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RESEARCH ARTICLE

Molecular and Immunological Detection of Hepatitis C Virus infection Among Blood Donors in Al-Muthnana Province- Iraq.

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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 from December 2012 into July 2013.Out of 5179 serum samples of blood donors of their 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 level ($P< 0.05$).

Reverse transcription real time polymerase chain reaction (RT-qPCR) was performed for direct and rapid detection 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 detection 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

INTRODUCTION

Hepatitis C virus (HCV) is the agent that causes Hepatitis C, a disease that affects around 130 million people worldwide (Shepard *et al*., 2005). HCV 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). HCV belongs to the Flaviviridae family and it is the only member of the Hepacivirus genus (Dustin and Rice, 2007). Phylogenetic analysis of HCV genomes has led to a classification of HCV into seven confirmed genotypes and 67 subtypes(Smith *et al*., 2014). Primary infection with HCV often shows only mild symptoms, but in the majority of patients, the infection becomes chronic. 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).

The prevalence varies markedly from one geographical area to another genotypes 1a and 1b being the most frequently encountered in Europe, United States, and Japan. HCV subtypes 2a and 2b are commonly found in North America, subtype 2c in Northern Italy, while genotype 4 is predominant in North Africa (especially in Egypt) and the Middle East; genotypes 5 and 6 are commonly reported in South Africa and Hong Kong, respectively. Genotype 3a

is endemic in South East Asia, and seems to be dominant in intravenous drug users (IDUs) in Europe and the United States (Timm and Roggendorf, 2007).

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).

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).

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).

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 study aimed to detect the HCV among blood donors by Immunological and 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- Muthanna province.

2- Study some epidemiological characteristic of HCV infections.

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

2-Materials & Methods :

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**.**

2-1-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 $^{\circ}$C until examined by Real-Time qPCR technique.

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

2-2-1-Principle of test

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.

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

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

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

2-2-2-2- 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.

2-2-2-3- 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.

2-2-3- 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

2-3-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.

2-3-1-Viral RNA extraction:-

Viral RNA was extracted from serum patient samples by using AccuZol^{TM} 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 percent 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.

2-3-2-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.

2-3-3- 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:

2-3-4- qRT-Real-Time PCR master mix preparation:-
RT-qReal-Time PCR master mix was prepared according to company instructions as the following table:

3- Results and Discussion:

Table (2-1): 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 (2-2): 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 (2-3): 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.

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

Real-Time PCR Thermocycler conditions were set according to kit instructions as following table (2-4):

Table (2-4): 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

2-3-5-1.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.

2-3-6- 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)

3-1. Seroprevalence of HCV infection:

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%(Table3-1) (Figure3-1).

Table (3-1): Positive number and total percentage of infected donors.

