Minstery of Higher Education
and Scientific Research
Al-Muthanna University
College of Science

Frequency of Hepatitis C Virus infection among blood donors in Al-Muthanna Province-Iraq

A Thesis

Submitted to the Council of the College of Science of AI-Muthanna University in Partial Fulfillment of the Requirements for the Degree of Master of Science in

Biology/ *Microbiology*

By

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B.Sc. AI- Muthanna University 2011

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2014 A.D

1435 A.H

بسم الله الرحمن الرحيم

﴿ وَعَلَّمَكَ مَا لَمْ تَكُنْ تَعْلَمُ وَكَانَ فَضْلُ اللَّهِ عَلَيْكَ عَظِيماً ﴾

صدق الله العلي العظيم

سورة النساء جزء من آية 113

Acknowledgments

First of all my deep thanks and gratitude are presented to Allah for every thing. Without His great assistance the work wouldn't have been finished and peace and mercy upon His messenger prophet Muhammed and His house hold.

I am sincerely indebted and grateful to the Deanery of Science College for the kind help, cooperation and encouragement during the fulfillment of this study.

I would like to express my great thanks and indebtedness to my supervisors Assist. Prof.Dr.Mohsen A. Alrodhan & Assist.Prof.Dr.Muhammed Oda Mallah for their guidance, patience , scientific directions, constant help and encouragement along of research period.

My deepest gratitude and appreciation extend to Dr. Hassan Hachim for his kindness and help in this work.

My great thanks to Dr. Laith A. /Head of biology department – college of science- Al-Muthanna University for his support and help.

Also, I would like to thank Mr. Hassan H. Al-Awadi and Fairs H. Kaleel in Blood bank laboratory-Samawa hospital for their kindness and help.

My great thanks go to Assist. Prof. Hazim Ismael in Baghdad University for his support to accomplish the statistical analysis.

I am especially indebted to my wife and I would like to express my sincere thanks for her endless help and encouragement.

Finally, I would like to extend my gratitude to all people who participated in this study.

Hameed

Abstract

This study was conducted to detect the prevalence of hepatitis C virus (HCV) infection in blood donors for the first time in AI-Muthanna province –Iraq during the period between Dec. 2012 and Oct. 2013.Out of 5179 serum samples of blood donors of age ranging from 20 to more than 60 year were 20 gave positive results by using enzyme linked immunosorbent assay (ELISA) in percentage of 0.386%.

The results of the study showed that the highest rate of infection was in age group of (31-50) years old with percentage of 35% while the results of seroprevalence of HCV infection in relation to the location of different geographical regions showed that highest infection rate was in Samawa 60% which is significantly different at ($P< 0.05$).

The results of infection rate according to the months of the year during the study period showed the results of Alanine aminotransferase (ALT) in seropositive patients as compare with control and different age group showed variability in the concentration which were 85.25± 17.38 ng/ml, 80.71± 16.o4 ng/ml, 103.28± 23.27 ng/ml and 64± 14.75 ng/ml in 20-30, 31-40, 41-50 and 51->60 years old respectively. On the other hand the results of ALT concentration among different age groups of seronegative control donors were 12.75 ±4.14 ng/ml, 13.14±4.27 ng/ml, 12.85±2.85 ng/ml and 15.5±5.31 ng/ml. There were significant difference among age groups of seropositive donors and with seronegative control donors, the highest ALT concentration was in age group 40-50 as103.28± 23.27 ng/ml.

Reverse transcription real time polymerase chain reaction (RT-qPCR) was performed for direct and rapid detect of hepatitis C virus infection using one step technique. The condition of viral RNA was designed in exicycler real time PCR system. The results of molecular detect showed that out of 20 seropositive samples 13 were positive for HCV in percentage of 65%. In conclusion HCV was recorded in AI-Muthanna province and RT-PCR technique was reliable, direct and rapid test in combination with serological test.

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List of Abbreviations

No	Abbr.	**Meaning**
1	ALT	Alanine amino transferase
2	Anti-HCV	Antibodies against HCV
3	AST	Aspartate transaminase
4	ATP	Adenosine triphosphate
5	C.O	Calculation of Cut-off value
6	CT	Threshold cycle
7	CD	Cluster of Differentiation
8	cDNA	Complentary Deoxy Ribonucleic Acid
9	CDC	Central of Disease Control
10	C	Core
11	DNA	Deoxy Ribonucleic Acid
12	DEPC	Diethyl Pyro Carbonate
13	ER	Endoplasmic Reticulum
14	E1	Envelope glycoproteins 1
15	E2	Envelope glycoproteins 2
16	EIAs	Enzyme Immunoassay
17	ELISA	Enzyme linked Immunosorbent Assay
18	F-protein	Frame shift protein
19	GBV-B	GB virus B
20	GAG	Glycosaminoglycans
21	GPT	glutamate Pyruvate transferase
22	GBV-C	GB virus C
23	HAV	Hepatitis A virus
24	HBV	Hepatitis B virus
25	HRP	Horseradish peroxidase
26	HCV	Hepatitis C Virus
27	HCC	Hepatocellula carcinoma
28	HHC	Hereditary haemochromatosis
29	HDLS	High density lipoproteins
30	Huh7 cells	Human hepatoma cells
31	HIV	Human immunodeficiency virus
32	IFN- α	Interferon Alpha
33	IPC	Internal Positive Control
34	ISDR	IFN α sensitivity-determining region
35	ISDR	IFN α sensitivity-determining region
36	IgM	Immunoglobulin M
37	IRES	Internal ribosomal entry site
38	IVDU	Intra venous drug user
39	kb	kilo Dalton

40	LDL	low density lipoprotein
41	LDL-R	low-density lipoproteins receptor
42	MHC	Major Histo-compatibility Complex
43	MSM	men who have sex with men
44	mRNA	messenger RNA
45	MW	Molecular Weight
46	NK	Natural killer
47	nm	Nanometer
48	NC	Negative Control
49	NANB	Non-A, non-B Hepatitis
50	Ng	Nan gram
51	NTC	Non – Template Control
52	NS	Nonstructural
53	OD	Optical density
54	ORF	Open reading frame
55	p7	Protein 7
56	PBS	phosphate buffer solution
57	pH	Potenza Hydrogen
58	PCR	Polymerase chain reaction
59	PTB	polypyrimidine tract-binding protein
60	RT-PCR	Reverse transcriptase –PCR
61	SD	Standard deviation
62	TMB	Tetra methyl benzidine
63	T-cell	Thymus cell
64	Th1	T Helper cell 1
65	Th2	T Helper cell 2
66	TL	T lymphocyte
67	RNA	Ribo Nucleic Acid
68	RdRp	RNA- dependent RNA polymerase
69	SPSS	Statistical packages for social science
70	SR-B1	scavenger receptor class B type 1
71	µL	Microlitre
72	SP	signle peptidases
73	SVR	sustained viral responses
74	SPP	signal peptide peptidase
75	ssRNA	single stranded RNA
76	UTR	untranslated region
77	3' - UTR	3'-untranslated region
78	5'-UTR	5'-untranslated region
79	USA	United States of America
80	WHO	World Health Organization

Chapter One

INTRODUCTION

1-Introduction:-

Hepatitis C virus (HCV) belongs to the *Flaviviridae* family and it is the only member of the *Hepacivirus* genus (Dustin and Rice, 2007). Most cases of viral hepatitis are caused by one of the following agents : Hepatitis A virus (HAV); Hepatitis B virus (HBV) ; Hepatitis C virus (HCV) ; Hepatitis D virus(HDV) ; Hepatitis E virus (HEV) ; Hepatitis G and F are newly discovered viruses, some viruses causing hepatitis as a part of general infection examples by Rubella virus , mumps virus, cytomegalic virus and Epstein-Barr virus and very rare by Herpes Simplex virus and yellow fever virus (Zuckerman *et al.*,2009;Brook *et al.*,2010 ; Mahy and Van Regenmortel, 2010). HCV is the agent that causes Hepatitis C, a disease that affects around 130 million people worldwide, primary infection with HCV often shows only mild symptoms, but in the majority of patients, the infection becomes chronic and leads to liver cirrhosis, which often results in liver cancer. HCV infection is currently one of the major reasons for liver transplantation in Europe and in the United States (Shepard *et al.*, 2005). Chronic HCV can arise to serious liver diseases such as liver cirrhosis and hepatocellular carcinoma (HCC) (Kanwal *et al.*, 2011). The prevalence varies markedly from one geographical area to another and within the population assessed, in Western Europe, HCV prevalence rates range from 0.4% to 3%. It is higher in Eastern Europe, the Middle East and in other countries such as Egypt (15%), Romania (6%), Pakistan (4.7%) and in Ukraine (4.0%) (Esteban *et al.*,2008 ; Negro and Alberti, 2011). HCV infection of the hepatocyte begins with a complex interaction of the virion with a series of cellular entry factors (Burlone and Budkowska, 2009). The viral particle is then internalized by clathrin-mediated endocytosis (Blanchard *et al,* 2006).

Successful response to treatment against HCV infection seems to depend on several factors, involving both, the virus and the host (Aronsohn and Reau 2009; Maekawa and Enomoto, 2009). The symptoms of acute hepatitis C infection include decreased appetite, fatigue, abdominal pain, jaundice, itching and flu-like symptoms (Tsang *et al,* 2008).

Chronic HCV infection leads to cirrhosis in about 10 to 20 percent of patients, increasing the risk of complications of chronic liver disease, including portal hypertension, hemorrhage and hepatocellular carcinoma (Jou and Muir, 2008).

Unlike ELISA in which antibodies are detected, the RT-PCR identifies the highly conserved antigenic areas of the virus present in the test sample. The method is further improved by the real time PCR in which TaqMan Chemistry is used (Meertens *et al.*, 2006). In this method oligonucleotide probe is constructed containing a reporter fluorescent dye on the 5' end and a quencher dye on the 3' end. The proximity of the quencher dye greatly reduces the fluorescence emitted by the reporter dye by fluorescence resonance energy transfer (Moradpour *et al*., 2007) . If the target sequence is present, the probe anneals downstream from the primer site and is cleaved by the 5' nuclease activity of Taq DNA polymerase as this primer is extended, in this way the quencher dye is separated and the primer is increased in length, that completes one cycle, with each cycle the reporter dye is cleaved and fluorescence is increased with each cycle the intensity of which is proportion to amount of amplification produced which can be graphically seen on computer screen and then easily interpreted (Barbeau *et al.*, 2004).

The study aimed to detect the HCV among blood donors by molecular technique at AL-Muthanna Province and the prevalence of HCV was unknown and unclear background of HCV epidemiology of the province, for that the present study suggested to carry out to achieve the following objective:-

1- Evaluate the role of HCV in hepatitis infection in blood donors of AL-Muthana province.

2- Study some epidemiological characteristic of HCV infections.

3- Evaluate the association of ALT Concentration and seropositive blood donors.

4- Study rapid and early detection of HCV infection by using indirect ELISA and efficient molecular technique real-time qRT-PCR as Confirmation diagnosis.

Chapter Two

Literatures Review

2-*Literature Review*

2-1- History:-

The term non-A, non-B Hepatitis (NANB) was introduced in the mid-1970s to describe inflammatory liver disease that is not attributable to infection with hepatitis A virus (HAV) or hepatitis B virus (HBV) (Alter *et al*., 1975).

The introduction of hepatitis C virus in 1989 illuminated many dark corners in the natural history of the infection formerly known as non-A, non-B hepatitis (D'Souza and Foster, 2004).

Choo *et.al.,* (1989) found that Hepatitis C Virus (HCV) was shown to be the cause of most cases of NANB, also this Virus is a blood-born pathogen that poses a significant threat to public health worldwide. A significant number of parentally transmitted viral hepatitis cases in 1980's could not be ascribed to any of the know hepatitis viruses (hepatitis A virus, hepatitis B virus and delta virus) (Feinstone *et al.,* 1975). Some Researchers transmitted the virus from patients of transfusion associated hepatitis to chimpanzees, demonstrating that the disease resulted from a transmissible agent. Portions of HCV genome were isolated, by screening complentary Deoxy Ribonucleic Acid (cDNA) expression libraries mode from Ribo Nucleic Acid (RNA) and Deoxy Ribonucleic Acid (DNA) of chimpanzees, infected with serum of NANB patient, the virus was given the new name hepatitis C virus, another breakthrough came in 1989 with the cloning of genome (Choo *et al.,* 1989) .

2- 2-Etiology:-

The *Flaviviridae* family is divided into three genera: *flavivirus*, *pestivirus*, and *hepacivirus*, *Flavivirus* include yellow fever virus, dengue fever virus, Japanese encephalitis virus and Tick-borne encephalitis virus. *Pestivirus* include bovine viral diarrhea virus, classical swine fever virus and Border disease virus, HCV belong to the genus of *Hepacivirus*, with at least 6 genotypes and numerous subtypes, is a member of the *hepacivirus* genus, which includes tamarin virus and GB virus B (GBV-B) and is closely related to human virus GB virus C (GBV-C) (Lindendach and Rice, 2001).

HCV is a small enveloped positive stranded RNA virus that belongs to the *Hepacivirus* genus in the *Flaviviridae* family (Lindenbach *et al*., 2007). The HCV particle consists of a nucleocapsid surrounded by a lipid bilayer in which the two envelope glycoproteins, E1 and E2, are anchored as a heterodimer which plays a major role in HCV entry (Lavie *et al*., 2007). A form of hepatitis was recognized that had features in common with hepatitis-B but which was seronegative for HBV marker it occurred after blood transfusion or injection of blood products, had an incubation period of about two days and was transmissible to chimpanzees, it seems more liable than HBV to cause severe liver damage, the agent is 60 nm in diameter and enveloped, although very different from HBV is transmitted in similar fashion but has greater tendency to cause chronic infections leading to cirrhosis and hepatocellular carcinoma (HCC)(Collier, 2000). The different genotypes have different geographical distributions and prevalence, with genotype 1 being the most prevalent genotype in North America and Europe while genotype 4 is most common in Egypt and North Africa and genotypes 5 and 6 are most common in South Africa and Hong Kong. Genotypes 2 and 3 are common in North America, Europe and Japan, but to a lesser extent than genotype 1, a high number of subtypes are evident in Africa and Southeast Asia, suggesting that this region may be the original source of

HCV as fewer subtypes are evident in Europe and North America (Smith and Simmonds, 1997).

2-2-1-Classification:-

Classification of the hepatitis C virus in to:

Family: Flaviviridae.

Genus: Hepacivirus.

Type species: Hepatitis C virus (Collier, 2006).

2 -3- Viral Genome:-

The HCV, positive-sense, single-stranded RNA genome is approximately of 9.6 kilo Dalton (kb) long. The sequence contains a 5'-untranslated region (5'- UTR) of 341 bases, a single open reading frame (ORF) coding for a polyprotein of 3,011 amino acids and a 3'-untranslated region (3'- UTR) of about 27 bases, a single polypeptide protein is cleaved into 10 mature structural and non-structural regulatory proteins (Figure 2-1) (Pawlotsky *et al.* , 1999). Structural components include the core (C) and two envelope (E1 and E2) proteins, six nonstructural (NS) proteins are NS2, NS3, NS4A NS4B, NS5A and NS5B) (Reed and Rice, 2000).

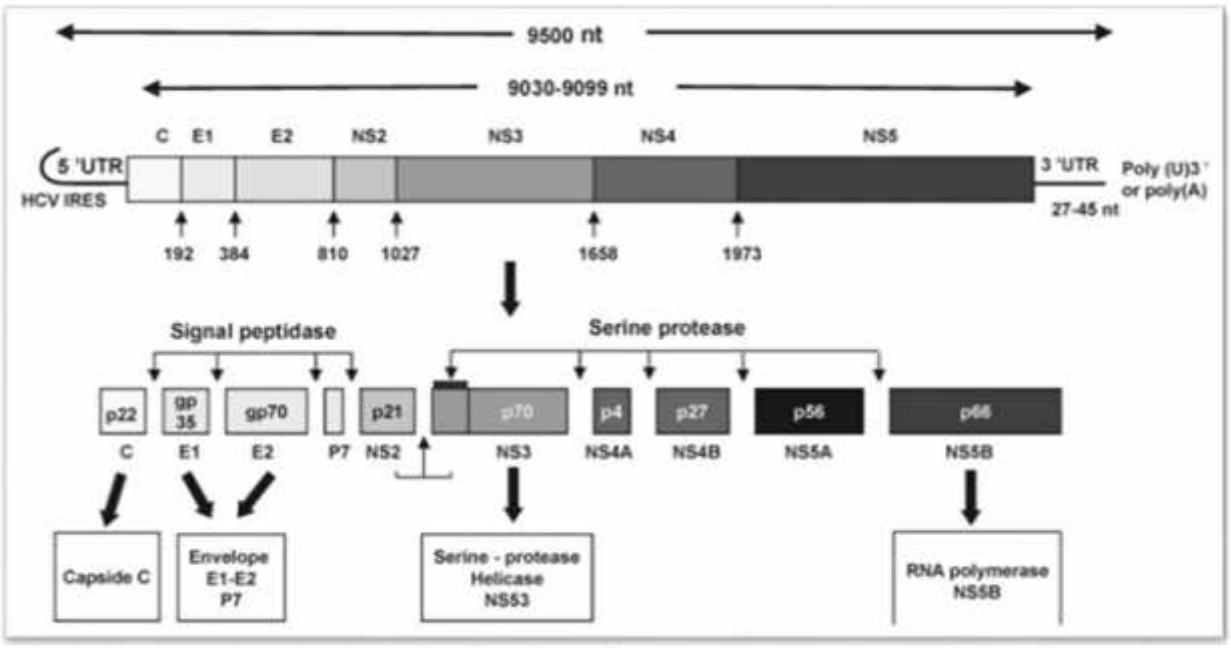

Figure 2-1:- HCV genome organization and processing of viral polyprotein (Pawlotsky *et al.* , 1999) .

