to Clinical and Laboratory Standards Institute (CLSI) guidelines to determine susceptibility of UTIs agents (11). The antibiotic disks (Gentamycin and tobramycin10µg, pipracillin 10-100 µg, ciprofloxacin 5 µg, cefotaxim 30 µg, imipenem 10 µg, chloromphicol 30 µg, , cefoxitin 30-µg, Ticarcillin-Clavulanic acid 75/10 µg Aztreonam 30 µg, (Mast Diagnostic, Bio analysis, UK).

DNA extraction:

DNA extracted from bacterial isolates using DNA extraction kit (Geneaid, Korea) according to company instructions. The integrity of isolated DNA was checked by 1% agarose gel electrophoresis and viewed using UV-trans illuminator.

Genotyping by PCR assay:

Specific primers used for Amplification of the genes *chuA, yjaA* and anonymous DNA fragment *TspE4.C2* by PCR (3) are shown in Table 1. These primers synthesized by Bioneer, Korea.

PCR reaction was conducted in 20 µl of a reaction mixture containing, 3µl upstream primer,3 µl of downstream primer, 4 µl of free nuclease water, 5 µl of DNA and 5 µl of Master mix powered in 0.2 ml thin walled PCR tube (Promega, CA) . Amplification was conducted using a Mastercycler (Eppendorf) programmed with 1 cycle at 94°C for 5 min; 30 cycles of 94°C for 30sec., 59°C for 30 sec., 72°C for 1min; a final extention f 72°C for 7min. was perforemed at the end of PCR. The amplified product was subjected to 1.5% agarose gel electrophoresis, and visualized under UV (Imagemaster VDS, Pharmacia Biotech, USA) after ethidium bromide staining.

Table 1: Primers used for the amplification of the genes used for *E. cloacae* genotyping

Primers	Primer sequence (5_–3_)	Amplicon size (bp)	Reference
chuA F	GACGAACCAACGGTCAGGAT	279	3
chuA R	TGCCGCCAGTACCAAAGACA		
yjaA F	TGAAGTGTCAGGAGACGCTG	211	3
yjaAR	ATGGAGAATGCGTTCCTCAAC		
TspE4C2 F	GAGTAATGTCGGGGCATTCA	152	3
TspE4C2 R	CGCGCCAACAAAGTATTACG		

Genotyping of bacterial isolates was done according to the grouping table (2), Phylogeny markers used in detection of *Enterobacter* phylogeny were *E.coli* phylogeny markers such as *YjaA* ,*TsPE4.C2* and, *chuA* (12). According to these markers, it was shown that bacteria isolated from human sources are classified phyelogenetically in to four groups: A, B1, and D (13) .

Group A: *YjaA*: positive; group B1:*TspE4C2* positive, group B2 : (*chuA+YjaA*: positive or *chuA+YjaA+TspE4.C2* : all positive), group D: *chuA+TspE4.C2* : positive (14).

Table 2: Phylogenic grouping of *E. cloacae* according to amplification of the genes used for genotyping

Phylogenic Groups	Genes		
	chuA	yjaA	TspE4.C2
subgroup A0 (group A)	-	-	-
subgroup A1 (group A)	-	+	-
group B1	-	-	+
subgroup B22 (group B2)	+	+	-
subgroup B23 (group B2)	+	+	+
subgroup D1 (group D)	+	-	-
D2 (group D)	+	-	+

Results and discussion

In total, (67) patients with clinical symptoms of UTI were investigated. There were 50 (76.12%) females and 17 (10.44%) males, with an age range of 1-4 years (mean, 2.14 years). Biochemical and bacteriological tests were used for identification of bacterial isolates as *Enterobacter cloacae***.**

Out of 76 patient's urine samples, 16 (21%) have *E. cloacae* infection. In Iraq no study conducted for prevalence of *E. cloacae* in children of Baghdad city. Other bacteria were also reported to cause UTI in children like including *E. coli*, Candida, *Enterococcus*, *Enterobacter*, and *Pseudomonas*(15).

