



## Novel Electron Spin Resonance-Enzyme Immunosorbent Assay for Detecting Occult Hepatitis B Infection in HCV Chronic Liver Disease

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### ABSTRACT

**Background:** Hepatitis B virus infection in patients who lack detectable hepatitis B surface antigen (HBsAg) is called occult hepatitis B infection (OHB). The very low level of HBV genome may hamper its detection by molecular techniques. Recently, a highly sensitive EIA utilizing a novel modified electron spin resonance (ESR) technique (modified ESR-EIA) was developed to detect HBsAg by measuring stabilized nitroxide radicals. **Aim:** to detect occult HBV infection, using ESR-EIA among HCV-related chronic liver disease (CLD) Egyptian patients who were seronegative for HBsAg by standard EIA. **Methods:** The study was conducted on two periods of time; in 1st period, 72 inpatients in Tropical Medicine Department of TBRI, were enrolled in the study. They were divided into two groups; 44 seropositive anti-HCV patients (Group I), 28 seronegative anti-HCV patients (Group II). Sera were subjected to virological assays for HBsAg, HBeAg, anti-HBc IgM, anti-HBc IgG, anti-HBs, anti-HCV and HCV RNA. We also examined serum HBV DNA by polymerase chain reaction (PCR) technique and real-time detection polymerase chain reaction (RTD-PCR). In the 2nd period; modified ESR-EIA was applied on 32 TBRI inpatients, 23 in Tropical Medicine Department (Group I) and 9 from hemodialysis unit (Group II) with HCV-related CLD. **Results:** OHB was detected in 18.1% and 86.9% of our patients in 2002 and 2006 respectively. In phase 1, there was a higher detection rate among HCV patients in Group I (25%) than Group II (7%), with higher prevalence (52.4%) in patients with positive HCV RNA in Group I versus those with negative HCV viremia (8%) in Group II. HBV DNA by either PCR or RTD-PCR was negative in all patients of both groups as the HBV viral load of the samples were below detectable level of the methods used; less than 100 copies/ml. None of 9 hemodialysis patients were positive for OHB. **Conclusion:** The newly developed quantitative ESR-EIA technique represents a great evolution for screening and diagnosing OHB in patients with CLD who are negative for conventional HBV-related serological markers. Moreover, investigation of chronic infection with a low HBV load and its clinical significance is considered to make a significant contribution to prevention and treatment. Detection of OHB would limit its nosocomial spread particularly in hemodialysis units and liver transplant recipients.

**Keywords:** Occult HBV, Electron spin resonance, Enzyme immunoassays, Chronic liver disease, HCV

### INTRODUCTION

Hepatitis B virus (HBV) infection is a serious health problem globally with more than 240 million chronic carriers despite the global vaccination program being launched since 1982. Chronic HBV infection is associated with increased risks of advanced liver diseases including cirrhosis, hepatocellular carcinoma and liver decompensation [1]. Occult HBV infection (OHB) is defined as the presence of a low amount of HBV DNA (less than 104 copies/mL)

in patients with negative hepatitis B surface antigen (HBsAg) serum/plasma [2]. The clinical implications of OHB involve different clinical aspects; First, OHB harbors potential risk of HBV transmission through blood transfusion, hemodialysis [3] and organ transplantation, Second, it may serve as the cause of cryptogenic liver disease, contribute to acute exacerbation of chronic hepatitis B, or even fulminant hepatitis, Third, it is associated with development of hepatocellular carcinoma [4]; Fourth, it may affect disease progression and treatment response of chronic hepatitis C. Escaped mutants of HBV might be as a result of post transcriptional effect of the mutation on HBsAg expression. In addition, it is a known fact that surface antigen mutation reduced effectiveness of diagnostics and allowed for humoral immune escaped thereby reducing vaccination effectiveness [5].

Hepatitis B surface antigen (HBsAg) is the main diagnostic marker for hepatitis B infection and for screening of donated blood [5]. Enzyme-linked immunosorbent assay (ELISA) and radio-immunoassay are standard test methods for detection of HBsAg [6]. In spite of using these sensitive screening assays in donors, post-transfusion HBV infection is not prevented completely [7]. Perhaps the amount of HBsAg contained in the peripheral blood of these HBsAg-negative HBV carriers is so small that the currently employed methods are not adequate [6]. However, with the improved ability of the polymerase chain reaction (PCR) to detect HBV DNA, HBV infection has been shown to be one of the causative agents in patients lacking HBV related serological markers [8,9]. Villa, et al. [10] reported that conventional serology gives partial information on the true occurrence of HBV infection in HBsAg negative patients, while HBV-DNA assay defines more accurately the HBV status.

Most reports agreed that there is a very low level of viremia in patients with OHB [11-13]. Serum HBV DNA was detected with very sensitive nested PCR techniques in only 60% of subjects positive for intrahepatic viral genome [11]. Real-time detection polymerase chain reaction (RTD-PCR) based on TaqMan chemistry is estimated to be  $10^4$  to  $10^5$  times more sensitive than the branched DNA (bDNA) assay for quantitation of HBV DNA [14]. Detection of HBV DNA is the key to OHB. However, its detection is hampered by the very low level of HBV genome in HBsAg negative individuals [12]. In addition, most PCR assays are not standardized [15]. The failure of diagnostic assays to detect HBV poses a major risk to recipients of blood transfusions or organ donations. A highly sensitive molecular method and an affordable and reliable serological test are required for the diagnosis of OHB [5].

Recently, a highly sensitive EIA utilizing a novel electron spin resonance (ESR) technique (ESR-EIA) was developed to detect HBsAg. According to the conventional ELISA system, the peroxidase activity in the peroxidase (POD)-antibody conjugates is measured by using a chromogen in the presence of hydrogen peroxide ( $H_2O_2$ ). In ESR-EIA technique, a new method to detect peroxidase activity was developed by Aoyama, et al. [16,17] in which the amount of a stable nitroxide radical that has been generated in the presence of  $H_2O_2$ , p-acetamidophenol (p-AP), and 4-hydrazonomethyl-1-hydroxy-2,2,5,5-tetramethyl-3-imidazoline-3-oxide (HHTIO) is quantitatively measured by using electron spin resonance spectroscopy. It has been reported that p-AP analogs are good reagents for a highly sensitive measurement of the POD activities [18]. Then, Matsuo, et al. [6] applied this technique to HBsAg detection. This ESR-EIA was approximately 10 times more sensitive than the standard EIA at detecting HBsAg with excellent reproducibility. It can detect lower levels of HBsAg (0.15 ng/ml) in the serum than peroxidase-chromogen method (1.2 ng/ml).

