

## The Necroinflammatory activity of HCV in liver biopsies of Iraqi patients that detected by *In Situ* hybridization technique.

**\*Raghad Harbi Al-azzawi\***

**Ghanima Sadiq Mohammed\***

**Nawal Mahdi Al-khalidi\*\***

Date of acceptance 30/6/2008

### Abstract:

*In situ* hybridization assay was performed from 22 chronic hepatitis-C virus(CHC) patients (9 males and 13 females) (all of them known to be positive for anti-CH antibodies and high transaminase levels(GOT,GPT)) in order to study the necroinflammatory activity and detection of HCV-RNA in the sections of liver biopsies. The results showed that the percentage of infected cells were ranged from (10-100%) with mean value of 61.77%. The HCV-RNA was detected in the cytoplasm of hepatocytes.

**Key words:** hepatitis-c virus, *In situ* hybridization, liver biopsies.

### Introductio:

Blood borne non – A, non – B hepatitis was first recognized in mid 1970s [1,2]. Hepatitis –C virus (HCV) was first identified by molecular cloning of the virus genome in 1989 [3].

HCV is a non – cytopathic hepatotropic member of the flaviviridae that cause acute and chronic hepatitis, and hepatocellular carcinoma [4]. HCV has been classified as a hepacivirus based on its genomic organization and genetic homology with pestiviruses and flaviviruses [5].

Several reports have described the detection of HCV RNA in liver tissue by the techniques of *in situ* hybridization and *in situ* PCR [ 6 , 7 , 8, 9] .

ISH technique was initially introduced in 1969 [10; 11], for the localization of DNA sequences. After 20 years Macvill described non – radioactive protocol to detect mRNA transcript in different tissue[12]. Currently the diagnosis is based on specific Ab detection (anti – HCV) in serum and

several nucleic acid – based technology are undertaken to detect the prevalence of circulating virus genome [13]. The detection of virus genome and viral protein in liver sections is an additional diagnosis procedure [14]. Nevertheless, the detection and exact localization of the HCV genome in the liver and in the other tissues may have a key role to understanding the pathogenesis history of HCV infection [15, 16].

For this purpose the aim of this study was:

Detection of HCV RNA by *in situ* hybridization assay in CHC sections of liver biopsies in Iraqi patients.

### Materials and Methods:

#### *In Situ* Hybridization Test:

Replication of the hepatitis C virus in the hepatocytes of liver biopsy was detected by using the RNA probe hybridization / Detection system – *In Situ* Kit that was a complete hybridization immunodetection system, incorporating the biotin-streptavidin amplification technology

\*Department of Biology/College of Science/uiversity of Baghdad

\*\*Gastroetrology and Hepatology Teaching Hospital/Baghdad

that recommended by manufacture (Maxim Biotech, Sanfrancisco).

**Principle:**

The method involved deproteinization of fixed tissue sections mounted on slides, hybridization of the target nucleic acid sequence with a RNA probe labeled with biotin, and detection of the hybridized probe by adding enzyme-conjugate streptavidin-AP followed by adding a suitable enzyme substrate (single component BCIP/NBT solution) which produced an intense blue signal appeared at the specific site of the hybridized probe that was visible by light microscopy.

This streptavidin-AP conjugate directly linked to the biotinylated probe, providing a rapid and highly sensitive detection method [17].

**Assay Procedure:**

**1- Tissue Preparation:**

- The slices were backed (after positioning the sections of liver biopsy on slides treated with egg albumin to adhere the tissues) in a vertical position at 70°C for 1 hour or 60-65°C overnight.
- The tissue sections were deparaffinised by immersing the slides sequentially in the following solutions at room temperature for (Xylene, 100% Ethanol, 95% Ethanol, 70% Ethanol, Deionized distilled water).
- The slides were placed in the boiling citric buffer (1X) for 3 minutes, boiled by hot plate, then, the slides were immediately transferred to deionized distilled water at 20-25°C and washed three times for 2 minutes each, then, the slides were dried.
- Enough (1X) proteinase K was added to cover the tissue sections and incubated for 10 minutes at 37°C.
- The slides were washed in deionized distilled water for 3 times, 2 minutes each.

- The slides were dehydrated by immersing them sequentially in the following solutions at room temperature (70% Ethanol, 95% Ethanol, 100% Ethanol).