Enterobacter cloacae were shown resistance to piperacillin, cefotaxim, ampicillin and sensitive to Gentamycin, ciprofloxacin, chlormphicol, impene, aztreonam and ticracillin clavulunic acid antibiotics. The resistance to the broad-spectrum antibiotics usually attributed to the random use of antibiotics in our country for different antibiotics which lead to antibiotic resistance for most bacteria isolated in hospitals and environment.(16).

PCR amplification of three genes (*chuA*, *yjaA* and *TspE4C2*) was used for determination of phylogenetic groups of *E. cloacae* (Figure1, 2 ,3 4). The results show that the most prevalence group is B (50%) and subgroup (B2) 50% of group B. Groups A, and D present with different ratios (Table3).

Table3: Distribution of *Enterobacter cloacae*. isolates according to phylogenetic groups and subgroups.

Isolate NO.	*chuA*	*yjaA*	*TspE4C2*	Phylogenetic group	Phylogenetic subgroup	Gender of patient
1	+	-	-	Group D	Subgroup D1	Male
2	+	-	+	Group A	Subgroup A2	Female
3	-	-	+	Group B	Subgroup B1	Female
4	-	+	-	Group A	Subgroup A1	Male
5	+	+	+	Group B2	Subgroup B3	Male
6	-	-	+	Group B	Subgroup B1	Male
7	-	+	+	Group B	Subgroup B2	Female
8	-	-	+	Group B	Subgroup B1	-
9	-	-	-	Group A	Subgroup A0	Female
10	-	+	+	Group B	Subgroup B2	Female
11	+	-	+	Group D	Subgroup D2	-
12	+	-	+	Group A	Subgroup A2	Female
13	+	-	+	Group A	Subgroup A2	Female
14	-	-	+	Group B	Subgroup B1	Female
15	+	-	-	Group D	Subgroup D1	Female
16	+	+	-	Group B	Subgroup B2	Male

Results of phylogenetic analysis showed that, the *YjaA* gene found in all groups B2 isolates and absents in group D isolates and it was present in all isolates of group A1 and absents in all isolates of group B1. While *TspE4.C2* is found in group B1 isolates, group B2 isolates, group D (sub group D2) and absent from all group A isolates.

The *chuA* gene was present in group B2 male and in all group D isolates, and absent in sub groups A1 and B1.The results of this study agreed with many studies like AL-Dhmoshi 2015(17) .

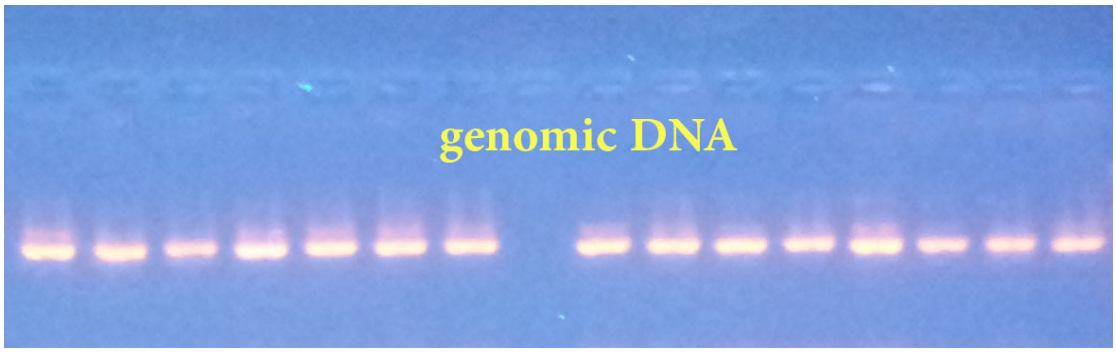

Figure (1): Genomic DNA of *Enterobacter cloacae*. On 1% agarose gel electrophoresis (70 voltage / hours) (lanes 1-15) DNA extracted from isolated bacteria.

