# An Investigation on the Prevalence of HCV RNA in Patients with Confirmed Chronic HCV Infection and Hepatocellular Carcinoma by *In situ* Hybridization

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## Abstract

**Background:** Infection with hepatitis C virus (HCV) is considered as the causative agent of chronic active hepatitis and one of the major risk factors implicated in the development of hepatocellular carcinoma (HCC). **Objective:** To investigate the prevalence of HCV RNA in formalin fixed of paraffin embedded liver samples by preparing the specific probe *in situ* Hybridization. **Materials and methods**: Thirty five formalin-fixed of paraffin-embedded liver samples obtained from Liver and Digestive System Hospital and private laboratories in Baghdad were included in this study. In addition, thirteen apparently normal liver autopsies were used as control group. Tissue blocks were processed and tissue sections were prepared on positive charged slides and used for the detection of HCV RNA by *in situ* hybridization (ISH) technique. **Results**: Hepatitis C Virus RNA positivity signal by used *In situ* hybridization were detected within cytoplasm and nuclei of hepatocyte. All apparently normal liver specimens showed negative results. The sensitivity of this technique was 89.28% for patients with chronic HCV infection and 90.09% for patients with hepatocellular carcinoma, while the specificity was 100%. In conclusion, probe prepare by *In Situ* hybridization method can be performed in routine pathology laboratories as confirmatory tool for the diagnosis of HCV infection.

**Key word:** Hepatitis C Virus, liver disease, reverses transcription, *in situ* hybridization, and polymerase chain reaction.

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## Introduction

Hepatitis C is a liver disease of humans caused by the hepatitis C virus (HCV), which is a member of the Flaviviridae family of enveloped positive-strand RNA viruses. HCV is classified into at least six main genotypes designated as 1 to 6 genotype and these genotypes showed diversities in their worldwide distribution, transmission and disease progression [1]. The HCV genome contains a linear and positive-strand RNA molecule of approximately 9,500 nucleotides encoding a single polyprotein precursor of approximately 3,000 amino

acids. The polyprotein is cleaved by both host and viral proteases to generate 3 putative structural proteins (Core, E1 and E2) and at least 6 nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) [2,3]. The main methods of infection are blood transfusions and unsafe medical procedures [4], the cause of transmission remains unknown in 20% of cases [5].

Hepatitis C viruses cause chronic hepatitis and hepatocellular carcinoma (HCC), which poorly understood mechanisms [6]. The infection is often asymptomatic, but chronic infection might lead to scarring of the liver and ultimately to cirrhosis that generally apparent after many years. In some cases, those with cirrhosis will go on to develop liver failure, liver cancer or life-threatening esophageal and gastric varices [7]. An estimated 130-170 million people worldwide are infected with hepatitis C [8]. Hepatocellular carcinoma is worldwide, the third cause of cancer-related death and one of the fastest growing malignancies in terms of incidence in western populations [9]. There is no study had focused on prepare probe specific for HCV from core region. Therefore, the objective of this study was to investigate the prevalence of HCV RNA in formalin fixed of paraffin embedded liver samples by preparing the specific probe *in situ* Hybridization.

## Subjects, Materials and Methods

**Study population:** The patients' liver samples were collected during the period from January 2010 till December 2011 from the archives of histopathology laboratories from Liver and Digestive System Hospital and private laboratories in Baghdad. Normal liver specimens were obtained from 13 persons collected from the Forensic Medicine Institute Archives after permission and used as control group. All patients had positive test for anti-HCV antibodies (third-generation ELISA). Thirty five formalin-fixed of paraffin-embedded liver tissue specimens were obtained from patients with chronic HCV infection and hepatocellular carcinoma. The age of patients was ranged from 17 to 65 years. 10 hepatocellular carcinoma cases that included four cases of moderate differentiated adenocarcinoma and six cases of poorly differentiated adenocarcinoma were included in this study.

Formalin-fixed of paraffin embedded tissue blocks were sectioned at 4$\mu$m thickness, sections were stained with Haematoxylin and Eosin, and mounted on charged slides to be used for *In situ* hybridization technique. The clinical diagnosis of the tissue blocks used in this study was primarily based on that obtained from histopathological records of liver biopsy samples and hospital laboratory records. This study was an attempted to take the first step in

prepare probe from core region and use for investigation the presence of HCV RNA in tissues of patients with chronic HCV infection and hepatocellular carcinoma, and study the relationship between this probe and different parameters such as age, gender, grade, histological active index (HAI) and stage.