The genome of the hepatitis C virus consists of one genome of virus 9.6 kb single stranded RNA molecule with positive polarity (Yu, 2007). Similar to other positive-strand RNA viruses, the genomic RNA of the hepatitis C virus serves as messenger RNA (mRNA) for the translation of viral proteins (Wakita, 2005). The linear molecule contains a single ORF coding for a precursor polyprotein of approximately 3000 amino acid residues flanked by two regulatory UTR (Heller, 2005) .

2- 4-Viral protein:-

Viral protein and their molecular weight function are summarized in table (2-1).

Table 2-1: Size and main function of HCV proteins, molecular weight (MW) in KD (Mauss *et al.*, 2013).

Protein	MW	Function
Core	21 KD	Capsid-forming protein. Regulatory functions in translation, RNA replication, and particle assembly.
F-protein	16-17KD	Unknown.
Envelope glycoprotein 1 (E1)	35 KD	Transmembrane glycoprotein in the viral envelope. Adsorption, receptor-mediated endocytosis.
Envelope glycoprotein 2 (E2)	70 KD	Transmembrane glycoprotein in the viral envelope. Adsorption, receptor-mediated endocytosis.
p7	7 KD	Forms an ion-channel in the endoplasmic reticulum. Essential formation of infectious virions.
NS2	21 KD	Portion of the NS2-3 protease which catalyses cleavage of the polyprotein precursor between NS2 and NS3

NS3	70 kd	NS2-NS3 protease, cleavage of the downstream HCV proteins . ATPase/helicase activity, binding and unwinding of viral RNA.
NS4A	4 KD	Cofactor of the NS3-NS4A protease.
NS4B	27 KD	Crucial in HCV replication. Induces membranous web at the ER during HCV RNA replication.
NS5A	56 KD	Multi-functional phosphoprotein contains the IFN α sensitivity-determining region (ISDR) that plays a significant role in the response to IFN α-based therapy.
NS5B	66 KD	Viral RNA-dependent RNA polymerase. NS5B is an error-prone enzyme that incorporates wrong ribonucleotides at a rate of approximately 10^3 per nucleotide per generation.

2- 5- Replication of HCV:-

2- 5-1-Attachment and entry:

The first step in a virus replication is the attachment of the infectious particle to the host cell, for which a specific interaction between a receptor on the cell surface and a viral attachment protein on the surface of the particle is required (Pileri *et al*., 1998). Before the virus association with the host cell the viral particles are associated with lipoproteins, which circulate in the blood stream (Evans *et al*., 2007).

Initial attachment between HCV and the host cell is with the low-density lipoproteins receptor (LDL-R) has been proposed as another potential attachment factor for HCV (Agnello *et al*., 1999). Use glycosaminoglycan's (GAG) and/or the low density lipoprotein (LDL)

receptor (LDL-R) as attachment factors for their initial binding to the host cell, after the initial attachment to the host cell, a virus binds to high affinity receptor(s) or specific entry factor(s), which are responsible for initiating a series of events eventually leading to the release of the viral genome into the cytosol. The first identified and best characterized entry factor is the tetraspanin Cluster of Differentiation 81 (CD81), which was initially shown to interact with HCV glycoprotein E2 (Pileri *et al*., 1998). HCV entry is strongly reduced in the presence of anti-CD81 antibodies or in CD81 knock-down hepatoma cell (Cocquerel *et al*., 2006).

The human scavenger receptor class B type 1 (SR-B1) is a protein expressed on the surface of the majority of mammalian cells. SR-B1binds high density lipoproteins (HDLS) and polysaccharides, allowing entry to the cell via intracellular compartment such as the gogli complex or endocytotic compartment (Vishnyakova *et al.,* 2003).

The binding of enveloped viruses to cell surface molecules is followed by fusion of the lipid envelope with a cellular membrane(Figure 2-2) (Tscherne *et al*., 2006). This process is tightly co-ordinated in time and space and requires drastic conformational changes in the fusion proteins, which are triggered by cellular factors, some viruses enter by fusing their envelope directly with the plasma membrane whereas others enter the target cells by endocytosis, for HCV, it has been shown that the particle enters the target cells by clathrin-mediated endocytosis (Blanchard *et al.,* 2006).
Furthermore, the acidic pH of endosomes triggers the fusion process probably by inducing conformational changes in the envelope proteins (Bartosch *et al*., 2003; Meertens *et al*., 2006).

After fusion of the viral envelope and an endosomal membrane, the viral genome is released into the cytosol (Hsu *et al*., 2003). It is worth noting that exposure of cell surface-bound virions to acid pH followed by a return to neutral pH does not affect HCV infectivity(Meertens *et al*., 2006; Tscherne *et al*., 2006).

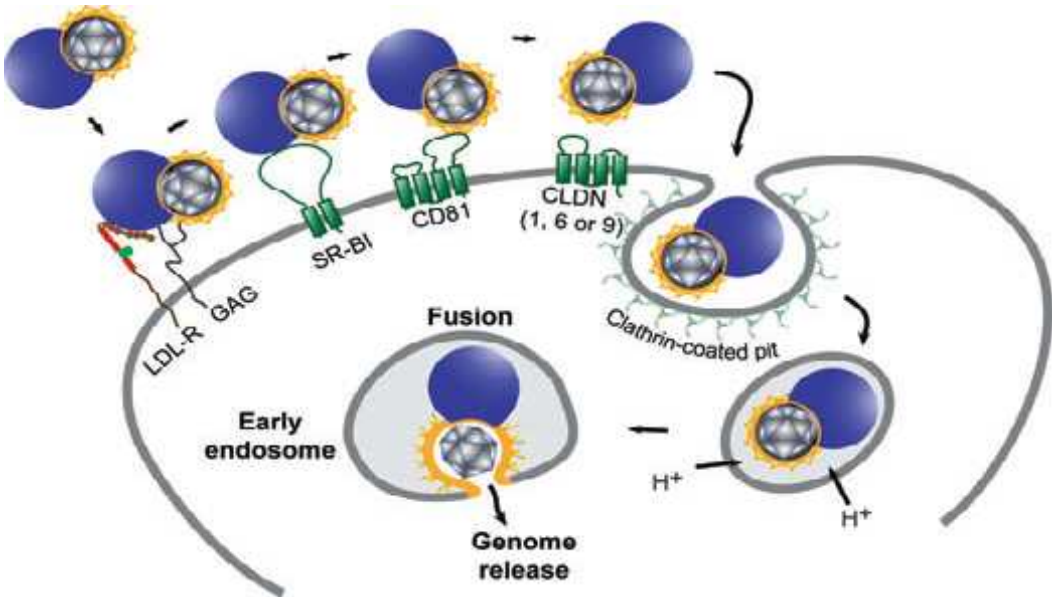

Figure 2-2 :-. Model of HCV entry into the hepatocyte (Evans *et al*., 2007).

2- 5-2-Translation of HCV:-

The HCV genome is a 9.6-kb uncapped linear single stranded RNA (ssRNA) molecule with positive polarity (Figuer2- 3) (Koutsoudakis *et al*., 2006). It contains 5′ and 3′ UTRs, including control elements required for translation and replication (Sarnow, 2003). The HCV UTRs flank an uninterrupted open reading frame encoding a single polyprotein of 3,011 amino acids, which is processed into structural (C, E1, E2, and p7) and nonstructural (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) proteins by host and viral proteases (Penin *et al.*, 2004).

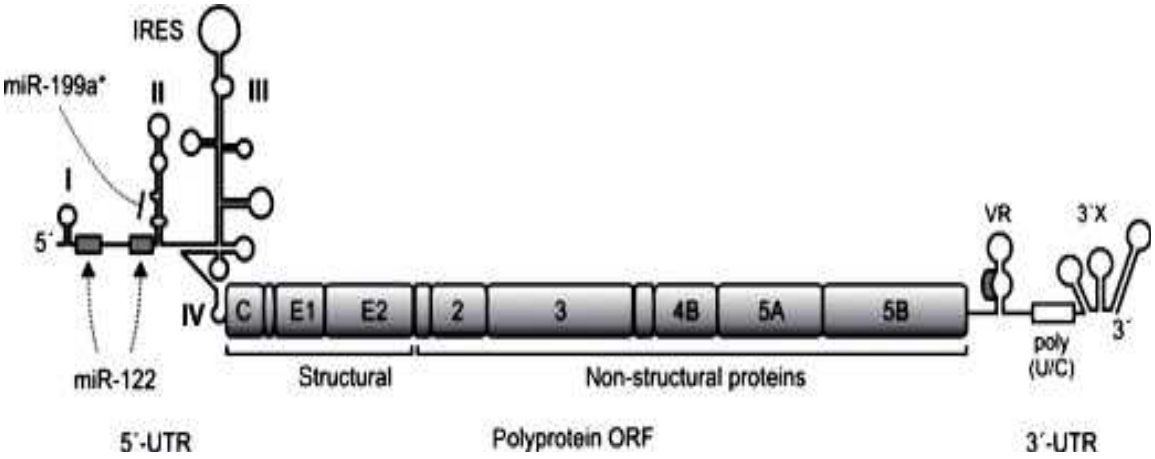

Figure2-3:- A Schematic representation of hepatitis C viral genome. (Niepmann , 2009) .

The HCV RNA genome does not possess a 5′-cap and the initiation of HCV protein translation occurs through a cap-independent, internal ribosomal entry site IRES-mediated mechanism(Tsukiyama-Kohara *et al.*,1992 ; Wang *et al.,* 1993). The HCV 5'-UTR is a highly structured region that contains four distinct domains (I, II, III and IV) and is highly conserved among different viral strains (Bukh *et al.*, 1992). Domains II and III contain the structural elements crucial for the initiation of translation whereas domain IV contains the initiation codon and part of the core-coding sequence (Brown *et al.*, 1992 ; Pestova *et al.*, 1998).The function of the HCV IRES is dependent upon its conserved secondary structure and partly on the primary sequence as demonstrated by mutational analysis (Fraser and Doudna , 2007) .

The translation of the HCV genome, which lacks a 5_ cap depends on an IRES within the 341-nucleotide 5' UTR (Ji *et al.*, 2004).Translation initiation begins with the direct recruitment of the 40S ribosomal subunit to the HCV IRES in the absence of any host translation initiation factors, a process that requires the basal portion of domain III (Otto and Puglisi, 2004; Hellen , 2009). Domain II partially occupies the exit channel and extends towards the P-site in the ribosomal subunit and this interaction induces a conformational change in the 40S subunit that is thought to result in the opening of the mRNA entry channel of the ribosome to allow stable accommodation of the HCV RNA (Lukavsky, 2009).

Translation of HCV RNA occurs at the rough ER and produces a single polyprotein which is cleaved co- and post-translation by both viral and cellular protease, to produce the structure and non-structure (Grakoui *et al.*,1993;Hijikata *et al.*,1993). HCV uses cellular protease called signal peptidases (SP) to cleave its structural protein: immature core protein, E1, E2, P7 (Santolini *et al.*, 1994). The cellular signal peptide peptidase (SPP) is responsible for the cleavage of the E1 single sequence the C-terminus of the immature core protein, resulting in the mature form of the core

(Mclauchlan *et al.*, 2002) The cleavage of NS2 and NS3 is accomplished through the action of a protease comprised of the NS2 and NS3 proteins themselves, a process described as auto cleavage (Figure2-4) (Reed and Rice, 2000). Whereas the NS3 serine protease cleaves the remaining functional proteins (Bartenschlager, 1999; Santoolini *et al.*, 1995).

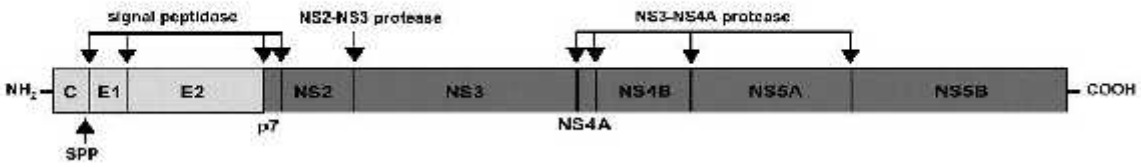

Figure 2-4 :-Cleavage sites within the HCV precursor polyprotein for SP, SPP and the viral proteases NS2-NS3 and NS3-NS4A, respectively (Mauss *et al.*,2013).

2- 5-3- Viral genome Replication:

Viral replication occurs after translation of the HCV genome from viral RNA to viral protein, HCV polymerase enzyme is located within the HCV NS5B protein, synthesis new strands (Lohmann *et al.*, 1997). This enzyme is called the RNA- dependent RNA polymerase (RdRp) (Behrens, 1996).

Simmonds *et al.*, (1993) Showed another feature of HCV replication is the rapid generation of virus variants, in fact based on the genomic variability in a small region of NS5B, HCV has been classified into at least six genotypes each with several subtypes.The UTR are forms stable secondary and tertiary structures. In the 5' end there is an IRES , which allows the RNA to bind to the ribosomes close to the start codon of the ORF. This connection maker enables translation of the ORF (Figure 2-5). In the 3′ end there is a conserved region important for RNA-replication (Bartenschlager and Lohmann, 2000). Initiation of RNA strand synthesis at the 3′-end of the plus and minus strands involves domain I of the 5′ UTR, which can form a G/C-rich stem-loop and the 3′ UTR (You *et al.*, 2004). A phosphorylated

form of polypyrimidine tract-binding protein (PTB) was found in the replication complex and PTB was shown to interact with two conserved stem-loop structures of the 3′ UTR, an interaction thought to modulate RNA replication (Luo, 2004). Importantly, inhibition of PTB expression by means of small interfering RNAs reduced the amount of HCV proteins and RNA in HCV replicon-harboring human hepatoma cells (Huh7 cells) (Chang and Luo, 2006).

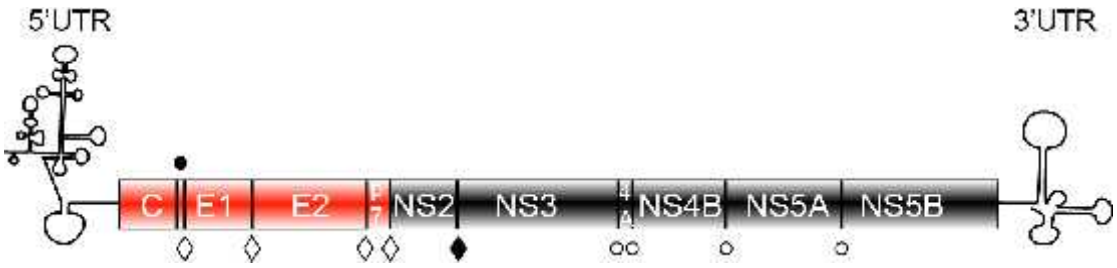

Figure2- 5:- Representation of the genetic organization of hepatitis C virus (Lohmann, *et al.*, 1999).

2- 5-4-Assembly and release:

After the viral proteins, glycoproteins and the genomic HCV RNA have synthesized these single components have to be arranged in order to produce infectious virion (Gastaminza, 2008). After that interaction between core protein and the 5-UTR of HCV RNA triggers the assembly of the capsid, and encloses genomic RNA within the capsid shell (Kunkel and Watowich, 2002; Ma *et al.*,2002). Findings suggest that viral assembly takes place within the Endoplasmic Reticulum (ER) and that lipid droplets are involved in particle formation (Miyanari *et al.*, 2007*;* Shavinskaya *et al.*, 2007).

Mahy and Van Regenmortel (2010) Found that the mechanism of budding for HCV is not understood, by comparison with the related *flaviviruses*, HCV is thought to acquire its envelope at the ER as the HCV glycoproteins localize predominantly to the ER, newly enveloped virions would be released from the cell in a process called exocytosis, the reverse of endocytosis (Serafino *et al.*, 2003). Alternately, viral release may involve

the HCV P7 protein. Previous research has shown that P7 can form pores or protein –lined tunnels on the plasma cell membrane called ion channels, which allow ions such as calcium, sodium and potassium to enter cell(Griffin *et al.*,2003). The P7 protein is a viable target for anti-HCV drug development (Figure2-6) (Pavlovic *et al.*, 2003).