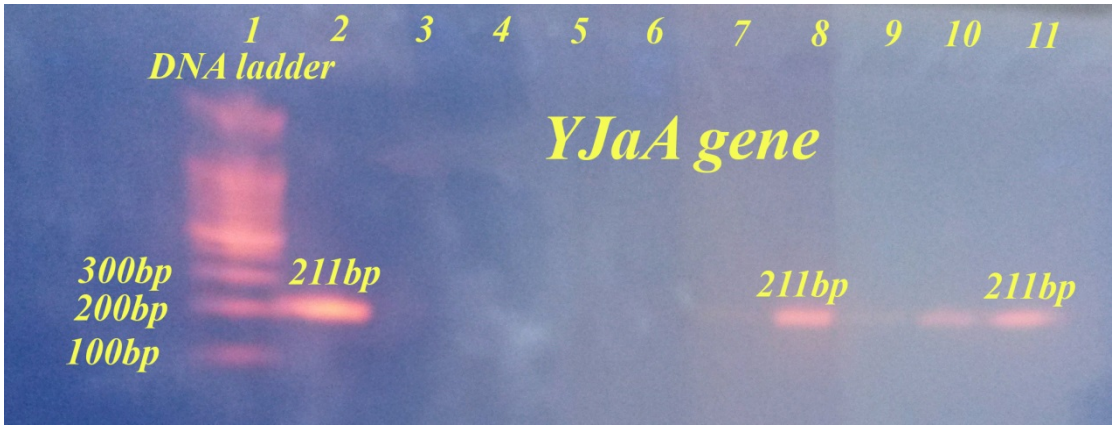

Figure (2): agarose gel electrophoresis of PCR of YJaA (bp=211) amplicon
Lane 1 Ladder (100 bp), Lane, 2, 3----------11 isolates of *Enterobacter cloac*

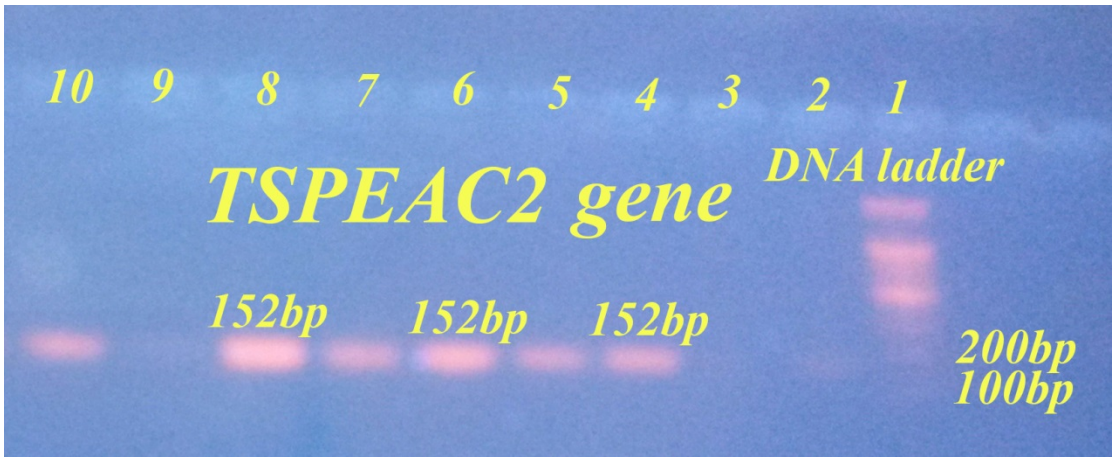

Figure (3): agarose gel electrophoresis of PCR of TSPEAC2 gene (bp=152) amplicon .
Lane 1 Ladder (100 bp), Lane, 2, 3----------10 isolates of *Enterobacter cloacae*

