Incidence of silent hepatitis b and hcv genotype among chronic hepatitis c patients

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ABSTRACT

This multi-center study was designed as a trial to explore the frequency of silent hepatitis B infection among hepatitis C patients and to determine the prevalent genotype of hepatitis C virus (HCV) in these patients. The study comprised 45 patients with post-hepatitic liver cirrhosis. All patients gave blood samples for estimation of liver function tests and ELISA estimation of serum levels of hepatitis B surface antigen (HBsAg) and anti-HCV antibodies; patients with HBsAg positive were excluded off the study. Qualitative detection of HCV RNA and HBV DNA by PCR (home-made PCR) and quantitative PCR for estimation of HCV viremia and HCV genotyping by RFLP technique were performed. The HCV-Ab was detected in all samples irrespective of its clinical severity class with a mean viremia level of 792336.7±400074.8; range: 134985-1957632 viral copy/ml as determined by quantitative PCR with a non-significant difference between clinical severity classes as regards viremia level. The HBV DNA was detected using qualitative PCR in 20 samples (44.4%); 4 class A, 7 class B and 9 class C samples with a significant increase of the frequency of silent HB in patients with class B ($X^2=5.446$, $p<0.01$) and C ($X^2=8.154$, $p<0.001$) in comparison to class A patients. Genotyping of HCV reported 41 samples (91.1%) with genotype-4 and 4 samples (8.9%) with genotype-1 with a prevalence rate of HCV genotype-4 was 91.1%. There was positive non-significant correlation between both HCV genotype and the presence of silent hepatitis B infection and clinical severity, however, using the receiver operating characteristic (ROC) curve analysis judged by the area under the curve (AUC) to evaluate the sensitivity and specificity of detection of silent hepatitis B infection and identification of HCV genotype as predictors of severe hepatitis showed a non-specific role for genotyping for prediction of severity with AUC=0.467, while the detection of HBV DNA using PCR in patients with HCV infection is a specific predictor of severity with AUC=0.617. It could be concluded that HCV genotype-4 is the most prevalent type in Egyptian Hepatitis C cirrhotic patients with an incidence of silent hepatitis B of 44.4% and its detection is a specific predictor of severe cirrhosis.
Key Word*: Hepatitis - PCR - Genotype*

INTRODUCTION

Hepatitis C virus is the major etiological agent of chronic hepatitis and liver disease worldwide, and it is a major cause of morbidity and mortality. In 80% of cases, infection with HCV results in chronic hepatitis, possibly leading to cirrhosis and hepatocellular carcinoma[1].

HCV is characterized by a high degree of nucleotide sequence variability. Overall, the heterogeneity of the viral genome ranges from 30 to 35% between different genotypes, although it varies with the specific region of the genome, and it has been

used to define three types of regions: highly conserved regions, e.g., the 5′ untranslated region, variable regions, e.g., envelope 1 [E1] and nonstructural 5b [NS5b] and hypervariable regions (HVR), e.g., HVR1 and HVR2 in E2[2]. Furthermore, because of errors of the RNA-dependent RNA polymerase, HCV has a high rate of mutation during replication, and it exists in the bloodstreams of infected persons as complex distributions of mutants known as viral quasi-species, which fluctuate during the course of the disease, mainly as a result of immune pressure. These coexisting mutant genomes always have a consensus, or master, sequence[3].

The classification HCV into six major genotypes has been accepted[4] and there is further intra-subtype and intra-subject heterogeneity[5]. The development of an effective HCV vaccine to protect humans from HCV infection and chronic liver disease is a public health priority, yet differences in antigenic epitopes in genotypes, subtypes, and quasi-species could make cross-protection unlikely[6].