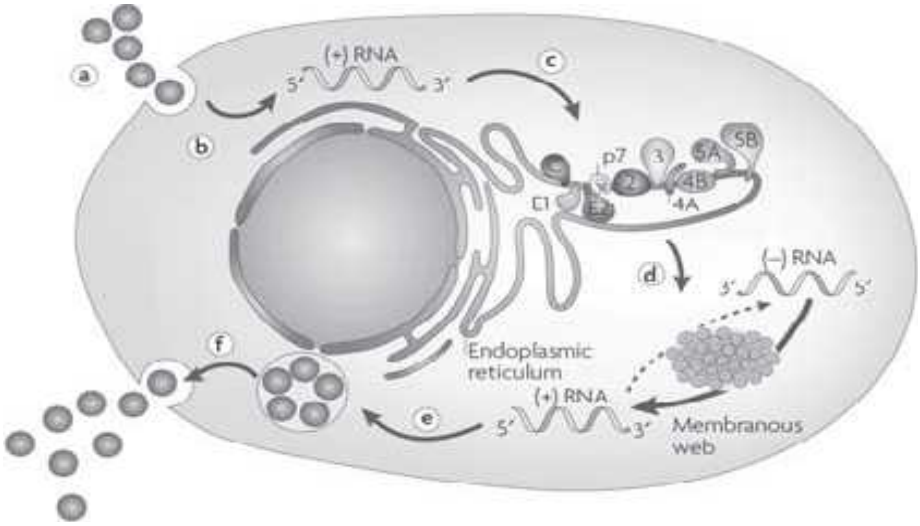

Figure 2-6:- Replication of HCV.

(a).Virus binding and internalization,(b). cytoplasmic release and uncoating, (c).internal ribosome entry site (IRES)-mediated translation and polyprotein processing, (d). RNA replication, (e).packaging and assembly, (f).virion maturation and release (Moradpour *et al.*, 2007).

2 -6-Epidemiology of HCV:--

2 -6-1-Prevalence:

World Health Organization (WHO) estimates that about 3% of the world's population has been infected with HCV and that some 170 million are chronic carriers at risk of developing liver cirrhosis and/or hepatocellular carcinoma (Wong and Lee, 2006). There are considerable regional differences: in some countries, e.g., Egypt, the prevalence is as high as 22% (WHO 2011).While in Africa and the Western Pacific prevalence is significantly higher than in North America and Europe (CDC 2012) .Estimated that there are 2-5 million HCV-positive people in Europe. In Europe and the United States chronic hepatitis C is the most

common chronic liver disease, the majority of liver transplants performed in these regions are for chronic HCV. It is difficult to determine the number of new HCV infections, as most acute cases are not noticed clinically. Recent numbers from Europe still show an ongoing epidemic of acute HCV especially among Intra venous drug user (IVDU) and men who have sex with men (MSM) (Rockstroh, 2012).There are six different major genotypes, and about 100 subtypes of HCV with varying presence in different parts of the world. Genotype 1-3 are spread worldwide, genotypes 4-5 are found mainly in Africa and genotype 6 is found in Asia (Schuppan *et al.*, 2003). WHO (1999) showed that in the Western world genotype 1 is predominate (60-90% of infected) and genotype 4 dominate in Egypt.

The prevalence of anti-HCV antibodies in Iraq is 7.1% in general population and 66.0% in Human immunodeficiency virus (HIV) infection hemophilia patients, for HCV genotypes, 1a, 1b, 4 and 4 mixed with 3a were detected, and HCV-1b was the most frequent genotype (AI-Kubaisy *et al.*, 2002*)*. The anti-HCV seroprevalence in pregnant women was 3.21% and correlated with the number of miscarriages and HCV-1b genotype (Chironna *et al.*, 2003*)*. HIV, HBV and HCV the three most common chronic viral infections all over the world, share similar transmission routes including sexual, blood-blood contact and injecting drug usage (Saravanan *et al.*, 2007). Co-infection with HIV and HCV and/or HBV is very common in certain population, such as intravenous drug users (IDUs) who often share the contaminated needles/syringes for intravenous drug injection (Koziel and Peters, 2007). It has been reported that the prevalence of HIV-HCV co-infection among IDUs can surpass 90% (Aceijas and Rhodes, 2007). Co-infection with HIV and hepatitis viruses has significantly increased morbidity and mortality of the HIV patients (Thio, 2009). Therefore, it is critical to investigate the prevalence of co-infection with HIV and HCV and/or HBV, especially among the IDUs considered to be a high-risk population of coinfection (Rotman and Liang, 2009). HCV infection is

associated with significant morbidity and mortality (Maier and Wu 2002). Worldwide, approximately 170 million people are infected with HCV, including 243,000 to 300,000 Canadians citizens (Zou *et al.*, 2000). There is a considerable geographical variation in seroprevalence of anti-HCV throughout the world, with approximately 1.3% in developed countries and 2.6% in developing countries (Parkin, 2006). About 25% of infected individuals spontaneously clear infection and 75% become chronically infected (Hoofnagle, 2002 ; Seeff, 2002). Within 20 to 30 years of infection, approximately 10% to 40% of HCV-infected individuals will develop cirrhosis (Thein H-H *et al.*, 2008).

2 -6-2-Routes of Transmission:

In earlier times, routes of virus transmission may have included variolation as protection against smallpox or have been associated with other culture-specific parenteral exchanges, hepatitis was also observed between 1910 and 1940 as a consequence of the use of unsterilized needles and syringes after injection with arsphenamine in the treatment of syphilis, or other injections (Mortimer, 1995). The HCV has been shown to be the major causative agent of parenterally transmitted non-A, non-B hepatitis (Choo *et al.*, 1989) . In developed countries, HCV is primarily transmitted through injecting drug use (Heintges *et al.*, 1997*).*

The problem of vertical transmission of hepatitis C is still not completely clear (Alte, 1994). Because of several reasons: the low prevalence of carriers in many countries, the methodology of viral RNA detection which, as instrument of epidemiological investigation, is neither widespread nor standardized, the need for a lengthy follow-up on new-born babies because even in cases of infection, HCV-RNA can be seen only intermittently (Otho *et al.*,1994). Although blood transfusion is the main way of HCV transmission, it is not the only one. It is recognized that hepatitis C virus infection can also occur through percutaneous injury using instruments with HCV-infected blood, such as, needles/syringes, piercing

and tattoos instruments, nail clippers, major/minor surgery and dental procedures(Idrees and Riazuddin, 2008). Besides the sexual transmission, although not being a classical and efficient route for HCV spread admitted, this possibility has not been rule out (Rè *et al.*, 2008). By the way, it is important to point out that 5% of all HCV cases occur by sexual route (Abou-Setta, 2004). and in 20% of cases, the agent transmission occurs by unknown route, although RNA-HCV detection has been achieved from the saliva, breast milk, urine, feces, semen and cervico-vaginal secretions (Abou-Setta, 2004). Additionally, it has been established that HCV per natal transmission is possible, and the risk is approximately 5%, but breast-feeding has not been widely reported (Thompson *et al.*, 2003).

2- 7- Pathogenesis:-

HCV infection is characterized by its propensity to evolve into chronicity and by a wide clinical spectrum, about 85% of patients infected by HCV will develop chronic infection and resolution of acute hepatitis C is observed in only 15% (Marcellin, 1999). HCV infects hepatocytes, it is still unclear whether the liver damage associated with HCV infection is the result of a direct cytopathic effect or is caused by a host immune-mediated cytolytic response, both processes are probably involved in causing hepatic damage (Houghton *et al.*, 1996). The severity of the liver disease varies widely from asymptomatic chronic infection, with normal liver tests and nearly normal liver, to severe chronic hepatitis, leading rapidly to cirrhosis and hepatocellular carcinoma,the mechanisms responsible for the persistence of HCV infection and for the liver lesions are not well understood, the lack of an efficient *in vitro* replication system or an animal model (the chimpanzee model is limited) has greatly hampered the study of these mechanisms, Chronic hepatitis C is characterized by portal inflammation, typically periportal hepatocellular necrosis, and fibrosis (Vander Poel , 1999). Factors that may affect the natural history of HCV infection are: Various cofactors such as presence of HBV, HIV and alcohol

intake appear to promote disease progression, chronic HBV / HCV co-infection (HBs-Ag and anti-HCV positive), co-infected patients have a higher risk of hepatocellular carcinoma than those who are only infected with one virus, however, it is unclear whether this high risk reflects a combined effect of the two viruses in the absence of interaction or some synergistic effect, the serological profile of anti-HBc alone / anti HCV positive is common (Harris *et al.*, 2001). An evidence suggests that the presence of anti-HBc alone might increase the risk of hepatocellular carcinoma among patients with chronic HCV infection, intake of more than 50 g alcohol / day accelerates progression to cirrhosis with a threefold risk increase, consistently normal Alanine amino transferase (ALT) levels are associated with slower fibrosis progression (Hourigan *et al.*, 1999). The size of the viral inoculums received may determine the course of disease, post transfusion cases may proceed more aggressively than infections associated with IDU, disease expression is related to viral expression, low levels of circulating HCV RNA are generally found in asymptomatic patients with normal ALT levels, experiments carried out with chimpanzees have shown that the administration of powerful immunosuppressants before and after virus inoculation prevents the development of acute hepatitis despite viremia in the animal and viral expression in the liver. Removal of the immunosuppressant triggered an immune response which resulted in the onset of acute hepatitis followed by virus elimination (Houghton *et al.*, 1996).

2- 8- Clinical Signs and Symptoms:-

2- 8- 1-Acute:

Acute hepatitis C refers to the first 6 months after infection with HCV, between 60% to 70% of people infected develop no symptoms during the acute phase, in the minority of patients who experience acute phase symptoms, they are generally mild and nonspecific, and rarely lead to a specific diagnosis of hepatitis C (Wasley *et al.*, 2008). The Symptoms of

acute hepatitis C infection include decreased appetite, fatigue, abdominal pain, jaundice, itching and flu-like symptoms (Tsang *et al.,* 2008). The hepatitis C virus is usually detectable in the blood within one to three weeks after infection by Polymerase chain reaction (PCR), and antibodies to the virus are generally detectable within 3 to 15 weeks, spontaneous viral clearance rates are highly variable and between 10–60% (Caruntu and Benea, 2006). Pepole infected with HCV clear the virus from their bodies during the acute phase as shown by normalization in liver enzymes alanine transferase (ALT) & Aspartate transaminase (AST),and plasma HCV-RNA clearance (this is known as spontaneous viral clearance).However persistent infections are common (Kamal, 2008). Most patients develop chronic hepatitis C, i.e., infection lasting more than 6 months (Villano *et al.,* 1999).

2- 8- 2-Chronic:

Chronic hepatitis C is defined as infection with the HCV persisting for more than six months. Clinically, it is often asymptomatic (without symptoms) and it is mostly discovered accidentally, the natural course of chronic hepatitis C varies considerably from one person to another. Although almost all people infected with HCV have evidence of inflammation on liver biopsy (Ngo *et al.,* 2006).

About 80% of those exposed to the virus develop a chronic infection (Nelson *et al.,* 2011). This is defined as the presence of detectable viral replication for at least six months, most experience minimal or no symptoms during the initial few decades of the infection, although chronic hepatitis C can be associated with fatigue, chronic infection after several years may cause cirrhosis or liver cancer (Rosen, 2011). The liver enzymes are normal in 7-53%, fatty changes to the liver occur in about half of those infected and are usually present before cirrhosis develops. Usually (80% of the time) this change affects less than a third of the liver (El-Zayadi, 2008). Worldwide hepatitis C is the cause of 27% of cirrhosis cases and 25% of HCC (Alter, 2007). About 10–30% of those infected develop cirrhosis over 30 years,

cirrhosis is more common in those also infected with hepatitis B, schistosoma, or HIV, in alcoholics and in those of male gender (Wilkins *et al.*, 2010). Liver cirrhosis may lead to portal hypertension, easy bruising or bleeding (enlarged veins, especially in the stomach and esophagus), jaundice and a syndrome of cognitive impairment known as hepatic encephalopathy (Ozaras and Tahan, 2009). Hepatic encephalopathy is due to the accumulation of ammonia and other substances normally cleared by a healthy liver, liver enzyme tests show variable elevation of ALT and AST, Periodically they might show normal results. Usually prothrombin and albumin results are normal, but may become abnormal, once cirrhosis has developed, the levels of elevation of liver tests do not correlate well with the amount of liver injury on biopsy, viral genotype and viral load also do not correlate with the amount of liver injury, liver biopsy is the best test (Ngo *et al.*, 2006).

2 -9- Diagnosis:-

2-9-1-Liver function tests:-

Laboratory liver tests are broadly defined as tests useful in the evaluation and treatment of patients with hepatic dysfunction, the liver carries out metabolism of carbohydrate, protein and fats, some of the enzymes and the end products of the metabolic pathway which are very sensitive for the occurred abnormality may be considered as biochemical marker of liver dysfunction, some of the biochemical markers are : serum bilirubin, alanine amino transferase, aspartate amino transferase, alkaline phosphatase and gamma glutamyl transferase. An isolated or conjugated alteration of biochemical markers of liver damage in patients can challenge the clinicians during the diagnosis of disease related to liver directly or with some other organs(Shivaraj *et al.*, 2009).

2- 9 -1-1- Alanine Transferase(ALT) and Aspartate Transferase (AST):-

Alanine aminotranferease (ALT) and aspartate aminotransferase (AST) are enzymes located in liver cells that leak out into the general circulation when liver cells are injured, these two enzymes were previously known as the SGPT (serum glutamic-pyruvic transaminase) and the SGOT (serum glutaic-oxaloacetic transaminase) (Hayashi *et al.,* 2003).These two transaminase enzymes may be reported on lab slips with both their new names and previous names or by their newer names only, ALT and AST are present in highest concentrations in cells from the liver, heart, skeletal muscles and red blood cells. Patients whose LFTs show a predominant rise in the transaminases have liver diseases that are characterized by hepatocellular damage (Gaze, 2007).

ALT is found predominately in the liver, with lesser quantities found in the kidneys, heart and skeletal muscle, as a result the ALT is a more specific indicator of liver inflammation than the AST, as the AST may also be elevated in diseases affecting other organs such as the heart or muscles **Alanine (Ala) + α-ketoglutarate ⇌ pyruvate + glutamate (Glu) (Hirotsu *et al.*, 2005).**

The AST is also elevated after a myocardial infarction, and during acute pancreatitis, acute hemolytic anemia, severe burns, acute renal disease, musculoskeletal diseases, and trauma, aspartate transaminase catalyzes the interconversion of aspartate and α-ketoglutarate to oxaloacetate and glutamate.

Aspartate (Asp) + α-ketoglutarate ↔ oxaloacetate + glutamate (Glu)

(Berg *et al.,* 2006). Levels of aminotransferases (ALT) in the blood indicate the degree to which liver membrane injury has resulted in an increased release of hepatocellular enzyme into the bloodstream, because ALT is more specific than AST for liver injury, ALT is used more often, in patients with risk factors for HCV infection and in whom there is no another explanation for increased enzyme levels, elevated aminotransferase levels are highly associated with HCV infection (Care, 2013).

2- 9 -1-2- Bilirubin test:-

Bilirubin is the catabolic product of haemoglobin produced within the reticulo endothelial system, released in unconjugated form which enters into the liver and converted to conjugated forms (Mauro *et al.,* 2006). Normal serum total bilirubin varies from 2 to 21µmol/L. The indirect (unconjugated) bilirubin level is less than 12µmol/L and direct (conjugated) bilirubin is less than 8µmol/L (Diana N.C., 2007). The serum bilirubin levels more than 17µmol/L suggest liver diseases and levels above 24µmol/L indicate abnormal laboratory liver tests (Wong *et al.,* 2004). In viral hepatitis, hepatocellular damage, toxic , levels of serum conjugated bilirubin is higher , hyperbilirubinemia in acute viral hepatitis is directly proportional to the degree of histological injury of hepatocytes and the longer course of the disease (Thapa and Anuj, 2007). Incomplete extrahepatic obstruction due to biliary canaliculi give lower serum bilirubin value than those occur with malignant obstruction of common bile duct but the level remains normal in infiltrative diseases like tumors and granuloma (Daniel and Marshal, 2007). Raised Serum bilirubin from 20.52 µmol/L to 143.64µmol/L in acute inflammation of appendix has been observed (Khan, 2006). The study shown that a high serum total bilirubin level may protect neurologic damage due to stroke (Perlstein *et al.*,2008).

2- 9 -1-3- Alkaline phosphatase (ALP):

ALP is present in mucosal epithelia of small intestine proximal convoluted tubule of kidney, bone, liver and placenta, it performs lipid transportation in the intestine and calcification in bone. The serum ALP activity is mainly from the liver with 50% contributed by bone, normal serum ALP is 41 to 133U/L . In acute viral hepatitis, ALP usually remains normal or moderately increase. Elevation of ALP with prolonged itching is related with Hepatitis A presenting cholestasis, tumors secrete ALP into plasma and there are tumor specific isoenzymes such as Regan, Nagao and Kasahara (Rosalki and Mcintye, 1999).

Hepatic and bony metastasis can also cause elevated levels of ALP, other diseases like infiltrative liver diseases, abscesses, granulomatous liver disease and amyloidosis may cause a rise in ALP, mildly elevated levels of ALP may be seen in cirrhosis, hepatitis and congestive cardiac failure (Simko, 1991). Low levels of ALP occur in hypothyroidism, pernicious anaemia, zinc deficiency and congenital hypophosphatasia , ALP has been found elevated in peripheral arterial disease, independent of other traditional cardiovascular risk factors (Cheung *et al.,* 2009).

2- 9 -1-4- Measurement Iron overload:

Also called Haemochromatosis, in medicine,iron overload indicates accumulation of iron in the body from any cause, the most important causes are hereditary haemochromatsis (HHC), a genetic disorder, and transfusion iron overload, which can result from repeated blood transfusion (Dlouhy and Outten, 2013). Organs commonly affected by haemochromatosis are the liver, heart and endocrine glands (Andrews, 1999).

