Occult HBV infection status among chronic hepatitis C and hemodialysis patients in Northeastern Egypt: regional and national overview

Mohamed Mandour[1], Nader Nemr[2], Atef Shehata[3],[4], Rania Kishk[3], Dahlia Badran[5] and Nashaat Hawass[2]

[1]. Department of Clinical Pathology, Faculty of Medicine, Suez Canal University, Ismailia, Egypt. [2]. Department of Endemic and Infectious Diseases, Faculty of Medicine, Suez Canal University, Ismailia, Egypt. [3]. Department of Microbiology and Immunology, Faculty of Medicine, Suez Canal University, Ismailia, Egypt. [4]. Department of Microbiology and Immunology, Faculty of Medicine, Jazan University, Jazan, Kingdom of Saudi Arabia. [5]. Department of Medical Biochemistry, Faculty of Medicine, Suez Canal University, Ismailia, Egypt.

ABSTRACT

Introduction: Occult hepatitis B infection (OBI) is considered to be one of the major risks for patients suffering from end-stage renal disease (ESRD) on regular hemodialysis (HD) and patients with chronic hepatitis C virus (HCV) infection. This study compared the prevalence of OBI among these two high-risk groups in the Suez Canal region, Northeastern Egypt, to obtain a better national overview of the magnitude of OBI in this region.

Methods: Serum samples were collected from 165 HD patients and 210 chronic HCV-infected patients. Anti-HCV antibody, hepatitis B surface antigen (HBsAg), total hepatitis B core (anti-HBc) antibody, and hepatitis B surface antibody (anti-HBs) were detected by enzyme-linked immunosorbent assay (ELISA). HCV RNA was detected using a quantitative real-time RT-PCR assay, and HBV was detected using a nested PCR.

Results: All patients were negative for HBsAg. A total of 49.1% and 25.2% of the patients in the HD and HCV groups, respectively, were anti-HBc-positive. In addition, more anti-HBs-positive patients were detected in the HD group compared to the HCV group (52.1% and 11.4%, respectively). Three cases were positive for HBV DNA in the HD group, while eighteen positive cases were detected in the HCV group. Both study groups showed significant differences in serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) level as well as anti-HBc, anti-HBs and HBV-DNA positivity.

Conclusions: OBI was more prevalent among chronic HCV patients than HD patients in the Suez Canal region, Egypt, with rates of 8.5% and 1.8%, respectively. However, more precise assessment of this infection requires regular patient follow-up using HBV DNA detection methods.

Keywords: Occult hepatitis B. HCV. Hemodialysis. Egypt. Anti-HBc.

INTRODUCTION

The continual existence of hepatitis B surface antigen (HBsAg) in blood has been established as a marker of overt hepatitis B virus (HBV) infection. Occult hepatitis B infection (OBI) is defined as the continuous existence of the HBV genome in liver tissues and/or serum in the absence of serum HBsAg. This phenomenon could result from the persistent presence of covalently closed circular deoxyribonucleic acid (cccDNA), which accumulates in the nuclei of hepatocytes and acts as a template for HBV transcription\(^1\). The risk of OBI transmission is decreased by the transfusion of blood components containing hepatitis B surface antibodies (anti-HBs)\(^2\)(\(^3\)).

Although Africa is considered to be a highly endemic region for HBV, it is considered to have an intermediate prevalence (2-6%) in Egypt. This discrepancy is mostly due to the introduction of HBV vaccination into compulsory immunization programs in Egypt in 1991 and the availability of this vaccine to populations at high risk for HBV infection. However, a considerable incidence of OBI has been reported in Egypt\(^4\).

OBI may occur in serologically positive patients with markers of prior HBV infection (anti-HBs and/or hepatitis B core antibody (anti-HBc) as well as serologically negative patients without markers of previous exposure. Generally, approximately 20% of OBI patients are serologically negative, whereas 80% are serologically positive for one or more markers of prior infection\(^5\). OBI is primarily spread among hemodialysis (HD) patients, individuals under going frequent blood transfusions, and intravenous drug abusers\(^6\)(\(^7\)(\(^8\)(\(^9\)(\(^10\)(\(^11\)(\(^12\)). Patients on chronic HD represent an important risk group for acquiring OBI, which may be attributed to the persistent exposure to blood-borne infections through the dialysis process and the need for frequent blood transfusions\(^13\).

Hepatitis C virus (HCV) infection suppresses not only HBV replication but also HBV surface protein expression...
HCV antibodies among HD patients was reported (21). There is an association between OBI infection and the existence of anti-HCV antibodies among HD patients and patients on regular HD due to end-stage renal disease (ESRD). The prevalence of OBI in HD patients varies markedly from one locality to another worldwide, with a range of 0% to 36% (13). In Egypt, the prevalence was reported at 4% in Minia and Assuit (Upper Egypt) and reached 26.8% among patients in Alexandria (North Egypt) (23) (24). Even with these recent data addressing the prevalence of OBI in patients on HD in Egypt, more comprehensive data may be needed to evaluate OBI among chronic HCV carriers (18) (19) (20) and to determine whether the prevalence differs following antiviral therapy, progression of hepatocellular carcinoma (HCC) and acute exacerbation of chronic hepatitis B (22).

