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ORIGINAL ARTICLE

Detection of occult hepatitis B virus among chronic hepatitis C patients



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Abstract *Background:* Concurrent infections with hepatitis B virus (HBV) and hepatitis C virus (HCV) are increasingly recognized in patients with chronic hepatitis. In Egypt, the last decade showed a remarkable decline in HBV infection associated with remarkable rise in HCV infection. The probable impact of occult HBV in patients with chronic HCV infection has been previously investigated and the evidence suggests a possible correlation with lower response to anti-viral treatment, higher grades of liver histological changes, and development of hepatocellular carcinoma. The aim of this study was to analyze the possible influence of occult HBV infection on the clinical outcomes in chronic HCV patients and to compare conventional and real-time PCR in detecting HBV DNA among Hepatitis B surface antigen (HBsAg) negative chronic HCV.

Methods: Sera collected from 100 chronic HCV patients (negative for HBsAg and positive for anti-HCV and HCV RNA) were tested for anti-HBc, anti-HBe and anti-HBs by ELISA, HCV-RNA viral load was determined by real-time PCR (TaqMan probe technique) and HBV DNA was detected with primers encoding the surface (*S*), core (*C*), polymerase (*pol*) and *X* genes. In addition, determination of liver enzymes including aspartate and alanine aminotransferases (AST, ALT) activities was performed.

Results: Fifty-eight percent of the study group were positive for anti-HBc. Meanwhile, only 18 cases (18%) were positive for the polymerase gene by nested PCR and were considered as occult HBV. Among these 18 polymerase gene positive patients (occult HBV) anti-HBc was detected among 9 (50%) of cases. Different gene profiles were noticed among the 18 polymerase gene positive patients.

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1. Introduction

Hepatitis B virus (HBV) and hepatitis C virus (HCV) infections account for a substantial proportion of cases of chronic liver disease including chronic hepatitis, cirrhosis, and liver cancer.¹ It is estimated that there are 240 million HBV carriers and 130–150 million HCV carriers worldwide.^{2,3} HBV and HCV are transmitted parenterally and share common routes of infection; as a result, infection with both viruses may occur.¹

The diagnosis of HBV infection is usually based on the detection of hepatitis B surface antigen (HBsAg), and the disappearance of this antigen indicates the clearance of HBV.⁴ However, it has been shown that HBV DNA can be detected in patients with chronic liver disease who are negative for HBsAg but positive for antibodies to hepatitis B core antigen (anti-HBc). This so-called occult HBV (OBI) infection has frequently been identified in patients with chronic HCV infection.^{5,6}

More recently, occult HBV infection has been considered to play a role in chronic hepatitis C virus infected patients, including the severity of HCV, related liver disease, poor response to anti-HCV treatment and development of hepatocellular carcinoma (HCC).^{7–9}

Owing to their distinct clinical course and heterogeneity, identification of patients who are candidates for therapy and selection of the optimal antiviral therapy is a challenge for clinicians.

The aim of this work was to study the possible influence of occult HBV infection on the clinical outcomes in the HBsAg negative chronic HCV patients and to compare conventional and real-time PCR in detecting HBV DNA among infected patients.

2. Patients and methods

A cross-sectional study was carried out, it included 100 consecutive patients (negative for HBsAg and positive for anti-HCV and HCV RNA) attending the Hepatology outpatient clinic and diagnosed by the Microbiology Department in Medical Research Institute, Alexandria, Egypt.

The study was carried out after receiving the approval of the Ethics Committee in the Medical Research Institute. All relevant information was collected from patients (after taking full consent) including personal data.

Sera were collected from all cases and were tested for antibodies against hepatitis B core antigen (anti-HBc), antibodies against hepatitis B e antigen (anti-HBe), antibodies against hepatitis B s antigen (anti-HBs) (Abbott Murex Diagnostic Division, Dartford, Kent, U.K) and determination of liver enzymes; aspartate aminotransferase (AST), alanine aminotransferase (ALT), total serum bilirubin and serum albumin.^{10,11}