HCV genotypes 4-6 are very common in geographic areas where chronic hepatitis C is highly prevalent. In Africa, circulating HCV is extremely heterogeneous[4]. Genotype 5 is endemic in Southern Africa. Genotype 1 circulates in North and Central Africa, and Genotype 2 has been found in North, Western, and Central Africa[7]. HCV Genotypes 1 and 4 are very heterogeneous in Cameroon, perhaps due to ancient infections. The homogeneity of HCV Genotype 2 indicates its more recent introduction from Western Africa[8].

Occult (silent, unapparent) hepatitis B infection is defined as the presence of HBV-DNA where hepatitis B virus (HBV) surface antigen (HBsAg) is absent. HBV-DNA positivity alone could be detected not only in crpytogenic hepatitis, hepatocellular carcinoma, recovered HBV infections and hepatitis C virus infections, but also in seronegative populations without symptomatic liver diseases. The mechanism, clinical outcome and risk of transmission of occult hepatitis B is not yet clearly defined[9].

Occult HBV infections are not restricted to areas of high HBV endemicity. It is now established that occult HBV infection among non-HCV patients suffering from chronic hepatitis varies from 20% to 30% in Europe, and in the context of HCV infection it varies from 20% in France up to 80% in Japan. During the course of HCV infection, occult HBV infection may worsen liver damage induced by HCV and reduce the response to HCV antiviral treatment. Occult HBV infection is a frequent phenomenon and HBV DNA testing with highly sensitive PCR in the clinical setting is therefore becoming of paramount importance[10].

Occult hepatitis B has been strongly implicated as a culprit that facilitates the development of hepatocellular carcinoma, typically in the background of cirrhosis. The finding of occult hepatitis B in noncirrhotic hepatocellular carcinomas among patients with hepatitis C who achieved antiviral sustained virologic response raises provocative theories regarding the

natural history of both of these viral hepatitis agents[11].

The present study was designed as a trial to explore the frequency of silent hepatitis B infection among hepatitis C patients and to determine the prevalent genotype of hepatitis C virus in these patients.

PATIENTS & METHODS

This multi-center retro-prospective study was conducted at Hepatology; National Liver Institute and Medical Biochemistry and Clinical Pathology Departments, Faculty of Medicine, Benha University. The study comprised 45 patients with post-hepatitic liver cirrhosis admitted or followed-up at Hepatology Department; all demographic and clinical data, routine investigations and result of liver biopsy data were captured out of patients' files, (Table 1). Patients enrolled in the study were intentionally chosen so as to include 15 patients of each severity class according to Child-Pugh classification of cirrhosis[12]. Patients with HBsAg positive were excluded off the study.

Sampling: A 10-ml venous blood sample was collected and equally divided in two dry sterile vacutainer tubes:

1. The first was allowed to clot in water bath at 37°C for 20 minutes and centrifuged, then serum was separated and divided into 2 aliquots; one aliquot was used for assay of liver function tests, hepatitis markers, the other aliquot stored at -20°C for assay of hepatitis C virus antigen (HCV-Ag) by ELISA.
2. The second tube was left to clot and centrifuged in the PCR unit and the serum was divided into 2 aliquots under strict sterile conditions and stored at -80°C to be used for PCR and for HCV genotyping.

Table (1): Demographic and clinical data

		Total	Class A	Class B	Class C
Number		45	15	15	15
Age (years)		42.4±5.5	41.4±6.1	43.3±3.9	42.5±6.4
Sex; M:F		32:13	11:4	10:5	11:4
Clinical data					
Jaundice		21 (46.7%)	0	6 (40%)	15 (100%)
Ascites		19 (42.2%)	0	4 (26.7%)	15 (100%)
Hematemesis		13 (28.9%)	0	3 (20%)	10 (66.7%)
Melena		14 (31.1%)	0	3 (20%)	11 (73.3%)
Liver					
	Normal	6 (13.3%)	6 (40%)	0	0
	Enlarged	16 (35.6%)	9 (60%)	7 (46.7%)	0
	Shrunken	23 (51.1%)	0	8 (53.3%)	15 (100%)
Enlarged Spleen		26 (57.8%)	3 (20%)	8 (53.3%)	15 (100%)
Edema lower of limbs		20 (44.4%)	0	5 (33.3%)	15 (100%)

