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Novel Approach for Quantification of Hepatitis C Virus in Liver Cirrhosis Using Real-Time Reverse Transcriptase PCR

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Abstract
Background Hepatitis C virus (HCV) infects nearly 3% of the population worldwide and is a major cause of acute and chronic infections leading to fibrosis, cirrhosis, and hepatocellular carcinoma. Current laboratory diagnosis of HCV is based on specific antibody detection (anti-hepatitis C virus (anti-HCV)) in serum. As HCV replicates in the liver cells, detection and localization of HCV RNA in liver tissue are vital for diagnosis.
Methods Ten biopsy samples diagnosed for cryptogenic liver cirrhosis, negative for the presence of anti-HCV and serum HCV RNA, were studied for analyzing presence of viral nucleic acid in liver tissues. Qualitative screening for HCV was done through ELISA while the nucleic acid analysis was performed through COBAS Amplicor. Detection of HCV RNA in liver tissue biopsies was performed following standard protocol of HCV detection kit (Shenzhen PG Biotech) with modifications using Light Cycler 2.0 (minimum detection limit 10 copies/ml).
Result Quantitative detection in liver biopsies following the modified method showed the presence of HCV RNA in three samples out of the ten studied.
Conclusion The results indicate that using Light Cycler 2.0, following the modified technique described, constitutes a reliable method of quantitative detection and localization of HCV in tissue in "serosilent" HCV infection.

Keywords Serosilent · Cirrhosis · HCV · Liver tissue · RT-PCR

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Introduction

Hepatitis C virus (HCV) is a global pandemic affecting 170 million persons. The infection results in a higher rate of chronic infection compared to HBV infection with approximately 80% of infected subjects showing viral persistence and chronic liver diseases.[1] In the last decade, HCV has emerged as a major causative agent of liver disease, resulting in acute and chronic infections that can lead to fibrosis,[2] cirrhosis,[3,4] and hepatocellular carcinoma.[5,6] Some 5–30% of individuals with HCV infection develop chronic liver disease. In this group, about 30% progress to cirrhosis, and in these, about 1–2% per year develop hepatocellular carcinoma. HCV-induced cirrhosis is also considered as a major indication for liver transplantation.[7] In India, the incidence of liver diseases is on the rise with hepatitis C contributing to the load singularly and in co-infection with HBV and HIV.[8–10] The

fragility of HCV RNA and the low levels of viral expression in infected tissues are a constant limitation in molecular assays for HCV characterization. It is however recognized as a key parameter for reliable diagnosis and treatment monitoring of HCV infection.

Determination of HCV RNA concentrations reduces the pre-sero conversion period in the diagnosis of HCV infection and supports management of interferon alpha-based therapies. Generally, laboratory diagnosis of HCV is based on specific antibody detection, anti-hepatitis C virus (anti-HCV) in serum.[11,12] Serological screening for viral presence by chemiluminescence technique has been reported by Dufour et al.[13]. In a Japan-based comparative study, Kita et al.[14] have demonstrated higher sensitivity of recombinant immunoblot assay (RIBA) III for anti-HCV antibody screening. According to guidelines given by Alter et al. in MMWR[15] for HCV testing and reporting, verifying the presence of anti-HCV minimizes unnecessary medical visits and psychological harm for persons who test falsely positive by screening assays. In a comprehensive review of diagnosis of patients suspected with chronic hepatitis C infection[16], it has been recommended that serological screening be followed by RIBA and reverse transcriptase PCR (RT-PCR) techniques for confirmation for viral presence. Detection of hepatitis C virus RNA in liver tissues by in situ hybridization and immuno-histochemical techniques is done in many labs around the world for enhanced sensitivity.[17,18] However, HCV infection of patients with abnormal liver function tests that are anti-HCV and serum HCV RNA negative but who have HCV RNA in the liver has been reported earlier from India.[19] Although treatment protocol in chronic hepatitis C infection in cirrhotic liver includes the use of liver biopsies, it is not commonly emphasized in cryptic cirrhotic liver.[20] Here, we report a novel approach to study "serosilent" HCV infection in cryptogenic cases of liver cirrhosis in the light of increasing evidence that a majority of cases with cirrhosis and HCV infection are at a greater risk of developing viral co-infections and hepatocellular carcinoma.

