Occult Hepatitis B Infection among Egyptian Chronic Hepatitis C Patients and its Relation with Liver Enzymes and Hepatitis B Markers

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Abstract: Background: Hepatitis B (HBV) and hepatitis C (HCV) viruses are the most common causes of chronic liver disease. Coinfection with HBV and HCV is not uncommon among individuals in HBV endemic areas. Occult HBV (OHB) infection is characterized by detection of HBV DNA in the serum or liver tissue of patients who test negative for HBsAg. This study aimed to evaluate the frequency of OHB infection among Egyptian patients with chronic HCV infection and its relation with liver function tests and HBV markers. **Methods**: Serum of 50 chronic HCV patients who tested negative for HBsAg and anti-HBc-IgM were analyzed for liver function tests and HBV markers using micro particle enzyme immunoassay kit (Axsym), in addition to quantitative detection of HCV RNA and HBV DNA by real time PCR. **Results:** HBV-DNA was detectable in serum of 10/50 patients (20%) with a mean of 374.8 IU/ml. Among OHB positive and negative groups, anti-HBc was detected in 100% and 80% (P=0.289), anti-HBs was detected in 20% and 57.5% (P=0.034) and HBeAg was detected in 60% and 0% (P=0.000), respectively. Also, the mean level of ALT and AST showed significant elevation in OHB positive group when compared to negative group, (p=0.000 for both). There was no significant correlation between the level of HBV-DNA and the levels of ALT and AST. **Conclusions:** OHB with low serum levels of HBV-DNA was observed in 20% of chronic HCV patients in Egypt and was associated with elevation in ALT and AST. HBeAg could be a useful maker for OHB prediction but a negative result doesn't exclude OHB infection, whereas negative anti-HBc-IgG may exclude such infection.
[Wafaa T. El-sherif, Sohair K. Sayed, Noha A. Afifi, Hussein A.EL-Amin. **Occult Hepatitis B Infection among Egyptian Chronic Hepatitis C Patients and its Relation with Liver Enzymes and Hepatitis B Markers.** *Life Sci J* 2012; 9(2):467-474]. (ISSN: 1097-8135). http://www.lifesciencesite.com. 69.

Key words: Occult hepatitis B, chronic HCV, liver enzymes, anti-HBc, anti-HBs, HBeAg.

1. Introduction

Hepatitis B virus (HBV) belongs to the Hepadnaviridae family of animal viruses, and its genome consists of a circular partially double-stranded DNA molecule of 3.2 kb in length which contains four overlapping reading frames that code for surface proteins (HBsAg), core proteins (HBc/HBeAg), the viral polymerase, and the transcriptional transactivator X protein (HBx) [1]. Hepatitis C virus (HCV) is classified in the Hepacivirus genus of the Flaviviridae family, and its genome is a positive-stranded RNA of 9.6 kb in length that encodes a large polyprotein that undergoes proteolytic processing by cellular and viral proteinases to generate the individual viral proteins [2]. Hepatitis B and hepatitis C viruses are the most common causes of chronic liver disease world-wide. Both viruses induce chronic hepatitis, which may progress to cirrhosis and eventually to hepatocellular carcinoma [3]. Due to shared routes of transmission, coinfection with HBV and HCV is not uncommon among individuals in HBV endemic areas [4].

Due to a lack of large scale population-based studies the exact number of HBV/HCV coinfected patients is unknown. Moreover, there may be underestimation of the true number of people with HBV/HCV coinfection as there is a well-known entity of occult HBV (OHB) infection in patients with chronic hepatitis C [5]. Occult hepatitis B infection is generally defined as the detection of HBV-DNA in the sera or tissues of subjects who have negative tests for HBsAg, with or without anti-HBc or antibody to HBV surface antigen (anti-HBs), outside the pre-seroconversion window period [6]. Occult hepatitis B virus infection is most frequently seen in patients with hepatitis B core antibody (anti-HBc) as the only HBV serological marker [7]. However, it is also reported in patients with hepatitis B surface antibody (anti-HBs) alone or even in those without any HBV serological marker [8,9].