**Preparation of primers:** Owing to its minimal variability the core gene was selected for the probe sequence. The probe sequences were designed using specific sense and antisense primers obtained from the HCV sequence database (http://hcv.lanl.gov) (Table 1).

**Table (1): Sequences of primers used for the synthesis of the HCV probe**

| Primer | Position | Direction | Sequence (5'-3') |
|---|---|---|---|
| DM51 | 71-95 | Forward | 5' GAAAGCGTCTAGCCATGGCGTTAGT-3' |
| DM50 | 292-311 | Reverse | 5'CTCGCAAGCACCCTATCAGG-3' |

**RNA extraction:** RNA for Hepatitis C virus was obtained from Dr. Robert Anderson's HCV diagnostic lab within the Division of Virology-National Institute for Biological Standards and controls, UK. RNA was extracted according to QIAampMinElute Virus spin Kit with the manufacturer's instructions (QIAGEN, Cat No. 57704, USA).

**Reverse transcription-polymerase chain reaction (RT-PCR) Procedure:** Hepatitis C virus RNA specific sense primer 5'GAAAGCGTCTAGCCATGGCGTTAGT-3' (nucleotides 71 to 95) and antisense primer 5'CTCGCAAGCACCCTATCAGG-3' (nucleotides 292 to 311) were used in this study. Preparation of the single-stranded complementary DNA (cDNA) of hepatitis C virus was performed using GoScript TM Reverse transcriptase according to the manufacturer's instructions with minor modifications (promega, Cat No, A5000, USA). Briefly, the reverse transcription template mix was prepared by mixing viral RNA, RNasin, Nuclease-free water, specific antisense primer to a final volume of 10µl. The mix was then heated for 5minute at 70°C before being placed on ice until was added to the RT reaction. Reverse transcription reaction contained included, in addition to the template mix, consist of 4µl of the GoScript$^{TM}$ 5X Reaction buffer, 4µl of the MgCl2, 1$\mu$l of the PCR Nucleotide Mix and 1$\mu$l of the GoScript$^{TM}$ Reverse transcriptase. The final volume was 20 µl and incubated at 37 for 1 hour. The produced cDNA was amplified PCR was performed, using 25µl QIAGEN HotstartTaq-Master Mix (Man no. 1010023), 4µl of the single strand cDNA solution, 1µl of each sense and antisense primers and nuclease-free water to a final volume of 50µl. This mixture was heated 95°C for 2 min then followed by 35 cycles of denaturation at 95°C for 30

Second, annealing at 60°C for 30 Second, and extention at 72°C for 40 Second, and a one cycle of final extension for 7 min at 72°C. The total volume of amplified products is 50µl were separated by 1% agarose gel electrophoresis. After athidium bromide staining, the cDNA fragment was identified under ultraviolet light with molecular weight 1 kb DNA ladder marker (Promega, Cat No. G5711. USA).

**PCR DIG Probe synthesis*:*** The non-radioactive probe was prepared using digoxigenin labeling procedure (Roche, Cat. No. 11093657910) preparation of reaction mix consist of two tubes, the first one DIG labeled experimental probe it's contain 5$\mu$l of the PCR buffer with MgCl2 10Xconc., 5$\mu$l of the PCR DIG probe synthesis mix, 1$\mu$l of the forward PCR primer, 1$\mu$l of the reverse PCR primer, 0.75$\mu$l of the enzyme mix and 3$\mu$l of the template cDNA, while the second (unlabeled control probe) contain the same component except 5µl of the dNTP stock solution instead of PCR DIG probe synthesis mix, then mix the reactions and centrifuge briefly to collect the sample for PCR machines programs. The PCR conditions consisted of an initial denaturation at 95°C for 2 min, followed by 35 cycles of denaturation at 95°C for 30 Second, annealing at 60°C for 30 Second, and extention at 72°C for 40 Second, finally 7 min at 72°C for final extension. The amplified products were separated by agarose gel electrophoresis.