A modification in ESR-EIA technique was developed by Togashi, et al. [19], with revolutionarily high sensitivity. This radical immunoassay is based on measurement of peroxidase activity in peroxidase-antibody conjugates, in which a stable nitroxide radical is generated in the presence of  $H_2O_2$ , p-acetamido phenol (p-AP), and 1-hydroxy-2,2,5,5-tetramethyl-3-imidazoline-3-oxide (HTIO). The radical immunoassay was able to detect 0.025 ng/ml HBsAg, with an undeterminable range of 0.01 ng/ml, while the lowest determinable level of EIA was 1.2 ng/ml and that of the chemiluminescence immunoassay was 0.2 ng/ml.

Co-infection with HBV and HCV viruses is frequent particularly in areas where the two viruses are endemic and among people at high risk for parenteral infections [20-22]. Co-infection of schistosomiasis, HBV and HCV is common in countries where schistosomiasis is endemic. Schistosomal infection is a risk factor of HBV infection that can increase the incidence of OHB [23]. Frank, et al. [24] concluded that the parenteral anti-schistosomal therapy (PAT) mass treatment campaigns had a major role in the spread of HCV infection throughout Egypt. There should be a suggested role for the PAT for the transmission of HBV infection as well [25,26].

Therefore, a study was conducted to investigate the prevalence of OHB using the novel modified electron spin

resonance technique (modified ESR-EIA) among HCV-related chronic liver disease (CLD) and hemodialysis patients who were seronegative for HBsAg by standard EIA.

### MATERIALS AND METHODS

Two studies were performed. The first study was conducted in 2002 in which the ESR-EIA was done for 72 patients with chronic liver disease who were seronegative for HBsAg by standard EIA. They were chosen from those attending outpatient clinic or inpatients in Tropical Medicine Department of Theodor Bilharz Research Institute. They were 46 males, their ages ranged from 33-70 with a mean of  $53.370 \pm 7.593$ , and 26 females, their ages ranged from 35-56 with a mean of  $47.923 \pm 5.433$ . They were divided into two groups; anti-HCV positive (44, Group I) and anti-HCV negative (28, Group II).

The second study was conducted in 2006 on 49 patients with chronic liver disease who were seronegative for HBsAg by standard EIA. They were chosen from those attending outpatient clinic or inpatients in Tropical Medicine department and hemodialysis unit of Theodor Bilharz Research Institute. They were 34 males, their ages ranged from 31-68 with a mean of  $51.91 \pm 6.82$  and 15 females, their ages ranged from 36-58 with a mean of  $46.843 \pm 5.132$ . They were divided into three groups; Group I (23), patients admitted to tropical medicine department with liver cirrhosis (4), hepatocellular carcinoma (4), chronic hepatitis (3), hepatosplenomegaly (3) and (9) liver cirrhosis associated with schistosomiasis, Group II (17), patient admitted to tropical medicine outpatients with elevated liver enzymes and Group III (9): patient admitted to hemodialysis unit. All patients were subjected to history taking and clinical examination with special stress on the stigmata of chronic liver disease, abdominal ultrasonography and upper endoscopy. Blood samples were collected from all patients and sera were stored at  $-85^{\circ}\text{C}$  immediately until subjected to analysis for viral markers for HBV and HCV.

Detection of serological markers for HBV and HCV by enzyme immunoassays (EIAs) according to the manufacturer's instructions was done in the two studies. The following markers were performed: hepatitis B surface antigen (HBsAg) (Murex version 3, Murex-Biotech Ltd. UK), hepatitis B surface antibody (anti-HBs) (ETI-AB-AUK-3 DiaSorin S.R.L., Italy), hepatitis B core total antibody (anti-HBc total) (ETI-AB-Corek-2 DiaSorin S.R.L., Italy), Hepatitis B core IgM (IgM anti-HBc) (ETI-core- IgM K-2 DiaSorin S.R.L., Italy), hepatitis Be antigen (HBe Ag) ( ETI-EBK-2 DiaSorin S.R.L., Italy), hepatitis C antibody (anti-HCV) (Murex anti HCV version 4 Murex-Biotech Ltd UK).

#### Detection of serum HBsAg by ESR-EIA and modified ESR-EIA technique

Phenoxy radicals are produced from the phenol compound p-AP by the action of horseradish peroxidase (HRP) in the presence of  $\text{H}_2\text{O}_2$  [18]. Hydroxylamine compounds of 4-hydrazonomethyl-1-hydroxy-2,2,5,5-tetramethyl-3-imidazoline-3-oxide (HHTIO) replaced by HTIO (4-hydrazonomethyl-1-tetramethyl-3-imidazoline-3-oxide) in modified ESR-EIA, are then converted to stable nitroxide radicals by oxidation of phenoxy radicals (Figure 1). Peroxidase activities are quantified by measurement of these stable nitroxide radicals by ESR spectroscopy [16,17,27]. The radical immunoassay method can amplify peroxidase activity by  $10^6$  times.

In the first study, the ESR-EIA was done as follows, an aliquot of 200  $\mu\text{L}$  serum was incubated at  $37^{\circ}\text{C}$  for 30 min with both 50  $\mu\text{L}$  of anti-HBs peroxidase conjugate (peroxidase-labeled monoclonal anti-HBs) and anti-HBs-coated beads (Auszyme II EIA kit, Abbott Laboratories, Chicago, IL). After the beads were washed with distilled water, they were incubated in a mixture of 160  $\mu\text{L}$  water, 30  $\mu\text{L}$  20 mM p-AP (Wako Pure Chemical Industries, Osaka, Japan), 100  $\mu\text{L}$  0.1 M 3-morpholinopropanesulfonic acid buffer (PH 6.5, 0.015%  $\text{H}_2\text{O}_2$ ) (Dojindo Laboratory, Kumamoto, Japan), and 10  $\mu\text{L}$  0.5 mM HHTIO (Aldrich Chemical, Milwaukee, WI) in dimethyl sulfoxide (DMSO) at room temperature for 30 min. This process produces phenoxy radicals. The enzyme reaction was stopped by adding 50  $\mu\text{L}$  100 mM  $\text{NaN}_3$  to the reaction mixture. The stable nitroxide radicals produced were quantified by using ESR spectroscopy (JES-FR30 ESR system, JEOL Datum Ltd., Tokyo, Japan). Two blanks, each consisting of 250  $\mu\text{L}$  HBsAg-negative control serum (human sera non-reactive for both HBsAg and anti-HBs) and three positive controls (HBsAg- EIA and ESR positive and PCR/RTD-PCR HBV DNA positive serum from Egyptian patient case number 22 and two control Japanese sera), were simultaneously assayed. The result was expressed as a signal to-noise (S/N) ratio, calculated by dividing the signal intensity of the sample by the averaged signal intensities of the negative controls (two blanks) (Figure 2). Reactions were further confirmed by a neutralization assay in which sera that tested positive were incubated with a human anti-HBs to bind to any HBsAg present [28]. If this neutralized serum ceased to react to the p-AP/HHTIO, the presence of HBsAg was confirmed.  $S/N$  ratio  $<1.488$  was considered negative,  $S/N \geq 2.181$  positive, and  $1.488 \leq S/N < 2.181$  to be undeterminable [6].