Data are presented as mean±SD, numbers; percentages are in parenthesis

Investigations:

1. Liver function tests were done using Integra 400 autoanalyzer (Roche Instrument Center AG, Rotkreue, Switzerland) to estimate serum bilirubin, total and direct[13], serum aspartate and alanine transaminases (AST & ALT) activities[14], total proteins and albumin levels[15], alkaline phosphatase[16], γ-glutamyl transferase (GGT)[17] and prothrombin time measurement[18].

2. ELISA detection of hepatitis B surface antigen (HBsAg)[19] using kits supplied by Sorin Biomedica Diagnostics (Diasorin, Italy) and anti-HCV antibodies were detected by Murex anti-HCV (version IV) by Murex VK47148, England[20]

3. Qualitative detection of HCV RNA by PCR (home-made PCR primer)[21].

4. Quantitative HCV-RNA PCR by COBAS AMPLICOR HCV monitor test, version 2.0 provided by Roche Diagnostic System, Inc. Hacienda Drive, Pleasanton, CA, USA

5. Qualitative HBV DNA by PCR using QIAamp DNA minikit provided by Qiagen, GmbH, D-40724, Hilden from Tahoma, France

6. HCV genotyping: by REFLP technique using kit materials provided by Invitrogen Tech-line, USA including 4 types of restriction endonucleases; Rsa 1 to recognize the sequence GT/AC and generates fragments with blunt ends, Hinf 1 to recognize the sequence G/ANTC and generates fragments with 5'-cohesive termini, MVa1 recognizes the sequence CC/GG and generates with 5'-cohesive termini and HaeIII recognizes the sequence GG/CC and generates fragments with blunt ends. Two mixtures were prepared of nested PCR product (10 μl each) with buffer and Rsa 1 and HaeIII enzymes in mixture 1 and MVa1 and Hinf 1 enzymes in mixture 2; and were digested for 2 hours at 37°C simultaneously then electrophoresis was done in a 4% metaphore gel (FMC Bioproducts). Type 4 bands are seen at 205 bp and type 1 at 144 bp[22].

Statistical analysis

Obtained data were presented as mean±SD, ranges, numbers and ratios. Results were analyzed using paired t-test and Chi-square test. Possible relationships were investigated using Pearson linear regression. Sensitivity and specificity of determination of HCV genotype and detection of silent hepatitis B as predictors of severe hepatitis were evaluated and confirmed using the receiver operating characteristic (ROC) curve analysis judged by the area under the curve (AUC). Statistical analysis was conducted using the SPSS (Version 10, 2002) for Windows statistical package. P value <0.05 was considered statistically significant.

RESULTS

Estimated liver function tests showed a significant (p<0.05) difference between patients included in the three studied classes, being worse in patients of Class C, (Table 2).

The HCV-Ab were detected in all samples irrespective of its severity class and qualitative PCR identified

HCV-RNA (Fig. 1) in all examined samples with a mean viremia level of 792336.7±400074.8; range: 134985-1957632 copies/ml as determined by quantitative PCR. There was a non-significant (p>0.05) difference between patients categorized according to the clinical severity class as regards the quantitative HCV-RNA viremia as quantitated using PCR, (Fig. 2).

The HBV DNA (Fig. 3) were detected using qualitative PCR in 20 samples (44.4%); of these 20 samples of silent hepatitis B, 4 samples belong to class A patients, 7 samples belong to class B patients and 9 samples were of class C. There was a significant increase of the frequency of silent HB in patients with class B (X^2=5.446, p<0.01) and C (X^2=8.154, p<0.001) in comparison to frequency detected in class A patients with a non-significant difference between classes B and C (X^2=0.174, p>0.05), (Fig. 4).