**Southern Blot Analysis:** cDNA samples were run on 1% agarose gels and capillary transferred onto nylon filters (Boehringer Mannheim) using 20X SSC (saline-sodium citrate 1X = 0.15M NaCl, 0.015M sodium citrate, pH 7.0). After washing with 1X SSC, filters were used for the detection of labeling efficiency.

**Determination of Labeling Efficiency:** All the working solutions were prepared according to the instruction of Roche Instruction Manual, Cat No. 11093657910 with slight modifications.The membrane was rinsed for 5 minutes in maleic acid buffer 0.1 Maleic acid, 0.15 Nacl, NaOH, (pH 7.5), and then incubated for 30 minutes with 100ml blocking solution (5ml of blocking stock add to 100ml Maleic acid buffer). The membrane was then treated with 20ml antibody solution (4$\mu$l of antibody solution add to 20ml blocking reagent) for 30 minutes, then washed two times with 100ml washing buffer 0.1M Maleic acid, 0.15M Nacl, 0.3% Tween 20 (pH 7.5) for 15 minutes each. After equilibration for 2-5 minutes in 20ml detection buffer 0.1M Tris-HCl, 0.1 M Nacl (pH 9.5) and the membrane was finally

incubated in 10ml freshly prepared color substrate (200µl of NBT/ BCIP add to 10ml detection buffer and kept in the dark. The reaction was stopped by washing the membrane with 50ml of sterile double distal water for 5 minutes then air dried.

***In situ* hybridization procedure:** The procedure of the *in situ* hybridization assay adopted by this study was carried out in accordance with the (Boehringer Mannheim, GmbH) with few modifications by used (DIG DNA Labeling and detection kit). Tissue section were dewaxed in xylene and rehydrated in graded ethanol solutions and finally distilled water, then treated with 30µl of proteinase K solution (1mg/ml) for 30 minutes at 37°C, washed in PBS and stopped the reaction by dipping the slides in 0.2% glysin in PBS for 30 Sec. The sections were incubated with 2X SSC for 10 minutes. One hindered micro liter of hybridization mixture containing of 50% formamide (sigma), 5% dextran sulfate (sigma), 50X Denhardt's solution (sigma), 0.25mg/ml of Escherichia coli tRNA (sigma), 0.5 mg/ml salmon sperm DNA (sigma) and 4X SSC, the final volume 10 ml, was placed on the tissue section and incubates at 37°C for 1 hour. Then 30µl of the probe (8µl of PCR DIG Probe mixed with 92µl of hybridization buffer) was applied on each tissue section after denaturation the probe at 95°C for 10 minutes, the slides were then covered with parafilm and seal with rubber glue and placed in humid chamber incubated at 95°C for 5 minutes to allow hybridization of the probe with the target nucleic acid, then incubated over night at 37°C. In the next day, carefully removed parafilm and soaked the slide in 2X sodium citrate-sodium chloride (SSC) for 10 minutes at room temperature, then added 30µl of blocking reagent for 1 hour at RT. Blocking reagent removed without washing, section were incubated with anti-digoxigenin conjugated with alkaline phosphatase, diluted 1 in 500 in 1X blocking reagent. After washes in detection buffer for 5 minutes, substrate consisting of nitrobluetetrazolium (NBT) and 5-bromocresyl-3-indolylphosphate (BCIP) was layered over the sections diluted 1:500 in detection buffer and one drop of levamizol (sigma), keep in dark at RT two hours, then stop the reaction by washing slides with distilled water, air dry and mount in Kaisers glycerin-gelatin mounting medium. Hybridization without DIG-labeled probes was used as a negative control. Finally the examination was done under light microscope at power 400.

**Statistical analysis:** Significant correlation between ISH result and various parameters were found based on Fisher's exact tests and Chi-square.

## Results

### Generation of DIG Labeled DNA Probe

A DIG-labeled DNA probe at 240bp by reverse transcription-PCR using the purified RNA of HCV was extracted from HCV sero-positive patients. The antisense primer, which starts within the 3'end of the HCV genome, was used for reverse transcription. The size of the obtained cDNA was estimated by amplification of was 240bp PCR products. These PCR products were analytical by 1% agarose gel electrophoresis (Figure 1).