Chronic hepatitis C virus infection is a leading cause of progressive liver fibrosis, liver cirrhosis and hepatocellularcarcinoma. Iron overload is frequently observed in cases of chronic hepatitis C and has been suggested as a negative prognostic factor for this disease(Sikorska *et al.,* 2011). Although the mechanisms leading to iron accumulation have not fully explained yet, both host and viral factors seem to contribute towards the development of this pathology(Bonkovsky *et al*., 1997). Better under-standing of the interplay between hepatitis C virus replication and expression of iron regulatory molecules may elucidate new and interesting targets for the effective treatment of chronic hepatitis C, iron overload in the liver induces oxidative stress leading to cell membrane damage, DNA instability and mutagenesis (Isom *et al*., 2009). Due to these effects, iron can be considered a proinflammatory, profibrogenic factor and a potential carcinogen, since the implementation of serological diagnostic tests for HCV identification, elevated serum iron-overload indices or appearance of

iron deposits in liver cells have been observed in 10-40% of patients with chronic hepatitis C and 50% of patients suffering both from Chronic hepatitis C and HCC (Piperno *et al*., 1995; Ludwig *et al*., 1997). In addition, feeding HCV-infected chimpanzees a diet with excess iron increased the level of ALT activity, histological changes that were observed provided further evidence of iron related exacerbation of liver injury (Bassett *et al*., 1999). Based on the above-mentioned observations, iron overload has been suggested as a negative prognostic factor of Chronic hepatitis C , with possibly influences on the increase in aminotransferase activity, exacerbation of inflammation, progression of liver fibrosis and decrease in antiviral therapy effectiveness (Metwally *et al*., 2004).

2- 9 -2-Serological tests (Antibody tests):

Diagnostic tests used for the detection of HCV infection include the HCV antibody enzyme immunoassay, recombinant immunoblot assay and quantitative HCV RNA polymerase chain reaction (PCR) (Ghany *et al.,*2009). A number of immunoassays has been developed to detect anti-HCV IgG in serum or plasma specimens. First-generation assays were based on a yeast-expressed recombinant protein containing an epitope from the NS4 region (C100-3) of the HCV genome, although these assays identified anti-HCV IgG in approximately 80% of patients with post transfusion hepatitis and led to the substantial reduction in transfusion-associated HCV infections, they lacked sensitivity and specificity (Barrera *et al.*, 1991). Second- and third-generation assays used a multiantigen format and included antigens from the core, NS3, and NS4 regions these modifications markedly improved sensitivity and specificity (Alter , 1992).The difference between the second- and third-generation assays is the inclusion of an additional antigen from the NS5 region (Barrera *et al.*, 1995). These assays reduced the window period observed in first-generation assays by an average of 5 weeks and permitted anti-HCV to be detected as early as 10 weeks after exposure, the diagnostic specificity of third-generation assays is

>99% (Colin *et al.,* 2001). The detection of anti-HCV antibodies in serum is based on the use of third-generation Enzyme Immunoassay (EIAs), that detect mixtures of antibodies directed against various HCV epitopes. Recombinant antigens are used to capture circulating anti-HCV antibodies onto the wells of microtiter plates, microbeads, or specific holders adapted to closed automated devices. The presence of anti-HCV antibodies is revealed by anti-antibodies labeled with an enzyme that catalyzes the transformation of a substrate into a colored compound (Pawlotsky, 1999). The optical density (OD) ratio of the reaction is proportional to the amount of antibodies in the serum sample, none the less, third-generation EIAs can yield false-negative results in patients who are undergoing hemodialysis or who are immunocompromised (Ghany *et al.,* 2009).Third-generation EIAs detect mixed antibodies against HCV core, NS3, NS4 and NS5 antigens. The target antigens are coated on microtiter plates, microbeads or holders designed for “closed” automated devices, the specificity of current EIAs is greater than 99%, there is no gold standard, so sensitivity is more difficult to determine. In routine use, more than 99% of immunocompetent patients with detectable HCV RNA are positive with current EIAs (Colin *et al.*, 2001). EIAs can be negative during hemodialysis and in profoundly immunodeficient patients despite ongoing HCV replication, but this is rare with the most recent tests (Thio *et al.,* 2000). In order to shorten the duration of the diagnosis of heaptitis C virus infection especially in preseroconversion period being capable of the detection of antibodies against NS 5 proteins means that a third generation reactive is very important for anti-HCV assays, because there remains a window period, estimated at 82 days with the second-generation assays, at 66 days with the third generation assays, between the infection and the detection of HCV antibodies (Courouce, *et al.,* 2000). NS5 enables the detection of HCV antibodies on an average of 26 days earlier in individuals with transfusion-transmitted HCV infection (Denoyel *et al.*, 2004).

2- 9-3-Molecular Test of HCV (PCR):-

2- 9-3-1- Reverse transcriptase (RT)-PCR:

PCR reactions are usually the reaction conditions must be varied to improve the efficiency, this is very important when trying to amplify a particular target from a population of other sequences, for example one gene from genomic DNA, or one cDNA from either a cDNA library or the products of a first strand cDNA synthesis reaction, this latter method of reverse transcribing mRNA and then PCR amplifying the first strand cDNA is called RT - PCR. (Turner *et al.,* 2005).

The first step is the synthesis of a complementary DNA copy of the target region of the RNA genome using RT primed by the antigenomic PCR primer or random hexadeoxyribonucleotides, the product of this reaction is a suitable target for amplification (RT-PCR), the concentration of virus in serum samples is often very low, so that the mass of product from the PCR reaction is insufficient for visualization on a stained gel, there for a second round of amplification (with nested primers) or detection of the primary product by southern hybridization is required, there is aconsiderable variation in nucleotide sequences among different isolates of HCV, and the 5′UTR, with seems to be highly conserved is the preferred target for diagnostic PCR (Zuckerman *et al.,* 2009).

2- 9-3-2-Real time PCR:-

Real time PCR the thermal cycler can determine the amount of product that has been made as the reaction proceeds, for example by detecting the increase in dye binding by the synthesized DNA, using a fluorometer, the advantages of real time PCR, apart from immediate information on the progress of the reaction, include high sensitivity, ability to cover a large range of starting sample concentrations, easy compensation for different efficiencies of sample amplification and the ease of processing many samples, since these do not necessarily need to be analysed at the end by, for example, gel analysis. Unfortunately the equipment is rather

costly(Turner *et al.*,2005). First began to develop real-time PCR machines in 1997, this type of instrument has an integrated fluorimeter to allow in tube real-time analysis of DNA template samples, a rise in fluorescence of the signal of each cycle indicates amplification (Lee, and Chen, 2008). The ability to monitor reaction progress has a number of advantages over endpoint analysis, as a result, real-time PCR has proven to be a powerful tool for genetic analysis (Kubista *et al.*, 2006). DNA detection simultaneous to amplification is preferentially achieved by the use of target sequence – specific oligonucleotides (probes) linked to two different molecules, a fluorescent reporter molecule and a quenching molecule (Barbeau, *et al.*, 2004). The first real-time fluorescent probes developed were 5' nuclease probes, which are commonly referred to by their proprietary name, TaqMan probes, a TaqMan probe is a short oligonucleotide (DNA) that contains a 5' fluorescent dye and 3' quenching dye, to generate a light signal (remove the effects of the quenching dye on the fluorescent dye), two events must occur. First, the probe must bind to a complementary strand of DNA at 60°C. Second, at this temperature, Taq polymerase, the same enzyme used for the PCR, must cleave the 5' end of the TaqMan probe (5' nuclease activity), separating the fluorescent dye from them quenching dye, a single TaqMan probe can be used for detection of amplified target DNA, if the intent of the assay is to differentiate a single nucleotide polymorphism from a wild type sequence in the target DNA, then a second probe with the complementary nucleotide(s) to the polymorphism and a fluorescent dye with a different emission spectrum are utilized (Chevaliez *et al.*, 2007). Thus, TaqMan probes can be used to detect a specific, predefined polymorphism under the probe in the PCR amplification product, for this application, two reaction vessels are required, one with a complementary probe to detect wild-type target DNA and another for detection of a specific nucleic acid sequence of a mutant strain, because TaqMan probes require 60°C for efficient 5' nuclease activity, the PCR is usually cycled between 95 and 60°C for

amplification. In addition, the cleaved (free) fluorescent dye accumulates after each PCR temperature cycle, and therefore can be measured at any time during the PCR cycling, including the hybridization step (Espy *et al.*, 2006). Assay for the detection of HCV –RNA is used to disclose viraemia HCV-RNA detection can be achieved using target amplification such as the branched DNA assay (Chevaliez and Pawlotsky, 2009). The classical techniques for viral genome detection and quantification are now being replaced by Real-time PCR assay (Chevaliez *et al.*, 2009). These assays have a broad dynamic range of quantification and more sensitive than classical PCR with lower limits of detection of 10-15 IU/ml , Real-time PCR can be fully automated and has become the technique of choice to detect and quantify HCV-RNA in clinical practice (Pawlotsky *et al.*, 2000). HCV-RNA becomes detectable within 1-2 weeks after initiation of infection. Detection and quantification of HCV-RNA is useful in clinical practice to detect and confirm HCV- infection and to monitor the virological response to antiviral therapy (Lavanchy, 2004).

2- 10-Treatment of HCV:-

The combination therapy with interferon-α and ribavirin, which is the most effective therapy known today, neutralizes the virus after 6 months in 40-50% of the infection cases with genotype 1 and in 80% of the infection cases with genotype 2 and 3(Schuppan *et al.*, 2003). Infections of HCV genotype 4 is, as genotype 1, relatively resistant to the interferon-α/ribavirin combination therapy (WHO, 1999).

The treatment with interferon-α and ribavirin has significant side effects and are quite expensive (Schuppan *et al.*, 2003). The side effects mainly derive from ribavirin as cough, shortness of breath, insomnia and haemolytic anaemia, despite these drawbacks and the fact that ribavirin is teratogenic and requires frequent dose modifications, the combination therapy is at least twice as effective as the mono therapy, if we compare the sustained viral responses (SVR), even from the economic point of view the

combination therapy is preferable preventing future costs, which else should occur in connection with chronic liver diseases (Mchutchison, 2002). Still, the therapies known today are not optimal and new approaches are needed. The HCV virus has a high rate of mutations and for example can be noted a mutation rate in the RdRp region at $5x10^{3}$/site per year, this fact together with the high genetic diversity and different genotypes in different parts of the world make developing a worldwide useful antiviral agent a great challenge, to be successful against HCV you probable will have to use combinations therapies with antiviral agents designed to bind to and to disable the functionality of functional proteins with conserved genetic regions (Locarnini and Bartholomeusz, 2002).

2-11- Immune response:-

2-11-1-Innate immune response:

The first mechanism of host defense against HCV infection is represented by the innate immune response, this consists of endogenous secretion of interferon and Natural killer (NK) cells, animal studies show that HCV causes early alterations in the expression of several hepatocytic genes, especially in those related to the response to interferon type 1. In acute phases of HCV infection, interferon 1 expression is one of the early manifestations of the innate immune response. Interferon type 1 (α and β) represents the first line of host defense against infections and has antiviral and immunomodulation effects (Heller and Rehermann , 2005).

Bertoletti and Ferrari, (2013) referred to the mechanisms of action of interferon type 1 include:

Down regulation of protein synthesis of the infected cells by inducing cellular protein kinases, increased expression of major histocompatibility (MHC) genes on the antigen presenting cells and target cells, inhibition of viral replication, Up regulation of NK cells, dendrites' cells and CD8 lymphocytes activity and induction of cell death by activating molecules involved in apoptosis.

Viral proteins also influence the activity of NK cells, thus proteins from HCV envelope bind to the surface of NK cells blocking their activation, cytokine secretion and cytotoxic activity, *in vitro* studies showed that NK cells from patients with HCV infection (but not from healthy individuals) have a lower capacity to activate the dendrites' cells (Rehermann and Nascimbeni, 2005). Dendrites' cells constitute a heterogeneous population of antigen presenting cells, playing important roles in antiviral immunity. They connect innate and adaptive immune responses. Signals from innate immune system (production of interferon type 1, interactions with NK cells) determine the maturation of dendrites cells, these are essential for launching antigen specific immune response, the role of the dendritic cells is to present the antigens to CD4 and CD8 lymphocytes, after the stimulation realized by mature dendrites cells , CD8 (and also CD4) T lymphocytes proliferate intensely (Bertoletti and Ferrari, 2003).

2-11-2-Adaptive immune response:-

2-11-2-1-Cellular immune response:

Currently, it is generally accepted that the intensity and persistence of specific HCV T lymphocytes response as well as the secreted cytokine profile determine the evolution of HCV infection, it has been demonstrated that an early, strong, polyclonal and multi specific (against several viral epitopes) response from CD4 and CD8 T lymphocytes is correlated with viral clearance (Orland *et al.,* 2001). Secreted cytokine profile may also influence the evolution of HCV infection, T- helper1 (Th1) cytokines stimulate cytotoxic CD8 lymphocytes while Th2 cytokines stimulate antibody production, thus, a Th1 cytokines secretion response has been associated with healing after acute infection ((Mondelli *et al.*, 2005). If the immune response is late, less efficient (of lower intensity), against a lower number of viral epitopes and does not persist for a sufficient period, HCV infection tends to become chronic, also, patients developing chronic infection have a Th2 cytokine secretion (Heller and Rehermann, 2005).

Data regarding the persistence of T lymphocyte response after acute infection is controversial and suggests that while CD4 T lymphocyte response persists several years after acute infection, CD8 T lymphocyte response fades in time (Orland *et al.*, 2001).

2-11-2-2-Humeral immune response:-

HCV specific antibodies become detectable in serum after the onset of cellular immune response and aminotransferase elevation (Heller and Rehermann, 2005). The first detectable antibodies in serum, HCV specific, are those that target NS3 region (anti c-33 antibodies) and core (anti 22c or anti capside antibodies). Later, region NS4 specific antibodies and those directed against envelope proteins (E1 and E2) appear (Orland *et al.*, 2001). Unlike HBV infection, in which surface antigen specific antibodies have neutralization capacity, in HCV infection a protective role of anti HCV antibodies has not been proven, furthermore in HCV infected patients, specific antibodies appearance is variable: early antibodies after exposure do not appear as in HBV infection, in which HBc IgM antibodies appearance is a marker of recent infection and, in some cases of HCV infection, specific antibodies may not appear at all, there are other important differences regarding the immune response between HCV and HBV infection: anti HCV antibodies levels are lower than HBV antibodies levels by at least 2 log and the profile of anti HCV antibodies is narrower than that in HBV infection. Also, anti HCV antibodies do not persist for the rest of the life, disappearing at 10-20 years after healing (Orland *et al.*, 2001).

Currently, the mechanisms of HCV persistence, despite specific antibodies and cellular immune response occurrence, are still not completely known, Viral eradication, viral recurrence although the majority of researchers consider that the patients cured after acute hepatitis C achieved viral eradication (Afdhal, 2004). Using PCR tests, sequences of RNA have been identified in serum and/or monocytes from peripheral blood of some patients at 5 years since the spontaneous healing of acute hepatitis, in these

patients, negative chains of HCV-RNA have been identified in the majority of peripheral monocytes, demonstrating that intermediary replicative forms of HCV may persist for many years after apparent healing, similar data have been obtained from patients with sustained virologic response after antiviral treatment. Although further confirmation is required, these data have major clinical implications, especially in cured patients in which HCV could reactivate if they would receive immune suppressor treatment – just like in occult HBV infection (Mondelli *et al.*, 2005). Another important aspect is represented by recurrence of viremia observed in patients in whom HCV-RNA tests have been negative for 4 months after aminotransferase normalization. Because disappearance of specific CD4 T lymphocyte response preceded HCV recurrence, this observation indicates that HCV is controlled but not completely eradicated in the first months after the (clinical) healing of acute hepatitis (Rehermann and Nascimbeni, 2005) .

2- 12- Prevention and Control:-

Viral hepatitis type C is a worldwide public health problem of major concern. Waiting for a safe and effective vaccine able to confer protection to susceptible individuals, public health challenges for controlling hepatitis C require implementation of primary prevention measures that reduce risks of acquiring/transmitting HCV infection, screening for safe blood and blood products, use of disposable syringes and needles and of universal precautions have dramatically reduced risk of infection in medical setting. Health education, counselling and testing of individuals at risk provide opportunities for controlling HCV infection (Zanetti *et al.,* 2003). HCV infection can only be prevented by avoiding contact with the virus, there is no vaccine and the heterogeneity of the virus makes it difficult to develop a conventional vaccine in the near future , no vaccine protects against contracting hepatitis C, or helps to treat it, vaccines are under development (Nevens *et al.*, 2003). The risk of infection can be reduced by avoiding.

Unnecessary and unsafe injections, unsafe blood products, unsafe sharps waste collection and disposal, use of illicit drugs and sharing of injection equipment, sharing of sharp personal items that may be contaminated with infected blood. Tattoos, piercings and acupuncture performed with contaminated equipment, education and counseling on options for care and treatment, immunization with the hepatitis A and B vaccines to prevent coinfection from these hepatitis viruses to protect their liver Early and appropriate medical management including antiviral therapy if appropriate and regular monitoring for early diagnosis of chronic liver disease (Fung *et al.*, 2008).