The prevalence of OBI in HD patients varies markedly and has failed to demonstrate any association between OBI and HCV (16) (17), whereas some studies have reported a high prevalence of OBI in chronically infected HCV patients (18) (19) (20). Similarly, a close association between OBI infection and the existence of anti-HCV antibodies among HD patients was reported (21). There is a broad range of clinical implications for OBI because it carries the potential for HCV transmission through blood transfusions, organ transplantations and HD. Moreover, OBI can result in fulminant hepatitis, poor responses to interferon/ribavirin (IFN/RBV) antiviral therapy, progression of hepatocellular carcinoma (HCC) and acute exacerbation of chronic hepatitis B (22).

Our study investigated the prevalence of occult hepatitis B in two groups of patients who were potentially immunocompromised and at higher risk for acquiring OBI (chronic HCV-infected patients and patients on regular HD due to end-stage renal disease (ESRD) using serological tests and nested polymerase chain reaction (PCR). A highly specific and sensitive nested PCR method was used to test for OBI.

**METHODS**

**Patients**

This study included two groups. The first group included 165 patients with ESRD who had been undergoing regular HD for more than 6 months and were HCV-negative (negative for anti-HCV antibody). The second group included 210 patients with chronic HCV infection. The patients were selected from two HD centers in the Suez Canal region, Northeastern Egypt. HCV diagnosis was based on the detection of serum anti-HCV antibodies and confirmed by the detection of serum ribonucleic acid (HCV/RNA). Patients with acute or chronic HCV infection (as determined by positive HBsAg), other causes of liver diseases (i.e., autoimmune hepatitis and continued alcohol abuse), or currently being treated with IFN and/or ribavirin were excluded. The medical history was collected for the entire study population, including HBV vaccination history and clinical and biochemical assessments. The study was conducted in accordance with the ethical standards of the Declaration of Helsinki. The study was approved by the research ethical committee of the Faculty of Medicine, Suez Canal University, and informed written consent was obtained from all patients included in this study.

**Samples**

Blood samples were collected from all patients. The serum was separated and stored at -80°C prior to analysis. Both alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were measured in all samples using a colorimetric method. All patients were screened for human immunodeficiency virus (HIV) 1 and 2 with commercial kits (AxSYM HIV 1/2gO, Abbott Diagnostics, Wiesbaden, Germany).

**Hepatitis C virus testing**

A third-generation enzyme-linked immunosorbent assay (ELISA; HCV 3.0 ELISA Ortho, Raritan, NJ, USA) was used to test for anti-HCV antibodies according to the manufacturer’s instructions. HCV RNA was detected using a quantitative real time reverse transcription polymerase chain reaction (RT-PCR) assay with a detection limit of 33.6IU/ml (Artus HCV RG RT-PCR, Qiagen, Hilden, Germany).

**Hepatitis B virus testing**

Serology: HBsAg was detected by ELISA (ETI-MAK-2 PLUS, Diasorin, Italy) and further confirmed with the Microparticle Enzyme Immunoassay (MEIA) kit (AxSYM HBsAg V2, Abbott). Other serological markers (i.e., anti-HBs and total anti-HBc) were screened using standard commercially available enzyme immunoassays according to the manufacturer’s instructions (ETI-AB-AUK PLUS kits and ETI-AB-COREK PLUS; DiaSorin, Saluggia, Italy, respectively).

**Nested PCR amplification of HBV region S**

DNA was extracted from 200µl of serum using the QIAamp Min Elute Virus Spin Kit (QIAGEN, Inc., Hilden, Germany) and re-suspended in 100µl of a storage buffer provided by the kit manufacturer.

According to the method of Sugauchi et al. (25), the S region of the HBV genome (681bp) was amplified using a nested PCR with four primers targeting the S region; the assay has a lower limit of detection of 100 copies/ml. The first PCR used the primer pair (nucleotide positions 18-958) (IS1) 5'-AACGCTTCTGCTAGATCCCAGAGT-3' and (HS4R) 5'-CATACTTTTCCAATCAATAGG-3'. The PCR was performed in a 25µl volume containing 5µl of DNA template, 12.5µl of One Taq® Quick-Load®, 2× Master Mix [20mM Tris-HCl, pH 8.9 at 25°C (New England Biolabs), 25µM of each primer, and 45 cycles of 96°C for 45 s, 55°C for 45 s and 72°C for 1 min.

The second PCR (nucleotide positions 414-989) used the primers (SB1) 5'- TGCCTGATGCTCATCTC-3' and (HS4R) 5'- CATACCTTTCCAATCAATAGG-3'. The PCR was performed in a 25µl reaction mixture volume containing 2µl of DNA template (from the first PCR product), 25µM of each primer, 12.5µl of One Taq® Quick-Load® 2× Master Mix (New England Bio labs), and PCR-grade water. The cycling conditions consisted of 7 min at 96°C and 45 cycles of 96°C for 45 s and 55°C for 45 s and 72°C for 1 min.