2.1. Quantitative detection of HCV RNA by real-time PCR

RNA extraction: RNA was extracted from serum specimens using a QIAamp viral RNA kit (Qiagen Inc., Valencia, California, USA) following manufacturer's instructions. Ten µl of extracted RNA was amplified by using 6 µl of HCV RG Master A and 9 µl of HCV RG Master B to bring the reaction

to a final volume of 25 µl, with the PCR MX3000 stratagene (Applied Biosystems Inc., Foster city, California, USA) system. Thermal Profile of amplification included the following: 50 °C for 30 min (reverse transcriptase step) followed by Ampli Taq activation step at 95 °C for 10 min, followed by a three step PCR protocol: 95 °C for 30 s and followed by annealing at 50 °C for 1 min and extension at 72 °C for 30 s, for 40 cycles with end point fluorescence detection.

2.2. Detection of HBV DNA

2.2.1. HBV DNA extraction

DNA was extracted from serum using QIAamp viral DNA mini kit (QIAGEN, Valencia, CA, United States) following manufacturer's instructions.

2.2.2. Detection of HBV-DNA by nested PCR using specific primers for pol gene¹¹

Sequences of primer pairs used for PCR to detect HBV genome¹¹

Primer name	Nucleotide sequence 5' to 3'	Seq id no
<i>Outer primers</i>		
HBPr 134 sense	GCTGCTATGCCTCATCTTC	134
HBPr 135 antisense	CA(G/A)AGACAAAAGAAAATTGG	135
<i>Inner primers</i>		
HBPr 75 sense	CAAGGTATGTTGCCCGTTTGTCC	75
HBPr 94 antisense	GG(T/C)A(A/T)AAAGGGACTCA(A/C)GATG	94

Outer amplification components included the following: 0.3 µl (30 pmol) of each of; outer primers, 12.5 µl universal PCR master mix 2-fold (GeneOn GmbH), 10 µl of Qiagen extracted DNA and H₂O was added to bring the reaction to a final volume of 25 µl. The DNA was amplified using the following profile: Ampli Taq activation for 95 °C for 10 min, followed by 40 cycles of PCR amplification; denaturation at 95 °C for 30 s, annealing at 45 °C for 30 s and extension at 72 °C for 30 s followed by final elongation at 72 °C for 10 min.

Nested amplification components included the following: 3 µl of outer amplified products to be re-amplified using 0.3 µl (30 pmol) of each of; inner primers, 12.5 µl universal PCR master mix 2-fold (GeneOn GmbH), and H₂O was added to bring the reaction to a final volume of 25 µl. The second round amplification profile was similar to the first round but only 35 cycles were carried out.

After amplification, Ethidium bromide stained, 2% agarose gel was prepared in TAE (Tris–acetate–EDTA, PH 8) buffer and used for detection of the 341 base pair positive band. Fifteen µl of PCR product was run on 2% agarose gel at 100 V for 45 min. A 100 bp DNA marker was used to identify the amplified bands.

2.2.3. Amplification of S, C and X genes of HBV by SYBR green real-time PCR (AB: Applied Biosystem) using specific primers¹²

	Nucleotide sequence 5' to 3'	Nucleotide position
<i>Surface gene</i>		
S-sense	AGAACATCGCATCAGGACTC	159–178
S-antisense	CATAGGTATCTTGCGAAAGC	642–623
<i>Core gene</i>		
C sense	CTGGGAGGAGTTGGGGGA	1730–1747
C antisense	GTAGAAGAATAAAGCCC	2503–2487
<i>X gene</i>		
X sense	CTAGCCGCTTGTGTTGCTCG	1282–1301
X antisense	TTATGCCTACAGCCTCCTAG	1666–1647

The amplification reaction was performed as follows:

For amplification of each gene: 0.3 µl (30 pmol) of HB s or c, or x gene sense and antisense primers, 12.5 µl syber green universal PCR master mix 2-fold (AB applied biosystem, United States), 10 µl of Qiagen extracted DNA and H₂O was added to bring the reaction to a final volume of 25 µl. After AmpliTaq activation at 95 °C for 10 min, the DNA was amplified for 40 cycles of PCR amplification. Each cycle entailed: denaturation at 95 °C for 15 s, annealing at 55 °C for 30 s and extension at 72 °C for 1 min. This was followed by melting curve analysis to determine the purity and specificity of the amplification product. The melting curve analysis profile was 95 °C for 1 min, 50 °C for 30 s, and 95 °C for 15 s.