Genotyping of HCV reported 41 samples (91.1%) with genotype-4, (Fig. 5) and 4 samples (8.9%) with genotype-1, (Fig. 6); with a prevalence rate of 91.1% for genotype 4, however, there was a non-significant difference between samples categorized according to clinical severity class as regards the HCV genotype distribution (X^2=0.474, p>0.05), (Fig. 7).

There was positive but non-significant correlation between both HCV genotype and the presence of silent hepatitis B infection and patients' age or gender or clinical severity class. However, using ROC curve analysis to evaluate the sensitivity and specificity of detection of silent hepatitis B infection and identification of HCV genotype as predictors of severe hepatitis showed a non-specific role for genotyping in prediction of severity with AUC=0.467, while the detection of HBV DNA using PCR in patients with HCV infection is a specific predictor of severity with AUC=0.617, (Fig. 8).

Table (2): Mean (±SD) of estimated levels of liver function tests

	Total	Class A	Class B	Class C
AST (U/L)	102±10.3	99.1±11.5	102.5±9	104.3±10.2
ALT (U/L)	76.9±11.2	74.6±10.2	75.9±11.5	80.2±11.7
Total bilirubin (mg/dl)	2.94±1.8	1.39±0.21	2.68±1.14*	4.75±1.65*†
Direct bilirubin (mg/dl)	1.67±1.28	0.61±0.25	1.37±0.59*	3.02±1.22*†
Total proteins (g/dl)	6.46±0.63	7.1±0.35	6.4±0.3*	5.8±0.42*†
Albumin (g/dl)	3.12±0.7	3.95±0.28	3.12±0.25*	2.29±0.26*†
Prothrombin concentration (%)	62.8±15	79.5±6.4	62.83±5*	46.2±6.6*†
Alkaline phosphatase (U/L)	127±43.4	89.3±15	127±23.5*	164.7±45.7*†
GGT (U/L)	52.8±18.9	64.8±18.9	52.8±12.4*	40.9±17.5*†

*: significant versus values estimated in samples of Class A patients
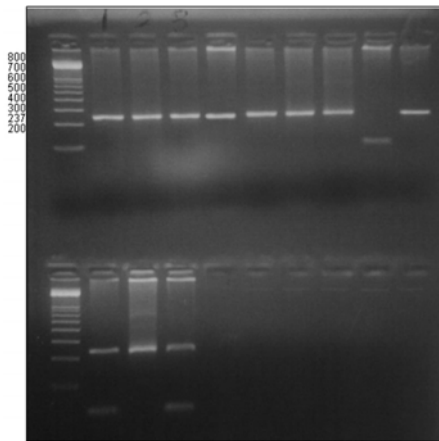†: significant versus values estimated in samples of Class B patients

Fig. (1): Electrophoretic analysis of HCV amplification product. HCV band=237 bp. Lane 2& 8: positive & negative controls, respectively. Lanes 3-7: samples positive for HCV. Lane 1: RNA standard size marker.
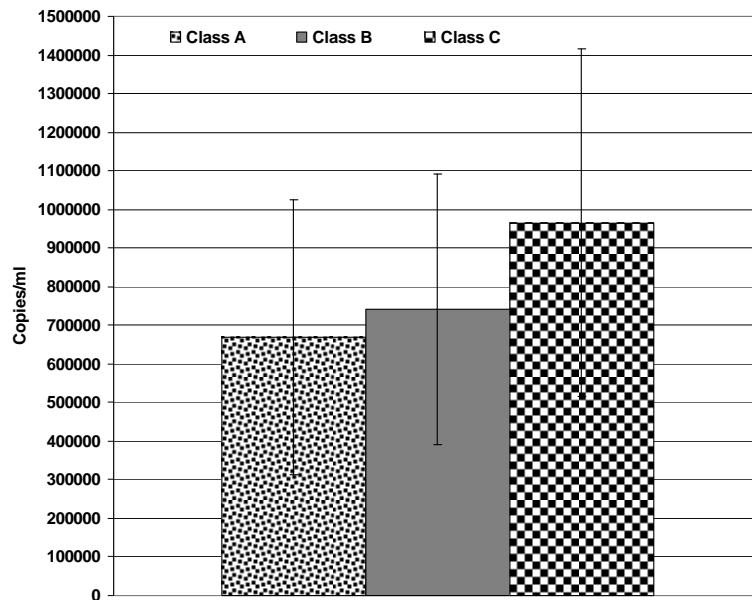