### Southern Blot Analysis

The DIG labeling efficiency was evaluated using southern blot analysis. The result revealed that the labeled probe was a single band of 240 bp. Total RNA from HCV was used to confirm the specificities of the HCV probe in order to analysis of population previously infected with HCV (Figure 2).

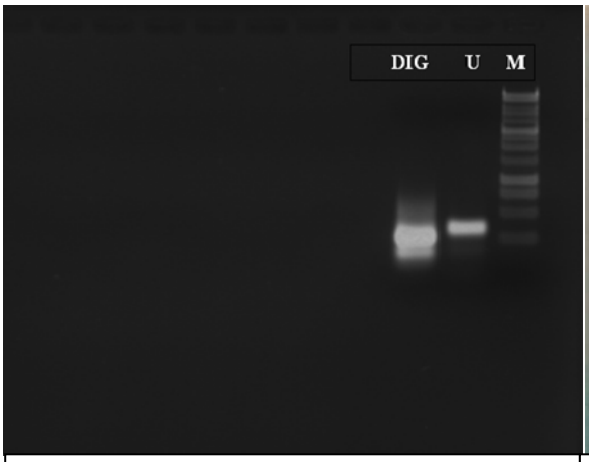


Figure 1: Depiction of PCR products obtained after RT with primer, analysis of amplified cDNA from HCV by 1% agarose gel electrophoresis, marker 1kb (Promega. USA).

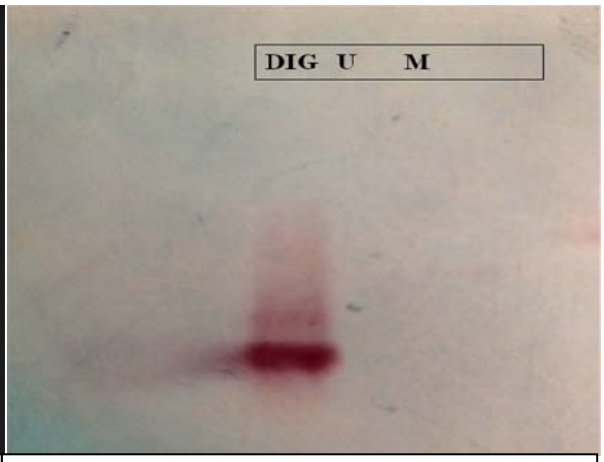


Figure 2: Southern blot on a nylon membrane of the same gel shown in Fig. 1. DIG labeling was detected using specific anti-DIG antibody.

### *In situ* hybridization

The anti-sense (anti-DIG) probe for HCV showed strongly signal in paraffin-embedded human liver section and positive staining appeared in the cytoplasm of infected cells, and around thenuclear membranes sometimes showed a positive reaction in the nuclei of hepatocyte (Figure 3 A, B).