3. Results

The 100 anti-HCV positive patients included in this study included 64 (64%) males and 36 (36%) females, with a male to female ratio of 1.7:1. Their age ranged from (20–60) years. Forty-five percent of them were in the (31–40) age group.

3.1. HCV RNA viral load

The majority of the 100 anti-HCV patients (85%) had high viremia (10⁵–10⁸) IU/mL, meanwhile, only (15%) had low level viremia of less than 10⁴ IU/ml. High HCV viral load (above

10⁵) was highly associated with abnormal liver functions (Table 1).

3.2. Occult HBV OR pol gene

Out of the 100 HCV RNA positive HBsAg negative patients, 58% were positive for anti-HBc. Meanwhile, only 18 cases (18%) were positive for the polymerase gene by nested PCR and were considered as occult HBV. Among the 18 polymerase gene positive patients (occult HBV, OBI) anti-HBc was detected among 9 (50%) of cases (Fig. 1).

3.3. Co HCV/HBV infection

94.5% of patients with dual HCV/occult-HBV infection had HCV RNA viral load of 10⁵–10⁶ IU/ml, compared to 80% of patients with single HCV infection. No significant association was found between HCV viral load and pol gene positivity. Interestingly 77% and 55% of the patients with dual HCV/occult-HBV infection had abnormal AST and ALT respectively, compared to 57% and 38% among patients with single HCV infection.

3.4. S, X and C genes

The 100 HCV positive patients were tested for the S, X and C genes by using SYBR Green real time, followed by dissociation curve analysis. Different gene profiles were noticed among the 18 polymerase gene positive patients with occult HBV infection. No significant association was found between the different gene profiles and abnormal AST and ALT levels (Table 2, Figs. 2–4).

3.5. Correlation between anti-core positivity, liver function tests and HCV viral load

The anti-core positivity was not significantly associated with the different liver functions. As, out of the 18 OBI patients, no difference among anti-HBc positive and anti-HBc negative patients was found as; 77.8% and 55.5% of either groups had elevated AST and ALT respectively (Table 3).

No significant association was noticed between anti-HBc positivity and HCV viral load as; 100% of anti-core positive compared to 89% of anti-core negative patients had HCV-RNA level of > 10⁵ IU/mL). However, only 11% of anti-core negative patients showed low HCV viremia (≤10⁴ IU/ml).

Table 1 Correlation between HCV RNA viral load, with abnormal liver functions among the 100 anti-HCV positive cases:

	No (%) N = 100	Abnormal ALT	Abnormal AST
HCV viral load ≤ 10 ⁴	15 (15%)	1 (6.7%)	4 (26.6%)
10 ⁵ – ≥10 ⁸	85 (85%)	45 (52.9%)	59 (69.4%)
Pearson Chi square		0.001*	0.002*

ALT: alanine aminotransferase, AST: aminotransferase.
FEp: Fischer exact test.
X2: Chi square test.
* Results ≤.05 are significant.