Different scenarios of infection have been described with HBV/HCV coinfection. Some patients may be inoculated with both viruses simultaneously and will present with acute hepatitis due to both viruses. In addition, HBV superinfection in patients with chronic hepatitis C, and HCV superinfection in patients with chronic hepatitis B have both been reported [10,11].

HBV or HCV can play the dominant role, HBV and HCV can inhibit each other simultaneously and they can alternate their dominance. Both viruses have the ability to induce seroconversion of the other. The chronology of infection may have a role in determining the dominant virus. However, the overall effect appears to be HCV suppression of HBV [12].

Occult HBV has been associated with more advanced fibrosis/cirrhosis and with a poor response to interferon [5], but not in all studies. Cirrhosis is considered to be the most important risk factor for HCC. Therefore, besides having a possible direct oncogenetic effect, occult HBV infection may increase neoplastic transformation in HCV-infected patients [13]. The oncogenicity of occult HBV infection is related to the transactivating role of the HBx protein and to the ability of HBV to integrate the host genome [14,15]. Indeed, some studies have shown that HBV DNA could be detected in HBsAg-negative patients with HCC [16,17].

Many patients with chronic HCV infection have fluctuating levels of serum alanine aminotransferase (ALT); it remains unclear whether co-infection with occult HBV may contribute to liver inflammation and ALT flare [18].

The aim of this study is to evaluate the frequency of OHB infection among Egyptian patients with chronic HCV infection and its relation with liver function tests and hepatitis B markers.

2. Patients and methods

Out of 220 chronic hepatitis C patients examined, fifty Egyptian patients with HCV infection (38 males, 12 females, with mean age of 38.70 ± 9.98 yr.) were recruited from Virology Outpatient Clinic, Assiut University Hospital from April 2010-June 2010. Criteria for inclusion were (1) HCV antibody and HCV RNA positive, (2) negative HBsAg and anti-HBc IgM, and (3) absence of signs for decompensation (ascites, encephalopathy, or gastrointestinal bleeding). Patients were excluded from the study according to the following exclusion criteria: (1) evidence for coexisting liver disease; (2) previous antiviral treatment; (3) serological evidence of concurrent infection with HIV; (4) presence of hepatocellular carcinoma; (5) patients on hemodialysis; (6) severe systemic disease. The participants were subjected to detailed clinical history, full physical examination and abdominal ultrasonography. The study was approved by the Ethics Committee of Faculty of Medicine, Assiut University and informed consent was obtained from all the participants.

Sample collection, serological markers assay

Laboratory investigations were performed for every subject. Five ml of venous blood were aseptically collected in plain tubes. The samples were centrifuged within 30 minutes at 3000rpm for 10 minutes, then the serum samples were collected, divided into aliquots and stored at -70^{o}C for further analysis. Liver function tests (serum ALT, AST and albumin) were performed using a chemical analyzer Hitachi 911 (Boehringer Mannheim, Germany). Serological markers of HBV [HBsAg, anti-HBs, anti-HBc IgM and IgG, and hepatitis B e antigen (HBeAg)], anti-HCV and anti-HIV were tested using commercially available micro particle enzyme immunoassay kits (AXSYM, Abbott Laboratories, Germany).

DNA and RNA extraction: DNA was extracted from 200 µl of serum with DNA extraction kit, catalog no.57704, and RNA was extracted from 140 µl of patient serum with viral RNA Mini Kit, catalog no.52904, according to manufacturer's procedure, (QIAGEN, GmbH, Germany).

Quantitative detection of HCV RNA and HBV DNA by real time PCR: In real-time PCR the amplified product is detected via fluorescent dyes. These are usually linked to oligonucleotide probes which bind specifically to the amplified product. Monitoring the fluorescence intensities during the PCR run (i.e. in real-time) allows the detection and quantitation of the accumulating product without having to re-open the reaction tubes after the PCR run [19].

Detection of HBV DNA was performed on 7500 fast real time PCR system (Applied Biosystems) using ready to use PCR kit supplied by artus® HBV TM PCR Kit (24), Version 1, catalog no. 4506163, QIAGEN GmbH, Germany. The HBV RG/TM Master contains reagents and enzymes for the specific amplification of a 134 bp region of the hepatitis B virus genome. The amplicon is detected by measuring the FAM® fluorescence. In addition, the artus HBV TM PCR Kit contains a second heterologous amplification system to identify possible PCR inhibition. This is detected as an Internal Control by measuring the JOE™ fluorescence.