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

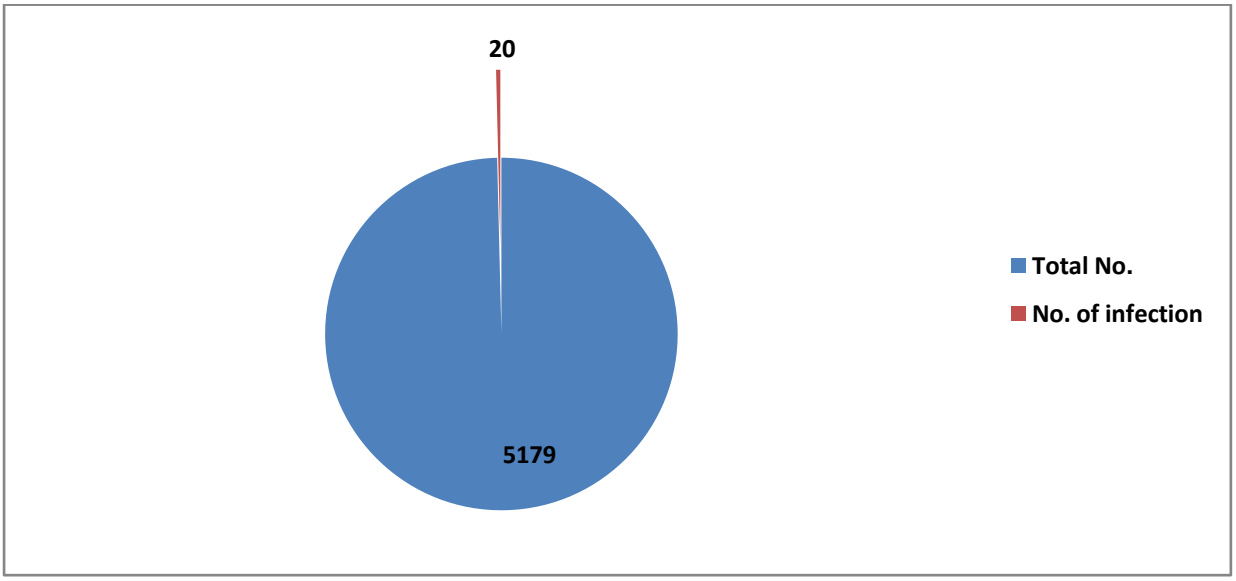

Figure (3-1): Total infection percent of HCV.

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).

3-1-1- 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 was10% . There was significant differences at (P <0.05) (Table 3-2).

Table (3-2): 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).

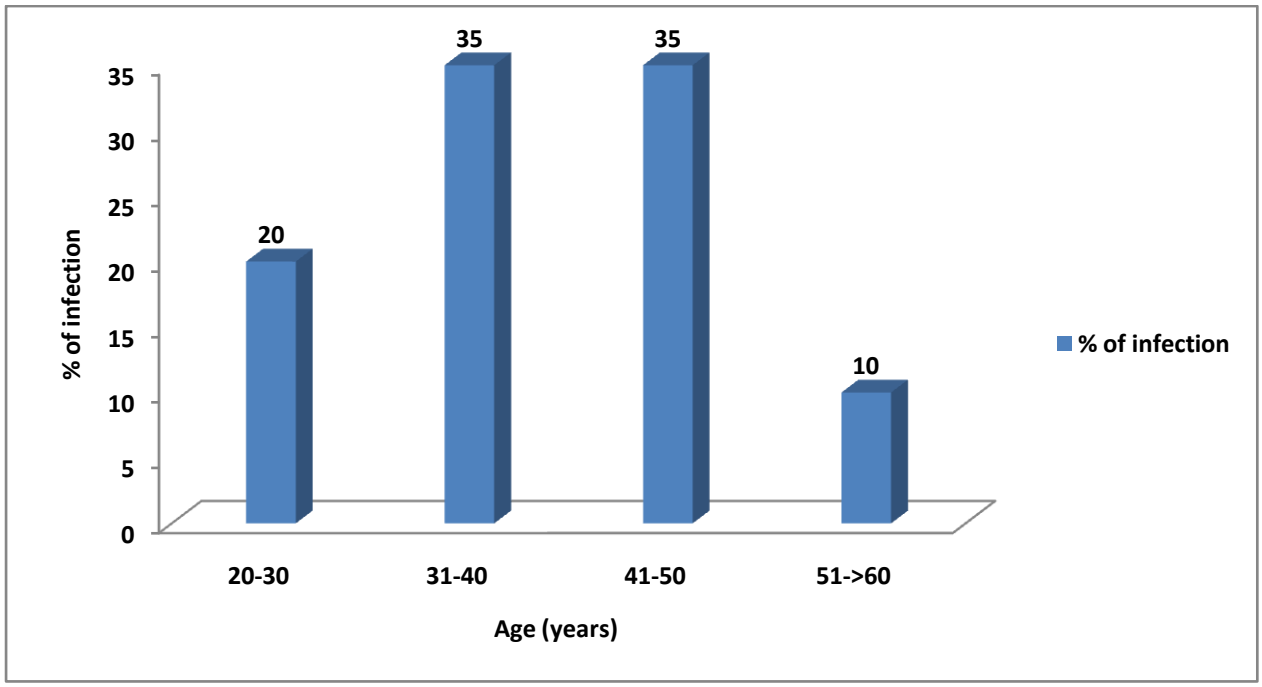

Figure (3-2): Seropositivity rate related to age of donors.

This study, which was carried out in adult population (20 years or older), showed an increasing prevalence of infection with age (Table 3-2),(Figure 3-2) , 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 31-40 and 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.