Fig. (2): Mean (+SD) of HCV-RNA viremia levels estimated in patients categorized according to Chlid severity class
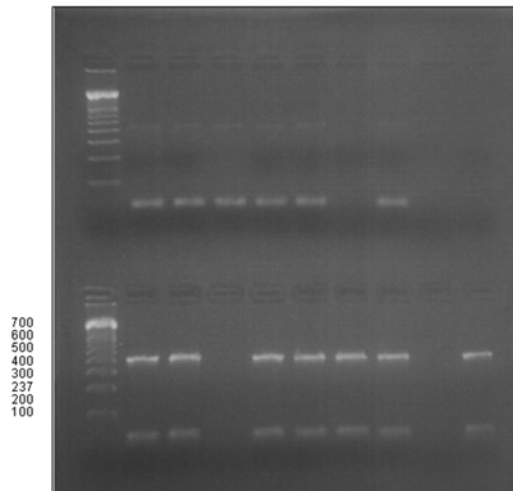
Fig. (3): Electrophoretic analysis of HBV amplification product. HBV band=400 bp. Lane 2 & 8: positive & negative controls, respectively. Lanes 4-7: samples positive for HBV. Lane 3: specimen negative for HBV. Lane 1: DNA standard size marker
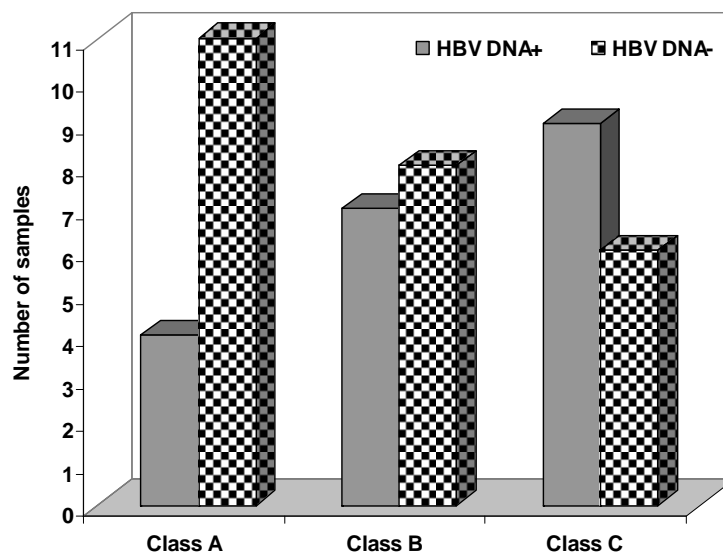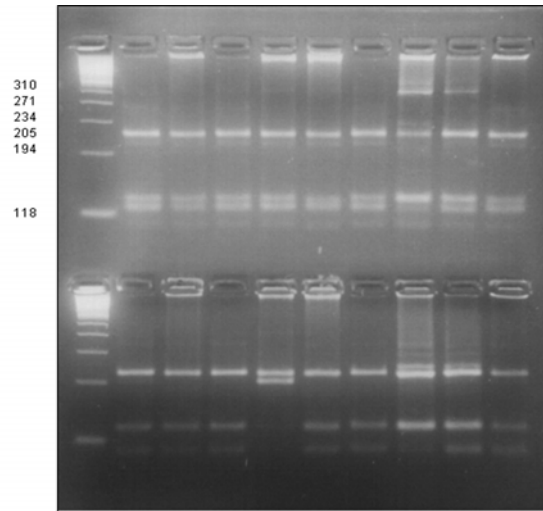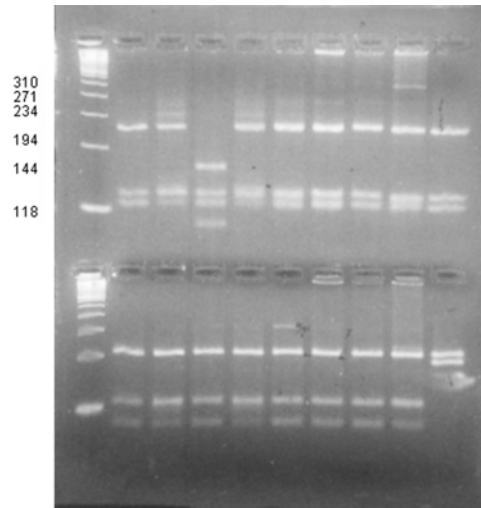