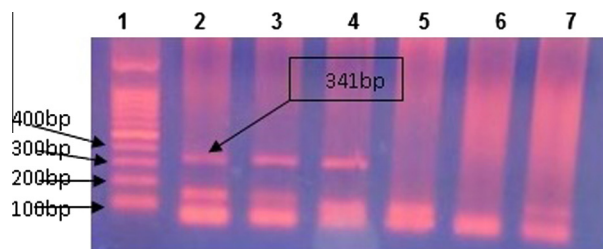


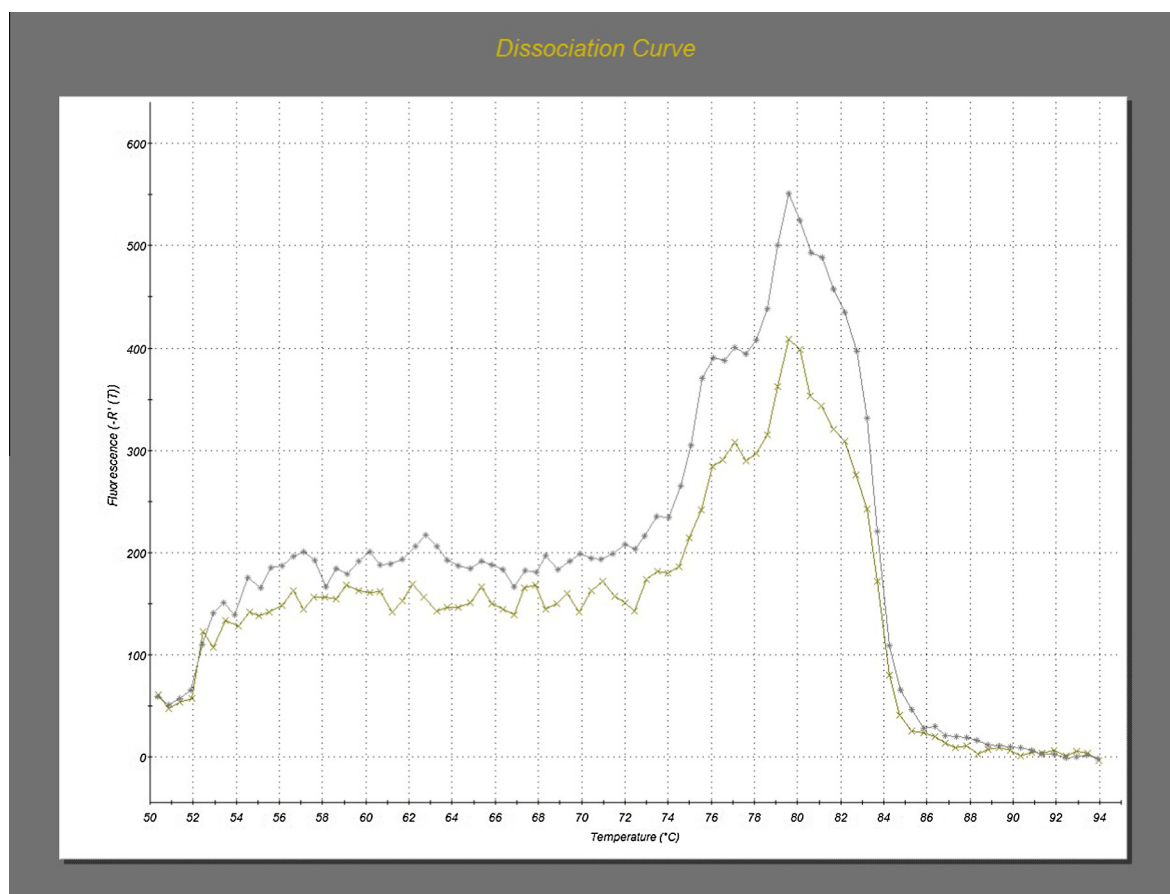
Figure 1 Gel electrophoresis of nested PCR product. Lane (1): DNA ladder – 100 bp. Lane (2): positive control. Lane (3, 4): positive samples. Lane (5, 6, 7): negative samples.

Table 2 Distribution of *S*, *X*, *C* and *pol* genes profile and liver enzymes among the 100 HCV positive patients.

Gene profile	No	%	AST		ALT	
			Normal	Abnormal	Normal	Abnormal
<i>S</i> + <i>C</i> + <i>X</i> + <i>pol</i>	1	1	1	0	1	0
<i>C</i> + <i>X</i> + <i>pol</i>	4	4	2	2	2	2
<i>S</i> + <i>pol</i>	2	2	2	0	1	1
<i>C</i> + <i>pol</i>	5	5	0	5	1	4
<i>X</i> + <i>pol</i>		1	1	0	1	1
<i>pol</i> gene alone	5	5	1	4	2	3
No gene detected	82	82	36	46	51	31
Total	100	100.0	FEp = 0.082		FEp = 0.488	

FEp: Fischer exact test.

X2: Chi square test.

*Results $\leq .05$ are significant.**Figure 2** Dissociation curve of *s* gene.