Detection of HCV RNA was performed on 7500 fast real time PCR system (Applied Biosynthesis) using ready to use PCR kit supplied by artus® HCV RG RT-PCR Kit 24, Version 1, catalog no. 4518263, QIAGEN GmbH, Germany. The HCV RG Master A and B contain reagents and enzymes for the reverse transcription and specific amplification of a 240 bp region of the HCV genome, and for the direct detection of the specific amplicon in fluorescence channel Cycling Green. In addition, the artus HCV RG RT-PCR Kit contains a second heterologous amplification system to identify possible PCR inhibition. This is detected as an internal control in fluorescence channel Cycling Orange.

Statistical analysis

Data were collected and analyzed by computer program SPSS "version 17" (The Statistical Package for the Social Science Program), Chicago, USA). All data were expressed as mean ± SD and percentages. Comparisons between two groups were analyzed by Student t-test and Chi square. Correlation between studied parameters was performed by Spearman rank correlation coefficient. P value < 0.05 was considered significant.

3. Results

Out of 220 chronic hepatitis C patients examined for HBsAg and anti-HBc IgM, 50 patients were negative for both markers and were included in this study. The demographic and laboratory data of the 50 patients are shown in table 1.

Rates of hepatitis B markers and HBV-DNA in total patients (Table 2)

Anti-HBc IgG was detected in 42/50 patients (84%), anti-HBs was detected in 25/50 patients (50%) and HBeAg was detected in 6/50 patients (12%). HBV-DNA was detected in 10/50 patients (20%) and its levels ranged from 133-722 IU/ml with a mean ± SD of 374.8 ± 216.3 IU/ml. Out of the 50 studied patients, eight patients (16%) were negative for all markers, nine patients (18%) were positive only for anti-HBc IgG, 23 patients (46%) were positive for anti-HBc IgG and anti-HBs, four patients (8%) were positive for anti-HBc IgG and HBV-DNA, four patients (8%)were positive for anti.HBc IgG, HBeAg and HBV-DNA and two patients (4%) were positive for all markers. According to presence or absence of HBV-DNA, patients were classified into OHB positive (10 patients) and OHB negative (40 patients) groups.

Demographic and laboratory data of OHB positive and negative groups

As shown in Table 3, there was no significant difference in the males to females ratio and the mean age of the two groups of patients. ALT and AST levels were significantly higher in the OHB positive group (P =0.000 for both). All OHB positive patients had ALT and AST levels ≥ 40 IU/L, while in the OHB negative group ALT and AST levels ≥ 40 IU/L were only observed in 10% and 22.5% of patients, respectively. There was no significant correlation between the level of HBV-DNA and the levels of ALT (r = 0.63, p = 0.051) or AST (r = 0.585, p = 0.075).

Hepatitis B markers in OHB positive and negative groups

Anti-HBc IgG was detected in all OHB positive patients (10/10) and 80% (32/40) of OHB negative patients. There was no significant difference between the rates of anti- HBc IgG in OHB positive and negative patients (P = 0.289). Anti-HBs was detected in 20% (2/10) of OHB positive patients and 57.5% (23/40) of OHB negative patients. There was a significant difference between the rates of anti-HBs in OHB positive and negative patients (P = 0.034). HBeAg was detected in 60% (6/10) of OHB positive patients and not detected in any OHB negative patient (0/40). There was a significant difference between the rates of HBeAg in OHB positive and negative patients (P = 0.000) (Table 4).

The levels of HBV-DNA in HBeAg positive patients ranged from 212-722 IU/ml (mean ± SD = 462 ± 227.11) compared to 133-356 IU/ml (mean ± SD = 244 ± 128.17) in those negative for HBeAg (p = 0.123).

Relation between HCV-RNA levels and OHB infection

As shown in table (5), there was no significant difference between the mean level of HCV-RNA in the OHB positive and negative groups (P = 0.114). None of the OHB positive and 7.5% (3/40) of OHB negative patients had levels of HCV-RNA ≤10.000 IU/ml. HCV-RNA levels ≥ 500.000 IU/ml were observed in 20% (2/10) and 5% (2/40) of OHB positive and negative patients, respectively. There was also no significant correlation between the levels of HBV-DNA and HCV-RNA (r = 0.304, p = 0.393).