In the second study, the modified ESR-EIA was done as follows; an aliquot of 240  $\mu$ L serum was incubated with both 60  $\mu$ L of anti-HBs peroxidase conjugate and a bead at 37°C for 30 min. After washing the beads with 0.01% W1A (Sigma Chemical Company, St. Louis, MO, USA) solved in distilled water 11 times, they were again incubated with 200  $\mu$ L of reagent (2 mM p-AP, 0.017 mM HTIO, DMSO and 33 mM MOPS, pH 6.5) and 100  $\mu$ L of 0.001% H<sub>2</sub>O<sub>2</sub> for 30 min. Then, 50  $\mu$ L of 100 mM NaN was added to the reaction mixture to stop the enzyme reaction (Figure 1). All the reagents were prepared with milli-Q water. HBsAg levels in the sera were determined by the radical immunoassay method, using an automated electron spin resonance (ESR) analyzer (Tohoku Seiki Industry, Yamagata, Japan), equipped with a pipettor, an incubator, a washer, and a reader station. The ESR spectroscopic settings for measurement were done automatically. The signal intensity of the middle-field component of the triplet nitroxide radical was measured. The result was expressed as the signal to noise (S/N) ratio, calculated by dividing the signal intensity of the sample (signal) by that of a paired HBsAg-negative serum sample (noise) (Figure 2). Two blanks, each consisting of 250  $\mu$ L HBsAg-negative control serum (human sera non-reactive for both HBsAg and anti-HBs) and three positive controls (HBsAg- EIA and ESR positive and PCR/RTD-PCR HBV DNA positive serum from Egyptian patient case number 22 and two control Japanese sera), were simultaneously assayed. S/N ratio  $\geq$  3.2 was considered positive.

### Detection of Serum HBV DNA

#### Nested PCR

In the first study 2002, it was performed in Theodor Bilharz research Institute according to Saber, et al. [29]. HBV genomic DNA was extracted from serum using guanidinium isothiocyanate phenol chloroform method and dissolved in distilled water. HBV DNA was detected by PCR using a set of nested primers designed for the core/precore region. Amplification products approximately (0.23 kb band) were visualized using 1.5% agarose gel electrophoresis. The detection limit of this system was 200 DNA copies/reaction. The primers used for the first PCR amplification were: 5'-GCT TTG GGG CAT GGA CAT TGA CCC GTA-3' and 5'-CTG ACT ACT AAT TCC CTG GAT GCT GGG TCT-3'. The primers used for the second PCR amplification were: 5'-GAC GAA TTC CAT TGA CCC GTA TAA AGA-3' and 5'-ATG GGA TCC CTG GAT GCT GGG TCT-3'.

Serum HBV DNA quantitation by real-time detection polymerase chain reaction (RTD-PCR) based on Taq Man chemistry system according to Loeb, et al. [30] was done in the first study 2002. It was performed in Second Department of Internal Medicine, Shimane Medical University, Izumo, Japan. The procedure permits the PCR amplification and detection of the amplified nucleic acid sequences in a single tube. A fluorescent dye-bound probe specific for HBV nucleic acid sequence allows the detection of the HBV PCR products as they are generated. Each successive PCR cycle results in the exponential amplification of the PCR product and Fluorescence intensity, which is detected by an ABI prism 7700 sequence detector system in real time. HBV DNA Extraction: Total DNA was extracted from 200  $\mu$ L of serum using QIAamp DNA blood Mini Kit (Qiagen GmbH, Germany) according to manufacturer's instructions. Purified DNA was resuspended in 50  $\mu$ L of 10 mM Tris HCL (pH 8.0) and 1 mM EDTA, and then stored at -20°C until PCR. A 10  $\mu$ L aliquot of DNA solution (40  $\mu$ L serum equivalent) was used for RTD-PCR. Amplification: using a set of primer which was designed against a conserved region of the HBV genome overlapping the genes encoding the X-protein and DNA polymerase, defined an amplicon corresponding to bases 1549 to 1653 of HBV genome, consisting of: HBV1F (5'-CCG TCT GTG CCT TCT CAT CTG-3'), HBV1R (5'-AGT CCA AGA GTC CTC TTA TGT AAG ACC TT-3'), and TaqMan probe HBV1TAQ (5'-CCG TGT GCA CTT CGC TTC ACC TCT GC-3'). Amplification Reaction Mixtures: consisted of 50  $\mu$ L containing: 1X Taq Man Universal Master Mix (PE Biosystems), 200 nM of primers and 200 nM of TaqMan probe. Thermal cycling conditions: were as follows, initial activation of uracil-N-glycosylase (UNG) at 50°C for 2 min to digest the product to destroy the potential carry-over contamination. Incubation for 10 min at 95°C allowed the activation of the Ampli Taq Gold DNA polymerase, inactivation of UNG and the denaturation of the nucleic acids. Subsequently, 40 cycles of denaturation were performed at 95°C for 15 seconds and annealing-extension at 60°C for 1 min were then carried out allowing the amplification-detection of HBV genomes. Detection and quantitation of PCR product: was performed with an ABI PRISM 7700 sequence detector system (PE Biosystems). The efficacy of RTD-PCR was evaluated by quantitatively measuring sequential levels of synthetic standard HBV-DNA. The detection limit of this system was as few as 100 DNA copies/reaction and a linear standard curve was obtained between 10<sup>2</sup> and 10<sup>4</sup> DNA copies/ reaction.