- The slides were dried by incubating them at 37°C for 5 minutes.

**2- Hybridization and Detection:**

- The working cRNA probe/hybridization solution was added to the tissue sections. A cover slip was placed over each slide. Positive and negative tissue controls and positive control probe should be included.
- The slides were placed in an oven at 70°C for 8-10 minutes in humidity chamber partially opened to denature the secondary structure of RNA.
- The slides were placed in humidity chamber and incubated at 37°C for overnight to allow hybridization of the probe with the target nucleic acid.
- The slides were soaked in 1X detergent wash buffer at 37°C until the cover slips fall down.
- 1-2 drops of RNase A (15µ/ml) were added to tissue section, incubate at 37°C (in humidity chamber) for 30 minutes.
- The slides were washed by protein block (prewarmed) at 37°C for 3 minutes, 3-times.
- The excess of buffer was wiped off carefully from around the tissue sections. Then 1-2 drops of conjugate were added to tissue sections and incubated at 37°C for 20 minutes (in humidity chamber).
- The slides were rinsed in detergent wash buffer for 5 minutes.
- The excess of buffer was wiped off carefully from around the tissue sections. Then, 1-2 drops of substrate were added to tissue sections and incubated at room temperature for 20 minutes in dark place.

- \* A blue colored precipitate was formed at the site of the probe in positive cells.
- The excess reagent was tapped off, and the slides were rinsed in 2-3 changes of distilled water.
- The slides were counterstained by using Nuclear Fast Red (NFR) for 30 seconds to study morphology.
- The slides were rinsed in distilled water for 1 minute.
- The slides were dehydrated by sequentially dipping the slides in graded alcohols: 95% ethanol, then twice in 100% ethanol, and then in xylene.
- The slides were mounted by DPX.
- The cells were examined microscopically under oil immersion lens for scoring.

## Results and Discussion:

Twenty – two HCV chronically infected patients ( 9 males , and 13 females ) who attended at the Gastroenterology and hepatology Teaching Hospital were investigated ,

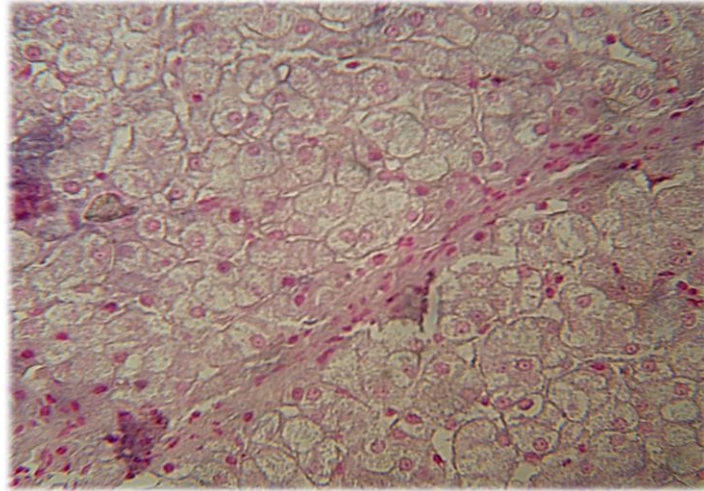
who were submitted to liver biopsies before starting antiviral therapy . All individuals were known to be positive for anti – HCV antibodies and high transaminases levels. The mean age of male patients was  $(38.5 \pm 21.5)$  years, and of female patients was  $(33 \pm 27)$  years. Liver biopsies from CHB patients used as negative tissue controls.

The necroinflammatory activity was detected with all liver sections, ranging from mild to moderate score that examined in the hospital laboratory.

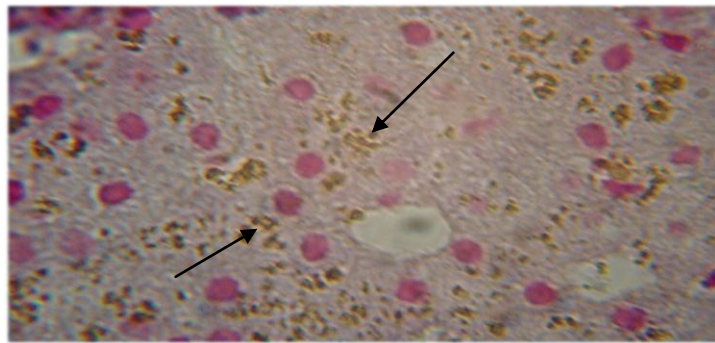
The results shows that the percentage of infected cells ranged from (10%) to (100 %) with mean value (61.77 %). The detection of HCV RNA by ISH was mainly found in the cytoplasm of hepatocytes (fig 1.1).