Table 1: Demographic and laboratory data of 50 HCV studied patients

Gender	
Male	38 (76%)
Female	12 (24%)
Age (years)	
Range	22- 48
Mean ± SD	38.70 ± 9.98
ALT (IU/L)	
< 40	36 (72%)
≥ 40	14 (28%)
Mean ± SD	37.54 ± 28.25
AST (IU/L)	
< 40	31 (62%)
≥ 40	19 (38%)
Mean ± SD	44.02 ± 29.87
Albmin (gm/dl)	
< 3.5	15 (30%)
≥ 3.5	35 (70%)
Mean ± SD	3.63 ± 0.60
HCV-RNA level (IU/ml)	
Range	9.000-790.000
Mean ± SD	191.105 ± 210.138

Table 2: Rates of HBV markers and HBV-DNA in total 50 studied patients

No of patients	Anti-HBc IgG	Anti-HBs	HBeAg	HBV-DNA
2 (4%)	+	+	+	+
23 (46%)	+	+	-	-
4 (8%)	+	-	+	+
4 (8%)	+	-	-	+
9 (18%)	+	-	-	-
8 (16%)	-	-	-	-
Total positive No (%)	**42 (84%)**	**25 (50%)**	**6 (12%)**	**10 (20%)**

Table 3: Demographic and laboratory data of occult hepatitis B (OHB) positive and negative patients

Criteria	OHB +ve (10 patients)	OHB –ve (40 patients)	*P* value
Gender			
Male	8 (80%)	30 (75%)	0.741
Female	2 (20%)	10 (25%)	
Age (years)			
Range	23-61	22-61	0.139•
Mean ± SD	43.75 ± 9.37	42.74 ± 9.61	
ALT (IU/L)			
< 40	0 (0%)	36 (90%)	0.000*
≥ 40	10 (100%)	4 (10%)	
Mean ± SD	78.80 ± 36.91	27.22 ± 11.95	0.000*•
AST (IU/L)			
< 40	0 (0%)	31 (77.5%)	0.000*
≥ 40	10 (100%)	9 (22.5%)	
Mean ± SD	92.60 ± 33.34	31.88 ± 10.39	0.000*•
Albumin (gm/dl)			
< 3.5	2 (20%)	13 (32.5%)	0.700
≥ 3.5	8 (80%)	27 (67.5%)	
Mean ± SD	3.75 ± 0.57	3.60 ± 0.61	0.486•

Chi-square test • Independent samples t-test * Statistical significant difference (P<0.05)

Table 4: The rates of hepatitis B markers in occult hepatitis B (OHB) positive and negative patients

Hepatitis B marker	OHB +ve (10 patients)	OHB –ve (40 patients)	*P* value
Total anti-HBc			
+ve (n=42)	10/10 (100%)	32/40 (80%)	0.289
-ve (n=8)	0/10 (0%)	8/40 (20%)	
HbeAg			
+ve (n=6)	6/10 (60%)	0/40 (0 %)	0.000*
-ve (n=44)	4/10 (40%)	40/40 (100%)	
Anti-HBs			
+ve (n=25)	2/10 (20%)	23/40 (57.5%)	0.034*
-ve (n=25)	8/10 (80%)	17/40 (42.5%)	

Chi-square test * Statistical significant difference (P<0.05)

Table 5: HCV-RNA levels in occult hepatitis B (OHB) positive and negative patients

HCV-RNA level (IU/ml)	OHB +ve (10 patients)	OHB –ve (40 patients)
≤10.000	0 (0%)	3 (7.5%)
>10.000- 500,000	8 (80%)	35 (87.5%)
>500,000	2 (20%)	2 (5%)
Mean ±SD•	285.280 ± 290.263	167.562 ± 182.195

Chi-square test (not applicable) • Independent samples t-test (P=0.114)

