

In Situ Localization of PCR Amplified cDNA for the Diagnosis of HCV Infection in Egyptian Liver Biopsies

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ABSTRACT

Hepatitis C virus (HCV), a major cause of chronic liver disease, is highly prevalent in Egypt. The morphological criteria for diagnosis of HCV in liver tissue are suggestive, while the definite diagnosis relies on direct detection of HCV RNA.

We attempted at localizing HCV genomic RNA in formalin fixed paraffin embedded liver tissue sections using the reverse transcription polymerase chain reaction in situ hybridization technique (RT *in situ* - PCR) versus standard in situ hybridization (ISH).

Liver biopsies from 43 patients with clinical and histological evidence suggestive of HCV infection were included. PCR amplified viral complementary DNA (cDNA) was detected in liver hepatocytes by *in situ* hybridization with a biotin labeled probe, in 32 cases, 32/43 (74.4%). The signal was seen as strong perinuclear reaction with occasional cytoplasmic reaction. From the 32 tissues found positive for HCV by RT in situ PCR, only 9 were positive using standard *in situ* hybridization, 9/32 (28.1%), with a weaker signal obtained.

The present study recommends applying RT *in situ* PCR as a sensitive method for the confident diagnosis of HCV in liver biopsies that might help to assess morphologic criteria. The objective diagnosis of HCV by RT *in situ* PCR could limit therapeutic lines to positive cases.

Key words: Chronic hepatitis - HCV RNA , *in situ* hybridization - RT -in situ PCR.

INTRODUCTION

HEPATITIS C virus (HCV), a positive sense, single-stranded RNA virus and member of the Flaviridae family, is a leading cause of chronic liver disease [29,36]. HCV is now recognized to be a major risk factor for hepatocellular carcinoma, utilizing either serological [30] or molecular assays for HCV detection [31,32,37].

Prevalence rates of hepatitis C virus (HCV) infection are approximately 0.5% to 2% in the general population of Western countries and the USA, while higher rates have been reported in some African and Southeast Asian countries. In

Egypt, serological tests for detection of HCV, have shown a relatively high prevalence rate of about 13.6% of hepatitis C infection even in some asymptomatic individuals [6,20].

Active schistosomal infection is not usually associated with anti-HCV status [1]. However, parenteral therapy of endemic schistosomiasis and blood transfusion are among the causes for the high prevalence [1,11,14,16,21]. In a recent study on 130 Egyptian patients, Kamal et al. [19], reported that active patients with concomitant HCV and schistosomiasis infection were characterized by more advanced liver disease, higher HCV RNA titers, predominance of HCV genotype 4, higher histologic activity, higher incidence of cirrhosis and hepatocellular carcinoma as well as a higher mortality rate.

Most HCV infections take a chronic course, the estimated rates ranging between 50% and 90%, and at least 20% of these patients will eventually develop cirrhosis. Although many patients do not present with clinical signs of active liver disease, consecutive liver biopsies have histologically proven progressive liver damage in more than half of the cases [19,29-31]. Interferon (IFN) is used for the treatment of chronic hepatitis C with only 50% of the patients responding to therapy and a mere 20% sustaining a response [18]. Long lasting elimination of HCV RNA was reported when IFN was coupled with another antiviral drug, ribavirin, in patients who relapsed after IFN therapy [10].

Diagnostic morphologic criteria for HCV infection in liver tissue are subjective and may be associated or overshadowed by other endemic parasitic infestations e.g. schistosomiasis. However, the detection of viral particles in liver cells could verify the diagnosis. HCV infection

is diagnosed by detection of HCV -specific antibodies in patient serum by serological assays. Since HCV replicates at a low level, detection of HCV genomic (positive-strand) RNA in serum requires molecular techniques such as reverse transcription polymerase chain reaction (RT-PCR) and branched chain DNA signal amplification assay for quantification of the viral load [38].

Although highly sensitive, these assays do not allow specific localization of HCV within the liver. Detection of HCV genome and gene products by *in situ* hybridization (ISH) technique [4,9,12,15,26,32,34], *in situ* amplification following reverse transcriptase (RT *in situ* PCR), with either direct or indirect incorporation of labeled nucleotides [23,24,33, 35] and immunohistochemistry (IH) technique [5,8, 17,22] have helped in identifying the site of viral replication, and defining host-viral interactions. However, these techniques were found to have their limitations thus producing conflicting results based on all three technique. In some studies, only a subset of biopsies from patients with hepatitis C stained positive for HCV RNA, and only a small percentage of hepatocytes appeared to be infected [4,22,23]. In other studies, HCV genomes were found in over 90% of biopsies from infected patients [2,12,13,25].

The aim of the present study was to localize HCV positive strand RNA in the livers of Egyptian patients with morphological criteria implicative of chronic hepatitis C by two *in situ* molecular techniques, RT *in situ* PCR in comparison to the standard ISH.

MATERIAL AND METHODS

The material in the present study consisted of formalin fixed paraffin embedded blocks of liver biopsies from 43 patients. Selection criteria included presence of clinical and histological evidence suggestive of hepatitis C. There was a male: female ratio of 3:1, mean age 45, and age range of 22-65 years.