Materials and Methods

The study was conducted on a total of 30 patients of which ten patients were known positive cases of HCV infection, ten patients of cirrhotic liver admitted in the Department of Surgical Gastroenterology, BMHRC, Bhopal, and ten negative control samples were included. Cirrhosis of liver was diagnosed based on clinical features, biochemical evidence, and histological features of cirrhosis. The inclusion criteria for patients investigated for the present study were cryptogenic cirrhosis (no defined cause of liver cirrhosis) and sero-negativity for anti-HCV and HCV RNA.

Routine investigations included hemogram, renal function tests, and various liver function tests such as serum glutamate oxalacetate transaminase, serum glutamate pyruvate transaminase, alkaline phosphatase, prothrombin time, serum bilirubin, and serum proteins with albumin to globulin ratio were estimated. Liver biopsy was done to establish the histologic diagnosis and etiology, as per ethical guidelines of the institutional review board. The serum samples collected from the patients were stored at −20°C until use. Liver biopsy was performed with a sterile disposable liver biopsy needle and the material stored in a −86°C freezer until RNA extraction.

Serologic Tests

The presence of antibodies to HCV (anti-HCV) was tested using COBAS core anti-HCV EIA II (M/S. Roche diagnostics GMBH, Penzberg, Germany—ELISA).

RNA Extraction and Quantification from Serum

Total RNA isolation from serum samples was done using standard protocol of Roche COBAS Amplicor kit. The amplicons were generated and quantified in COBAS as per standard protocol following all the necessary instructions from the supplier.[21] The obtained values have a sensitivity of 1,620 copies/mL (600 IU/mL as per manual).

RNA Extraction from Liver Tissue

Total RNA isolation was done with slight modification of omitting a step of tissue wash with phosphate-buffered saline as is the protocol for fresh tissue using TRIzol reagent. Frozen tissues were taken directly in 1 mL of cold TRIzol and immediately homogenized for 60 s with a rotor power homogenizer to quickly dissociate the tissue. To this tissue, 40–200 μl of chloroform was added and mixed by vortexing for 15 s. The sample was then incubated for 15 min at RT followed by centrifugation at 1,200×*g* for 15 min. The upper aqueous layer was transferred to a new tube, and 500 μl of isopropanol was added. The samples were centrifuged at 900×*g* at 4°C for 10–30 min to pellet RNA. The supernatant was carefully removed, and 1 mL of 70% ethanol was added and vortexed on low for 5–10 s to wash the pellet thoroughly. Then, centrifugation was done at 600×*g* at 4°C for 5 min to re-pellet RNA. The supernatant was carefully removed, and the pellet was air-dried at room temperature for 5–10 min. The pellet was dissolved in DEPC-dH_2O (30-μL) by gentle pipetting and incubated at 55°C for 5–10 min.

Nucleic Acid Analysis

The quantification of HCV RNA in liver tissue biopsies was done using Shenzhen PG Biotech kit on Roche Light

Cycler 2.0 with minimum detection limit of 10 copies/ml. The kit is recommended for HCV detection from serum or plasma but was used in the present study to work on HCV RNA isolated from tissue samples with excellent results as observed and compared with known viral loads of positive controls. Amplification was done as per original protocol of the kit using master mix, RT-PCR enzyme, and PCR enhancer added to processed sample as well as prepared control. Quantification was done by selecting F1 channels and making adjustments for "fit points," "proportional," and "noise band" selections. Standard curve was created by putting up the concentration of correction controls 1 to 4 and Ct values measured. Result interpretation was done as per listed values of the kit.

HCV Genotyping

Following the quantification, identification of HCV genotypes was done using Linear Array HCV genotyping kit (Roche Diagnostics, Mannheim, Germany) as per the supplier's protocol. Briefly, HCV RNA was amplified through COBAS Amplicor (Roche Diagnostics, Mannheim, Germany) using biotinylated primers, and the amplicon was subjected to hybridization with the genotype specific probes attached on genotyping strips. After hybridization, the attached amplicon was washed and an enzyme (Horse radish peroxidase) was added followed by the addition of a substrate (3,3′-tetramethylbenzidine + H_2O_2) to develop a band which is specific to a particular genotype. The developed bands were analyzed in order to identify a genotype using a reference strip.[22,23]

Results

Quantitative RT-PCR analysis of liver biopsies by the method modified for frozen tissues was performed on ten samples negative for the anti-HCV and serum HCV RNA. Of these, six were males and four were females with an age range of 29 to 59 years. The "serosilent" patient group that was evaluated showed a preponderance of males and a plausible correlation with increasing age which was also the case in other institutional registered cirrhotic liver patients.