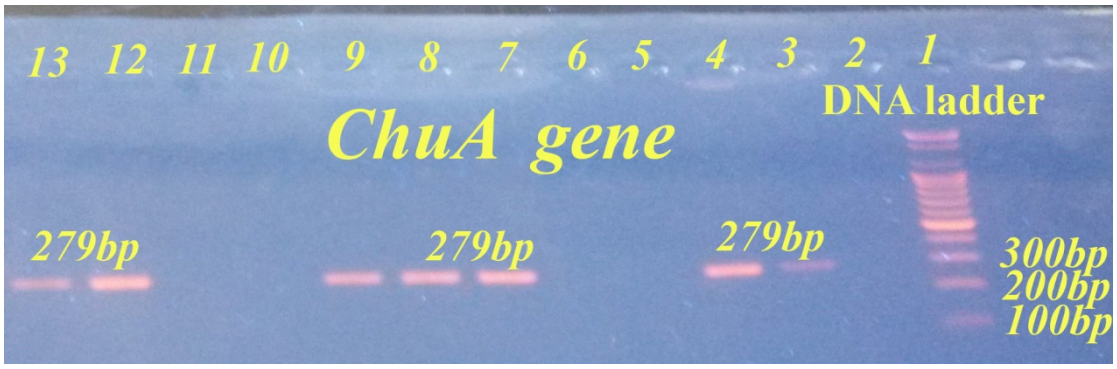

Figure (4): agarose gel electrophoresis of PCR of ChuA gene (bp=279) amplicon
Lane 1 Ladder (100 bp), Lane, 2, 3----------13 isolates of Enterobacter cloacae

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Modified technique to increase Adenovirus DNA load for diagnosis of gastroenteritis infections by conventional PCR

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Abstract: Ninety stool samples were collected from patients with gastroenteritis to diagnose adenovirus infections among diarrhea cases by conventional PCR. The patients were grouped into three age groups (<1 year), (1-6 years) and (>6-15) years. Adenovirus positive cases were only one when DNA was isolated by the traditional kit technique, but after using the modified technique they became 8, the percentage of gastroenteritis due to adenovirus infection was (9%), and these cases were distributed among all age groups of the patients. The modified technique of DNA isolation used may be better than the traditional kit technique as it yields higher viral DNA loads.

Keyword: *Adenovirus*, Gastroenteritis and Conventional PCR.

تقنية محورة لزيادة تركيز الحامض النووي للفيروس الغدي لتشخيص التهابات المعدة والامعاء بوساطة فحص تفاعل البلمرة المتسلسل التقليدي

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الخلاصة: تم جمع تسعين عينة براز من المرضى الذين يعانون من التهاب المعدة والأمعاء لتشخيص الاصابة بالفيروس الغدي بين حالات الإسهال بوساطة فحص تفاعل البلمرة المتسلسل التقليدي. تم تقسيم المرضى إلى ثلاث فئات عمرية (<1 سنة)، (1-6 سنوات) و (> 6-15) سنة. كانت الحالات الإيجابية للفيروس الغدي واحدة فقط عندما تم عزل الحامض النووي بالتقنية التقليدية ولكن بعد استخدام التقنية المحورة اصبحت 8، نسبة الاصابة بالالتهابات المعوية بسبب الفيروس الغدي كانت (9٪) وكان توزيع هذه الحالات بين جميع الفئات العمرية للمرضى. ان التقنية المحورة لعزل الحامض النووي المستخدمة قد تكون أفضل من التقنية التقليدية لانها اعطت تراكيز اعلى من الحامض النووي الفيروسي.

Introduction

Gastroenteritis is a medical condition caused by inflammation of the gastrointestinal tract, which involves both the stomach and the intestine, it is also called infectious diarrhea. Its symptoms include combination of diarrhea, vomiting, abdominal pain and cramping, dehydration may occur as a result (1). Acute gastroenteritis is a worldwide health problem, it has been reported as a main factor for childhood morbidity and mortality worldwide (2). Adenovirus is considered as one of the most important etiological agents of serious viral gastroenteritis among infants and young children less than five years, however, it is believed that adenovirus is the second common agent in infantile gastroenteritis,after rotavirus(3,4-5).