4. Discussion

HBV infection is one of the major global health problems. Worldwide, HBV is the primary cause of cirrhosis and HCC and is one of the ten leading causes of death.¹³

Occult HBV infection has frequently been identified in patients with chronic HCV infection.^{14,15} This occult infection

may be associated with more severe liver damage and even the development of hepatocellular carcinoma (HCC).^{16,17}

This study included 100 HCV RNA positive patients; 64 (64%) were males, 36 (36%) females, with a male to female ratio of 1.7:1. The age of the study group ranged from (20–60) years, 45% of them were in the (31–40) age group. The majority of anti-HCV patients (85%) had high viremia ($> 10^5$ IU/ml), meanwhile, only (15%) had low level viremia

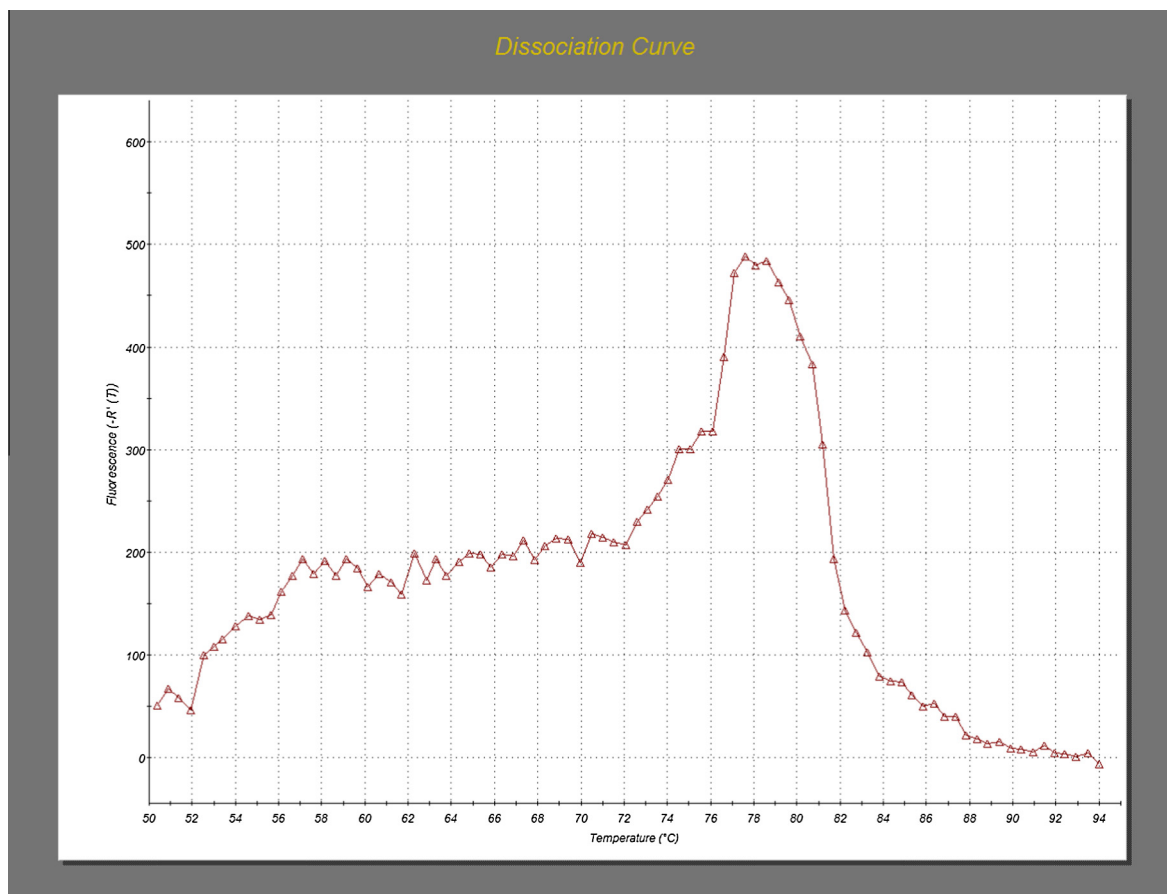


Figure 3 Dissociation curve of *c* gene.

of ($\leq 10^5$ IU/ml). Abnormal serum ALT and AST were found to be statistically associated with high HCV viral load.