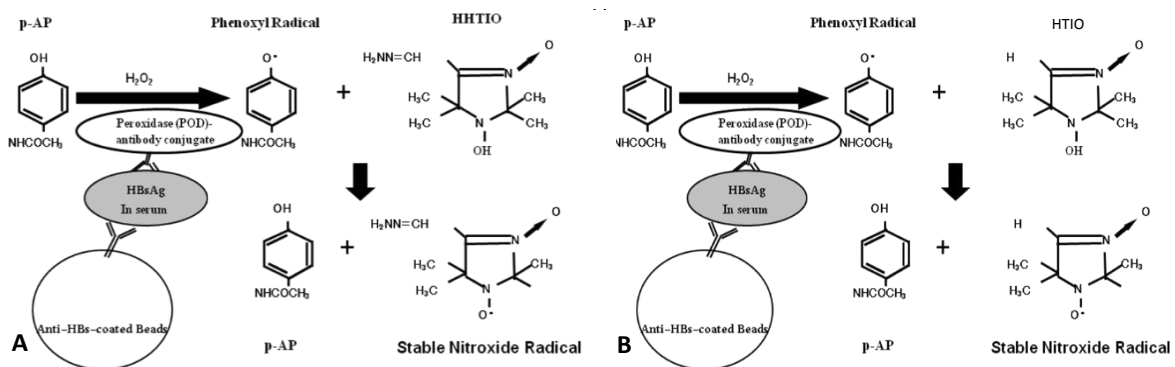
Serum HBV DNA quantitation by RTD-PCR according to Abe, et al. [14] was done in the first study 2002. It was performed in the Centre for Molecular Biology and Cytogenesis (SRL), Tokyo, Japan. It is similar to the above mentioned RTD-PCR method with the following differences: the set of primer was also derived from X region; consisting of: Forward primer, HBXF1 (5'-ACG TCC TTT GTT TAC GTC CCG T-3'), nt 1414-1435; Reverse primer HBXR1 (5'-CCC AAC TCC TCC CAG TCC TTA A-3'), nt 1744-1723 and TaqMan probe HBXP1 (5'-TGT CAA CGA CCG ACC TTG AGG CAT A-3'), nt 1681-1705. Denaturation was performed by 53 cycles at 95°C for 20 seconds. Detection and quantitation of PCR product was performed with an ABI PRISM 7700 sequence detector system (Perkin Elmer, Foster City, Calif). The detection limit of this system was 200 copies/ml and a linear standard curve was obtained between  $10^1$  and  $10^8$  DNA copies/reaction.

HBV DNA was measured using a real-time direct test for HBV (HBV-Direct Mag, JSR, Tokyo, Japan), was done in the second study 2006 which combines the use of a DNA extraction system based on magnetic beads coated with polyclonal anti-HBsAg and the real-time detection method [14,15]. The PCR primers and probe used were designed using Primer Express software (Applied Biosystems, CA, USA) and were available for eight HBV genotypes (A-H) on the basis of alignment with their sequences [14]. The detection limit of the test is  $1.0 \log_{10}$  copies per ml [19].

Detection of Serum HCV RNA by nested reverse transcription PCR was done in the first study 2002 (Amplicor HCV test, version 2.0 Roche Diagnostic System, Inc., USA). HCV RNA was extracted using acid guanidinium thiocyanate-phenol chloroform single step method [31]. HCV-RNA was detected by qualitative nested RT-PCR using two sets of primers within the 5' non-coding region. Amplification products were analyzed using 2% agarose gel electrophoresis [32].

### Statistical analysis

Descriptive statistics and statistical analysis of all data were calculated with the computer package Epi-Info 6.0, and  $P < 0.05$  were considered statistically significant. Our data shown in tables were expressed as arithmetic mean (as a measure of location)  $\pm$  the standard deviation (as a measure of dispersion) and positivity percent. One-way analysis of variance (Kruskal-Wallis), the LSD "t" test was used to determine the significance of associations between groups. Chi square (Mantel-Haenzel, Bartlett's test) and Fisher's exact test were used for significance testing of proportional data. Histogram was used to illustrate the frequency distribution of occult HBV infection among studied groups and scatter diagram was used to show the distribution of S/N ratios among positive and negative samples and the cut-off value.



**Figure 1 (A):** Phenoxy radicals are produced from p-AP (p-acetamido phenol) by the action of POD in the presence of  $H_2O_2$ . **(B):** HHTIO (4- hydrazone methyl-1- hydroxy -2,2, 5,5-tetramethyl-3-imidazoline-3-oxide) in ESR-EIA, replaced by HTIO (4-hydrazone methyl-1-tetramethyl-3-imidazoline-3-oxide) in modified ESR-EIA, is converted to stable nitroxide radical by oxidation of phenoxy radicals. HBsAg is quantified through measuring these stable nitroxide radicals with ESR spectroscopy

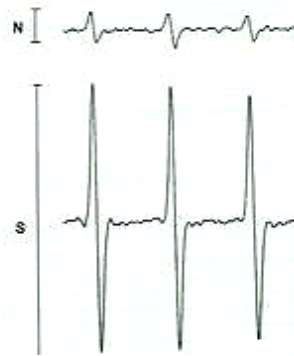


Figure 2 S/N ratio is calculated by dividing the signal intensity of the sample (S) by the signal intensity of negative control (N)

**RESULTS**

**Detection of serum HBsAg by ESR-EIA and modified ESR-EIA technology**

In 2002, serum HBsAg was detected by ESR-EIA in 13 (18.1%) out of 72 HBsAg negative patients by standard EIA. It is clear that the S/N ratio in HBsAg-positive cases was at high level of  $6.109 \pm 6.177$  (mean  $\pm$  SD) compared to its low level of  $1.172 \pm 0.310$  in HBsAg-negative ones. The difference between means was statistically significant ( $P < 0.0001$ ).

In 2006, Occult HBV infection was detected by modified ESR-EIA in 30 (61.2%) out of 49 HBsAg negative patients by standard EIA with highly significant difference between Group I (91%) and Group II (29.41%) or Group III (44.44%) with  $P < 0.0000001$  and  $P < 0.01$  respectively. Distribution of S/N ratios and their relative means in 4 positive controls, 21 HBsAg ESR seropositive patients (Group I), 5 HBsAg ESR seropositive patients (Group II) and 4 HBsAg ESR seropositive patients (Group III) are shown in Tables 1 and 2; Figure 3. The S/N ratio in HBsAg-positive cases was at high level of  $272.45 \pm 25.499$ ,  $3.45 \pm 15.410$  and  $4.4205 \pm 1.243$  (mean  $\pm$  SD) in Group I, II, III respectively compared to its low level in HBsAg-negative cases of  $2.583 \pm 1.183$  in Group I, III and  $1.172 \pm 0.310$  Group II. The difference between means was statistically significant ( $P < 0.0001$ ).