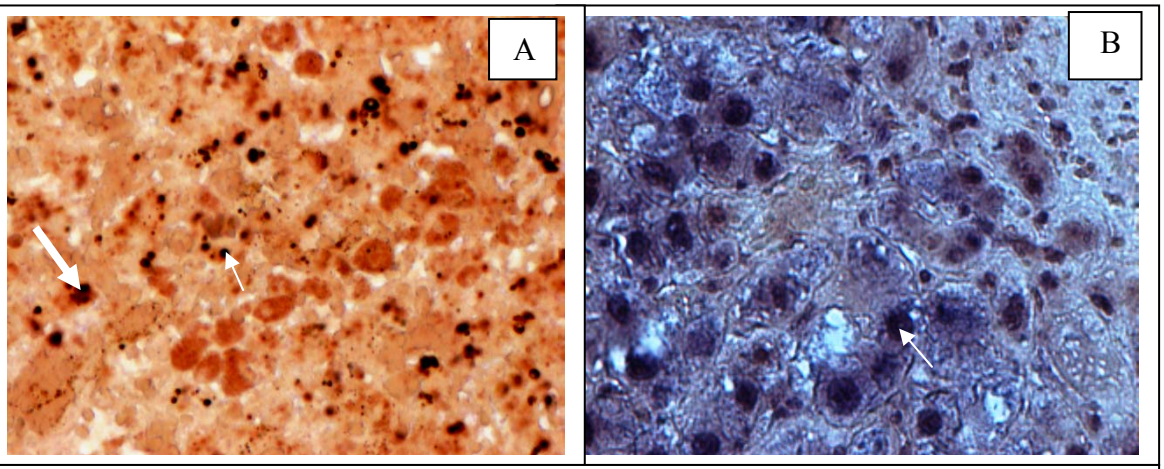


Figure 3: Liver tissue showed the positive cells occur after used anti-DIG probe of intracytoplasmic (thick arrow) and intranuclear (thin arrow), A (magnification power, 200X), B (magnification power, 400X).

**Relationships between ISH and Different Parameters**

There was no statistically significant association between HCV RNA positivity by ISH and parameters of patients with chronic HCV infection such as agegroup <= 40 was 13 (59.09%). The percentage of males was 68.18 % which more than females 31.08%. According to the stage of fibrosis, results of the patient's, it found that 2 patients (66.66%) had stage 1/6, while 1 patient (33.33%) had stage 3/6, also there was no correlation could be obtained between HCV RNA positivity and histological active index (Table 2). The sensitivity of this technique was 89.28% for patients with chronic HCV infection, while the specificity of this technique was 100%.

According to the *in situ* hybridization results of the patient's hepatocellular carcinoma, it found that expression of HCV probe occur in age group > 40 and male (100%), also it found that 4 patients (44.44%) had moderately-differentiation or grade II while poorly-differentiation or grade III, included 5 patients (55.55%). However, there was no statistical significant differences were found among them ($p<0.01$) (Table 3). The sensitivity of this technique was 90.09% for patients with hepatocellular carcinoma, while the specificity of this technique was 100%.

**Table 2: Expression of HCV-ISH in patients with chronic HCV infection and apparently healthy control group**

| Variables | | Expression of HCV probe | | Comparison of Significance | |
|---|---|---|---|---|---|
| | | Negative | Positive | p-value | Sig. |
| Age | <= 40 | 3 (100%) | 13 (59.09%) | 0.280 | N.S. |
| | > 40 | 0 | 9 (40.90%) | | |
| Gender | Male | 0 | 15 (68.18%) | 0.052 | N.S. |
| | Female | 3 (100%) | 7 (31.08%) | | |
| HAI | 3/18 | 0 | 2 (9.09%) | 1.000 | N.S. |
| | 4/18 | 2 (66.66%) | 5 (22.72%) | | |
| | 5/18 | 0 | 10 (45.45%) | | |
| | 6/18 | 0 | 1 (4.54%) | | |
| | 7/18 | 0 | 1 (4.54%) | | |
| | 8/18 | 1 (33.33%) | 2 (9.09%) | | |
| | 9/18 | 0 | 1 (4.54%) | | |
| Stage | 0 | 0 | 3 (13.63%) | 0.554 | N.S. |
| | 1/6 | 2 (66.66%) | 4 (18.18%) | | |
| | 2/6 | 0 | 4 (18.18%) | | |
| | 3/6 | 1 (33.33%) | 5 (22.72%) | | |
| | 4/6 | 0 | 1 (4.54%) | | |
| | 5/6 | 0 | 2 (9.09%) | | |
| | 6/6 | 0 | 3 (13.63%) | | |
| Control | | 13(100%) | 0 | | |

**Table 3: Expression of HCV probe in patients with HCC and apparently healthy control group**

| Variables | | Expression of HCV probe | | Comparison of Significance | |
|---|---|---|---|---|---|
| | | Negative | positive | p-value | Sig. |
| Age | <= 40 | 1 (100%) | 4 (44.44%) | 1.000 | N.S. |
| | > 40 | 0 | 5 (55.55%) | | |
| Gender | Male | 1 (100%) | 9 (100%) | 1.000 | N.S. |
| | Female | 0 | 0 | | |
| Grade | I | 0 | 0 | 1.000 | N.S. |
| | II | 0 | 4 (44.44%) | | |
| | III | 1 (100%) | 5 (55.55%) | | |
| Control | | 13 (100%) | 0 | | |