Human adenoviruses (HAdVs) are non-enveloped, icosahedral with linear dsDNA genome. They are categorized in 7 species (A-G), which include 52 recognized serotypes (6). Rapid diagnosis of human adenovirus infections was achieved by PCR in the recent years. The advantages of detecting viral DNA by PCR include speed, sensitivity, ability to detect non-infective particles, and potential elimination of toxic effects of the specimen or contaminating microorganisms. However, conventional PCR has the risk of carrying over contamination due to open handling with its products, and results are only qualitative (7-8). Real-time PCR is a sensitive and quantitative procedure for the detection of adenovirus infections, it shows higher sensitivity than conventional PCR in detecting HAdV DNA in positive clinical samples with low viral loads. The sensitivity of the conventional PCR can be enhanced by performing an additional nested PCR amplification (7-9).

Diarrhea is a major cause of illness and death in Iraqi children. Many reports indicated rotavirus infection however, little data is recorded about adenovirus in Iraq, and so this project was undertaken to compare between tow techniques of DNA isolation used for the diagnosis of adenovirus and to investigate the percentage of infection by this virus among local gastroenteritis patients.

Materials and Methods

Samples: Stool samples were collected from 90 patients (63 males and 27 females) with ages ranging between (<1-15 years) admitted to the central teaching hospital of pediatric in Baghdad with symptoms of acute gastroenteritis during the period from November 2013 till April 2014. A total 45 stool samples from apparently normal children, which were both age and sex matched, were used as control group. Each stool sample was taken

once and stored undiluted at -20˚C until the time of analysis.

Polymerase Chain Reaction (PCR):

1-Isolation of DNA from samples was performed using traditional kit technique: (MagaZorb® DNA Mini-Prep Kit, Promega, USA) was used for DNA isolation. This traditional kit technique is based on the specific interaction between nucleic acids and proprietary magnetizable particles in the presence of specially formulated buffer reagents (10). Isolation of DNA was done according to the instructions of the manufacturer.

2- Isolation of DNA from samples using the modified technique: some modifications were done on the traditional kit technique, involved the preparation of the stool samples before DNA isolation (Fecal cell suspension was prepared using less amount of PBS, shaken with glass beads in a shaker, then subjected to centrifugation, in order to concentrate the sample, after that the supernatant was used to proceed with the steps of the isolation). Other changes in reagents volumes and additions were performed (11). These modifications were done according to WHO Lab Manual (12).

3-Measurment of DNA concentration and purity: the concentration and purity of the isolated DNA samples (isolated by the traditional kit technique and by the modified technique) were measured by NanoDrop spectrophotometer (Thermo scientific, Germany) (13).

4-PCR assay: (GoTaq Green Master Mix kit, Promega, USA) which is a premixed ready-to-use solution was used for amplification of DNA templates by conventional PCR.

Adenovirus primers for the adenovirus DNA amplification by conventional PCR were used from Rezaei *et al*. (2012) (2) and supplied by(Alpha DNA,Canada). The details of primers are shown in table 1.

Table 1- Details of primers sequence.

Primer	Sequence (5'→ 3')	Gene	Species	Amplicon
Ad1	TTCCCCATGGCTCACAACAC	Hexon	A to F	482 bp
Ad2	CCCTGGTAGCCGATGTTGTA	Hexon	A to F	482 bp

Total volume of 25µl reaction mix was prepared which contains 12.5µl of GoTaq® Green Master Mix, 1µl of each primer, 5µl of DNA template and 5.5µl of Nuclease-Free Water. A negative control (without DNA template) was included in every experiment. PCR was performed by thermal cycler (Biometra, Germany) after optimization of PCR conditions which shown in table 2.

Table 2- PCR conditions.

Name of cycle	Temperature °C	Time	No. of cycles
Initial denaturation	95	2 min	1 cycle
Denaturation	94	50 sec	30 cycles
Annealing	55	50 sec	30 cycles
Extension	72	50 sec	30 cycles
Final extension	72	5 min	1 cycle
Hold	4	24 h	1 cycle

The PCR products were analyzed by electrophoresis on a (1.5%) agarose gel containing ethidium bromide, at 100 volt for 60 minutes and after that were visualized under ultraviolet light by Gel Electrophoresis system and UV trans-illuminator (Amersham/USA). The 100-bp DNA ladder (Promega/USA) was a size marker to estimate the length of products. A positive PCR reaction was expected to produce a 482 bp band.