**Table 1 Distribution of S/N ratios and their relative means in in HBsAg EIA, ESR and PCR/RTD-PCR HBV DNA seropositive controls, HBsAg ESR seropositive and seronegative Patients (2002)**

Groups	Case No	S/N ratio	Mean $\pm$ SD
HBsAg EIA, ESR and PCR/ RTD-PCR HBV DNA seropositive controls	Case no 22	31.782	12.785 $\pm$ 16.469
	PC 1	4.032	
	PC 2	2.54	
HBsAg ESR seropositive Patients (no.13)	19	(+) 8.02	6.109 $\pm$ 6.177
	38	(+) 23.124	
	49	(+) 13.010	
	116	(+) 9.661	
	135	(+) 5.460	
	156	(+) 2.340	
	164	(+) 2.810	
	166	(+) 3.130	
	226	(+) 2.270	
	244	(+) 2.200	
	250	(+) 2.182	
	276	(+) 2.820	
	352	(+) 2.410	

Representatives from 59 HBsAg ESR seronegative patients	6	(-) 0.847	1.172 ± 0.310
	9	(-) 0.621	
	14	(-) 0.683	
	25	(-) 0.914	
	56	(-) 0.979	
	66	(-) 0.837	
	103	(-) 1.391	
	104	(-) 1.119	
	153	(-) 1.100	
	172	(-) 1.070	
	191	(-) 1.190	
	252	(-) 1.070	
	253	(-) 1.020	

The distribution of S/N ratios of the negative samples did not overlap that of the positive ones. The cut-off value could be established theoretically as  $S/N \geq 2.181$  and  $\geq 3.2$  in the years of 2002 and 2006 respectively.

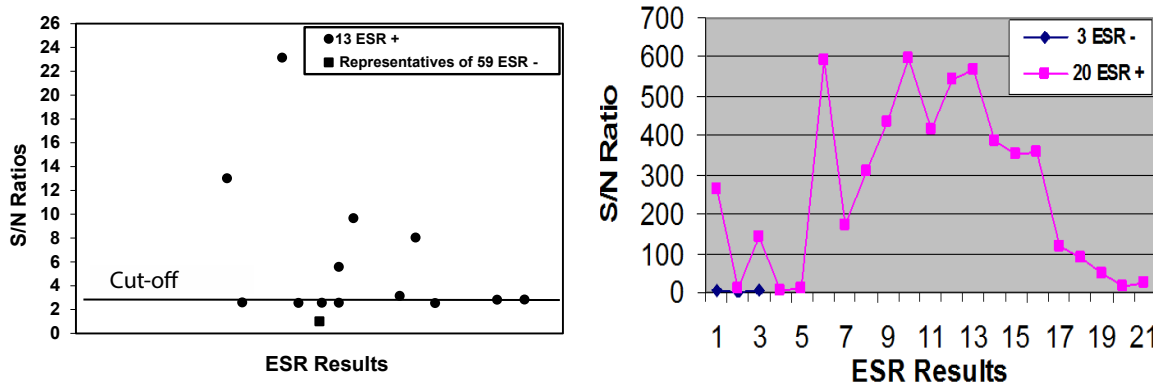


Figure 3 The Distribution of S/N ratios of the negative samples did not overlap that of the positive ones. The cut-off value could be established as  $S/N \geq 2.181$  (2002) and  $S/N \geq 3.2$  (2006)

Table 2 Distribution of S/N ratios and their relative means in HBsAg EIA, ESR and PCR/ RTD-PCR HBV DNA seropositive controls and HBsAg ESR seropositive patients (2006)

Group	Case No	S/N ratio	HBV DNA (Log IU/ml)	Case No	S/N ratio	HBV DNA (Log IU/ml)	Mean ± SD
Controls	Case no 14	588.503	-	-	-	-	263.340 ± 16.469
	Case no 19	305.672					
	PC 1 (0.1)	20.649					
	PC 2 (1.0)	138.531					

Group I	1	264.44	-	15	542.685	1.8	272.45 ± 25.499
	2	9.804	-	16	568.503	1.2	
	3	143.82	-	17	386.526	-	
	4	12.099	-	18	354.809	2.2	
	5	591.532	-	20	359.876	1.4	
	6	172.53	-	21	116.236	1.1	
	7	308.984	-	22	88.438	-	
	8	435.387	-	23	50.134	-	
	9	599.182	-	24	15.044	-	
	10	414.802	-	25	24.836	-	
	11	3.614	-	-	-	-	
Group II	61	3.44	-	-	-	-	3.45 ± 15.410
	62	3.804	-				
	63	4.82	-				
	75	5.099	-				
	77	3.532	-				
Group III	5	3.219	1.4	-	-	-	4.4205 ± 1.243
	9	3.436	-				
	14	4.067	-				
	15	7.16	-				

#### Detection of serum HBV DNA by PCR/RTD-PCR

In 2002, the results of HBsAg-seropositivity by the ESR-EIA in 13 cases weren't consistent with the PCR results. These 13 cases were all negative for serum HBV DNA by either PCR or RTD-PCR. While the 3 HBsAg seropositive controls were positive for serum HBV DNA by both PCR and RTD-PCR. The viral load of HBV DNA for sample number 22 was  $4.3 \times 10^3$  copy/mL (173 copy/reaction) on using Loeb, et al. [30] method, and had only slight level of HBV  $\times$  region DNA with 530 copies/mL on using Abe, et al. [14] method. Other samples were below detectable level of the methods used; less than 100 copies/mL and 200 copies/ml respectively (Table 3).

**Table 3 Distribution of S/N ratios and their relative means in HBsAg ESR seronegative patients (2006)**

Groups	Case No	S/N ratio	HBVDNA (Log IU/ml)	Mean ± SD
Group I	12	2.009	-	2.583 ± 1.183
	13	2.835	-	
Group II	37	0.847	-	1.172 ± 0.310
	40	1.668	-	
	49	1.466	-	
	51	3.019	-	
	60	2.236	-	
	64	3.436	-	
	68	2.025	-	
	70	1.07	-	
	72	0.914	-	
	76	1.391	-	
	78	1.119	-	
	79	1.1	-	
Group III	1	1.465	-	2.583 ± 1.183
	2	1.668	-	
	3	1.466	-	
	6	2.236	-	
	13	2.025	-	

In 2006, Poor agreement was detected between modified ESR-EIA and PCR (concordance=51.020%, Kappa coefficient <0.40). The results of HBsAg-seropositivity by the ESR-EIA in 30 cases weren't consistent with the PCR results. Among the 30 cases that were HBsAg-seropositivity by the ESR-EIA, only 5 cases were positive for serum HBV DNA by PCR. While the 2 HBsAg seropositive controls were positive for serum HBV DNA by PCR (Figure 4).