3-1-2- 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 3-3))(Figure 3-3).

Table (3-3): 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).

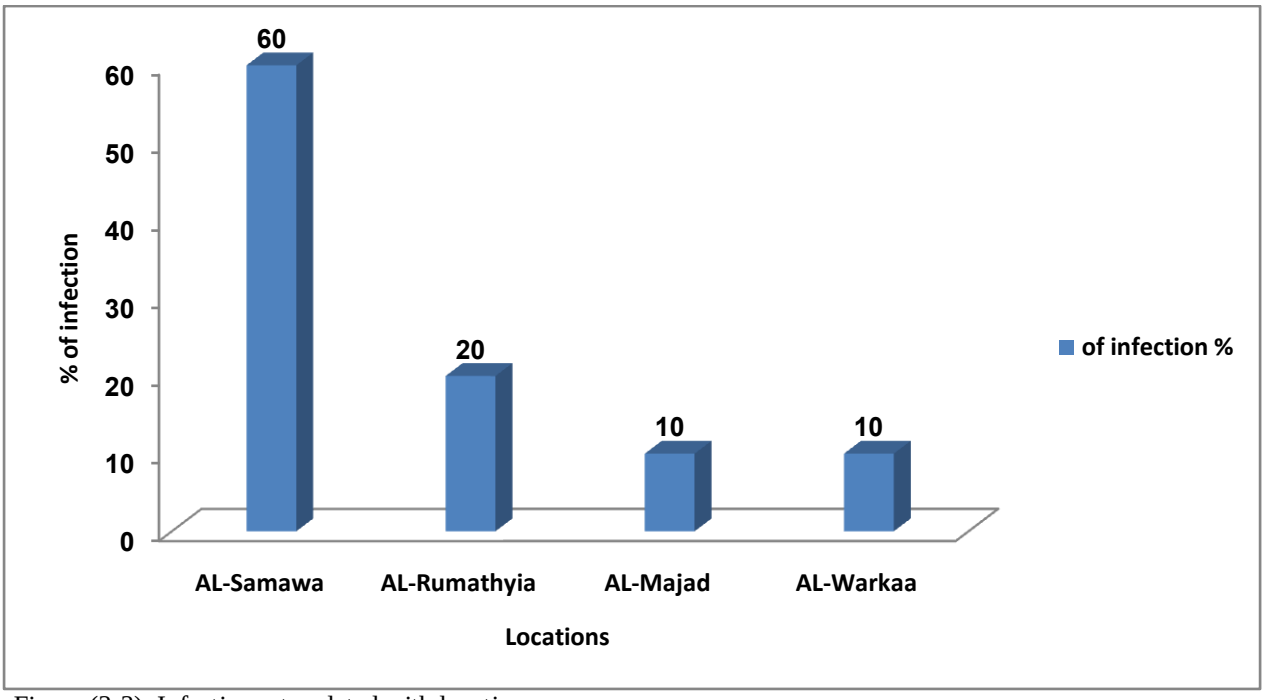

Figure (3-3): Infection rate related with location.

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 (3-4) 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 3-4).

3-1-3 - 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 Decmber , Januray , Faburay, March,April, May and June 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 December. and Januray. 5%. There was significant differences at (P <0.05) (Table 3-5) (Figure 3-4).

Table(3-5): Number and Infection percent of HCV Infection in Months.

Months-year	No. of donors	No. of infection	% of infection
December	695	1	5
January	655	1	5
February	820	3	15
March	697	8	40*
April	845	3	15
May	758	2	10
June	709	2	10
Total	5179	20	100

*Significant differences at (P <0.05).