Chapter Three

Materials and Methods

3-1- Materials:-

3-1-1- Equipments and Instruments:

Table (3-1): The equipments and instruments that used in this study with their companies and countries of origin:

No.	Equipment & instrument	Companyl/ Origin
1	Autoclave	Memmert/Germany
2	High Speed Cold centrifuge	Techne (USA)
3	Oven	Memmert/Germany
4	Sensitive Balance	Sartorius/Germany
5	Water Bath	Memmert/Germany
6	Vortex	Cyan/ Belgium
7	Micropipettes 5-50, 0.5-10, 100-1000µl	Cyan/ Belgium
8	Nanodrop	THERMO/ USA
9	Exicycler Real-Time PCR	Bioneer/ korea
10	Eppendorf tubes	Bioneer/ korea
11	Multichannel pipette	Cyan/ China
12	Exispin vortex centrifuge	Bioneer/ Korea
13	Elisa Reader	Bio Tech(USA)
14	Elisa Washer	Bio Tech(USA)
15	Micrometer stage	England

3-1-2- The kits used in this study with their companies and countries of origin:- (www.dialab.at)

Table (3-2): Third generation ELISA (IgG) HCV Kit (DIALAB,Qsterreich)

NO.	Components	Volume
1	Micro well plate	1 block (96 wells)
2	Positive control	1 Vial (0.2 ml, antibodies diluted in protein stabilized buffer containing preservatives: o.1% proclin300).
3	Negative control	1 Vial (0.2 ml, protein stabilized buffer containing preservatives: o.1% proclin300).
4	Enzyme conjugate	1 Vial (13ml, Horseradish peroxidase (HRP)-Conjugated rabbit anti-human IgG antibodies).
5	Substrate solution A	1 Vial (8ml of 3, 3', 5, 5'-Tetramethyl-benzidine (TMB) dissolved in citric acid).
6	Substrate solution B	1 Vial (8ml of urea peroxide solution)
7	Stop solution	1 Vial (0.5M sulphuric acid,8ml,readey to use)
8	Sample diluents	1 Vial (13ml,protein-stabilized buffer, casein and sucrose solution)
9	Wash buffer	1Vial, 50ml,PH7.4,PBS(Containing Tween 20 as a detergent, concentrate[20x])
10	Cardboard plate cover sheets	2

Table (3-3): The Transaminase kit ALT (www.BioMerieux.com, France) :-

NO.	Reagent	Components
1	Reagent 2	GPT substrate/ Phosphate buffer pH 7.5 , 95 mmol/L, Alanine 200mmol/L and α ketoglutarate 2mmol/L.
2	Reagent3	Color reagent/ 2.4 dinitrophenylhydrazine 1mmol/L and HCl 0.1L/L.
3	Reagent4	Standard/ pyruvate.

Table (3-4): AccuZol™ Total RNA extraction kit (www.bioneer.com, Korea).

NO.	Reagent	Volume
1	Trizol	100ml

Table (3-5): AccuPower ® HCV Quantitative RT-PCR Kit (www.bioneer.com, Korea).

NO.	Content	Volume
1	HCV Quantitative RT-PCR PreMix	1block (12 strip, 96 tubes).
2	HCV Standard RNA(2×10^2~ 2×10^6 Copies/ml)	5ml
3	Internal Positive control RNA	1ml
4	PCR Grade Water (NTC)	5ml
5	PCR Grade Water	44ml

3-1-3- Chemicals:-

Table (3-6): The solutions with their companies and countries of origin used in this study:

No.	Chemical	Company and Origin
1	Ethanol	BDH (England)
2	Isopropanol	BDH (England)
3	DEPC water	Bioneer/ Korea
4	Free nuclease water	Bioneer/ Korea
5	Phospho buffer solution (PBS)	Bioneer/ Korea

3-2- Methods:-

3-2-1- Patients samples :

This study was carried out from December 2012 to July 2013, a total of 5179 Individuals were donors in the central blood bank at AL-Muthanna Province

3-2-2- Blood samples collection:

A Sample of 5ml of fresh blood was drawn from individual and collected in a sterile plastic tube, left to clot at room temperature then centrifuged at 2000 rpm for 10 minutes, then serum was collected in sterile tube and examined by ELISA Assay to detect anti HCV then stored at –20 c until examined by Real—Time qPCR technique.

3-2-3-Investigation of anti HCV antibodies (IgG) in serum by third generation ELISA test (DIALAB):-

3-2-3-1-Principle

Anti-HCV enzyme immunoassay kit was a qualitative determination of Abs to HCV (anti-HCV) in human serum samples, diluted patient's sample (serum) was added to microtiter wells precoated with purified antigen mimicking the core, NS3, NS4, NS5 gene segments of HCV genome, these peptides have been shown to react and bind with the predominant classes of anti-HCV Abs present in HCV positive serum.

After incubation, peroxidase- conjugated anti-human IgG Ab was added to form a detectable complex, and then, substrate was added to form a colored complex. The intensity of color was proportional to the amount of anti-HCV

present in the sample, then, the reaction was stopped by the addition of acid and the resulting color intensity can be read spectrophotometrically at 450 nm.

For the detection of antibodies to HCV antigens, ELISA (DIALAB) was used as following of the manufacture.

3-2-3-2-Preparation of reagents (according to manufacturer's instructions):

3-2-3-2-1- Preparation of washing solution

Washing solution was prepared from 20X concentrated solution by diluting it to 20-fold with distilled water at room temperature.

3-2-3-2-2- Preparation of conjugate solution:

A proper amount of concentrated conjugated was diluted by conjugate buffer solution (ratio is 2:100) in accordance with the number of wells which has been used.

3-2-3-2-3- Preparation of substrate solution:

A proper amount of concentrated Tetra methyl benzidine (TMB) was diluted by substrate buffer solution (with 2:100) in accordance with the number of wells which has been used; this was repeated for each Test plate. This solution is stable for 4 hours at room temperature but should be prepared again if the color of the solution turns blue.

3-2-3-3- ELISA Procedure (according to manufacturer's instructions):

1-The reagent and sample were allowed to reach room temperature (18-30 ^{0}C) for at least 15-30 minutes.

2- Wash buffer concentrate was checked for the presence of salt crystals. If crystals have been formed in the solution was resolubilized by warming at 37 ^{0}C until crystals were dissolved.

3- Stock wash buffer was dilute 1to 20 with distilled or deionized water. Using only clean vessels to dilute the wash buffer .

4- Numbering of wells: The strips needed was place on strip-holder and well was numbered including three negative control as B1, C1, D1 and two positive control as E1, F1 and one Blank as A1, neither samples nor HRP-conjugate was added into the Blank well .

5- Hundred µl of specimen diluent was added into each well except the blank.

6- Ten µl of Positive control, Negative control and Specimen was added into their respective wells. By using a separate disposal pipette tip for each specimen , Negative and positive control as to avoid cross-contamination. The plate was mixed by tapping the plate gently.

7-Incubation: The plate was covered with plate cover and was incubated at 37^{0}C for 30 minutes.

8-Washing (1): After incubation, The plate cover was removed and discarded and each well was washed 5 times with diluted wash buffer. The wells allowed soaking for 30-60 seconds after washed cycle, the strips plate was turned onto paper or clean towel, and tapped it to remove any remainders.

9-Hundred µl of HRP-Conjugate was added to each well except the Blank.

10- The plate was covered with the plate cover and incubated for 30 minutes at 37 ^{0}C.

11-Washing (2): At the end of the incubation, the plate cover was removed, each well was washed 5times with diluted wash buffer as in step 6.

12-Coloring: Fifty µl of chromogenic B and 50 µl of chromogenic A Solution was dispensed into each well including the Blank and mix by tapping the plate gently. The plate was incubated at 37 ^{0}C for 15minutes avoiding light.

13- Stopping reaction: By using a multichannel pipette or manually. A fifty µl stop solution was added into each well and was mixed by tapping the plate gently. Intensive yellow color was developed in positive control and anti-HCV Positive sample wells.

14-Measuring the Absorbance: The plate reader was Calibrated with the Blank well and the absorbance was read at 450nm. The Cut-off value was calculated and the results were recorded .

The absorbance was read within 5minutes after stopping of the reaction.

15- The mean absorbance value for three negative controls.

Calculation of Cut-off value (C.O.) =*NC + 0.12

Positive: ratio absorbance ≥ cut-off

Negative: ratio absorbance < cut-off

3-2-4- Determination of serum Alanin Transaminase :-

3-2-4-1-Principle

The coloric determination of ALT activity according to the method of Reitman and Frankel, (1957) was used as follows:

$$\text{Alanin} + \alpha\text{- Ketoglutarate} \xrightleftharpoons{\text{ALT}} \text{Pyruvate} + \text{glutamate}$$

The pyruvate formed was measured in its derivative form, 2,4 dinitrophenyl hydrazone. The formation of pyrovate was spectrophotometrically measured at 490-520 nm wave length.

3-2-4-2- Procedures of ALT tests:-

1-One ml of R2 (phosphate buffer pH7.5, alanine and α- ketoglutarate)was added to ALT tubes. Then the tubes were incubated for 5 minutes at 37◦C.

2-A volume of 200µl of serum was added to each tube.

3-The tubes were mixed and incubated for 30 minutes at 37◦C.

4-One ml of R3 (2, 4 dinitrophenyl- hydrazine) was added to each tube.

5-The tubes were mixed and left at 25◦C for 20 minutes.

6-Ten ml of NaOH (0.4N) was added to each tube.

7-The tubes were mixed and measured spectrophotometrically at 505 nm within 5 minutes.

8-The normal value should be ≤ 40 units/ml for GPT.

3-2-5-Molecular Study:-

In this study, Reverse Transcription Real-Time PCR technique was used for detection of hepatitis C virus in patient's serum. This technique was carried out according to instruction of kit of manufactures.

3-2-5-1-Viral RNA extraction:-

Viral RNA was extracted from serum patient samples by using AccuZol™ Total RNA extraction kit (Bioneer, Korea) and done according to company instructions as explained in the following steps:

1. Two hundred and fifty µl of serum samples were transferred by sterile pipette into sterile and clean 1.5ml Eppendorf tube, then 750 Accuzol reagent mixed by vortex.
2. Two hundred Chloroform µl were added to each Eppendorf tube and shaken vigorously for 30 seconds.
3. The mixture was incubated on ice for 5 minutes.
4. After that, the mixture was centrifuged at 12,000 rpm, 4C°, for 15 minutes.
5. Supernatant was transferred to a new Eppendorf tube, and 500µl isopropanol was added.
6. The mixture was mixed by inverting the tube 4-5 times and incubated at 4C° for 10 minutes.
7. The mixture was centrifuged at 12,000 rpm, 4C°, for 10 minutes.
8. The supernatant was discarded.
9. Eighty per sent Ethanol was added into each tube and mixed by vortex, then centrifuged at 12,000 rpm, 4C° for 5 minutes.
10. The supernatant was discarded and the RNA pellet was left to dry at room air for 5 minutes.
11. After that Diethyl Pyro Carbonate (DEPC) water (50µl) was added to RNA pellet tubes and mixed by vortex to dissolve the RNA pellet.

12. The extracted RNA sample was kept at -20 freezers.

3-2-5-2-Estimation of RNA extraction from serum samples:-

The extracted viral RNA and total RNA from serum patient samples were estimated by using Nanodrop spectrophotometer that used in the measurement of the RNA concentration and purity at absorbance 260/280 nm at ratio 1.8 as pure RNA, and done as the following steps:

1-The appropriate application (RNA) was chosen after the opening up the Nanodrop software.

2- A dry Chem-wipe was taken and cleaned the measurement pedestals several times. Then carefully pipet 1µl of ddH2O onto the surface of the lower measurement pedestals .

3- The sampling arm was lowered and clicking OK to initialize the Nanodrop, then cleaning off the pedestals and 1µl of the appropriate blanking solution was added as black solution which is the same elution buffer of RNA samples.

4- After that, the pedestals were cleaned and pipet 1µl of RNA sample for measurement.

3-2-5-3- Quantitative Reverse Transcription Real-Time PCR:-

RT-qReal-Time PCR was performed for detection and quantification of viral loads of hepatitis C RNA virus by using **AccuPower ® HCV Quantitative RT-PCR Kit** that contained specific primers and probe for hepatitis C RNA virus. This technique was carried out according to kit company instructions as following preparation:

3-2-5-4- qRT-Real-Time PCR master mix preparation:-

RT-qReal-Time PCR master mix was prepared according to company instructions as the following table:

Table (3-7): Quantitative Standard curve positive control RT- qPCR master mix .

RT-qPCR master mix	Volume
HCV Standard RNA (2×10^2~2×10^6)	5µL
Internal Positive Control (IPC)	1µL
PCR Grade Water	44µL
Total	**50µL**

Table (3-8): Samples of RNA RT- qPCR master mix .

qRT-PCR master mix	Volume
Sample RNA template	5µL
Internal Positive Control (IPC)	1µL
PCR Grade Water	44µL
Total	**50µL**

Table (3-9): Negative control RT- qPCR master mix:

qRT-PCR master mix	Volume
Non – Template Control (NTC)	5µL
Internal Positive Control (IPC)	1µL
PCR Grade Water	44µL
Total	**50µL**

This RT-qPCR master mix reaction components that are mentioned in the table above were added into standard qPCR tube contained reverses transcriptase, specific primer and probe premix, then all strips tubes were mixed and centrifuged for 3000 rpm for 3 minutes in exispin vortex centrifuge, after that transferred into exicycler Real-Time PCR thermocycler.

3-2-5-5- Real-Time PCR Thermocycler conditions:-

Real-Time PCR Thermocycler conditions were set according to kit instructions as following table (3-10):

Table (3-10): Real-Time PCR Cycle.

Step	Condition	Cycle
Reverse transcriptase	95 °C 15 min	1
Pre-Denaturation	95 °C 5 min	1
Denaturation	95 °C 5 sec	45
Annealing/Extension	55 °C 30 sec	
Detection (Scan)	FAM-BHQ: Target	TAMRA-BHQ: IPC

RT-PCR Data analysis:

RT-PCR data analysis was performed by calculating the threshold cycle (CT) value represented the +ve amplification of alleles gene in RT cycle numeral.

3-2-6- Statistic analysis:-

Statistical analysis was conducted to determine the statistical differences among different groups using differences among different groups using ready – made statistical design statistical package for social science. Probabilities of ($P \leq 0.05$) were considered statistically significant.(SPSS version 13).

Also Statistical analysis was conducted by using ready-made (Standard curve Expert 1.3) (Sorlie, 1995)

Chapter Four

Results & Discussion

4-1- Seroprevalence of HCV infection :-

Hepatitis C virus infection is a worldwide health problem, causing chronic hepatitis in approximately 85% of the cases, with a frequent progress to severe forms of liver damage like cirrhosis and hepatocellular carcinoma (Levrero, 2006). Serologic tests for detection of HCV antibodies are important first-line tests in screening and diagnosis of HCV infection, the presence of anti-HCV antibody in serum and plasma reflects exposure to the virus and may indicate an acute or chronic infection (Mahy and Van Regenmortel, 2010).

The present study was the results of serological examination by indirect Enzyme Linked Immunsorbent Assay (ELISA) for detection of Antibodies of HCV in central blood bank in Al-Muthanna province showed that 20 out of 5179examined donors were positive in percentage 0.386%(Table4-1) (Figure4-1-Appendixes).

First-generation ELISA used the c100-3 epitope of an NS protein (NS4), the sensitivities of these ELISA were low for a high-prevalence population (approximately 80%), and rate of false positive was high for low prevalence population like blood donors (Richter, 2002). This led to development of more sensitive and specific second-generation ELISA that incorporation additional antigens from the core, NS3, and NS4 regions these modifications markedly improved sensitivity and specificity (Zuckerman *et al.*, 2009).

The latest , third-generation ELISA detect antibodies against HCVcor,NS3, NS4 and NS5 antigens the diagnostic specificity of third-generation assays and sensitivity rates is approximately 99% (Colin *et al.*, 2001).

Table (4-1): Positive number and total percentage of infected donors.

Total No. of samples	No. of positive samples	Percent %
5179	20	0.386%

The results of the present study revealed that the prevalence of HCV was estimated as (0.38%), and this result is less than those in previous studies conducted in Iraq by Noaman (2012), AL-Badry (2011) , AL-Saaedi (2001), and Abdul-Aziz *et al.,* (2001). They reported the prevalence in Diyala province (1,15%) , in Thi-Qar province (1.4 %) , in AL- Diwania (0.45), and in Kirkuk province (0.93) respectively, and more than results obtained by Tawfeeq (2013) in Babylon province (0.29%), Amin (2011) in Musol (0.07%) and Hussein (2010) in Sulaimania (0.1%) and this result was in agreement with the results obtained by Hanan *et al*., (2011) in Baghdad province (0.3%) and Abdul-Kareem *et al.*,(2001) in AL-Najaf province (0.34%).