Fig. (4): Samples distribution according to the result of PCR amplification of HBV DNA categorized according to Child severity class

Fig. (5): HCV Genotype-4, Φ x 174 DNA/Hae III fragments.
Genotype 4 equals 205 bp.

Fig. (6): HCV Genotype-1, Φ x 174 DNA/Hae III fragments.
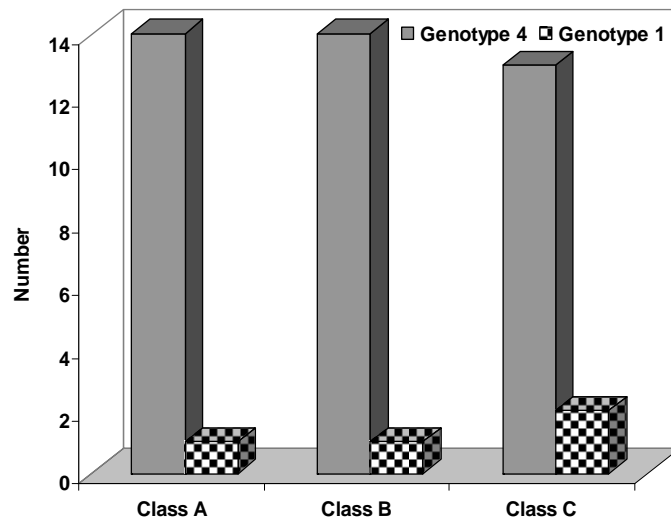**Genotype 4 equals 144 bp.**

Fig. (7): Frequency of specimens grouped as HCV genotype in patients categorized according to Child severity class
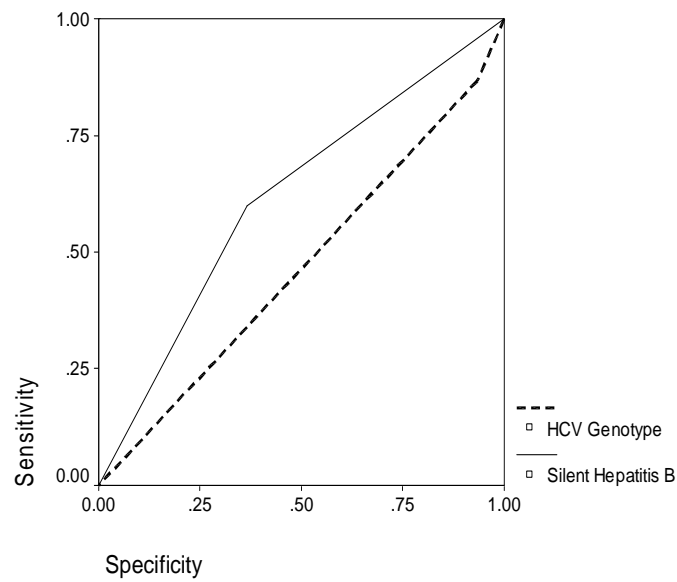