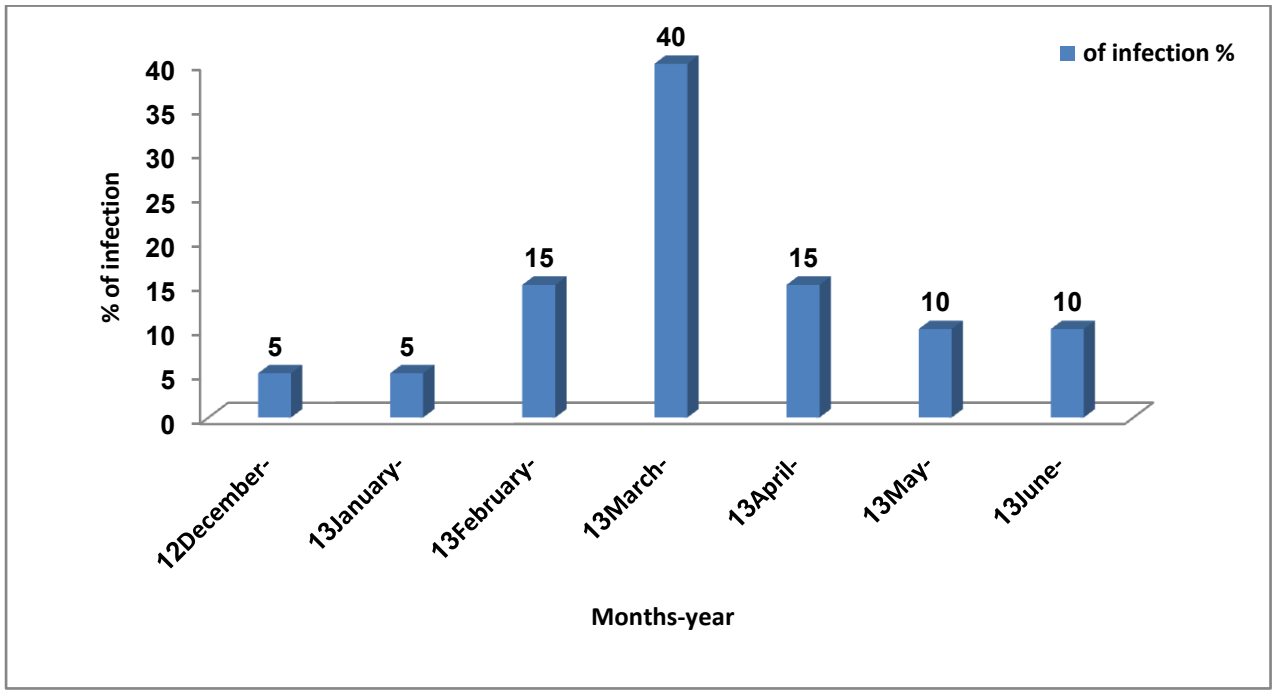

Figure (3-4): Relation of seropositivity rate and months of year.

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 3-5). 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).

3-1-4- 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 3-6).

Table (3-6): Comparison between concentration of HCV Antibodies in control and infected HCV patients (ng/ml) according to age.

ELISA age group	Control mean OD	(normal) 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) (Rotman and Liang , 2009) .

3-2- 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 2n after n cycles, in practice, 20–40 cycles are commonly used. (Turner *et al*., 2005).

3-2-1- 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 3-5).

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 3-6).