This result is in agreement with Carvallo et al [16] who found that the localization of ISH signals was cytoplasmic or perinuclear, and HCV RNA detected in 8 of 10 patients (80 %).

**A**



**B**



**Fig 1.1 : ISH result of HCV in hepatocytes .A- control of HBV biopsy (40X). B- the specificity of riboprobe for HCV infection (100X).stained with (Nuclear Fast Red (NFR))**

In some studies, only a subset of biopsies from patients with HC stained positive for HCV RNA, and a small percentage of hepatocytes appeared to be infected [6,18]. In other studies, HCV genomes were found in over 90 % of biopsies from infected patients [19], suggesting that HCV infection may be more widely disseminated in human liver than previously thought. Previous studies have reported a wide range in the percentages of hepatocytes positive for HCV RNA in positive liver; the reports have also presented conflicting data on the correlation among hepatic RNA, serum RNA, and hepatocellular injury. Reports suggesting no relationship between the level of HCV RNA in the liver and degree of hepatocellular injury have led to the hypothesis that HCV may not be a cytopathic virus [5,20,21,22]. However, other reports have suggested a correlation between HCV RNA and, or HCV antigens in the liver and the degree of liver injury [7,23,24].

Chang et al [5] reported that the positive signals were located outside the nuclei, confirming that the signals resulted from In situ hybridization (IS) detecting of cytoplasmic RNA and not plasmid DNA. They showed a cytoplasmic localization of the granular signals which represents HCV genomic RNA and 73 % of hepatocytes stained positive for HCV genomic RNA by ISH. Rodrigueux – Iñigo et al [25] found that the percentage ranged from 0.04 % to 83.3 %.

Revie et al [26] reported that the ISH results suggest that the positive strand of HCV is synthesized at or migrates to the plasma membrane and that the negative strand remains in the cytoplasm. RNA – dependent RNA polymerase contains a transmembrane segment which is anchored in the membrane. Non – structural proteins

and positive strand RNA have also been found associated with the plasma membrane. These results suggest that the site where HCV is fully assembled is probably in or near the plasma membrane of the infected cells. Probably HCV – RNA is synthesized in the cytoplasm and migrates to the plasma membrane for the final assembly. The completed virion is then released into the extra cellular space.

The association between histological index activity, serological markers, serum and liver HCV – RNA content could be used to assess the effectiveness of anti – viral in CHCV [27], and ISH providing a solid basis for diagnosis and control of patients under anti – viral therapy.

Carvallo et al [16] reported that ISH performed constitutes a reliable method of HCV detection and localization of HCV in tissue, since it allows identification of specific region of the viral genome with high confidence. On the other hand, comparing results between HCV RNA detection in serum and liver by RT – PCR and ISH, respectively, a lower sensitivity of the second method was observed. This could be expected since ISH does not include amplification of viral genome.

## References:

1. Prince, A.M.; Brotman, B.; Grady, G.F.; and Kuhns, W.J. 1974. Long incubation posttransfusion hepatitis without serological evidence of exposure to hepatitis B virus. *Lancet*; 2: 241-246.
2. Feinstone, S.; Kapikian, A.Z.; Purcell, R.H.; and Holland, P.V. 1975. Transfusion – associated hepatitis not due to viral hepatitis type A or B. *N Engl. J Med.* ; 292: 767-770.
3. Choo, Q.L.; Kuo, G.; Weiner, A.J. 1989. Isolation of a cDNA clone