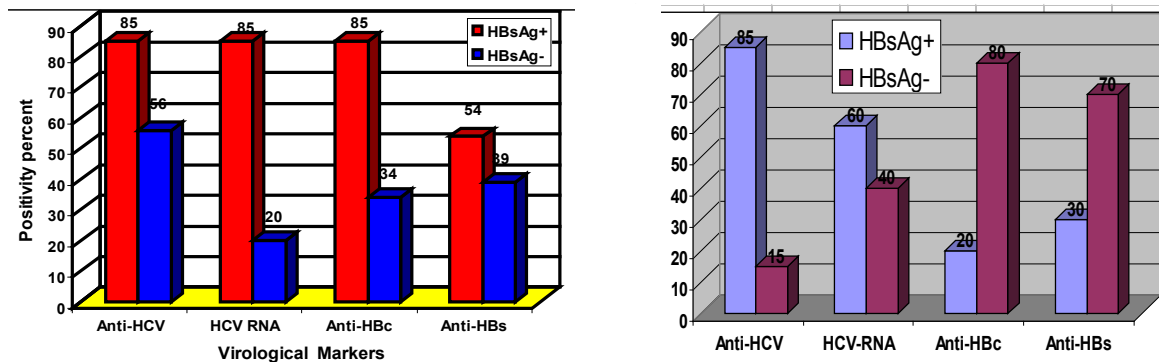


Figure 4 Frequency distribution of virological markers in patients with versus without occult HBV infection

**Correlation of ESR-EIA HBsAg with other virological markers**

In the two studies, although anti-HBs was more frequently detected in occult HBV cases, yet the association was insignificant ( $P > 0.05$ ). Regarding HBV markers, in the first study 2002, anti-HBc total was significantly associated with HBsAg seropositive cases (occult HBV infection) than seronegative ones ( $P < 0.001$ ) while there was no significant ( $P > 0.05$ ) association detected in the second study (Table 4). The results of HBeAg, and anti-HBc IgM, assays were negative in all patients. Regarding HCV markers, anti-HCV and HCV RNA were more frequently detected in occult HBV infection in 2002 ( $P < 0.05$ ,  $P < 0.0001$  respectively), while in 2006, anti-HCV was more frequently detected in Group I ( $P < 0.05$ ) with no significant association in the other studied groups.

Table 4 Demographic and virological data in patient groups with versus without HBsAg (2002)

Groups	No (%)	HBsAg Results	No (%)	Age Mean ± SD	Sex M/F	Anti-HBc No (%)	Anti-HBs No (%)	HCV RNA No (%)
I	44	HBsAg +	11 (25) <sup>a</sup>	51.759 ± 7.394	8/3	9 (82) <sup>a</sup>	5 (45)	11 (100) <sup>c</sup>
	-61	HBsAg -	33 (75)	47.842 ± 5.336	22/11	14 (42) <sup>b</sup>	14 (42)	10 (30.3) <sup>d</sup>
II	28	HBsAg +	2 (7) <sup>b</sup>	50.333 ± 7.276	0/2	2 (100)	2 (100)	0 (0)
	-39	HBsAg -	26 (93)	53.742 ± 6.235	16/8	6 (23)	9 (35)	6 (30)
Total	72	HBsAg +	13 (18.1)	51.403 ± 7.340	8/5	11 (85)	7 (53.8)	11 (85)
	-100	HBsAg -	59 (81.9)	49.023 ± 5.36	38/21	20 (34)	23 (40)	16 (27)

a: vs b:  $P < 0.05$ ; c vs d:  $P < 0.0001$

**Demographic and virological data in patient groups**

In 2002, HBsAg was more frequently detected in HCV cases (Group I) than without co-infection (Group II) ( $P < 0.05$ ). HCV RNA prevalence rate was significantly higher in patients with versus without OHB in Group I ( $P < 0.0001$ ). No statistically significant differences were found between the two groups regarding gender or age distribution. HCV RNA were detected in 21 (47.7%) out of 44 anti-HCV seropositive patients. Eleven (52.4%) out of them, were found to be positive for HBsAg by ESR-EIA. Only two (8%) out of 26 HCV RNA negative patients in Group II, were positive for HBsAg ( $P < 0.01$ ). Two HBsAg and anti-HCV negative cases (7.1%) out of 28 patients in Group II, were positive for HCV RNA. Although anti-HBs was more frequently detected in 19 (43%) out of 44 co-infected cases than in 11 (39%) out of 28 patients with seronegative anti-HCV, yet such association was insignificant.

Table 5 Demographic and virological data in seronegative HBsAg patient groups (2006)

Groups	No (%)	Age Mean ± SD	Sex M/F	Anti-HBc No (%)	Anti-HBs No (%)	HCV RNA No (%)
I	23 (46.8)	52.36 ± 6.54	17/6	1 (4.5)	8 (36.4)	8 (36.4)
II	17 (34.6)	49.91 ± 5.82	07/10	0 (0)	5 (29.4)	7 (41.2)
III	9 (18.6)	51.63 ± 7.413	07/2	0 (0)	3 (33.3)	4 (44.4)
Total	49 (100)	51.27 ± 5.231	34/15	1 (2.1)	16 (32.7)	19 (38.8)

In 2006, no statistically significant differences were found between the two groups regarding gender or age distribution (Table 5). Among Group I, there was higher prevalence (100%) of occult HBV infection in patients with HCVAb seropositivity versus seronegativity (33.3%),  $P < 0.05$ . There was higher prevalence (100%) in patients with HCV viremia versus those without (87%).

## DISCUSSION

Chronic HBV infection is the primary cause of cirrhosis and hepatocellular carcinoma and is one of the ten leading causes of death. Traditionally, people with chronic HBV infection have been identified with blood tests for HBV antigens and antibodies using conventional standard EIAs. OHB is defined as the presence of HBV DNA detectable by sensitive PCR among individuals testing negative for HBsAg [10,17-20]. It was found that HBV can persist as occult infection three decades after acute, apparently self-limited hepatitis B [18].