As viral DNA levels in occult hepatitis B are very low, the identification of occult hepatitis B is strongly dependent on both the sensitivity and the specificity of the assay. Current technologies used for DNA detection are as follows: nested-PCR, real-time PCR, and transcription mediated amplification (TMA). Primers must be specific for different HBV genomic regions and complementary to highly conserved (genotype shared) nucleotide sequences.^{11,18}

Based on several studies, conventional nested PCR using specific primers targeting the *pol* gene was found to be a highly sensitive technique for detection of OBI.¹⁹ Based on these data, the 100 chronic HCV patients enrolled in the current study were tested for anti-HBc and *pol* gene was amplified using nested PCR.

In this specific high-risk group, several studies have been conducted worldwide. The available data on the frequency of occult HBV infection are widely divergent. OBI seems to be highly prevalent in Asia.²⁰ Although the prevalence of occult hepatitis B in patients with chronic hepatitis varies, the highest prevalence is among hepatitis C patients.²¹ There is general agreement that patients infected with HCV should be considered as a category of individuals with high prevalence of occult hepatitis B.²² The incidence of OBI in HCV patients varies greatly, ranging from 0% to 52%.²³

In the current study, out of the 100 CHC patients 18 cases (18%) were positive for the *pol* gene by nested PCR (OBI).

Similar results were reported in several studies.^{24–28} Also, our results agree with that reported among Mediterranean countries since HBV-DNA was detectable in about one-third of HBsAg negative HCV carriers in the Mediterranean basin.^{29,30}

In 2011, Selim et al.,²⁵ reported that 23 (38.3%) of 60 chronic Egyptian hepatitis C patients had detectable HBV-DNA, despite the absence of circulating HBsAg.

In Lebanon, an overall rate of 16.3% of occult HBV was detected among chronic HCV patients.³⁰ Georgiadou et al.,³¹ demonstrated that almost one quarter of HCV-positive/HBsAg-negative Greek patients had detectable HBV-DNA by PCR. Higher prevalence was reported in Far East Asian countries by using PCR, as the prevalence of OBI in Chinese patients with chronic HCV infection was 41.9%.³²

The difference in the reported prevalence may be due to a multitude of reasons. This difference is attributed mainly to geographical variation regarding HBV endemicity in these countries. Risk factors for hepatitis B infection and the biomarkers of hepatitis B infection are different in the studied populations, and also, different in terms of sensitivity and detection limit of the assay used (standard/nested PCR, real-time PCR, etc.). The number of HBV DNA domains examined and the biological compartment explored (liver, plasma, or both) play an important role.

There have been significant advances in understanding the molecular mechanisms underlying occult HBV infection in the last decade; the stability and long-term persistence of viral