- derived from a blood – born non – A, non – B viral hepatitis genome . Science ; 224 : 359 – 362.
4. Lindenbach, B.D.; and Rice, C.M. 2005. Unravelling hepatitis C virus replication from genome to function. Nature: 436(7053): 933-38.
  5. Chang, M.; Marquardt, A.P.; Wood, B.L.; Willmas, O.; Cotler, S.J.; Taylor, S.L.; Jr, R.L., C.; and Gretch, D.R. 2000. In situ distribution of hepatitis C virus replication – intermediate RNA in hepatitis tissue and its correlation with liver disease. J.Vitol. ; 74(2) : 944 – 955.
  6. Blight, K. ; Trowbridge, R. ; Rowland, R. ; and Gowans, E. 1992. Detection of hepatitis C virus RNA by in situ hybridization. Liver; 12 : 286 – 289.
  7. Haruna, Y.; Hayashi, N.; hiramatsu, N.; Takehara, T.; Hagiwara, H.; Sasaki, Y.; Kasahara, A.; Fusamoto, H.; and Kamada, T. 1993. Detection of hepatitis C virus RNA in liver tissues by in situ hybridization technique. J. Hepatol.; 18: 96-100.
  8. Nuova, G.J.; Lidonnici, K.; Mac Connell, P.; and Lane, B. 1993. Intracellular localization of polymerase chain reaction (PCR)-amplified hepatitis C cDNA. Am.J. Surg. Pathol.; 17: 683-690.
  9. Felgar, R.E.; Montone, K.T.; and Furth, E.F. 1996. Arapid method for the detection of hepatitis C virus RNA by in situ hybridization . modern Pathol.; 9: 696-702.
  10. John, H.A.; Birnstiel, M.L.; and Jones, K.W. 1969. RNA-DNA hybrids at the cytological level. Nature; 223: 582-587.
  11. Pardue, M.L.; and Gell, J.G. 1969. Molecular hybridization of radioactive DNA to the DNA of cytological preparations. Proc. Natl. Acad. Sa. USA; 64: 600-604.
  12. Macville, M.V.; Van Dorp, A.G.; Wiesmeijer, K.C.; Dirks, R.W.; Fransen, J.A.; and Raap, A.K. 1995. Monitoring morphology and signal during non-radioactive *in Situ* hybridization producers by reflection – contrast microscopy and transmission electron microscopy. J. Histochem. Cytochem.; 43: 665-674.
  13. Schiff, E.R.; de Medina, M.; and Kahn, R.S. 1999. New perspectives in the diagnosis of hepatitis C. Semin Liver Dis.; 19: 3-15.
  14. Walker, F.M.; Dazza, M.C.; Dauge, M.C.; Boucher, O.; Bedel, C.; Henin, D.; and Lehy, T. 1998. Detection and localization by in situ molecular biology techniques and immunohistochemistry of hepatitis c virus in livers of chronically infected patients. J. Histochem. Cytochem.; 46: 653-660.
  15. Qian, X.; Guerrero, R.B.; Plummer, T.B.; Alves, V.F.; and Lioyd, R.V. 2004. Detection of hepatitis C virus RNA formation-fixed paraffin- embedded sections with digoxigenin- labeled cRNA probes. Diag. Mol. Pathol.; 13: 9-14.
  16. Carvalho, H.S.; Baptista, M.L.; Pinto, M.A.; Silva, M.; Takiya, C.M.; Chagas, V.; Pannain, V.; Coelho, H.S.M.; and Yoshida, C.F.T. 2005. Detection of hepatitis C virus RNA by in situ hybridization in paraformaldehyde fixed biopsies. Mem. Inst. Oswaldo. Cruz. , Rio de Janeiro; 100 (3) : 269 – 272.
  17. Lewis, F.A.; Griffith,S.; Dunncliffe, R.; Wells, M.; Dudding, N.; and Bird, C.C. 1987. Sensitive in situ hybridization technique using biotin- strptavidin-polyalkaline phosphatase complex. J.Clin.Pathol.; 40: 163-166.