Several possibilities have been hypothesized beyond the occurrence of OHB. First, mutations of HBV-DNA sequence which could result in impaired antigen production by the virus [32], a diminished rate of replication, [33] or facilitate viral persistence [12]. Second, integration of HBV-DNA into host's chromosomes was evidenced by the high frequency of persistence of fragmented HBV genome detectable by PCR for varying times in liver tissue. Likewise, the presence of circulating fragmented HBV-DNA may be secondary to HBV subgenomic expression of the integrated HBV-DNA [12,34]. Third, infection of peripheral blood mononuclear cells by HBV. Fourth, masking by anti-HBs in immune complexes [35]. Fifth, selective deficiency of the host immune response against HBV which could keep the virus in a latent state [12,36]. Lastly, interference of HBV by other viruses especially HCV [37]. Brechot, et al. [15] have provided very strong evidence that most cases of OHB are related to very low levels of HBV (low HBV load) rather than to HBV mutants that do not express or produce aberrant HBV surface protein. Full-length genome analysis has shown that multiple alterations in the HBV genome may have a synergistic effect in down-regulation of HBsAg production, making it difficult to establish a specific mutation in a particular gene [38].

In areas where HBV infection is highly endemic, vertical, and horizontal transmissions are now a social health problem [25]. Although the prevalence OHB is speculated to be high in these endemic areas, the PCR method cannot be used routinely for screening large numbers of samples because of the complexity of sample preparation, the expense of reagents including *Taq* polymerase, and the time taken for sample processing. Moreover, the sensitivity of PCR analysis depends on DNA concentration, selection of PCR primers, and assay conditions [26]. Thus, PCR analysis of OHB is not suitable as a universal screening method [27,28].

The role of HBV in these serologically negative cases is controversial, raising the question of whether the standard markers for HBV infection (HBsAg and anti-HBc) are insensitive, or sensitive research assays compared with HBV DNA by PCR [39]. Therefore, we expected that our radical immunoassay system would be able to screen the majority of OHB carriers conveniently because of its high sensitivity. Testing our samples by real-time PCR for HBV DNA validates the rationality of the radical immunoassay test by detecting the degree of agreement between the two techniques.

In our study, the incidence of OHB was 18.1% and 61% in 2002 and 2006 respectively. There are contradictory reports on the incidence of occult HBV infection in different parts of the world. In Egypt, the prevalence of OHB infection has been reported to vary from 23.1% [15] to 31.2% [40] and up to 51.4% [41] of standard EIA HBsAg negative, RTD-PCR serum HBV DNA positive patients. In USA, it has been reported to vary from 0% to 30% [15]. Even higher rates were reported in Japan (53.8%, 70%) and in Israel (73%) [15]. On the other hand, lower rates were reported in France (5.5%) [42], and in India (3.2%) [43], where HBV is moderately endemic. So, the prevalence of HBsAg-negative HBV infection should also be adjusted by the baseline prevalence of overt HBV infection in the same geographical area [37]. The discrepancy in the reported incidence of OHB might be attributed to variation in the quantity of viremia in different patients, the endemicity of HBV infection, or difference in the sensitivity of the methods used for detection of either HBsAg or viral genome [22].

Poor agreement was detected in the present study between modified ESR-EIA and PCR. In the first period, serum HBsAg was detected by ESR-EIA in 13 (18.1%) out of 72 HBsAg negative patients by standard EIA. These 13 cases were all negative for serum HBV DNA by either PCR or RTD-PCR. In 2006, among the 30 cases that were HBsAg-seropositivity by the ESR-EIA, only 5 (17.2%) cases were positive for serum HBV DNA by PCR. In accordance to our results, two Japanese studies showed that serum HBsAg examined by ESR-EIA was positive with prevalence rates of 11.5% and 45% of patients with chronic HBV and acute/fulminant hepatitis respectively, in whom standard EIA did not detect HBsAg [6,39]. Matsuo, et al. [6] reported that HBV DNA was not detected by nested PCR in 33.3% of patients who were positive for HBsAg by ESR-EIA. The choice of primers could represent another important factor. It was reported that detection of HBV DNA using a PCR based on primers derived from the pre S1 and pre-core region

should be included in the diagnosis of HBV infection [44]. In order to make the results comparable among studies, a standardized PCR technique should be employed [45].

This discrepancy in our results may be attributed: In one hand to lowering of HBV viral load of the samples to below the detectable level of the methods used (less than 100 copies/ml). On the other hand, the ability to standardize the PCR method as there was no synchronous relationship between the level of HBV DNA and that of HBVsAg i.e., a relatively high level of HBV DNA may find with low level of HBVsAg and vice versa. The extremely small amount of HBV may not be able to present antigens and elicit immune responses [37]. Most reports agreed that there are very low levels of viremia in patients with occult HBV infection [11,41]. Most of our patients had low levels of HBsAg, as shown by the low S/N ratio. However, it would be expected to be more sensitive to use a system with detection limit less than 100 DNA copies/reaction [37,41]. A finding that highly recommends the urgent need for highly sensitive diagnostic tools to detect such low viraemic infection on routine basis.

Although PCR is highly sensitive for detecting HBV DNA, the expense of *Taq* polymerase, the process of DNA purification, and the meticulous precautions for avoiding contamination still preclude PCR as a routine method for examining blood samples. Improved methods of detecting serum HBsAg can contribute to better diagnosis and detection of HBV carriers. So, the newly developed highly sensitive modified ESR-EIA technology represents a great evolution for detection of HBsAg. This method requires as short a time as four hours and its procedures are relatively simple. It has advantages over conventional EIA because it reveals both occult HBV infection in patients with acute liver injury and prolonged seropositivity of HBsAg in chronic HBV carriers in whom HBsAg became negative by conventional EIA [6].

Therefore, Aoki, et al. [39] recommended the use of ESR-EIA for prevention of viral transmission especially in the settings of both blood donation and organ transplantation. They also reported that HBV carriers who became negative for HBsAg by standard EIA and negative for HBV DNA by PCR were still positive for HBsAg by the ESR-EIA method. This may be because of the low amounts of HBsAg that are transcribed from integrated viral DNA during long-term infection, but circulating viral DNA would not be detected because viral replication would have ceased.