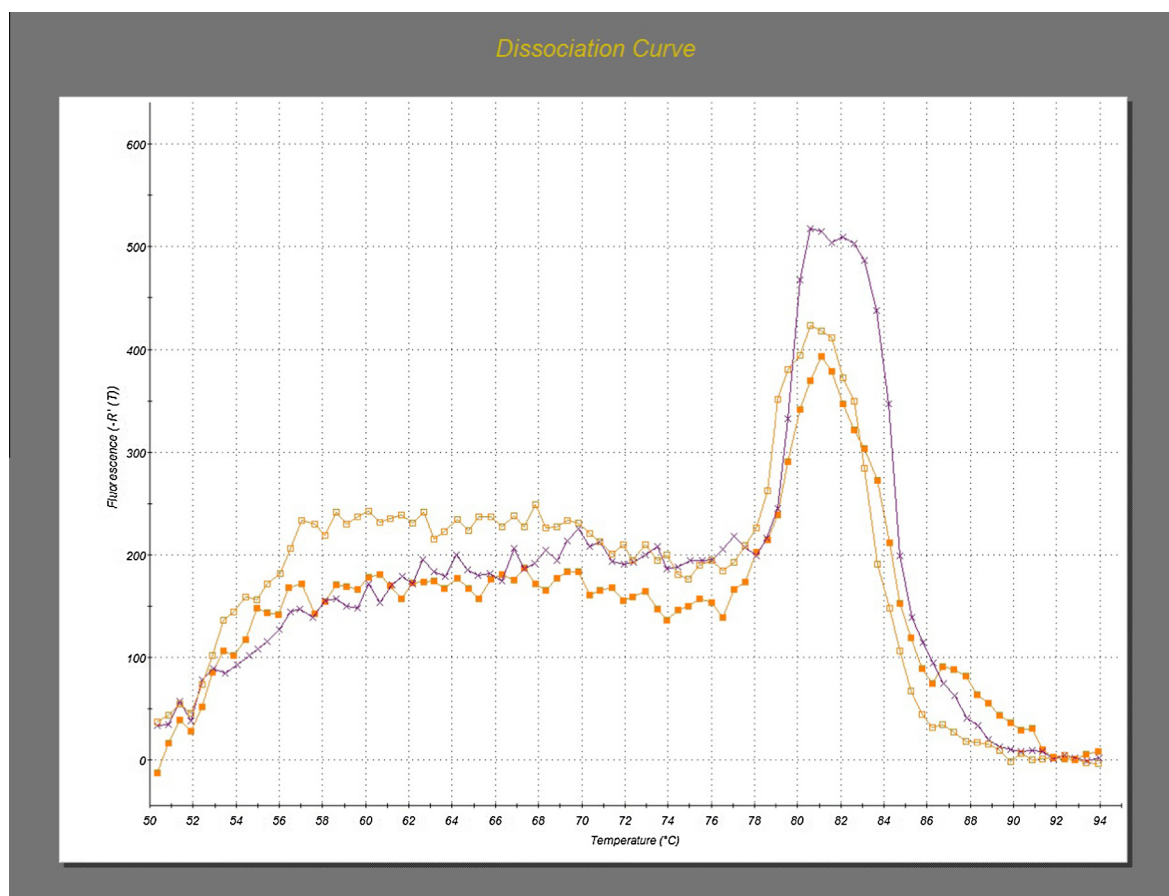


Figure 4 Dissociation curve of x gene.

Table 3 Distribution of liver function tests and anti-HBc among the 18 OBI patients.

	Anti-core +ve						Anti-core -ve						Total	Fischer exact <i>P</i> value
	Normal		Abnormal		Total		Normal		Abnormal		Total			
	No	%	No	%	No	%	No	%	No	%	No	%		
<i>Liver function Dist</i>														
AST	2	22.2	7	77.8	9	100.0	2	22.2	7	77.8	9	100.0	18	1
ALT	4	44.5	5	55.5	9	100.0	4	44.5	5	55.5	9	100.0	18	1

anti-HBc: antibodies against hepatitis Be antigen, ALT: alanine aminotransferase, AST: aminotransferase.

FEp: Fischer exact test.

X2: Chi square test.

*Results $\leq .05$ are significant.

covalently closed circular DNA (cccDNA) molecules together with the long half-life of hepatocytes imply that HBV infection, once it has occurred, may possibly continue for life. There is persistence of low HBV-DNA levels and lack of detectable HBsAg in patients with OBI remains largely unknown although the condition is probably multifactorial. Both host and viral factors may be involved.²⁹⁻³⁴

Among the host factors, the host immune response may play a role in keeping viral replication at low levels characteristic of OBI or hindrance of HBsAg through circulating immune complex. Likewise, hepatic cytokines such as TNF- α and IFN- β may inhibit viral replication and activation. Mean-

while, reduced HBV viremia may result from extra-hepatic HBV replication such as that takes place in polymorph-nuclear cells (PBMCs). Patients with long-standing abnormal results of liver function tests with unknown etiology may have HBV-DNA in their PBMCs in the absence of HBV markers, serum HBV-DNA.³⁵

Regarding viral factors any mutation that changes the antigenicity of the HBsAg or that decreases the synthesis of this antigen or blockage of free HBsAg secretion may be the cause of the lack of detection of HBsAg in patients with OBI (HBV variants).³⁶ Additional mechanisms for OBI have been thoroughly investigated, emphasizing that integration of viral

sequence may alter HBsAg expression and decrease HBV replication. Mutations in the regulatory regions of the HBV genome or the polymerase domain or in the *s* gene cause HBsAg to be undetectable by commercial assays.^{11,37}

Early studies reported a higher prevalence of HBV-DNA detection in anti-HBc-positive than in anti-HBc negative patients.^{38,39} In this study the *pol* gene was equally detected among both anti-HBc positive (50%) and anti-HBc negative patients (50%).