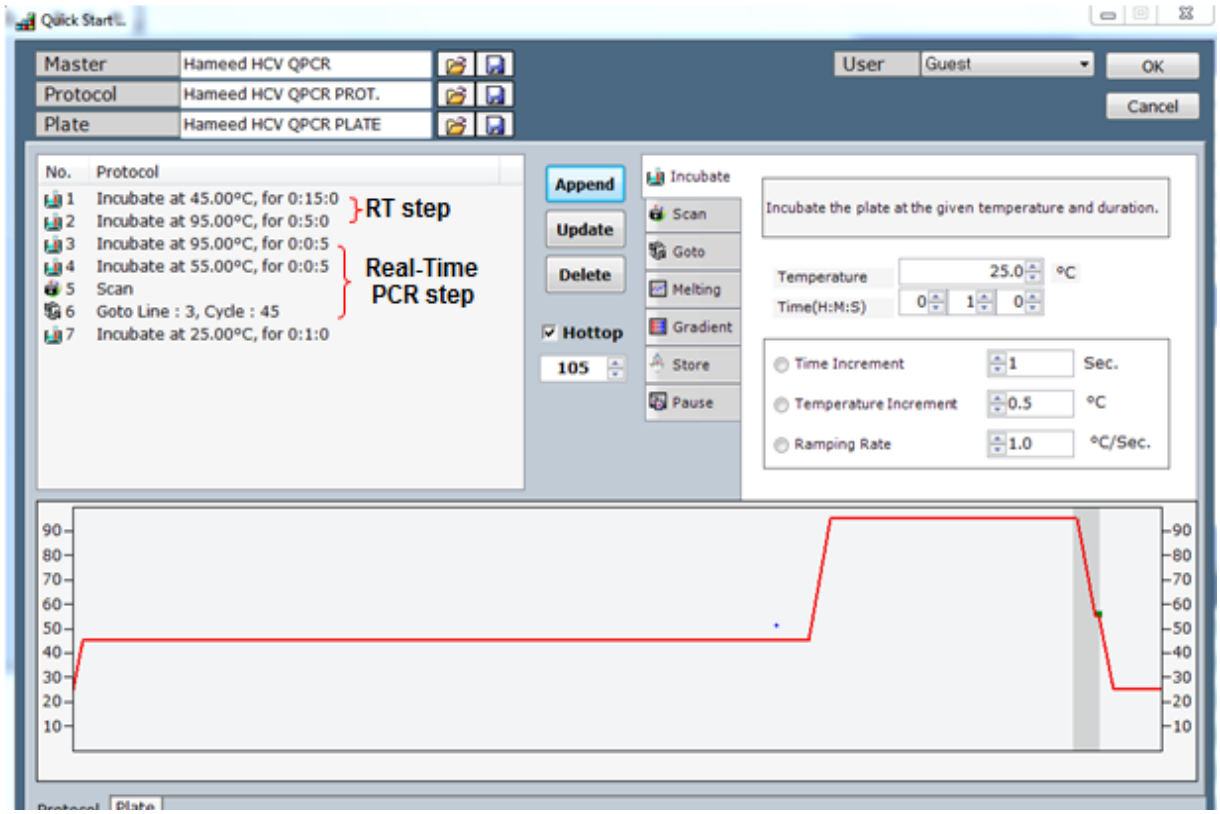

Figure (3-5): Quantitative Reverse Transcription Real-Time PCR Thermocycler condition for HCV in Exicycler Real-Time PCR system.