## Discussion

The use of *in situ* hybridization method for detection HCV genome in liver tissues and core protein considered semi conservative region. The core protein, which is located at the N terminus of the polyprotein, is considered a component of viral capsid. It is phosphorylated,

has both nuclear and cytoplasmic localization and possesses several distinct functions. It acts as a regulatory protein that positively or negatively modulates the cellular or viral promoters, although the molecular mechanism of this transaction is still not fully understood. Additionally, it interacts with a wide spectrum of cellular factors such as apolipoprotein, lymphotoxin- β receptor, tumor necrosis factor-type 1 receptor, heterogeneous nuclear ribonucleoprotein K, p53, RNA helicase and p21/WAF1. In most cases, the core protein might affect the biological functions of its targeted proteins [11].

Diagnostic methods in HCV infection involved the determination of anti-HCV antibodies using the ELISA immunoassay and examination of HCV RNA with either reverse transcription (RT)-PCR [12]. However, a simple, sensitive and specific assay for the HCV RNA detection is very useful for the early diagnosis of acute HCV infection because the serological window for detecting of anti-HCV antibodies, which approximately in 2-7 weeks [13,14]. Liver tissue biopsy considered as important investigatory tool of patients with viral disease, since it is used in the current study of PCR molecular techniques for development of the probe synthesis to confirm the presence of Hepatitis C virus in liver tissues.

Many investigations have used in the different regions of HCV genome for cDNA synthesis and PCR amplification in order to detect the viral genome by *in situ* hybridization, Negro *et al*. (1992) who used a non isotopic *in situ* hybridization (NISH) for detection of HCV RNA by synthetic oligonucleotide complementary to bases 252-301 of the highly conserved 5' non coding region of HCV genome. Kayhan *et al*. (16) who prepared the digoxigen in-labeled cDNA probe from the 3' non structural region (NS5) of hepatitis C virus to performing non isotopic *in situ* hybridization of liver tissue. While Sandres-sanue *et al*. (17) who performed comparison study between used non-commercial assay (uses non-structural 5b (NS5b) region and Trugene 5' NC genotyping kit by used PCR techniques.

More sensitive assay for HCV RNA identification is nested RT-PCR and real-time PCR [18,19]. A set of nested primers from the 50 noncoding region was designed according to the previous reports [20]. Sheehya *et al*. (21) who used construct near full-length cDNA clones to each genotype to performing assembly PCR, while Kim *et al.* (22) who used gene-specific primers of NS3 and NS5A as templates for prepared cDNA by real-time PCR. Wang *et al.* (23) used highly conserved sequence in the 5' un-translated region of HCV by real-time PCVR and nested RT-PCR. On the other hand, otherresearchers used a double nested PCR (DN-PCR) to detect on viral genome [24,25].

The performance of RT-PCR procedure in this study to prepare probe specific for the detection of HCV RNA using paraffin embedded tissue section by *In situ* hybridization. The HCV sequences of this RNA are derived from conserved area of the HCV genome (core area). In this study, we have focused on chronic infection and hepatocellular patients to indicate the specificity and sensitivity of the probe. The sensitivity of this technique was 89.28% for patients with chronic HCV infection and 90.09% for hepatocellular carcinoma tissues and these results was higher comparing with the previous report by Felgar*et al.* (26) who reported that the percentage of sensitivity is 60%, while the specificity of this technique was 100%.

In this study, HCV RNA was not detected in all patients who were tested positive for third generation of ELISA, this finding was in agreement with other researcher indicate the same results [26,28]. It can be explained by the possibility that the tissue sections (in cases with positive serum HCV RNA) might be taken from non infected areas and accordingly, these areas showed no HCV RNA-positive hepatocytes [26] ormight be related with used too small amount of HCV RNA in the liver tissue.Regarding the cell types infected by HCV positive signals for ISH were detected in the liver cells, no staining was found in the bile duct epithelium or infiltrating mononuclear cells, this result was in agreement with other studies [29,31]. Hepatitis C virus RNA positive signals showed in the cytoplasm and nucleus of infected cell and this finding was also detected by other investigators [30,32].In conclusion, the use of ISH method for detection of HCV RNA of liver tissue showed highlysensitive and specificity that consider best method of monitoring and indication of viral infection.

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