Statistical Analysis: Statistical Package for the Social Sciences (SPSS) was used for data analysis. T test was used to compare the significant differences between samples in this study, probability value of $P<0.05$ was considered statistically significant (14).

Results and Discussion

The DNA that was first isolated according to the manufacturer instructions of the isolation kit, and subjected to PCR, showed that only one sample out of the 90 stools was positive for adenovirus, however it showed a very light band of DNA at 482bp (Figure 1).

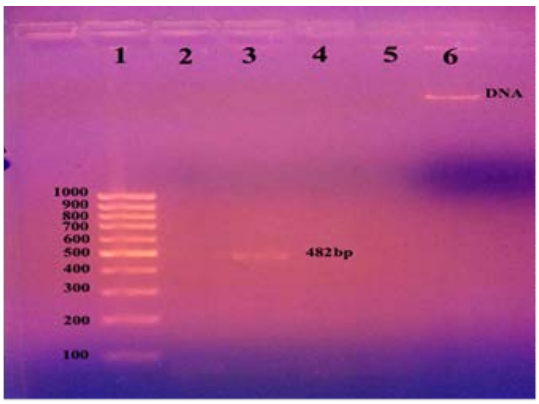

Figure 1- Agarose gel electrophoresis of PCR for +ve adenovirus sample before the modifications of DNA isolation procedure. Lane (1) 100bp Ladder, Lane (2)–ve sample of HAdV DNA, Lane (3) +ve sample of HAdV DNA at 482bp, Lane (4). Empty, Lane (5) total DNA of HAdV –ve sample and Lane (6) total DNA of HAdV +ve sample.

It was suspected that the viral DNA concentration was low to give clear obvious band on gel, and according to what was reported by Allard *et al*. (2001) (9), that the sensitivity of the conventional PCR can be enhanced by performing an additional nested PCR amplification, and therefore a second run of PCR was done for the samples by using the same primers and the product of the first run as a template (Figure 2).

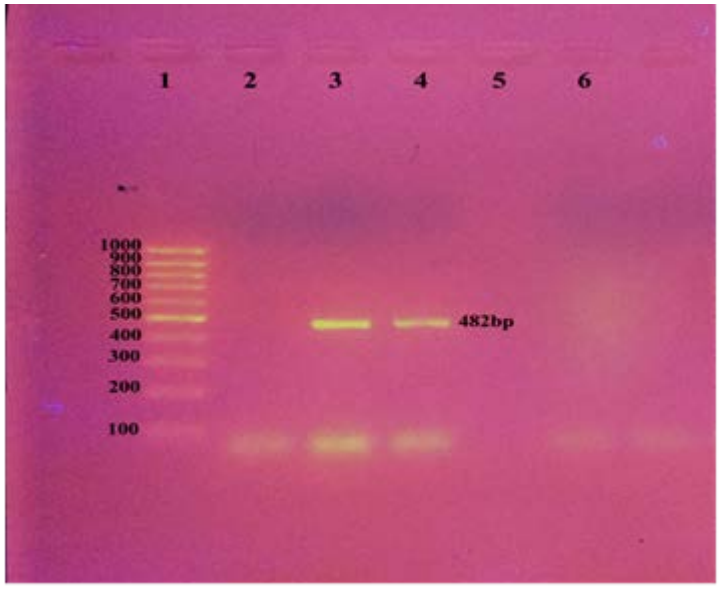

Figure 2- Agarose gel electrophoresis of PCR for +ve adenovirus sample after a second run of PCR. Lane (1) 100bp Ladder, Lane (2) Negative control, Lane (3) Positive control, Lane (4) +ve sample of HAdV DNA, Lane (5) Empty and Lane (6) –ve sample of HAdV DNA.