Ramia S et al.,³⁰ detected HBV DNA in 41% of anti-core positive patients compared to 7.1% of anti-core negative chronic HCV patients

Detection of HBV DNA from serum or liver samples is considered the gold standard for the diagnosis of occult HBV infection. Experts have recently recommended the use of highly sensitive nested PCR or real-time PCR assays that can detect fewer than 10 copies of HBV DNA for the diagnosis of occult HBV infection. In addition, testing for multiple targets on the HBV genome increases HBV DNA detection rates.³⁷

In the present study, we tested two technical procedures regarding their specificity and sensitivity: real-time PCR namely SYBR Green (amplifying *S*, *C* and *X* genes) and nested PCR with primers targeting the region encoding the *pol* gene.

Using three sets of primers, specific for HBV *surface*, *core* and *X* genes, different gene profiles were noticed among the 18 chronic HCV patients with occult HBV infection as follows: the 4 genes (*pol*, *S*, *C*, *X*) combination was only detected among one patient (5.5%), (*pol*, *C*, *X*) gene combination was detected in 4 patients (22.3%). However *pol* and *S* genes, *pol* and *C* genes and *pol*, *X* genes were detected solely in 2 patients (11%), 5 patients (27.8%), and one patient (5.5%) respectively. On the other hand, 5 cases (27.8%) positive for *pol* gene, were negative for the other 3 genes.

Different results were reported by Kader et al.⁴⁰ among Sudanese blood donors as there were two main profiles, namely the presence of the *s*, *c*, *x* genes together in 33.3% of the blood donors or the presence of *x* gene in addition to the core gene in 38% of their study group.

Our results disagree with those of Alice et al.⁴¹ who reported that HBV Cobas TaqMan real-time PCR seems completely appropriate for exploration of occult hepatitis.⁴²

On the other hand, several studies reported that the use of COBAS TaqMan HBV PCR assay to screen OBI has not provided information similar to that reported using nested PCR.⁴³⁻⁴⁶ Fujiwara et al.,⁴⁷ used a real-time PCR for quantitation of HBV-DNA. They reported that the detection rate was slightly lower than that in other studies using different techniques. The specificity of the results was usually enhanced by using nested PCR with two rounds of amplification and primers amplifying different targets.

The gold standard test for detection of OBI is the amplification of HBV-DNA. At present, the optimal standard for diagnosis is the analysis of HBV-DNA extracts performed by real-time, nested PCR techniques. False results of these assays could be avoided by choosing PCR primers that span at least three genomic regions of the HBV genome such as the *s*, *x* and *c* genes, and validation should require detection from at least two regions of the genome. This suggestion is not usually fulfilled, and only one segment of a region is amplified.^{12,34,48}

Patients with both HBV and HCV infections may show a large spectrum of virological profiles.⁴⁹ HCV infection can

suppress HBV replication, as demonstrated by studies showing that patients with chronic hepatitis B who are coinfecting with HCV have lower HBV-DNA levels, decreased activity of HBV-DNA polymerase, and decreased expression of HBsAg and HBcAg in the liver.^{50,51} Moreover, patients with chronic HBV infection who become superinfected with HCV can undergo seroconversion of HBsAg. This effect may be mediated by the host immune response (via the induction of cytokines such as IFNs) or by a direct effect of HCV proteins. Direct interference mediated by HCV proteins can occur in vivo only if both HBV and HCV coexist in the same hepatocyte.⁵²

Several studies^{53,54,15} emphasized on the clinical impact of silent HBV in patients suffering from chronic liver disease as a result of HCV and reported that higher levels of the liver disease severity were seen. It has been suggested that HBV replication accounts for many of the liver enzyme flares in patients coinfecting with HCV and occult HBV, and is associated with fibrosis/cirrhosis.^{55,56} However, in the current study, no significant association was found between OBI and the liver function tests either in the anti-core positive or anti-core negative patients.

5. Conclusion

From this study we can conclude that occult hepatitis B is observed in a considerable number of hepatitis C patients in Egypt. Routine serological profiles are not always reliable in determining status of HBV infection. Prospective studies are also required to establish the relative risk of HCC among individuals with HBsAg negative chronic liver disease and HCV coinfection.

Conflict of interest

None declared.

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