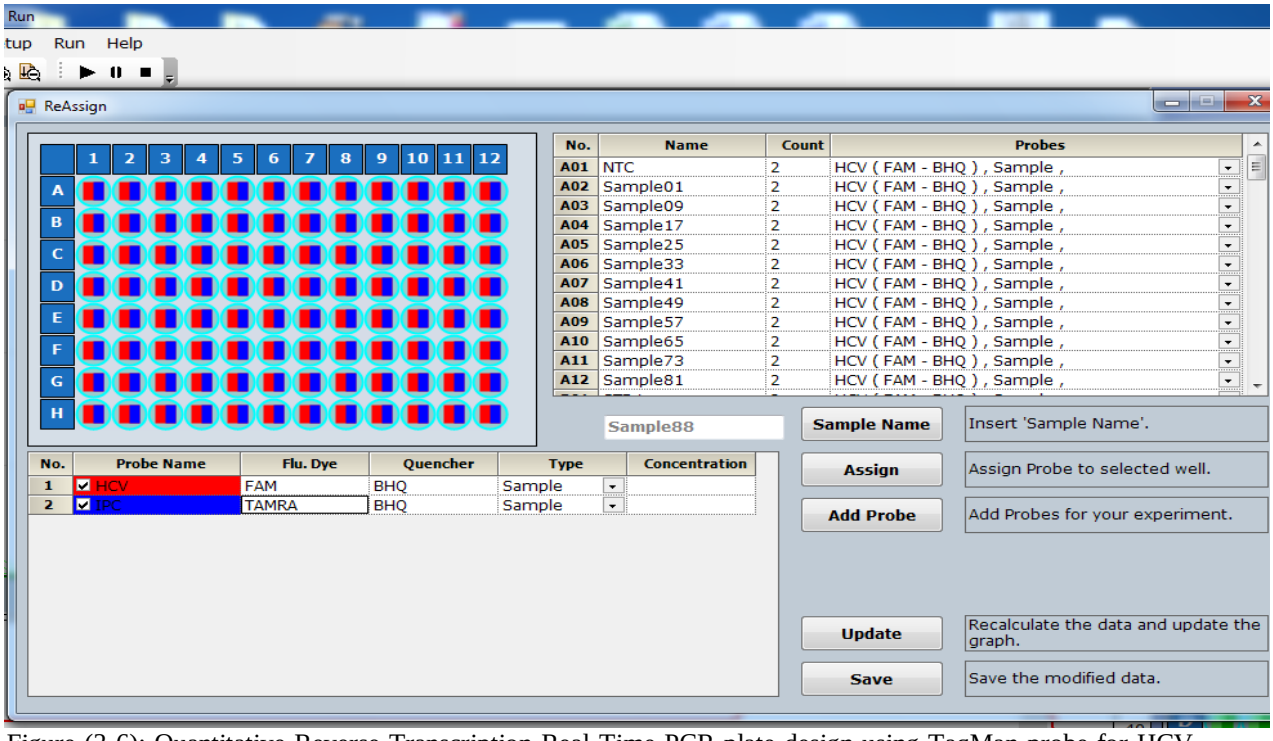

Figure (3-6): Quantitative Reverse Transcription Real-Time PCR plate design using TaqMan probe for HCV samples in Exicycler Real-Time PCR system.

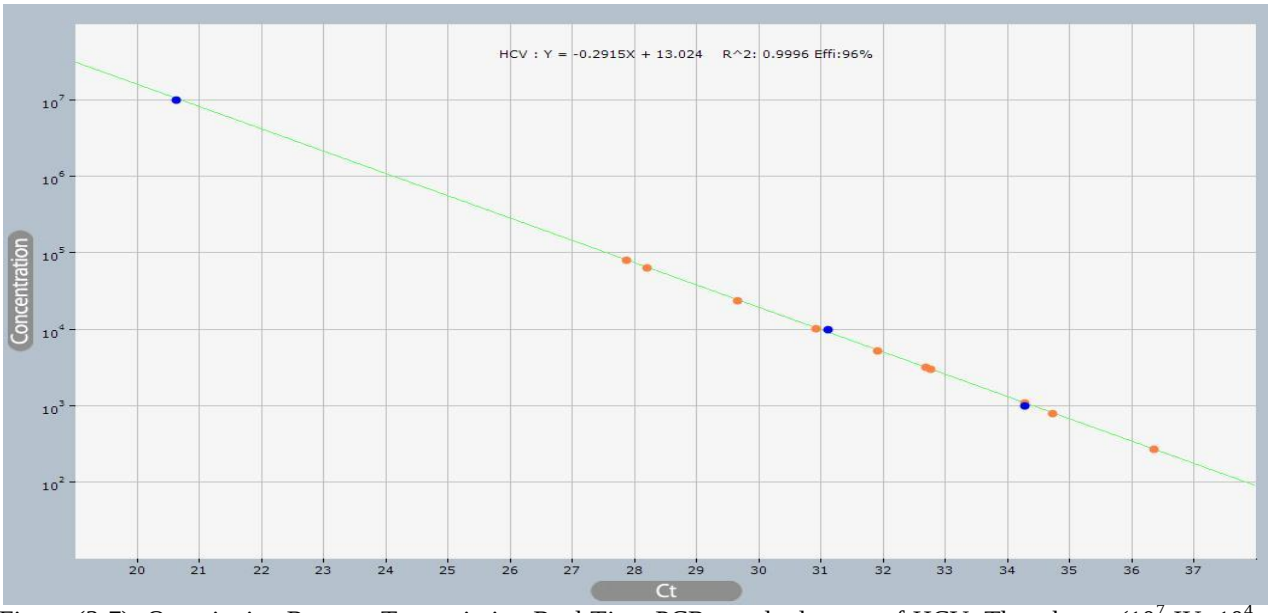

Figure (3-7): Quantitative Reverse Transcription Real Time PCR standard curve of HCV. That shown (10^7 IU, 10^4 IU ,& 10^2 IU HCV Standard) was used for detection of unknown viral load in HCV positive samples.

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 (3-7) .

Table (3-7): 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 negative samples represents three internal positive control and three HCV negative samples (Figure 3-8).