The positive sample in the first run showed thicker band after the second run, while the other samples remained negative. Therefore some modifications in the DNA isolation procedure were made in order to increase the total DNA yield of the samples and to increase the concentration of the viral DNA. Total DNA concentration was measured by nanodrop technique in order to ensure that the DNA concentration was increased after the modifications done in the isolation procedure, and to ensure that the samples contain adequate DNA to be tested by conventional PCR. DNA concentration was increased among all the samples, results are shown in table 3.

Table 3- The concentration of DNA in gastroenteritis patients and control group samples as measured by nanodrop technique before and after modifications of DNA isolation procedure.

Samples	Before	After
	Mean ± S.D DNA con. ng/µl	Mean ± S.D DNA con. ng/µl
Patients +ve	(88.7 ± 6.5)	(124± 21)
Patients –ve	(56.8 ± 5)	(72.5 ± 5)
Control group	(14.4 ± 7.8)	(40 ± 7.5)
P-value	**P<0.05**	***S**

***S = Significant**

***There was a significant differences between concentrations of DNA in samples before and after the modifications.**

When PCR was performed with the DNA samples that isolated by the modified procedure, the first sample was positive as previously shown to have a thicker and brighter band of DNA, another sample which was previously negative turned positive with obvious band of DNA (Figure 3).

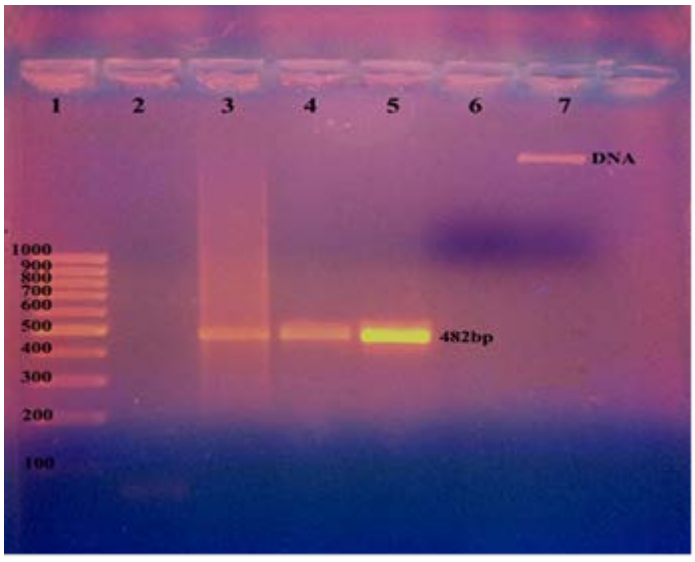

Figure 3- Agarose gel electrophoresis of PCR for +ve adenovirus samples after the modifications of DNA isolation procedure. Lane (1) 100bp Ladder, Lane (2) Negative control, Lane (3) Positive control, Lanes (4 and 5) +ve samples of HAdV DNA, Lane (6) Empty and Lane (7) Total DNA of +ve HAdV sample.

After that the procedure was repeated with all the samples, and another 6 samples that were previously negative turned positive with obvious bands of DNA, however only 8 out of the 90 samples showed to be positive to adenovirus (Figure 4).