Three of the patient samples out of the total ten which were analyzed by RT-PCR showed the presence of HCV RNA with a sensitivity of 30% and a specificity and positive predictive value of 100%. The positive samples showed a great disparity in viral load ranging from 4.59×10^5 to 2.38×10^2 (Table 1), addressing the serendipity of sero-negative results in cases with cryptic cirrhosis. Out of the remaining seven samples, all were found to be below detection limit.

HCV genotyping of the positive samples showed the presence of genotype 3.

Discussion

Nucleic acid amplification has become one of the most valuable tools in clinical medicine, especially for the diagnosis of infectious diseases with PCR being the most widely used because of its reliability. The availability of effective antiviral therapy for hepatitis C has increased the need for molecular detection and quantification of hepatitis C viral particles.[24] Because of the use of viral kinetics during polyethylene glycol–interferon–ribavirin therapy and the development of specific new anti-hepatitis C virus (anti-HCV) drugs, assessment of the efficacy of anti-HCV drugs needs to be based not on end-point PCR assays but on real-time PCR.[25]

Pan et al.[12] have emphasized the importance of diagnosis and treatment of HCV infection using real-time fluorescent quantitative reverse transcription polymerase chain reaction and nested reversed transcription polymerase chain reaction by evaluating sera of two groups each having 180 patients. In a report based on viral hepatitis markers in sera samples

Table 1 Data showing the finding of RT-PCR of liver biopsies

Sequence no.	Patient's code	Sex/age	Anti-HCV	HCV RNA serum	HCV RNA liver
1	63/RES	F/29	Negative	Negative	2.38×10^2
2	93/RES	M/36	Negative	Negative	BDL
3	126/RES	M/59	Negative	Negative	BDL
4	221/RES	M/35	Negative	Negative	BDL
5	222/RES	F/33	Negative	Negative	BDL
6	234/RES	M/57	Negative	Negative	4.59×10^5
7	256/RES	F/49	Negative	Negative	1.01×10^3
8	279/RES	M/56	Negative	Negative	BDL
9	284/RES	F/49	Negative	Negative	BDL
10	353/RES	M/58	Negative	Negative	BDL

BDL below detection limit

of 69 patients with cirrhosis, Irshad et al.[26] have reported sero-positivity for HCV by RT-PCR in supplementation with other clinical parameters. There is extensive documentation of viral HCV RNA detection through RT-PCR, even in sero-negative cases.[27,28] In one study by Tashkandy et al.,[29] 316 serum samples were subjected to HCV antibody detection by ELISA and RIBA tests and HCV RNA detection by RT-PCR assay. The false positivity of HCV-Ab by ELISA and RIBA, when compared with RT-PCR, was reported as 5%, 3.9%, and 0% for blood donors, hemodialysis patients, and HIV–HCV co-infected cases, respectively. While comparing ELISA with RT-PCR, the false positivity was 10%, 5.9%, and 0%, respectively, for blood donors, hemodialysis patients, and HIV–HCV co-infected cases, thus delineating the importance of using the RT-PCR for HCV RNA to avoid false-negative results. In a comparative analysis of HCV RNA detection by RT-PCR and LAMP PCR in serum samples, Esfahani et al. implied that the sensitivity of the latter is more enhanced and specific, but designing a highly sensitive and specific primer set is crucial for performing LAMP amplification.[30] In recently reported series of formalin-fixed, paraffin-embedded liver samples by RT-PCR, Gruppioni et al. cited that the assessment of hepatitis C virus (HCV) RNA in liver tissues was clinically relevant in cases where histology, liver function tests, and HCV serology were insufficient for a definitive diagnosis of HCV-related hepatitis.[31]

However, the site specificity for liver biopsy in cases of latent HCV infection can be an issue for the surgeon with only portal spaces being documented as eligibility criteria for the biopsy in various studies.[32,33] The need for serial biopsies in cirrhotic liver cases for in situ localization of HCV is also ruled out on the basis of histological homogeneity of the cirrhotic tissue and evidence from studies based on viral detection after liver transplants.[34,35]

Therefore, real-time PCR remains a cost- and time-effective molecular technique of choice accompanied by sensitivity and specificity for diagnosis and prognosis of HCV, especially latent cases. During the present investigation, it is for the first time that sero-negative anti-HCV and HCV RNA cases of cryptic cirrhosis have been found positive for HCV RNA in frozen liver tissue samples. The observed results indicate that the approach described here is a reliable and must-do method of quantitative detection and localization of HCV in tissue, since it allows identification of specific regions of the viral genome with high confidence.