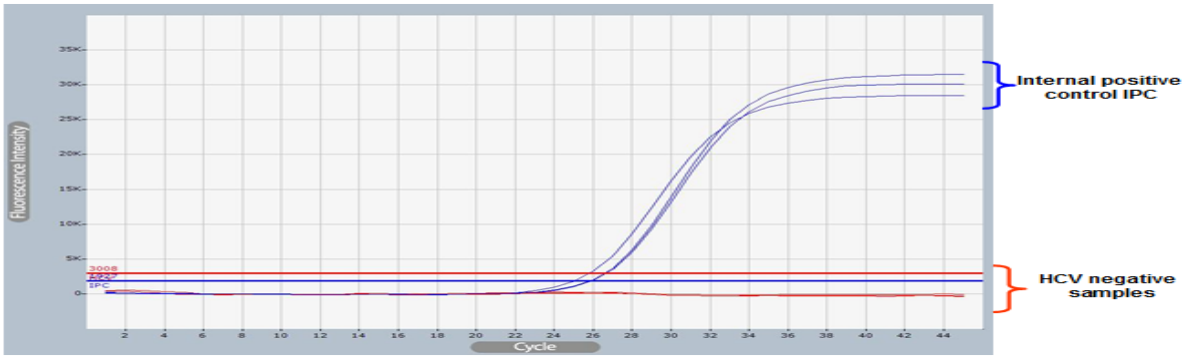

Figure (3-8): The amplification plot of Quantitative Reverse Transcription Real Time PCR of positive HCV that shown the internal positive control and HCV negative samples in 45 qPCR cycles.

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 3-9).

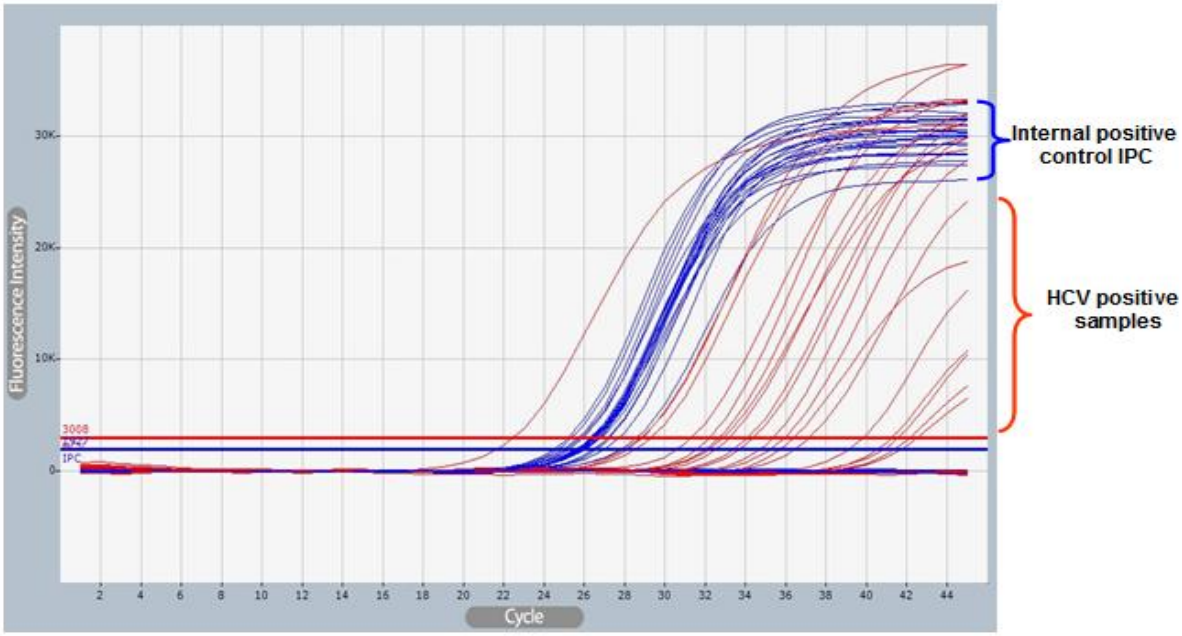

Figure (3-9): The amplification plot of Quantitative Reverse Transcription Real Time PCR of positive HCV. That shown the internal positive control and HCV positive samples in 45 qPCR cycles**.**

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 3-7, Figure 3-9). 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).

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