18. Lau, J.Y.; Krawczynski, K.; Negro, F.; and Gonzales Peralta, P. 1996. In situ detection of hepatitis C virus- a critical appraisal. *J.Hepatol.*; 24: 43-51.
19. Gastaldi, M.; Massacrier, A.; Planells, R.; Robaglia Schlupp, A.; Portal Bartolomei, I.; Quilici, F.; Fiteni, J.; Mazzella, E.; and Cau, P. 1995. Detection by in situ hybridization of hepatitis C virus positive and negative RNA strands using digoxigenin – labeled cRNA probes in human liver cells. *J. Hepatol.*; 23: 509-518.
20. Nouri- Aria, K.T.; Alexander, G.M.; Margin, S.; Anderson, M.G.; Eddleston, A.L.; and William, R. 1988. Abnormal T-cell activation in chronic hepatitis B viral infection: a consequence of monocyte dysfunction. *J. Immunol.*; 64: 733-738.
21. Negro, F.; Krawczynski, K.; Quadri, R.; Rubbia-Brandt, L.; Mondelli, M.; Zarski, J.; P.; and Hadengue, A. 1998. Detection of genomic- and minus- strand of hepatitis C virus RNA in the liver of chronic hepatitis C patients by strand-specific semiquantitative reverse- transcriptase polymerase chain reaction. *Hepatol.*; 29: 536-542.
22. Negro, F.; Giostra, E.; Krawczynski, K.; Quadri, R.; Rubbia-Brandt, L.; Mentha, G.; Colucci, G.; Perrin, L.; and Hadengue, A. 1999. Detection of intrahepatic hepatitis C virus replication by stand-specific semiquantitative RT-PCR: preliminary application to the liver transplantation model. *J. Hepatol.*; 29: 1-11.
23. Gretch, D.R.; Bacchi, C.E.; Corey, L.; DelaRosa, C.; Lesniewski, R.R.; Kowdley, K.; Gown, A.; Frank, I.; Perkins, J.D.; and Carithers, R.L. 1995. Persistent hepatitis C virus infection after liver transplantation : Clinical and Virological features. *Hepatology* ; 22: 1-9.
24. Sansonno, D.; Cornacchiulo, V.; Lacobelli, A.R.; Gatti, P.; Di Stasi, M.; and Dammacco, F. 1995. Demonstration and distribution of HCV RNA sequences by *in situ* hybridization and HCV- related proteins by immunohistochemistry in the liver tissue of patients with chronic HCV infection. *Pathobiology*; 63: 239-248.
25. Rodrigueus- Inigo, E.; Bartolome, J.; deLucas, S.; Manzarbeitia, F.; Pardo, M.; Arecena, C.; and Gosavez, J. 1994. Histological damage in chronic hepatitis C is not related to the extent of infection in the liver, *Am. J. Pathol.*; 154: 1877-1881.
26. Revie, D.; Braich, R.S.; Bayles, D.; Chelyapov, N.; Khan, R.; Geer, C.; Reisman, R.; Kelley, A.S.; Prichard, J.G.; and Salahuddin, S.Z. 2005. Transmission of human hepatitis C virus from patients in secondary cells for long-term culture. *Viol. J.*; 2: 37.
27. Arrojo, I.P. ; Pareja, M.O. ; Orta, M.D. ; Luque, F.N. ; Lamas, M.C. ; Gordo, F.S. ; Mancha, I.V. 2003. Detection of a healthy carrier of HCV with no evidence of antibodies for over four years. *Transfusion*; 43: 953 – 957.

دراسة الفعالية الالتهابية التنخرية للفايروس نمط-ج في خزعات الكبد للمرضى  
العراقيين والكشف عنها باستخدام تقنية In Situ hybridization

غنيمة صادق محمد\*

رغد حربي الغزاوي\*

نوال مهدي الخالدي\*\*

\* قسم علوم الحياة- كلية العلوم- جامعة بغداد.

\*\* مستشفى الجهاز الهضمي وامراض الكبد التعليمي-بغداد.

الخلاصة:

تم اجراء اختبار *in situ hybridization* لـ 22 مريضا مصابا بالتهاب الكبد الفيروسي نمط (ج) لـ ( 9 ذكور و 13 انثى ) (مسبقا اظهروا جميعهم نتائج موجبة لاضداد anti-Hc IgG وارتفاع مستوى انزيمات GPT,GOT) وذلك من اجل دراسة الفعالية الالتهابية التنخرية والكشف عن وجود الحامض النووي الرايبوس لفايروس التهاب الكبد نمط (ج) (HCV-RNA) في المقاطع النسيجية لخزعة الكبد. اظهرت النتائج بان نسبة الخلايا المصابة تتراوح ما بين 10% الى 100% وبمعدل (61.77%)، وان الحامض النووي الرايبوس قد تم الكشف عنه في سايتوبلازم الخلايا الكبدية.

الكلمات المفتاحية: التهاب الكبد الفيروسي نمط-ج ، تقنية *In situ hybridization* ، خزعات الكبد.