The prevalence of anti-HCV antibodies in some countries among normal population where (2.4%) anti-HCV positivity were found in Yemen, Sudan (1.9%) , Egyptian blood donors(19 %) (Hussain *et al.*,2008). Kuwait (0.8%) (Ameen *et al.*, 2005). Iran (0.13%) per 100,000 Iranian blood donations) (Kafi-abad *et al.*, 2009). Lebanon (0.6%) (Irani-Hakime *et al.*, 2001). Jordan (0.9%) (Al-Gani, 2011). Japan (0.49%). China (1%) (Tanaka *et al.,* 2004). Belgium(0.87%) (Van Damme *et al.,* 2002). Italy(3.2%) (Bellentani and Tiribelli 2001). France (1.3%) (Theodore and Mazen 2006). The HCV prevalence among the blood donors in Kosovo is(0.3%) (Fejza and Telaku 2009).The World Health Organization estimates that the worldwide prevalence of HCV infection is approximately 3% with significant geographical and ethnical variations, possibly due to the presence and frequency of risk factors associated to the transmission of HCV inside a community.

The small percentage (0.386%) probably due to sampling from healthy donors only and not receiving the donors that proven to have a previous infection compared to most researches that take samples from various study groups example (blood donors ,Thalassemic patients, Renal dialysis, Medical staff, polycythaemia patients) (AL-Badry, 2011).

4-1-1- Seroprevalence of HCV Infection in relation with Age.

The results of seroprevalence of HCV infection by using indirect ELISA in relation to the different age groups 20-30 years , 31-40 years ,41-50 years and 51->60 years old were 20% ,35% ,35% and 10% respectively, the highest rate of the seropositivity was in age groups31-40 years and 41-50 years 35% and the lowest rate of seropositivity was in age groups 51->60 years old 10% . There was significant differences at (P <0.05) (Table 4-2).

Table (4-2): Seroprevalence of HCV infection in different Age of donors.

Age (years)	No. infection	% of infection
20-30	4	20
31-40	7	35*
41-50	7	35*
51->60	2	10
Total	20	100

* Significant differences at (P <0.05).

This study, which was carried out in adult population (20 years or older), showed an increasing prevalence of infection with age (Table 4-2),(Figure 4-2-Appendixes) , significant inter age group differences in the prevalence of HCV among our blood donors with the lowest rate of 20% in the age group of 20-30 years and 10% in the age group of 51->60 years old. High rates of HCV infection of 35% was found within the age range of 31-40and 41-50 years old. This distribution is similar to work done by Arora *et al*., (2011) was described in countries like Japan and Italy, where the estimated peak incidence of infection was 30 to 50 years age and also similar to report of work done by Abdulamir, (2012) who documented Percent of prevalence according to age group as 34% of patients 40-49 years old , 32% which represents 30-39 years, 20% over 50 years old, 14%for the age range 20-29 years old in Iraq and Damulak *et al*., (2013) who documented the highest age prevalence of HCV among blood donor above 35 years in Nigeria.

4-1-2- Seroprevalence of HCV Infection in relation with Location.

The results of seroprevalence of HCV infection by using indirect ELSA in relation with location of different geographical study area, that the prevalence rate of infection in Samawa , Rumathya , Majad and Warkaa were 60%, 20%, 10% and 10% respectively. The highest percent of seropostively was in Samawa 60% and the lowest was in majad and warkaa 10%. There was significant differences at (P <0.05) (Table 4-3).

Table (4-3): Seroprevalence of HCV infection according of location.

City	No. Infection	%
AL-Samawa	12	60 *
AL-Rumathya	4	20
AL-Majad	2	10
AL-Warkaa	2	10
Total	20	100

* Significant differences at (P <0.05).

The prevalence of HCV according to location in Samawa city was 60% and the lowest percent of infection in AL-Rumathya was 20%, Al-majad and Al-warkaa was 10% (Table 4-3)(Figure 4-3- Appendixes).This result was in agreement with the results given by AL-Badry (2011), Mahmood (2005) and AL-Saaedi (2001). The high percent age of spread hepatitis in Samawah city over the rest of other regions in the province may be because of the fact that the city is more developed than other regions or because of the many donors from Samawa are more than those from other sites or probably because of the use of razor blades and tattoos is more in Al-Samawah furthermore, large number of Hospital staff are in contact with infected pepole and contaminated tools.

Table (4-4) Comparison between concentration of control and infected HCV patients (ng/ml) according to location.

City	Control (normal)		HCV
	mean OD	mean ng/ml	mean ng/ml
AL-Samawa	<0.193	Non	55.83+14.38 A
AL-Rumathya	<0.193	Non	77.86+12.11B
AL-Majad	<0.193	Non	34.85+13.28 C
AL-Warkaa	<0.193	Non	22.68+9.082 D

Different letters A,B are significant at (P<0.05) to compression rows.

Depending on location the results by ELISA show that the highest concentration of HCV (ng/ml) in AL-Rumathya city was 77.86+12.11 ng/ml and the lowest concentration in AL-Warkaa city was 22.68+9.082 ng/ml with significant differences at (P<0.05) (Table 4-4).

4-1-3 - Results of Seroprevalence infection of HCV in relation with months of year.

The results of seropositivity of HCV infection by using ELSA in relation with different months of year showed that the seropositivity in Dec-12 , Jan-13 , Fab-13, March-13 ,Apr-13, May-13and Jun-13 was 5%, 5%, 15%, 40%, 15%, 10% and 10% respectively. The highest seropositivity percent was in March 40% while the lowest seropositivity percent was in Dec. and Jan. 5%. There was significant differences at (P <0.05) (Table 4-5).

Table(4-5): Number and Infection percent of HCV Infection on Months

Months-year	No of infection	% of infection
Dec	1	5
Jan	1	5
Feb	3	15
Mar	8	40*
Apr	3	15
May	2	10
Jun	2	10
Total	20	100

* Significant differences at (P <0.05).

Hepatitis C Virus infection takes place all year round, that our study results showed that the peak of infection was observed in March 2013 the rate of seropositive was 40% and low seropositive percent in December 2012- January - 2013 was 5% (Table 4-5)(Figure 4-4-Appendixes). This result is in agreement with the results obtained by Arora *et al.,* (2011) in India that they found the peak of HCV infection was in March because of the change in temperature .

The biggest obstacle most people with Hepatitis his face during the cold and flu season is October-march of more than infection of months the virus of hepatitis (Lok and McMahon, 2007). The reason is the large number of flu virus to gather, which is a catalyst for the development of virus and the reason that causes disturbance in the immune system during the period to gather and allow him to obtain secondary infection (Heung *et al.,* 2002). During flu season is increasingly taking acetaminophen drugs, a staple in the pharmaceutical arsenal for cold and flu symptoms, acetaminophen is a widely used over-the-counter pain reliever and fever reducer, as one of the leading causes of liver toxicity from an accidental overdose, acetaminophen-containing drugs warrant an additional level of caution for people with Hepatitis C (Daly *et al.,* 2008). Unfortunately, acetaminophen is either the primary or auxiliary ingredient in a

majority of medications for cold and flu. The medical community is fully aware that too much acetaminophen can injure the liver. acetaminophen toxicity is one of the most common causes of poisoning worldwide and common cause of acute liver failure (Larson *et al.*, 2005).

4-1-4- Results of HCV antibody concentration .

The results of HCV antibody concentration in relation to age groups by using indirect ELSA as compared with control, showed that the concentration in age groups 20-30 , 31-40 , 41-50 and 51->60 years were 69.84 ng/ml , 44.27 ng/ml, 43.21 ng/ml and 46.53 ng/ml respectively.

The highest concentration of HCV antibody was in age group 20-30 years old 69.84±11.74 ng/ml and the lowest concentrations was in age group 41-50 years old 43.21±6.71 ng/ml with significant differences at (P<0.05) (Table 4-6).

Table (4-6): Comparison between concentration of HCV Ab in control and infected HCV patients (ng/ml) according to age.

ELISA age group	Control (normal) mean OD	mean ng/ml	HCV Ab mean ng/ml
20-30	<0.193	Non	A 69.84±11.74
31-40	<0.193	Non	B 44.27±7.53
41-50	<0.193	Non	B 43.21±6.71
51->60	<0.193	Non	B 46.53±8.95

Differences letters A,B are significant at (P<0.05) to compression rows.

The viral persistence in infected people may be because the weak antiviral immune response to viral antigens or because the high rate of HCV genetic variability is thought to assist the persistence of the viral infection or may be found co-infection example (HBV, HIV) .

4-1-5- Results of ALT Detection in seropositive patients.

The results of ALT concentration in seropositive patients as compared with control group showed that the concentrations among different age groups of seropssitive patients 20-30 years , 31-40years , 41-50 years and 51->60 years were 85.25± 17.38, 80.71± 16.o4, 103.28± 23.27 and 64± 14.75 respectively as compare with 12.75 ±4.14, 13.14±4.27, 12.85±2.85 and 15.5±5.31 of normal control donors. The highest ALT concentration was in age group 41-50 and the lowest concentrate was in age group 51->60 and there was a significant difference between age group and control at (P<0.05) (Table 4-7.Figure 4-5) .

Table (4-7) Comparison between concentration of HCV in control and infected HCV patients (ng/ml) by ALT according age.

Age (Years)	HCV Patient (mean+SD) ng/ml	Control (mean+SD) ng/ml
20-30	85.25±17.38 A,a	12.75±4.14 A,b
31-40	80.71±16.04 A,a	13.14±4.27 A,b
41-50	103.28±23.27 B,a	12.85±2.85 A,b
51->60	64±14.75 C,a	15.5±5.31 A,b

Different letters a,b,c are significant at (P<0.05) to compression columns.
Different letters A,B are significant at (P<0.05) to compression rows.

Table (4-8) Concentration of control HCV (unit/ml) by ALT at 505 nm.

ALT concentration (unit/ml)	0	25	50	83	126
O.D at 505nm	0.2007	0.2132	0.2257	0.2447	0.2637

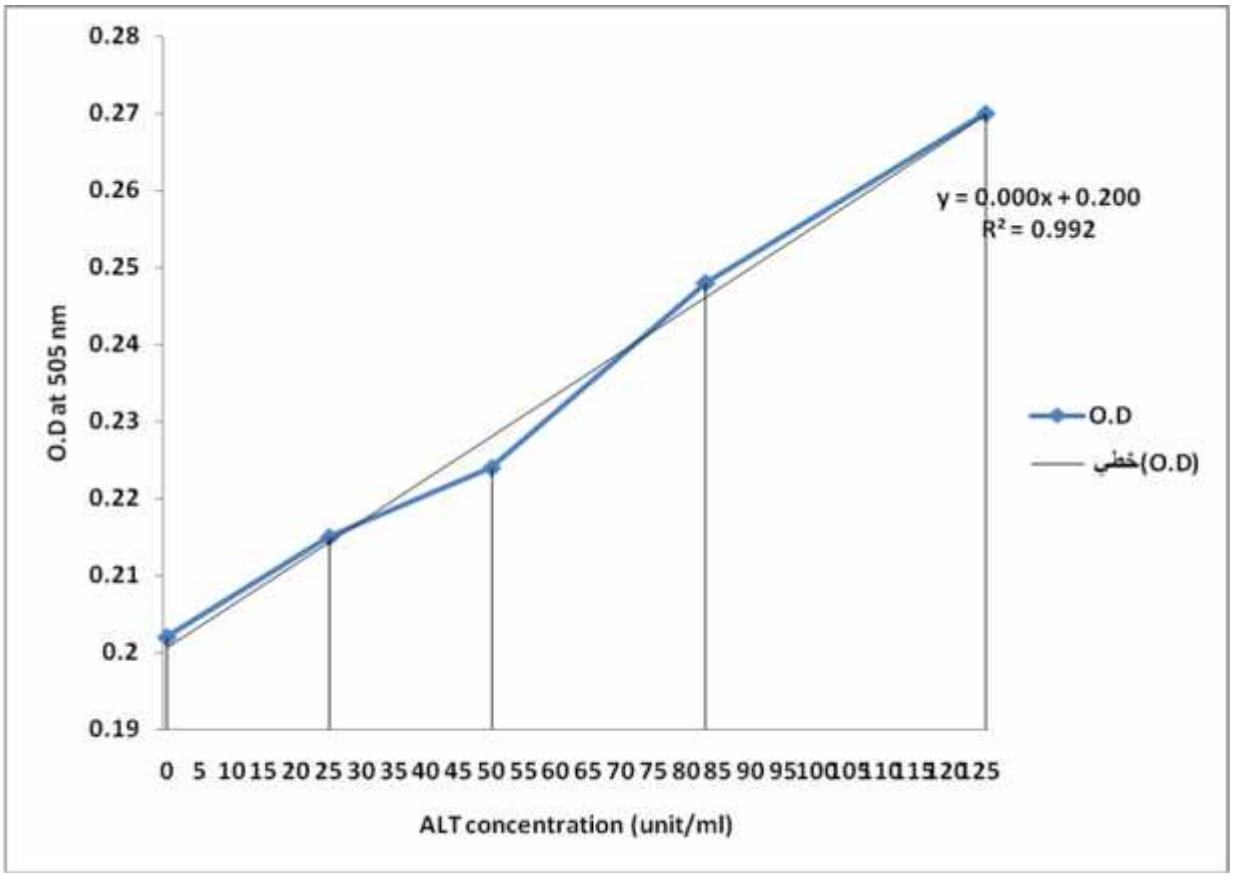

Figure (4-5): Standard curve of ALT.

ALT is found in kidney, heart, muscle and the greater concentration in liver as compared with other tissues of the body. ALT is purely cytoplasmic catalysing the transamination reaction (Mauro *et al.*, 2006) . Any type of liver cell injury can reasonably increase ALT levels, elevated values up to 300 U/L are considered nonspecific (Diana N.C., 2007). Marked elevations of ALT levels greater than 500 U/L are observed most often in people with diseases that affect primarily hepatocytes such as viral hepatitis, ischemic liver injury (shock liver) and toxin-induced liver damage. Despite the association between greatly elevated ALT levels and its specificity to hepatocellular diseases, the absolute peak of the ALT elevation does not correlate with the extent of liver cell damage (Kallei *et al.*, 1964). Viral hepatitis like A, B, C, D and E may be responsible for a marked increase in aminotransferase levels, the increase in ALT associated with hepatitis C infection tends to be more than that associated with hepatitis A or B

(Marcellin, 1999). Moreover in patients with acute hepatitis C serum ALT is measured periodically for about 1 to 2 years (Mauro *et al.*, 2006). Persistence of elevated ALT for more than six months after an occurrence of acute hepatitis is used in the diagnosis of chronic hepatitis .Elevation in ALT levels is greater in people with nonalcoholic steatohepatitis than in those with uncomplicated hepatic steatosis (Sheth *et al.*, 1997). In the recent study the hepatic fat accumulation in childhood obesity and nonalcoholic fatty liver disease causes serum ALT elevation, moreover increased ALT level was associated with reduced insulin sensitivity, adiponectin and glucose tolerance as well as increased free fatty acids and triglycerides (Burgert *et al.*, 2006). Presence of Bright liver and elevated plasma ALT level was independently associated with increased risk of the metabolic syndrome in adults (Shen *et al.*, 2005). ALT level is normally elevated during 2nd trimester in asymptomatic normal pregnancy (Bacq *et al.*, 1996). In one of the studies, serum ALT levels in symptomatic pregnant patients such as in hyperemesis gravidarum was 103.5U/L, in pre-eclampsia patients was 115U/L and in haemolysis with low platelet count patients showed 149U/L. However in the same study ALT rapidly drops more than 50% of the elevated values within 3 days indicating the improvement during postpartum (Wong *et al.*, 2004). One of the recent studies has shown that coffee and caffeine consumption reduces the risk of elevated serum ALT activity in excessive alcohol consumption, viral hepatitis, iron overload, overweight, and impaired glucose metabolism (Everhart and Ruhl, 2005).

4-2- Results of molecular technique :-

HCV infection can be confirmed by using highly specific reverse transcriptase polymerase chain reaction (RT-PCR) for HCV RNA detection. This test can detect HCV RNA in serum within 1-2 wks following exposure (Thakral, *et al.*, 2006). Real time – qPCR was used to amplify a sequence of DNA using a pair of oligonucleotide primers each complementary to one end of the DNA target

sequence. These are extended towards each other by a thermostable DNA polymerase in a reaction cycle of three steps: denaturation, primer annealing and polymerization. The reaction cycle comprises a 95°C step to denature the duplex DNA, an annealing step of around 55°C to allow the primers to bind and a 72°C polymerization step, Mg2+ and dNTPs are required in addition to template, primers, buffer and enzyme (Jackson, *et al.*, 2004). In the first cycle, the target DNA is separated into two strands by heating to 95°C typically for around 60 seconds, the temperature is reduced to around 55°C (for about 30 sec) to allow the primers to anneal to the template DNA, the actual temperature depends on the primer lengths and sequences, after annealing, the temperature is increased to 72°C (for 60–90 sec) for optimal polymerization which uses up dNTPs in the reaction mix and requires Mg2+, in the first polymerization step, the target is copied from the primer sites for various distances on each target molecule until the beginning of cycle 2, when the reaction is heated to 95°C again which denatures the newly synthesized molecules, in the second annealing step, the other primer can bind to the newly synthesized strand and during polymerization can only copy till it reaches the end of the first primer, thus at the end of cycle 2, some newly synthesized molecules of the correct length exist, though these are base paired to variable length molecules. In subsequent cycles, these soon outnumber the variable length molecules and increase two-fold with each cycle, if PCR was 100% efficient, one target molecule would become 2*n* after *n* cycles, in practice, 20–40 cycles are commonly used. (Turner *et al.*, 2005).