4. Discussion

The frequency of OHB in HCV patients varies greatly, ranging from 0%-52% [20,21]. In our study, 20% of patients with chronic HCV had OHB. 20% of this group were females and 80% were males, comparable to 25% females and 75% males in the OHB negative group. There was no significant difference in the mean age of the two groups. Similar results were obtained by **Shavakhi *et al.*** [22] who detected OHB in 19.4% of chronic HCV patients; 25% of them were females and 75% were males. Out of the non-infected subjects, 19.3% were females and 80.7% were males. The two groups were not significantly different in respect of sex and age. **Fujiwara *et al.*** [23] have also detected HBV-DNA in 19.5% chronic HCV patients. Comparable results were reported by **Kanbay *et al.*** [24] and **Ramia *et al.*** [25] who detected HBV-DNA in 14.2% and 16.3% of HCV positive patients.

On the other hand, higher frequency was obtained by **Shetty *et al.*** [26] who detected OHB in 28% of patients with HCV cirrhosis. **Torbenson *et al.*** [27] have also detected OHB in 45% of injection drug users with chronic HCV. Lower percentage was indicated by **Ismail *et al.*** [28] who found that 6.3% of hemodialysis patients with HCV infection had occult HBV. Another study was conducted by **Emara *et al.*** [29] who detected OHB in 3.9% of Egyptian chronic HCV patients under pegylated interferon/ribavirin therapy (using COBAS® TaqMan®HBV Test) and this lower frequency was attributed to the effect of treatment on the HBV.

Occult HBV is characterized by the presence of ongoing viral replication with very low levels of viremia (<200 IU/ml), and negativity for HBsAg, while the so-called 'false' OHB with higher levels of HBV-DNA that are negative for HBsAg are usually due to the occurrence of mutations of the HBsAg sequence that may alter the recognition by some immunoassays [30]. In our study, the mean level of HBV-DNA in serum was low (374.8 ± 216.3 IU/ml). **Noborg *et al.*** [31] reported that blood HBV-DNA level in HBsAg-positive subjects was high (10^4 to 10^8 copies/mL), but in those with OHB it was below 10^2 copies/ml. This suggests that many OHB patients would be serum PCR-negative and the use of liver tissue may be more helpful in detecting OHB infection. Recent definitions of OHB infection included liver HBV DNA positivity as a prerequisite for considering OHB infection [32], but examination of liver tissue is not always applicable in clinical practice and that is why highly sensitive PCR assays with low detection limit should be used in diagnosis of OHB infection [33].

The reasons for persistence of low levels of HBV-DNA in the absence of detectable HBsAg remain largely undefined, both host and viral factors are important in suppressing viral replication and keeping the infection under control. Low levels of viral replicative activity may result from the presence of defective interfering particles or to mutations in transcription control regions or the polymerase domain leading to inefficient replication in conjunction with the discordant release of HBsAg by the hepatocytes [34]. Additional mechanisms include (i) formation of immune complexes; [35] (ii) mutations affecting the '*a*' epitope of the S gene that encodes amino acid residues within the HBsAg coding region rendering the virus undetectable; or (iii) coinfection with hepatitis delta virus or HCV resulting in downregulation of HBV replication and a reduction in antigen synthesis [36].

In standard practice, the best diagnostic test to assess liver inflammation is liver biopsy, but ALT has been used as a surrogate marker for liver inflammation. In chronic HCV patients with OHB, fluctuation of HBV-DNA might directly affect the ALT level, and such an accumulation might increase the severity of liver disease in OHB infected patients [37]. In this study, ALT and AST levels ≥40 IU/L were detected in all OHB positive patients compared to 10% and 22.5% of the OHB negative group, respectively. The mean levels of ALT and AST were significantly higher in the OHB positive group (P =0.000 for both). In accordance with our results, **Shavakhi *et al.*** [22] found that OHB was observed in a considerable number of HCV patients in Tehran and was associated with elevation in liver enzymes. Serum AST and ALT were higher in patients with OHB and this finding may have clinical implication. In some studies, there was an elevation in transaminase in OHB patients, and it was associated with progression to cirrhosis [38,21]. However, in other studies on HCV patients with OHB, no elevation in ALT and ALT was found, and surprisingly, histological changes and cirrhosis in the OHB group were the same as HCV only group [39-41]. **Selim *et al.*** [18] have also found that HBV DNA was detected in 13.3% of patient with normal or slightly high ALT, while in those with ALT flare, HBV DNA was detected in 63.3% of patients (p<0.001). They have concluded that presence of OHB, with its added deleterious effect, must always be considered in chronic HCV patients, especially those with flare in liver enzymes.