OHB has frequently been identified in patients with HCV-related chronic liver disease [11]. In our study, Anti-HCV and HCV RNA were more frequently detected in OHB (Group I) than without co-infection (Group II) in 2002 ( $P<0.05$ ,  $P<0.0001$  respectively), while in 2006, anti-HCV was more frequently detected in Group I ( $P<0.05$ ) with higher prevalence in patients with HCV viremia versus those without and no significant association in the other studied groups. In a study in Seuz Canal University hospital in Egypt, OHB was detected in 41% in association with chronic HCV patients [41]. HBV genome was detectable in 21% of HBsAg negative Austrian patients with chronic hepatitis C [22]. In a Spanish study, HBV DNA was found in 49% of HCV RNA positive and 64% of HCV RNA negative sera of HBsAg negative anti-HCV positive patients [46]. Interestingly, a significant high incidence (90%) of OHB was detected in liver biopsy samples of Japanese patients with chronic hepatitis C [47]. It has been reported that HCV core protein can bind to HBV RNA polymerase II involved in HBV replicative cycle and suppress gene expression and replication causing OHB [48]. Co-infection produces mutual inhibition between both viruses with subsequent reduction in HCV RNA and HBsAg titers so that they escape detection by standard immunoassays [49]. Such suppressive effect might explain the previously underestimated rate of coinfection based on detection of HBsAg and anti-HCV markers [26]. Cacciola, et al. [11] demonstrated a significant correlation of OHB with cirrhosis among HCV infected patients. Human hepatoma cell line was successfully transfected by full-length HBV-DNA genome cloned from patients with HCV and OHB co-infection, indicating replicating capacity of HBV in these patients [37]. Integrated HBV was detected in some patients with HCV infection; in these patients, the integrated DNA was associated with accelerated hepatocarcinogenesis [49]. El-Sherif, et al. [50], reported that Egyptian chronic HCV patients have a high prevalence of OHB. Despite the reciprocal inhibition between hepatitis viruses, co-infection might promote liver damage [38]. So, chronic HCV patients that do not respond to anti-viral treatment and devolve rapid clinical deterioration may respond appropriately to HBV treatment even in absence of HBV markers with surprising clinical improvement. It is recommended treating patients who have moderate to severe chronic hepatitis as evidenced by  $>2$ -fold elevation of ALT levels or significant findings on liver biopsy associated with HBV DNA  $>105$  copies/mL. Patients may have detectable HBeAg associated with wild type virus or HBeAg-negative from infection with the precore or core promoter mutant virus. In general, the same treatment criteria should be applied to patients who are HBC/HCV dually infected as are applied to monoinfected patients. Initiation of treatment, as with both HBV

and HCV, is recommended in patients with active chronic hepatitis or cirrhosis prior to decompensation [51].

Serological markers of past HBV infection are frequently detectable in HBsAg negative patients with Chronic Hepatitis C [21,22]. HCV infection blocks all circulating viral expression of HBV replication, but anti-HBc remains in serum and HBV DNA persist in the hepatocytes resulting in an increased severity of liver disease with a higher risk of developing HCC [38]. The significance of anti-HBc as a predicting factor for OHB remains unknown. Many studies reported that, the incidence of detectable HBV-DNA is much higher in patients with anti-HBc than in those without any HBV markers [11,21,37,41]. Our study in 2002, revealed significantly higher frequency (85%) of anti-HBc in patients with versus without OHB ( $P < 0.001$ ) in accordance with El-Sherif, et al. [50], El-Gohary, et al. [47] and Hassan, et al. [52] in Egypt, reported a detection of anti-HBc in 22.5%, 41% and 43% in chronic HCV patients respectively. However, our study in 2006, anti-HBc total was insignificantly associated with HBsAg seropositive cases than seronegative ones ( $P > 0.05$ ). Ramezani, et al. [53] reported that, anti-HBc did not predict OHB in low-risk individuals. Other studies reported that, demographic data, biochemical parameters of patients, and serological markers for HBV were shown to be unhelpful in distinguishing HBV DNA positive from negative for OHB [53,54].

Patients undergoing hemodialysis are at risk of infection with both hepatitis B virus (HBV) and hepatitis C virus [54,55]. OHB were demonstrated in this setting, and this involves further concerns regarding possible transmission and pathogenic consequences [56]. The rate of HBsAg positive patients on regular dialysis in the developed world is currently low, but outbreaks of HBV continue to occur. The diffusion of HBV in dialysis units in developing countries is higher, although available information is not abundant [57,58].

In our study, no OHB was detected among hemodialysis patients in accordance with that of Gwak, et al. [59] in Korea who reported that, the prevalence of OHB was 0%. In an Egyptian study performed by Abu El Makarem, et al. [60], it was reported that, 4% of the hemodialysis patients had OHB with no significant difference in the prevalence of OHB between hemodialysis patients with or without HCV co-infection. Youssef, et al. [61] in Ismailia, Egypt reported that, 4.7% were positive for HBsAg, and 72.9% positive for HCV-Ab and HBV-DNA was detected in 65% of cases negative for HBsAg and HCV-Ab. Recently, Helaly, et al. [62] detected OHB in 32% of chronic hemodialysis patients in Alexandria, Egypt and a significant association was found between the presence of HBVDNA and anti-HCV positivity. Di Stefano, et al. [57] demonstrated OHB in 26.6% by HBV DNA that was more frequent when anti-HBcAg antibodies were detected alone (72%) than when associated with anti-HBsAg antibodies (31%). Among HCV-seropositive patients, OHB was observed in 66% among them the association of the seropositivity of HCV, anti-HBcAg and HBV DNA was found in 93%. On multivariate analysis, HCV seropositivity and the presence of anti-HBs were still respectively correlated to the presence and absence of OHB.

## CONCLUSION

The newly developed quantitative ESR-EIA technique represents a great evolution for screening and diagnosing HBV Infection in patients with chronic liver diseases who are negative for conventional HBV-related serological markers. It is anticipated that the radical immunoassay method will become a powerful tool for worldwide prevention of vertical and horizontal transmission of OHB, including cases with a low virus load. The radical immunoassay method has high sensitivity for HBsAg detection. This method demonstrated that a low concentration of HBsAg was present in healthy volunteers and a higher percentage of patients with non-B chronic liver diseases. The present findings clearly demonstrate that infection with HBV, including OHB, is far more prevalent than previously thought, as a result of increasing the sensitivity of HBsAg detection to below the present limit. Moreover, investigation of chronic infection with a low HBV load and its clinical significance is considered to make a significant contribution to prevention and treatment. Initiation of treatment, as with both HBV and HCV, is recommended in patients with active chronic hepatitis or cirrhosis prior to decompensation. Detection of OHB would limit its nosocomial spread particularly in hemodialysis units and liver transplant recipients.

## DECLARATIONS

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#### Conflict of Interest

The authors and planners have disclosed no potential conflicts of interest, financial or otherwise.

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