4-2-1- Reverse Transcription Quantitative Real-Time Polymerase chain reaction (RT- qPCR) test:-

RT-qPCR technique was performed for direct detection of Hepatitis C virus (HCV) patient serum by one step technique and the condition was designed in the Exicycle Real time PCR system (Figure 4-6).

Reverse Transcription qRT-PCR was condition using specific Taq-Man probe for detecting of HCV in serum sample and FAM dye and BHQ quencher (Figure 4-7).

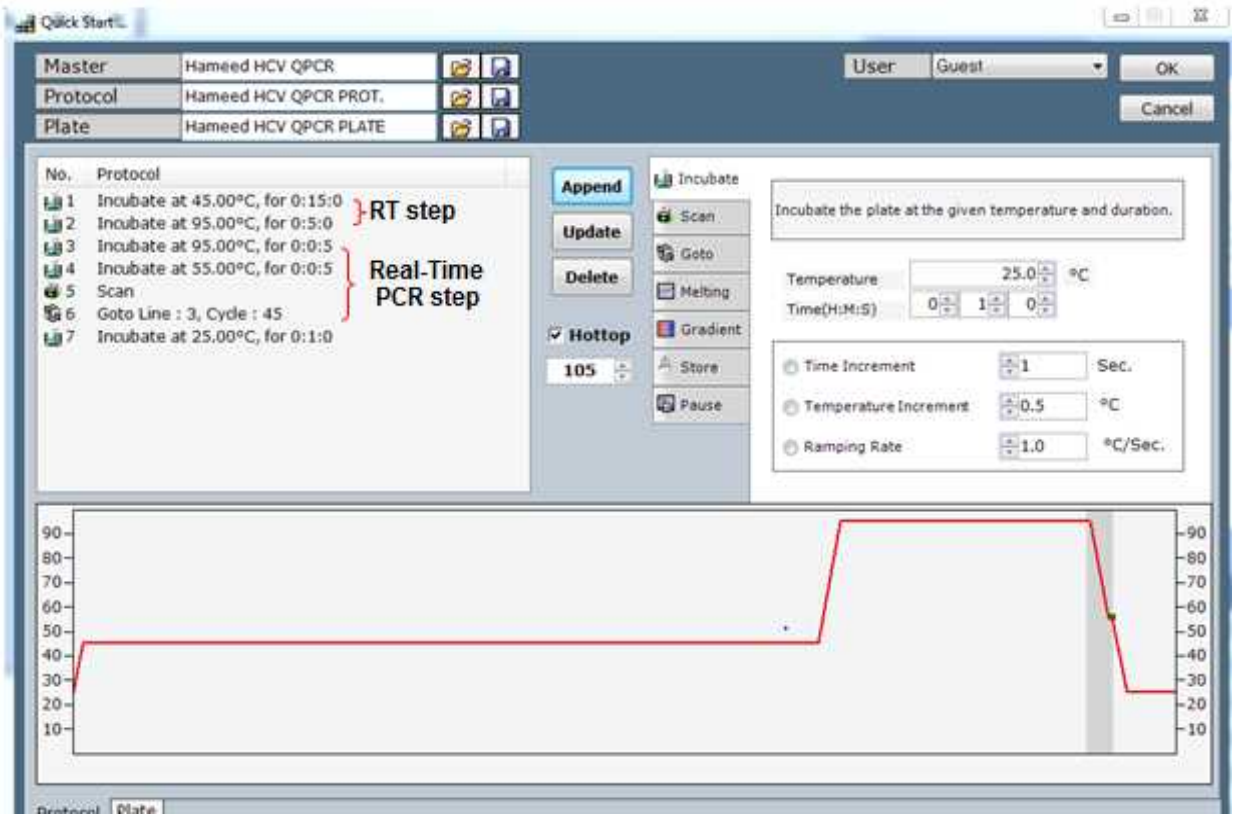

Figure (4-6): Quantitative Reverse Transcription Real-Time PCR Thermocycler condition for HCV in Exicycler Real-Time PCR system.

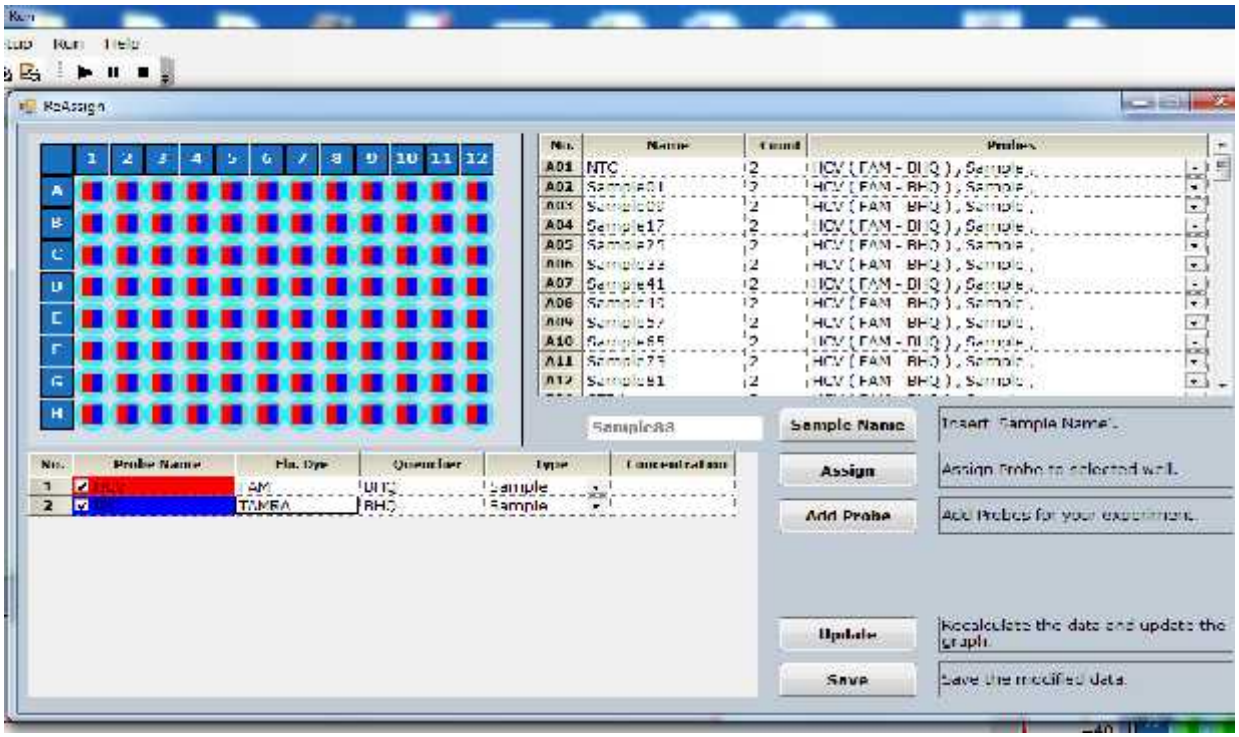

Figure (4-7): Quantitative Reverse Transcription Real-Time PCR plate design using TaqMan probe for HCV samples in Exicycler Real-Time PCR system.

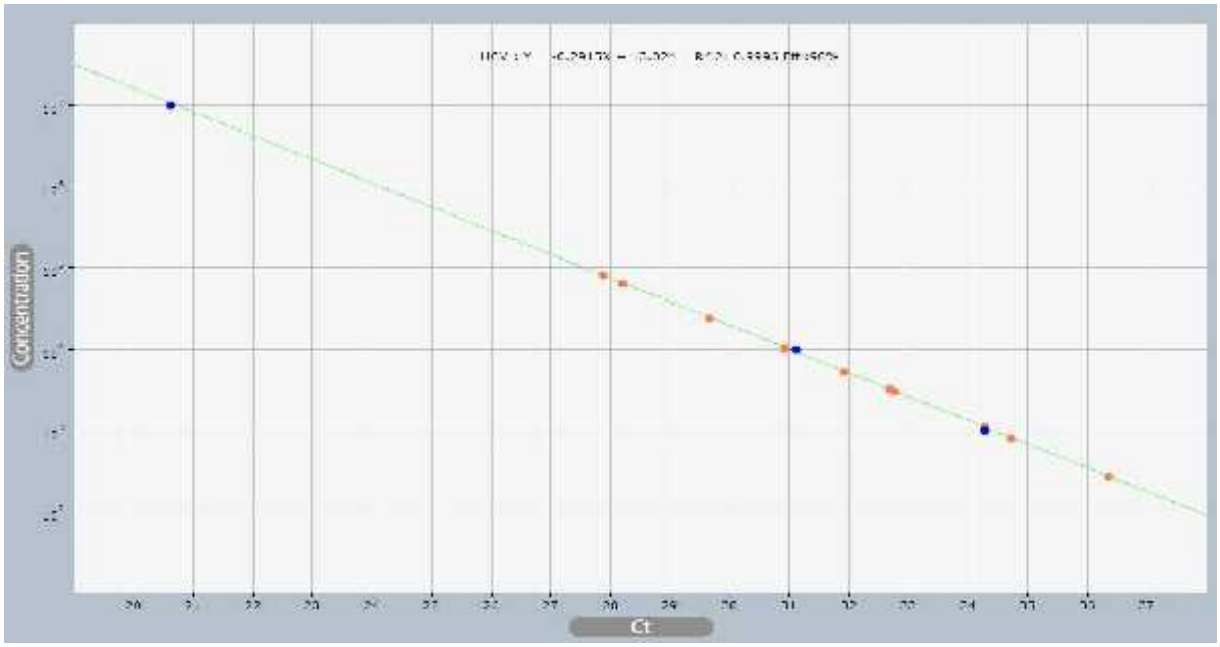

Figure (4-8): Quantitative Reverse Transcription Real Time PCR standard curve of HCV. That shown (10^7 IU, 10^4 IU ,& 10^2 IU HCV Standard) was used for detection of unknown viral load in HCV positive samples.

4-2-2- Results of molecular detection of HCV by using (RT- qRT- PCR):-

The results of detection of HCV in seropositive showed that out of (20) positive serum samples (13) samples were positive for HCV infection in percentage of (65%) Table (4-9) :

Table (4-9): percentage of positive HCV in serum samples by RT-PCR.

Result	No. of tested samples	Percent %
Positive	13	65%
Negative	7	35%
Total	20	100%

The amplification plot of control positive and control negative represents three internal positive control and three HCV negative samples (Figure 4-9).

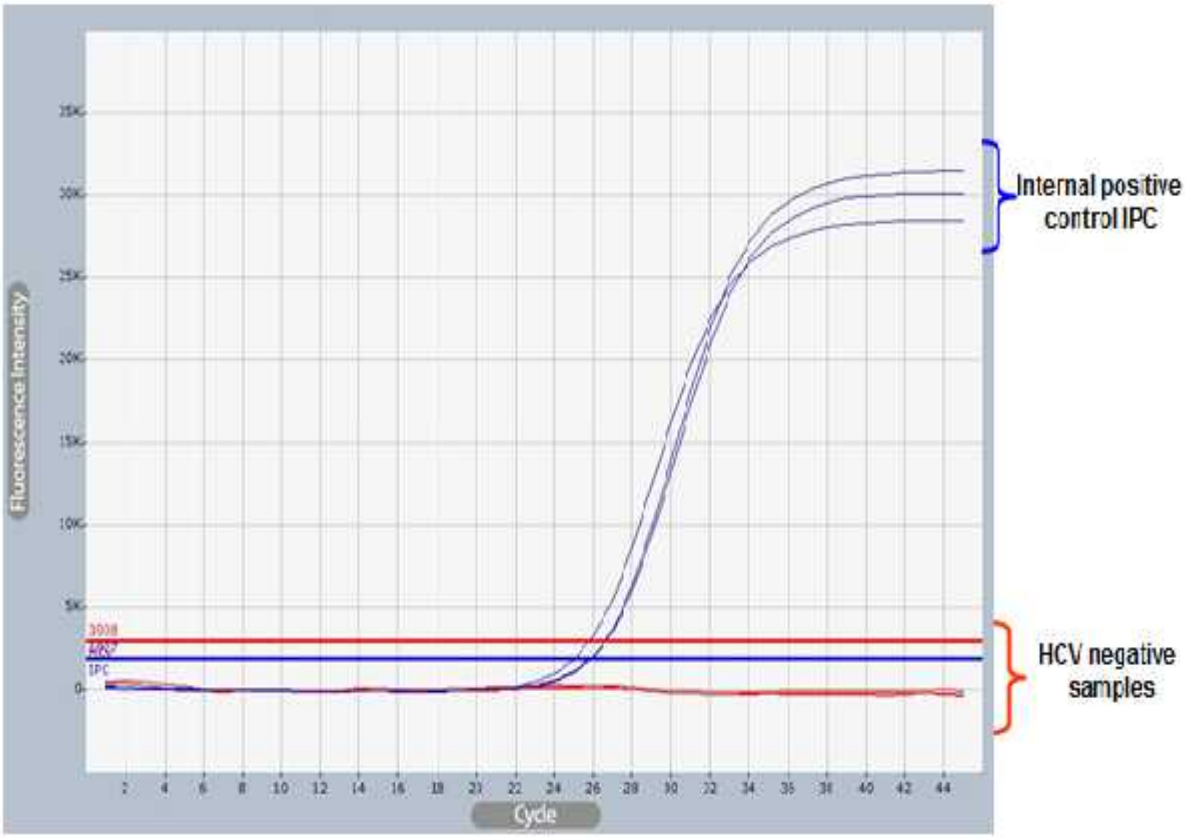

Figure (4-9): The amplification plot of Quantitative Reverse Transcription Real Time PCR of positive HCV. That shown the internal positive control and HCV negative samples in 45 qPCR cycles.

The results of amplification plot of tested samples represent (13) HCV positive results and 7 negative results as compared with HCV positive samples and positive control (Figure 4-10).

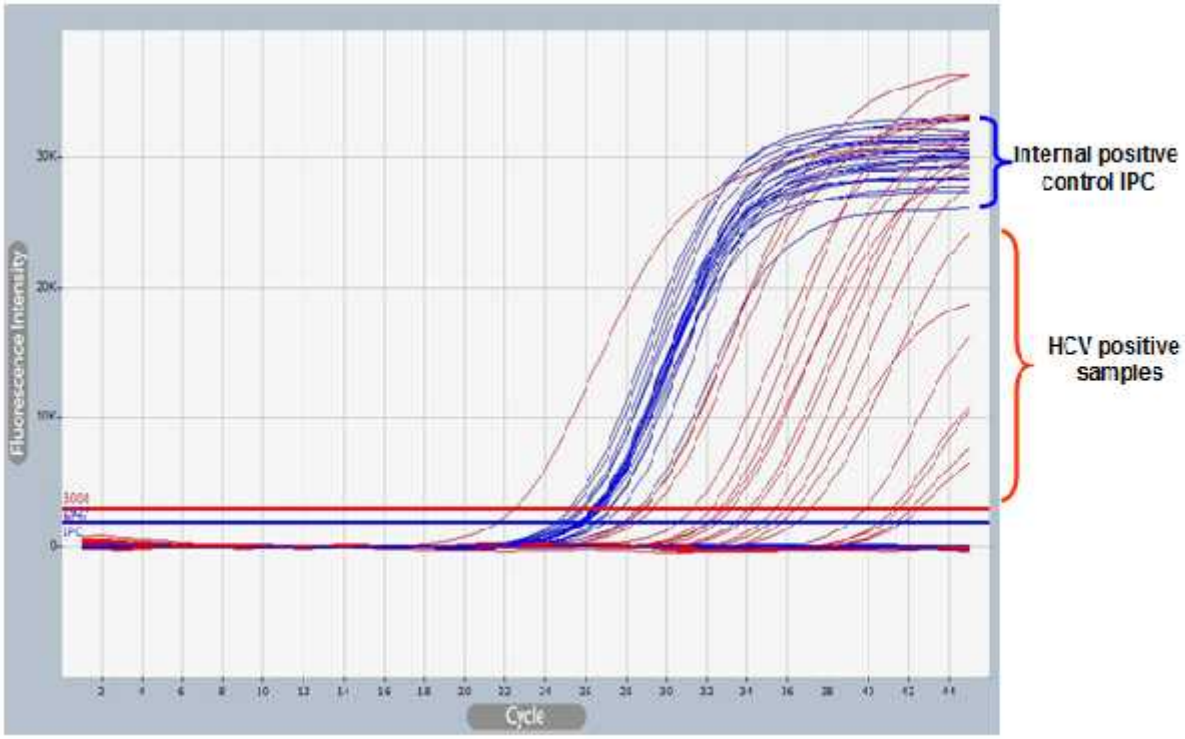

Figure (4-10): The amplification plot of Quantitative Reverse Transcription Real Time PCR of positive HCV. That shown the internal positive control and HCV positive samples in 45 qPCR cycles**.**

In this study, HCV infection was found only in 20 samples of the patient serum by ELISA and ALT tests but RNA HCV was detected in 13 samples of them by RT-q PCR and 7 samples of HCV-RNA negative (Table 4-9, Figure 4-10). Where the results of this study showed a significant difference between ELISA and Real time qPCR techniques and this results were in agreement with other studies obtained by Tawfeeq (2013) Babylon province, AL-Badry (2011) Thi-Qar province, Ali and Lal, (2010) Pakistan, who found difference between ELISA and PCR results. And this significant difference between both techniques may be due to the false positive ELISA for anti HCV can be seen in patients who have cured from the virus. Meanwhile, after the acute infection or by therapy and as such may be positive on ELISA which may indicate past

infection, patients with autoimmune hepatitis and other hyperglobulinemic states give false positive tests (Hinrichsen *et al.*2002).