It is documented that epidemiology and other risk factors have a major role to play in the incidence of liver cirrhosis associated HCV infection and its management. In a major survey from Canada, there was an estimated increase in prevalence of HCV infection by sex and age group. HCV prevalence was 64% greater in males than in females (0.95% versus to 0.61%). HCV prevalence generally, though not always, increased with increasing age. HCV incidence among men overall was 0.034%, almost twice that among females with 0.018%. Findings of older age at HCV infection, male gender, and heavy alcohol intake being linked to a more rapid cirrhosis have been reported in literature.[36,37]

Although the subject group in our study, showed a preponderance of males and a similar picture emerged for the registered cirrhotic liver patients, the sample size of the present study was too small to draw any conclusive evidence on gender bias and correlation of age to viral infection as also the preponderance of genotype 3.

Conclusion

Detection of HCV in liver tissue is an important tool to study "serosilent" HCV infection in cryptogenic cases of liver cirrhosis as well as a definitive investigative method for long-term clinical management of HCV cases, especially in the light of increasing evidence that a majority of cases with cirrhosis and HCV infection are at a greater risk of developing co-infections and hepatocellular carcinoma. The present investigation arraigns that cryptogenic cirrhosis patients had underlying HCV infection which lead to the diseased state, a clinical development that is well documented in the literature. Further studies, comprising a larger sample size, keeping in view the age and gender bias, are being undertaken to plan a stratagem for elucidation of viro-pathological mechanisms inclusive of assessment of viral genotype(s), even in patients without evidence of circulating HCV.

The ethical guidelines as per norms of Indian Council of Medical Research were strictly adhered to. The study was approved by the Institutional Review Board, BMHRC, Bhopal, India.

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Discussant

Dr. Robert C. G. Martin (Louisville, KY): Dr. Maudar and colleagues from the Department of Surgical Gastroenterology Bhopal Memorial Research Center present their small pilot study evaluating patients who are serum negative for HCV RNA but are liver tissue positive for hepatitis C virus. This is an interesting study with the potential to further risk stratify the small subset of patients who are

infected with hepatitis C virus but who sero convert and demonstrate no viral load within the serum. This is an interesting concept with potential for improvement in the management of patients with hepatitis C vaccine but is inherently limited by the small number of patients, that being three, who actually have this type of serosilent HCV infection. I have three questions for the authors. One, probably being the most important, that being what is or what do we know, is the true clinical relevance of patients who are serosilent hepatitis C RNA patients with only active RNA within the liver. Do we truly know that those patients are at a greater risk for hepatocellular carcinoma vs. potentially normal control? The second is in regard to the validation of the RT-PCR technique. How do you know that you are not amplifying other types of RNA? What type of specificity control have you, indeed, evaluated and presented?

Third is a follow-up to question 1 in the fact that if you truly believe that the serosilent HCV patient is at risk for hepatocellular carcinoma, what type of screening modality would you recommend for this unique subset of patients?

Closing Discussant

Dr. Kewal Krishan Maudar: Query 1: Do we know the true clinical relevance of patients who are serosilent hepatitis C RNA patients with only active RNA within the liver? Do we truly know that those patients are at a greater risk for hepatocellular carcinoma vs. potentially normal control?

Response: There is an emphatic clinical relevance of patients who are serosilent hepatitis C with active RNA only within the liver, since according to the literature, they are at a higher risk for developing HCC, after varying latency periods (depending on individual response), because chronic HCV infection progresses to liver fibrosis, cirrhosis, and finally HCC. This fact is highly supported by various studies as cited by us (References 5 and 6, also Castello et al. 2010).

Query 2: In regard to the validation of the RT-PCR technique: How do you know that you are not amplifying other types of RNA? What type of specificity control have you, indeed, evaluated and presented?

Explanation: The technique of real-time PCR that has been standardized in the laboratory involves the use of highly specific primers for the amplification of a specific region of the HCV genome and the analysis is based on FRET probes. The controls consisted of known negative liver tissue samples and known positive samples of HCV infection.

Query 3: If the serosilent HCV patient is at risk for hepatocellular carcinoma, what type of screening modality would you recommend for this unique subset of patients?

Explanation: Screening of liver tissue of serosilent HCV patients through quantitative real-time PCR is the only practical approach for diagnosis and management of patients with latent HCV infection, as early detection increases the chances of application of curative treatments.