Fig. (8): ROC curve analysis of sensitivity and specificity of determination of HCV genotype and detection of silent hepatitis B

DISCUSSION

More than 170 million people throughout the world are infected with hepatitis C virus (HCV). Persistence of the virus in the liver leads to chronic hepatitis in 70 % of infected patients, which can progress to cirrhosis and liver cancer[23]. This single-stranded RNA virus is genetically extremely heterogeneous because of its rapid replication and the poor fidelity of its RNA-dependent RNA polymerase, encoded by the NS5B region[24].

Studies on the taxonomy of HCV based upon phylogenetic analyses of nucleotide sequences indicate that there are six HCV genotypes (1–6), each of which may have various subtypes. The virus population in an individual is defined as quasispecies[5]. Several methods are used for genotyping, based on analysis of portions of the genome (5'NC, NS5B, core, E1)[25]. Knowledge of HCV variability is crucial for clinical and epidemiological analyses. Prediction of sustained virological response and choice of treatment duration depend on genotype[23].

The current study was designed as a trial to explore the frequency of silent hepatitis B infection among hepatitis C patients and to determine the incidence genotype of hepatitis C virus in these patients

The HBV DNA was detected in 20 samples (44.4%), such frequency of silent hepatitis B infection in HCV cirrhotic patients with HBsAg negative goes in hand with the previously reported results in literature. *Georgiadou et al.*[26] investigated HCV patients for the presence of occult HBV in an attempt to determine the frequency and importance of this phenomenon and reported that HBV-DNA was detected in 26.2% of HCV-infected patients and HBV-DNA was neither associated with HBV markers, nor with epidemiological and virologic data and concluded that one quarter of HCV-infected patients had occult HBV infection.

Ghisetti et al.[27] reported a frequency rate of occult hepatitis B of 64% in HCV patients who were HBsAg negative and undergoing transplantation surgery. *Fujiwara et al.*[28] reported that hepatitis B virus DNA was amplified in 19.5% of the HCV-related chronic liver disease. *Honarkar et al.*[29] detected hepatitis B virus-DNA in 22% of chronic liver disease patients and *Datta et al.*[30] reported occult HBV in 53.6% of 28 HBSAg negative samples. These different figures could be attributed to several factors; patients from countries highly endemic for HBV are more likely to develop occult HBV infections, the nature of biological material tested, with a higher proportion for liver compared to serum specimen and the method of detection, including PCR primer selection.

On contrary to the obtained results; *Khattab et al.*[31] assessed the prevalence and clinical consequences of occult HBV infection in chronic hepatitis C patients undergoing antiviral therapy and found occult HBV genomes were in the serum of four of 53 (7.5%) patients, unrelated to anti-HBc status and concluded that the prevalence of occult HBV co-infection among patients with chronic

hepatitis C was low and independent of the presence of markers of previous HBV infection. These low figures could be attributed to the effect of antiviral therapy; in agreement with this attribution, *Hasegawa et al.*[32] who found that occult HBV may be sensitive to interferon (INF) therapy although HBV is not completely eradicated

The ROC curve analysis showed that presence of occult HB infection could be a specific predictor of severe liver derangement despite the positive non-significant correlation with clinical class of severity of liver disease. That finding agreed with *Pollicino et al.*[33] who reported that occult HBV is a risk factor for development of hepatocellular carcinoma and the potential mechanisms whereby overt HBV might induce tumor formation are mostly maintained in cases of occult infection. *Raimondo et al.*[34] reported that coinfection by HBV and HCV may have considerable clinical relevance and is generally believed to be a factor favoring the progression of liver fibrosis toward cirrhosis and the development of liver cancer.