Real-Time PCR technology is based on the ability of detection and quantification of PCR products, or amplicns, as the reaction cycles progress. Higuchi and colleagues introduced this technology (Templeton *et al.*, 2003). It became possible by including of a fluorescent dye that binds to the amplicon as it is made , initially a fluorescent dye, SYBR green I (A), was used to detect the amplicons. SYBR green I binds the double stranded, DNA amplicon and fluorescences upon illumination with UV light, in TaqMan PCR (B), the oligoprobe contains a fluorescent marker and chemical group that quenches fluorescent of oligoprobe until the dye is liberated by 3' exonuclease activity of the Taq DNA polymerase, in TanqMan PCR an intact "internal" fluorogenic oligoprobe binds to target DNA sequence, internal to the PCR primer binding sites, this oligoprobe possesses a reporter dye that will fluorescence and a suppressor dye known as quencher that prevent fluorescent activity via Fluorescence Resonance Energy Transfer (FRET), after each PCR cycle when the doublestranded DNA products are made, a measure of fluorescence is taken after the fluorogenic probe is hydrolytically cleaved from the DNA structure by exonuclease activity of the *Thermus aquaticus* DNA polymerase (Pehler *et al.*, 2004) .

The advantages of real-time PCR over conventional PCR include:

Wide dynamic assay range, allowing maximum sensitivity, Objective, quantitative results, High degree of reproducibility, Rapid turnaround time and Minimizing contamination risk (Lobert *et al.*,2010).

Chapter Five

Conclusions

and

Recommendations

Conclusions

1- Hepatitis C virus is one of causes among blood donors indicator of Al-Muthanna province.

2- The epidemiological features of HCV were associated with age groups, different locations and different months of the year.

3- There was a close relation between the concentration of ALT and Seropositive individuals .

4- RT-PCR indicates the circulation of the virus in blood stream of some seropositive individuals and gives confirmative results in relatively short time.

Recommendations

1-Further epidemiological , immunological and Molecular studies can be done to understand the pathogenesis and epidemiology of clinical acute and chronic in apparent HCV infection in Al- Muthanna.

2- Use the RT-PCR as confirmative diagnostic technique as well as ELISA test.

3-The prevalence of HCV infection in blood donors indicates the need for application of prevention programs and control measures in AL- Muthanna.

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Appendixes

Appendix A:- Questionnaire of donors.

-Names of the donors which have HCV infection from through diagnosis by ELISA Tech.(Positive HCV)

NO.	Donors name	Age	Title

Appendix B:-

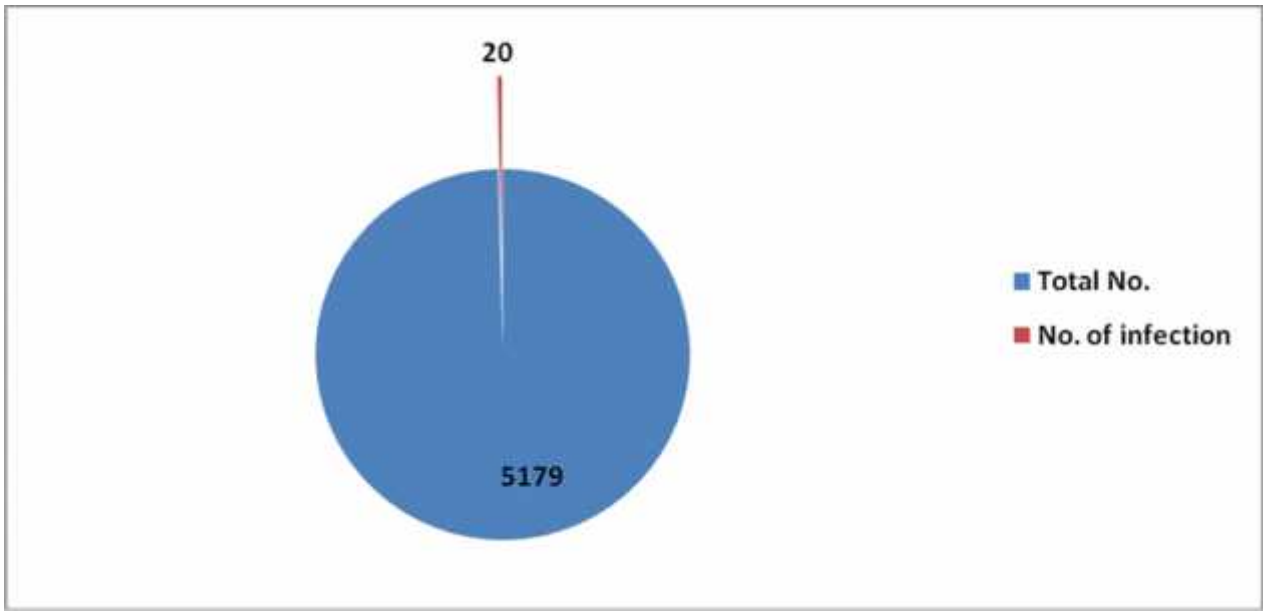

Figure (4-1): Total infection percent of HCV.

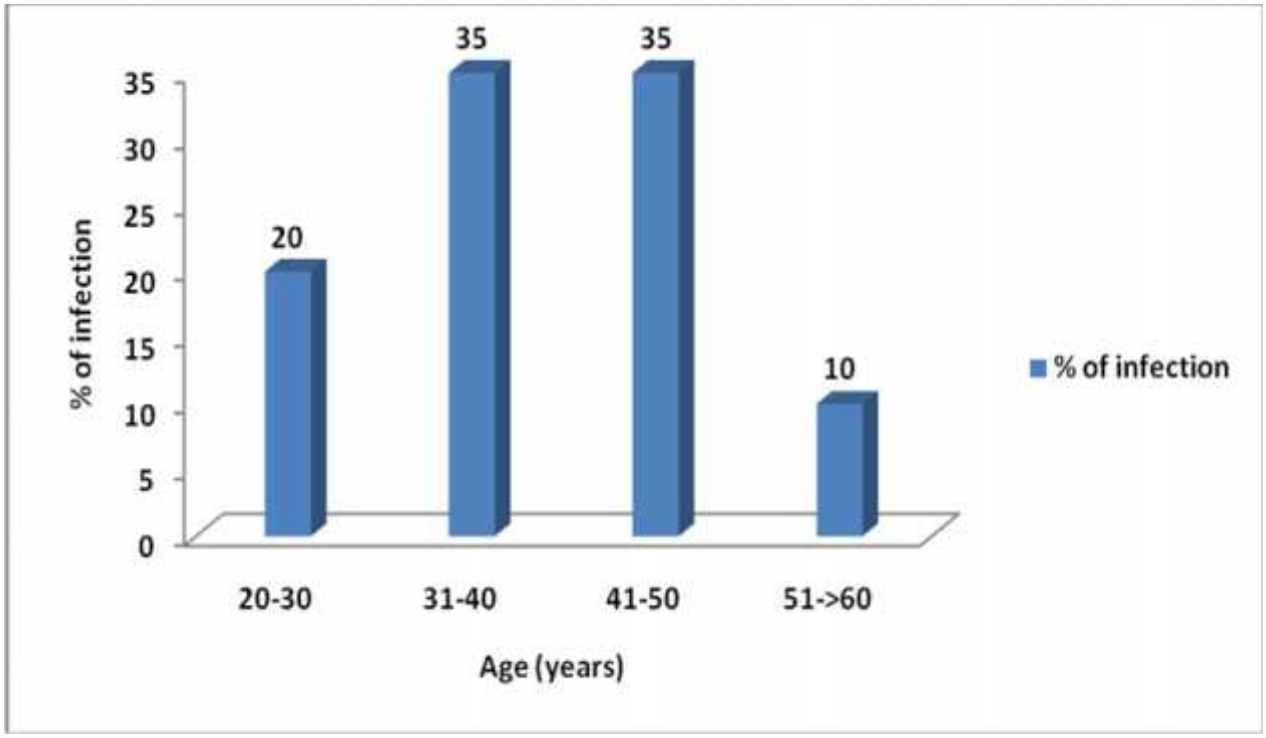

Figure (4-2): Seropositivity rate related to age of donors.

Appendix:-C

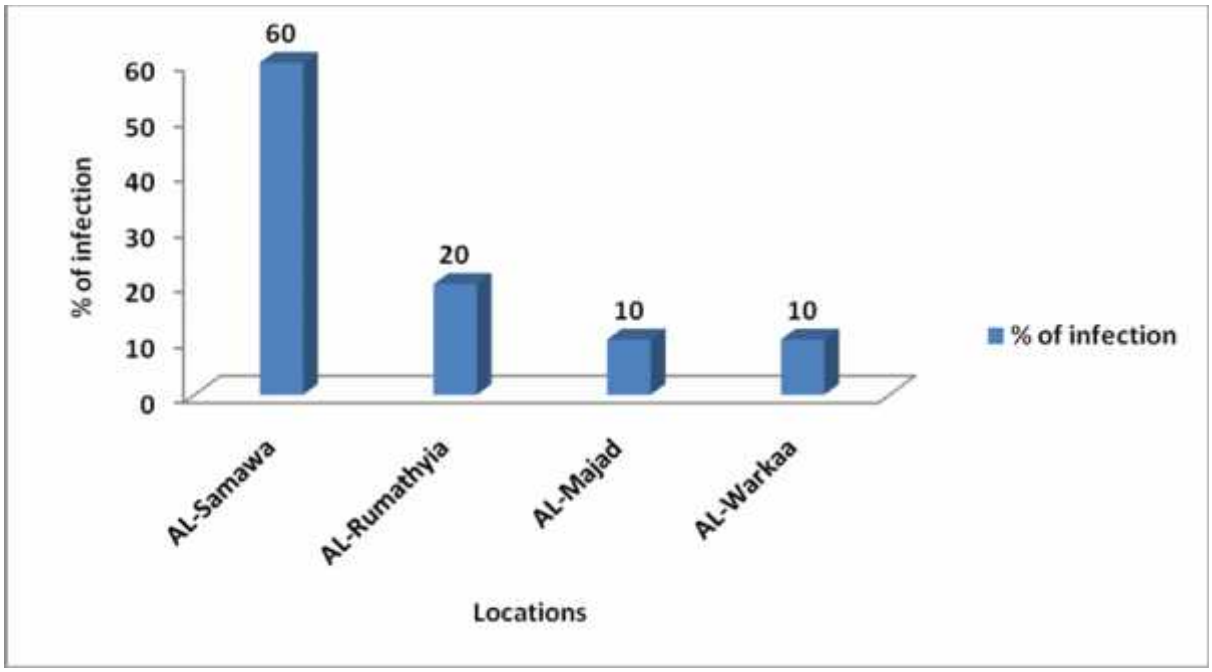

Figure (4-3): Infection rate related with location**.**

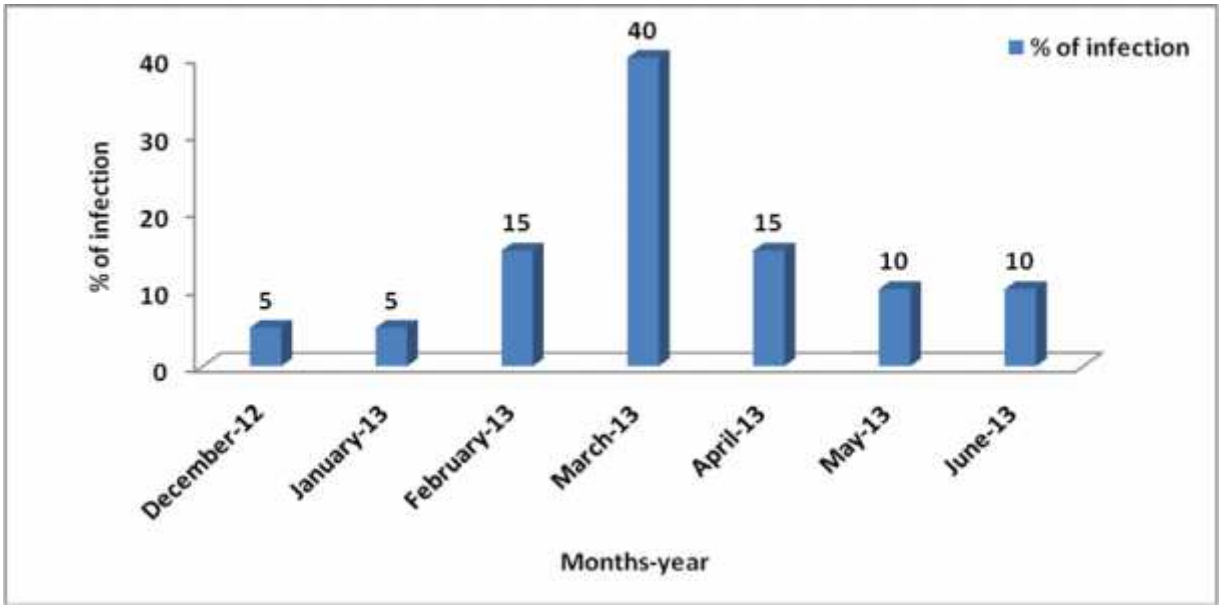

Figure (4-4): Relation of seropositivity rate and months of year

الخلاصة

صممت الدراسة الحالية للتحري عن انتشار الإصابة بفيروس التهاب الكبد الفيروسي (ج) (HCV) لعينة من متبرعين الدم لأول مرة في محافظة المثنى والذين تراوحت أعمارهم من 20 إلى أكثر من 60سنة وللفترة من كانون الأول 2012 إلى تشرين الأول 2013. تم فحص 5179 عينة دم للتحري عن أضداد الفيروس باستخدام فحص المقايسة المناعي المرتبط بالإنزيم الغير المباشر(Indirect ELISA). أظهرت النتائج أن هناك 20 عينة أعطت نتائج موجبة وبنسبة % 0,379وقد كانت أعلى نسبة انتشار على أساس العمر كانت للفئة العمرية (31-50)سنة وبنسبة 35% بينما كانت أدناها في الفئة العمرية (50- >60) في حين سجل معدل انتشار الإصابة نسبة إلى الموقع الجغرافي إن أعلى النسب كانت في مدينة السماوة 60% بينما كان أدناها في قضائي الوركاء والمجد 10% مع وجود اختلافات معنوية وعلى مستوى احتمالي $p<0.05$. أما نتائج انتشار الإصابة حسب أشهر السنة المشمولة بالدراسة فقد سجل شهر آذار أعلاها 40% بينما كان أدناها في شهري كانون الأول 2012وكانون الثاني 2013 وبنسبة 5%. كانت نتائج فحص إنزيمات الكبد (ALT)للأشخاص الذين أعطوا نتائج موجبة لفحص (ELISA) مقارنة مع الفئات العمرية المختلفة و مجموعة الأشخاص الذين اعطو نتائج سالبة للفحص المصلي (ELISA) والذين اعتبروا كمجموعة سيطرة ، أظهرت اختلافا في تركيز هذا الإنزيم بين الفئات العمرية من جهة ومجموعة السيطرة من جهة أخرى إذ كانت نتائج فحص ALT للفئات العمري 20-30 ، 31- 40، 41-50 و51الى أكثر من60سنة كانت,85.25±17.38ng/ml، 80.71±16.o4 ng/ml ، 103.28± 23.27 ng/ml وng/mL 14.75 ±64 على التوالي فيما كانت في مجموعة السيطرة حسب الفئات العمرية اعلاة 12.75 ± 4.14 ng/ml ، 13.14 ± 4.27 ng/ml ، 12.85 ± 2.85 ng/ml و 15.5±5.31 ng/ml مع وجود اختلافا معنويا بين الفئات العمرية من جهة وكذلك للأشخاص في مجموعة السيطرة وعلى مستوى احتمل $P<0.05$.

لقد صمم الفحص الجزيئي الاستنساخ المنعكس لتفاعل سلسلة البلمرة للوقت الحقيقي (Reverse transcription RT-PCR) للتحري السريع والمباشر عن فيروس التهاب الكبد الفيروسي (ج) (HCV)وعن طريق استخدام تقنية الخطوة الواحدة (On step technique) في جهاز Exicycler real time PCR إذ كانت نتائج هذا الفحص الجزيئي إن من بين 20 مصل موجب لفحص ELISA الغير مباشر 13 موجبة وبنسبة 65% ويمكن إن نستنتج إن الإصابة HCV لمتبرعين الدم في محافظة المثنى منتشرة وبنسبة قليلة كما إن فحص تفاعل سلسلة البلمرة في الوقت الحقيقي وعن طريق الاستنساخ العكسي لجينات الفيروسي يعد فحص سريع وموثوق ويمكن تعزيز الفحص المصلي ELISA بة.

وزارة التعليم العالي والبحث العلمي
جامعة المثنى
كلية العلوم

انتشار الإصابة بفايروس التهاب الكبد نمط(C) بين المتبرعين بالدم

في محافظة المثنى - العراق

رسالة مقدمة إلى
مجلس كلية العلوم ـ جامعة المثنى كجزء من متطلبات نيل درجة
ماجستير في علم الأحياء المجهرية .

من قبل

حميد فرهود عبيد

بكالوريوس علوم الحياة / جامعة المثنى *2011*

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