As regard HBV markers in OHB positive and negative patients, this study shows that anti-HBs was detected in 20% of OHB positive and 57.5% of OHB negative patients (p = 0.034). Anti-HBc IgG was detected in all OHB positive and 80% of OHB negative patients (p = 0.289). Therefore, positive anti-HBc IgG can not be a predictor for OHB infection but a negative result may exclude it as none of the OHB positive group was anti-HBc IgG negative. In agreement with our findings, concerning anti-HBc, **Fujiwara *et al.*** [23], **Marusawa *et al.*** [42] and **Fukuda *et al.*** [21] found that all the occult HBV-positive patients were positive for anti-HBc. Different results were observed in other studies; **Shetty *et al.*** [26] reported that (63%) of OHB positive patients were positive for anti-HBc, whereas only 42% of patients without OHB tested positive for anti-HBc. In a study conducted by **Emara *et al.*** [29], OHB could not be predicted by serological markers of HBV infection, where only 2 out of the 6 patients with detectable HBV DNA had anti-HBc antibodies, and none had anti-HBs antibodies. **Selim *et al.*** [18] did not find any association between the presence of HBV DNA and various serology markers of HBV infection. **García-Montalvo** and **Ventura-Zapata** [43] detected anti-HBs in 62.5% of samples from OHB positive blood donors, and no significant difference was observed between HBV DNA positivity and anti-HBs levels.

Our study shows that HBeAg was detected in six patients, all of them had detectable HBV-DNA. Six of ten OHB positive patients (60%) and none of the OHB

negative patients were HBeAg positive (p = 0.000). Therefore, positive HBeAg could be a predictor for OHB infection but a negative result doesn't exclude it. **Yuan *et al.*** [44], revealed that HBeAg was not detected among 30 blood donors with OHB. **García-Montalvo** and **Ventura-Zapata** [43] detected HBeAg in 1/24 (4.16%) blood donors with OHB. These contradictory results could be attributed to the difference in studied subjects as they were blood donors and not chronic HCV patients. The positivity for HBeAg could be attributed to a superinfection in a naturally immune subject or to reactivation of a latent infection; the mutated virus had a reduced fitness and was therefore able to replicate only at low levels, resulting in a mild form of OHB infection.

In our study, the mean levels of HBV-DNA in HBeAg positive patients (462 ± 227.11) was higher (but not statistically significant) than in those negative for HBeAg (244 ± 128.17).

In this study, there was no significant difference between the mean levels of HCV-RNA in the OHB positive and negative groups (P = 0.114) and there was also no significant correlation between the levels of HBV-DNA and HCV-RNA (r = 0.304, p = 0.393). Discrepancies in results were observed in other studies. **Rodrı´guez-I´n˜igo *et al.*** [45] revealed that serum HCV RNA concentration was significantly lower in the six patients with OHB infection than in patients with chronic HCV without HBV infection. To the contrary, **Liu *et al.*** [46] reported that patients with HCV/HBV dual infection were noticed to have high HCV RNA load than those with HCV mono-infection. This seems to be applicable to genotype 4, where HBV DNA positive patients showed higher baseline HCV viral load than HCV monoinfected patients. Suppression of the dominant virus -usually HCV predominates over HBV- may be associated with flares of the non-dominant virus.

OHB with low serum levels of HBV-DNA was observed in 20% of chronic HCV patients in Egypt. It was associated with elevation in ALT and AST. HBeAg was detected in 60 % of OHB patients and negative in all OHB negative patients. Hence, it could be a useful maker for OHB prediction but a negative result doesn't exclude OHB infection, whereas negative anti-HBc-IgG may exclude such infection.

Recommendations:

Testing for HBeAg should be added to other markers that are routinely screened to detect OHB infection. In HCV patients, the likelihood of a HBV DNA test increases with elevated AST and ALT levels. Hence, screening for HBV-DNA at a time of elevated liver enzymes using highly sensitive PCR assays, with low detection limit, may be a good focal point for detection of OHB.

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4/25/2012