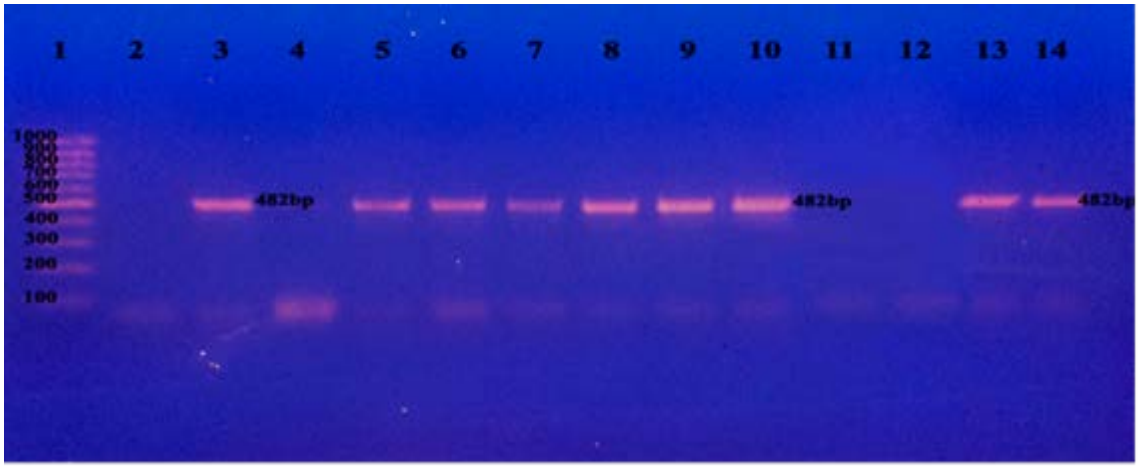

Figure 4- Agarose gel electrophoresis of PCR for all +ve adenovirus samples after the modifications of DNA isolation procedure. Lane (1) 100bp Ladder, Lane (2) Negative control, Lane (3) Positive control, Lanes (4, 11 and 12) -ve samples of HAdV DNA and Lanes (5, 6, 7, 8, 9, 10, 13 and 14) +ve samples of HAdV DNA.

From the present results, it was found that the conventional PCR showed lower sensitivity to the low viral DNA concentrations. That was agreed with other previous studies. A previous study in Brazil, indicated that from the 61 stool samples positive by a combined enzyme immunoassay for rotavirus and adenovirus (EIARA) was developed as a double-antibody sandwich assay, 59 were available in sufficient amounts for testing by PCR, all 59 (100 %) were positive by the generic PCR for human adenoviruses and 57 (97 %) by the species-specific PCR, four samples were initially negative for species-specific PCR but positive by generic PCR for human adenoviruses. These samples were inoculated onto HEK-293 cells and two were found to be positive and characterized as species C, probably due to the increased virus concentration (15). In another study in Germany of rapid and quantitative detection of human adenovirus DNA by Real-Time PCR, twenty-two stool samples were tested in parallel by real time PCR and conventional PCR giving identical results in 21 samples (10 positive and 11 negative), the results of the study showed that the higher viral load in positive samples by real time PCR, the thicker bands of conventional PCR amplicons, while samples with concordantly positive

results but lower virus loads in real time PCR, showed faint bands of conventional PCR amplicons and were unable to be typed, and one sample was negative by conventional PCR and was positive by real time PCR (7). A study done in Iran, in which the detection of immobilized amplified products in one phase system (DIAPOPS) method for detection of types 40 and 41 adenoviruses in stool samples of diarrheal children by solid phase PCR used, the comparison between conventional PCR and DIAPOPS results showed a significant increase in sensitivity of the DIAPOPS test, 6 samples shown to be negative by conventional PCR, were demonstrated positive by DIAPOPS (8).

Adenovirus positive cases were 8 and the percentage of adenovirus gastroenteritis infections was (9%), these cases were distributed among all the age groups of the patients (Table 4).

Table 4- Distribution of Adenovirus positive cases among gastroenteritis patients and control group samples according to age.

Samples	Total No.	Age groups (years)	No. of Adenovirus +ve (No. = 8) (%)
Patients	90	<1	2 (25)
		(1-6)	3 (37.5)
		(>6-15)	3 (37.5)
Control group	45	(<1-15)	0 (00)

A study in Iraq showed that the percentage of HAdV-F is (9.3%) in children under 5 years of age when detected by real time PCR (16). A study in Iran showed that among 100 stool samples of children under age of 5 years suffering from acute gastroenteritis analyzed by PCR, 8 samples were positive for adenoviruses and also positive for enteric adenoviruses (2).

In this research, the modified technique of DNA isolation used was better than the traditional kit technique as it yielded higher viral DNA loads.

More optimizations of the conventional PCR methods and modifications of DNA isolation from stool samples are recommended. However we suggest the use of the real time PCR as it is rapid, having a high sensitivity as compared with conventional PCR, and it has an added advantage of quantification.

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