Histopathologic evaluation: Haematoxylin and eosin stained paraffin sections were microscopically examined for histopathologic assessment. Morphological selective criteria of liver biopsies included the presence of lymphoid aggregates, bile duct degeneration, focal necrosis and apoptosis, mild steatosis, granulomas and mallory bodies.

cDNA - RNA ISH:

ISH was done as previously published [24]. Briefly, the hybridization cocktail contained 10% formamide, 150 mM NaCl, 10% dextran

sulfate, and the biotin oligoprobe at 50 ng/ml. The probe and target RNA were heated at 100°C for 5 min, followed by hybridization at 37°C for 2 hours. The slides were washed in a solution containing 0.2% bovine serum albumin and 300 mM NaCl at 40°C for 10 min, then incubated for 30 min in the antibiotin antibody - alkaline phosphatase conjugate (Boehringer Mannheim) diluted 1:50. The alkaline phosphatase - based detection method uses the chromogen nitroblue tetrazolium, which in the presence of 5-bromo-4 chloro-3-indoyl phosphate, yielded a purple blue precipitate at the site of hybridization. The counterstain, nuclear fast red, stained nuclei and cytoplasm pale pink.

RT in situ PCR:

RT *in situ* was performed according to the method described by Nuovo, et al., 1993 [27]. Briefly, in preparation for cDNA synthesis, tissue section were digested with trypsin (2mg/ml, 37°C, Promega, USA) for 30-90 min, treated overnight with an RNAase- free DNAase solution made according to the manufacturer's recommendations (10 U/tissue section, 37°C, Boehringer Mannheim). The tissues were included directly on the glass slide at 42°C for 30 min with 10 ul of a solution which contained the downstream primer (1 uM) and reverse transcriptase made according to the manufacturer's protocol (RT PCR kit, Promega, USA). The PCR solution contained 4.5 mM MgCl₂, 200 uM dNTPs, and 1 uM of the primers. The primers and biotin labeled oligoprobe sequence (Promega, USA) were prepared as formerly published [24]: JH51: antisense: 5' CCCAACA-CTACTCGGCTA 3'; JH52: antisense: 5' ACT-CTTGCGGCCGCAGCGCCAATC 3'; JH93: sense: 5' TCCGCGGCCGCACTCCACCATG-AATCACTCCCC 3'. Biotin labeled probe Alx 89:5' CCATAGTGGTCTGCGGAACCGGTG-AGTACA 3'; cDNA: nucleotide 8 to 268. After an initial denaturation step of 94°C for 3 min., 15 cycles were performed using the following protocol: annealing/extension at 55°C for 2 min. and denaturation at 94°C for 1 min. Successive 3 min. washes in xylene and 100% ethanol removed the mineral oil and xylene, respectively. Biotin containing PCR product was detected as described above.

RESULTS

RT in situ PCR:

PCR amplified Hepatitis C viral cDNA was detected in liver hepatocytes in 32 cases, 32/43 (74.4%), Table (1) and Fig. (1). A strong perinuclear signal with or without cytoplasmic reaction was detected Fig. (2).

ISH:

Only 9 cases were positive by standard ISH out of the 32 cases found positive for HCV by RT *in situ* PCR, 9/32 (28.1%), Table (2), Fig. (1). A weaker perinuclear signal was obtained using standard ISH than that achieved with RT *in situ* PCR.

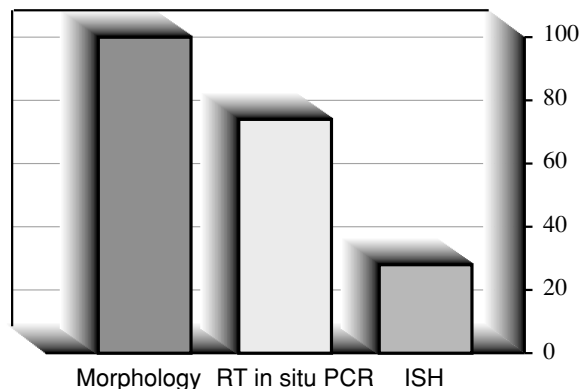
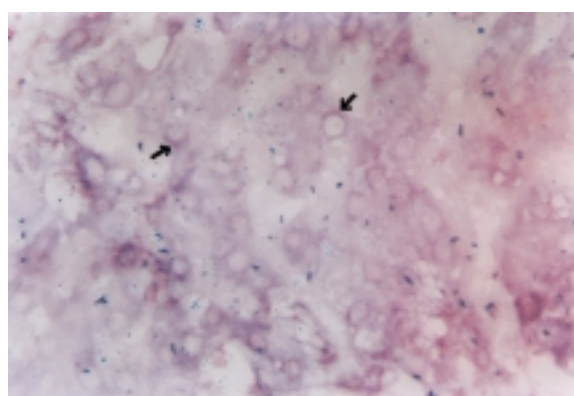
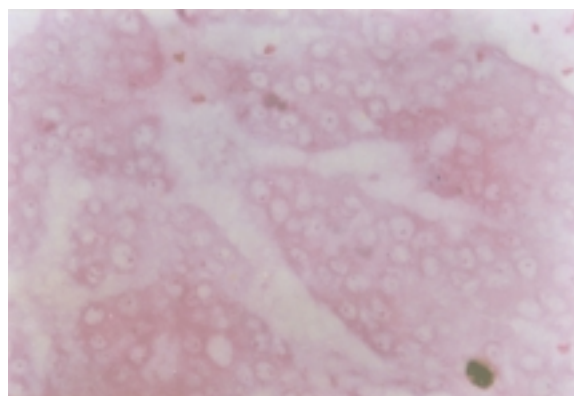