Genotyping of HCV reported 41 samples (91.1%) with genotype-4 and 4 samples (8.9%) with genotype-1; these figures agreed with that reported by *Elsaway et al.*[35] who reported HCV genotype 4 in 91% of samples of their series collected from Dakahlia provinces, and with *Genovese et al.*[36] who reported that only HCV genotype 4 was isolated from patients with chronic liver disease in Alexandria District and the other previously determined genotypes were variants of genotype 4 due to its heterogeneity.

Also, *Derbala et al.*[37] reported that Egypt has a high prevalence rate of hepatitis C infection and as much as 90% is genotype 4 and the poor response of genotype 4 in Egypt to different forms of IFN therapies may be related to an intrinsic resistance to the direct antiviral effect of IFN.

It could be concluded that HCV genotype-4 is the most prevalent type in Egyptian Hepatitis C cirrhotic patients with a prevalence of silent hepatitis B of 44.4% and its detection is a specific predictor of severe cirrhosis.

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الالتهاب المزمن في المرضي المصابين بفيروس س

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تم عمل هذه الدراسة كمحاولة لاكتشاف معدل انتشار الالتهاب الكبدي الكامن بفيروس ب في مرضي الالتهاب الكبدي المزمن بفيروس س وقد شملت هذه الدراسة ٤٥ مريض بتليف الكبد عقب الالتهاب الكبدي الفيروسي. وتم عمل تحليل وظائف الكبد و اختيار الأجسام المضادة للفيروس س و الأنتيجين للفيروس ب بطريقة الأليزا وقد تم استبعاد جميع المرضي الحاملين للأنتيجين الخاص بفيروس ب من تلك الدراسة و تم تقسيم المرضي إلي ثلاث مجوعات و تم القياس الكيفي لفيروس س و فيروس ب بطريقة تفاعل البلمرة المتسلسل و كذلك القياس الكمي لفيروس س و النوعية الجينية له بطريقة أر ال أف ب. وقد تم قياس الفيروس س بغض النظر عن الحالة الإكلينيكية للمريض و كان مستوي الفيروس يتراوح بين ١٣٤٩٨٥ و ١٩٥٧٦٣٢ نسخة من الفيروس لكل ١ مللي دم.

و قد تم التحديد الكمي للفيروس ب – دي إن أ بطريقة البلمرة في ٢٠ عينه (٤٤.٤ %) ، (٤) من المجموعة أو (٧) من المجموعة ب و (٩) من المجموعة (س) مع زيادة ذات دلالة إحصائية في المجموعة (ب) ، (س) مقارنة بالمجموعة (أ).

وقد تم تحديد النوعية الجينية للفيروس س من نوع رقم ٤ في ٤١ عينه (٩١.١%) مقارنة بالنوع الجيني رقم ١ في ٤ عينات (٨.٩ %) و قد كان هناك ارتباط بدون دلالة إحصائية بين النوعية الجينية للفيروس س و التهاب الفيروس الكبدي ب الكامن و شدة الحالة الإكلينيكية للمريض.

وباستعمال تحليل المنحني لاستكشاف الحساسية و الخصوصية لتحديد الالتهاب الفيروسي الكامن ب و النوعية الجبنبة للفيروس س كمؤشر لتحديد شدة الالتهاب الكبدي تبين عدم وجود دور محدد للعينة الجينية لفيروس س لتحديد شدة المرض.

ولكن تم تحديد دور محدد للفيروس ب الكامن في مرضي الالتهاب المزمن للفيروس س كمؤشر رقم ٤ لتحديد شدة الالتهاب الكبدي.

يمكن استنتاج انتشار النوعية الجينية للفيروس س رقم ٤ في مرضي الالتهاب الكبدي المزمن بالفيروس س في مصر مع انتشار الالتهاب الكبدي الكامن بالفيروس ب و الذي يمكن إعتبارة مؤشر للتليف الكبدي.