Fig. (1): Column chart showing hepatic morphology suggestive of chronic hepatitis due to HCV in 100% of the studied cases versus 74% positive cases by RT *in situ* PCR and 28% by ISH.



A



B

Fig. (2): (A) RT *in situ* PCR localization of HCV in hepatocytes shown by perinuclear staining and occasional cytoplasmic staining. (B) Negative RT *in situ* PCR result with no perinuclear staining denoting absence of HCV in hepatocytes.

Table (1): Hepatic morphology versus RT *in situ* PCR among 43 liver tissue sections.

	Number	% positive	% negative
Hepatic Morphology	43	100	0
RT <i>in situ</i> PCR	32	74	26

Table (2): RT *in situ* PCR versus ISH.

	Number	% positive	% negative
RT <i>in situ</i> PCR	32	100	0
ISH	9	28.1	72

DISCUSSION

To our knowledge, this is the first report on attempting to localize HCV positive strand RNA applying ISH molecular techniques on Egyptian liver biopsies with morphologic sign insinuating of chronic hepatitis due to HCV. In this study we were able to localize HCV genomic RNA in 32/43 (74.4%) in the hepatocytes of Egyptian liver biopsies, using RT *in situ* PCR method. Out of these 32 liver biopsies that had detectable HCV positive strands, only 9 were positive by standard ISH. These findings are in agreement with previous reports [23,24,33,35], highlighting the fact that a marked increase and hence lower threshold and higher sensitivity in the rate of detection of the HCV positive strand RNA was reached following the addition of PCR amplification. The detection rate by RT *in situ* PCR is estimated to be as low as a single copy per cell in comparison to ten copies per cell detected by standard ISH. In hepatitis C infection, hepatocytes as well as kupffer cells contain <10 copies of viral genomes per cell [27]. This further illustrates the diagnostic importance behind the utilization of RT *in situ* PCR over standard ISH in HCV genomic detection.

In the current study we have demonstrated that the hepatitis C viral cDNA amplified by PCR in the hepatocytes was strongly perinuclear. Our results are in conformity with previous findings describing viral localization at the perinuclear membrane [24,27] or within the nuclei of hepatocytes [15,26].

We also noted that with RT *in situ* PCR, a diffuse and occasional staining within the cytoplasm, always associated with the nuclear signal in hepatocytes which is consistent with findings in other studies [26,27,35]. Recent IH studies produced similar sites of nuclear localization as with RT *in situ* PCR, yet IH is far less sensitive and produces much weaker im-

munoreactive intensity [5,35]. Other reports have restricted HCV localization to the cytoplasm [3,27]. Differences in HCV site localization by IH is mainly due to differences in monoclonals and their different HCV target sites. The significance of signal obtained in the cytoplasm is still doubtful, it might be a diffusion type artifact [24] or a background staining [35].

In this study we managed to obtain consistent results by performing RT-PCR assay after HCV RNA extraction from formalin-fixed, paraffin-embedded liver biopsies, we confronted no difficulties with sensitivity in the RT *in situ* PCR, confirming other earlier reports [7,24,35]. On the other hand, Park, et al., 1996 [28], postulated that formalin-induced crosslinks may inhibit extension by RT probably due to loss of HCV RNA copies following RNA extraction after dewaxing at high temperature, leading to false-negative results.

In other reports, the superior sensitivity of tissue RT *in situ* PCR has proved even more efficient than serological testing or even RT PCR for HCV RNA, for monitoring interferon therapy response, since chronic active hepatitis C was demonstrated in absence of detectable serum HCV RNA. [7,35]. Studies that showed discrepancy between serum RT-PCR and *in situ* PCR results could be interpreted either by lack of sensitivity of serum RT-PCR (level of detection about 100 HCV genomes/ml) while in RT *in situ* PCR could detect a very low viral copy number per cell, such as a single copy [35], or perhaps that the level of viral replication in liver is minimal for detection in the serum. This viral form, probably in a latent state, may explain the relatively high chronic hepatitis C relapse.

Conclusion:

The value of RT *in situ* PCR for localization of genomic HCV RNA in liver biopsies emphasized in this study highlights the need for implementation of this technique in monitoring infected patients livers before and after IFN-therapy as it is clear that it allows for HCV localization not evident by other methods.

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