The second PCR (nucleotide positions 414-989) used the primers (SB1) 5'- TGCCTGATGCTCATCTC-3' and (HS4R) 5'- CATACCTTTCCAATCAATAGG-3'. The PCR was performed in a 25µl reaction mixture volume containing 2µl of DNA template (from the first PCR product), 25µM of each primer, 12.5µl of One Taq® Quick-Load® 2× Master Mix (New England Bio labs), and PCR-grade water. The cycling conditions were the same as those described for the first PCR.

Positive and negative controls were run along with the clinical samples. DNA from anHBsAg-positive patient with a high viral load (under treatment) was used as a positive DNA control, while DNA from a healthy blood donor was used as a negative reference. The PCRs were performed using an Eppendorf Master Cycler nexus PCR thermal cycler. Proper precautions were taken to avoid cross contamination.
Gel electrophoresis: The resulting PCR amplicons were electrophoresed in 1.5% agarose gels in 1× Tris-borate-ethylene diaminetetraacetic acid (EDTA) buffer and stained with ethidium bromide. The images were captured using the Syngene G: Box documentation system (Syngene, UK).

Statistical analysis

Statistical Package for Social Science (SPSS) version 16 was used for data processing and analysis. The results are expressed in ranges, percentages and mean (SD). For comparisons between the study groups, the Student’s t-test was employed for quantitative variables, and the Chi-square test was used for categorical (qualitative) variables. P values less than 5% were considered to be statistically significant.

RESULTS

General characteristics of the studied populations

The current study included two groups. The first group included 165 patients with ESRD who received regular HD. These patients ranged from 12 to 76 years in age with a mean (SD) of 44.35 years (13.40 years); additionally, 53.9% of the patients were male and 46.1% were female. The second group included 210 patients with chronic HCV infection. These patients ranged from 17 to 56 years with a mean (SD) of 40.61 years (9.09 years); additionally, 67.2% of the patients were male and 32.8% were female. There were significant differences in age and gender between the study groups.

The ALT values ranged from 5 to 90 with a mean (SD) of 20.17 (11.89) in the HD group and from 5 to 246 with a mean (SD) of 53.43 (33.67) in the chronic HCV group. The AST means (SD) were 21.93 (10.98) and 51.07 (30.01) in the HD and HCV patients, respectively. Both the ALT and AST levels were significantly different between the HD and chronic HCV groups (Table 1).

Hepatitis B serological status

All the patients were negative for HBsAg. Positive cases for total anti-HBc were significantly higher in the HD group compared to the HCV group (49.1% and 25.2%, respectively). Moreover, the number of positive cases for anti-HBs was higher in the HD group compared to the HCV group (52.1% and 11.4%, respectively). The results revealed significant differences in anti-HBc and anti-HBs seropositivity in both groups, with p values < 0.01 for both markers (Table 1).

In both study populations, males were more common than females among the anti-HBc-positive cases. Table 2 shows that serum ALT and AST levels were significantly higher in the HCV group, where as the HD group showed more positive anti-HBs cases than the HCV group (63% and 18.9%, respectively).

Nested PCR results

All the DNA samples extracted from patients in the current study were subjected to nested PCR targeting the S region of the hepatitis B genome. The resulting amplified product was approximately 681bp in size, as shown in Figure 1. Highly significant variation was observed in positive cases between the two groups (p value <0.01); only 3 cases were positive for HBV DNA in the HD group, while 18 cases were positive in the HCV group (Table 1). The OBI prevalence was 1.8% and 8.5% in the HD and HCV groups, respectively. The characterization of HBV DNA-positive cases in both study groups is shown in Table 3.

All three cases in the HD group were negative for anti-HBs, and only two cases were positive for anti-HBc; in contrast, more than half (55.5%) of the cases in the HCV group were positive for anti-HBs, and all cases were positive for anti-HBc. There were no significant differences in age, gender, ALT and AST levels, and anti-HBc and anti-HBs positivity between the HBV DNA-positive cases from both study groups.

| TABLE 1 - Demographic and laboratory data of the study group populations. |
|--------------------------------------------------|------------------|-----------------|------|
|                                                   | Group 1 (n=165 patients) | Group 2 (n=210 patients) | P value |
| Age (years), mean (SD)/(range)                    | 44.4 (13.4)/(12-76)   | 40.6 (9.1)/(17-56)     | < 0.01 |
| Sex (n,%), male                                   | 89 (53.9%)            | 141 (67.2%)           | 0.01  |
|                                                  | 76 (46.1%)            | 69 (32.8%)            |       |
| ALT in IU/L, mean (SD)/(range)                    | 20.2 (11.9)/(5-90)    | 53.4 (33.7)/(5-246)   | < 0.01 |
| AST in IU/L, mean (SD)/(range)                    | 21.9 (11.0)/(5-57)    | 51.1 (30.0)/(5-231)   | < 0.01 |
| Total anti-HBc positivity (n, %)                  | 81 (49.1%)            | 53 (25.2%)            | < 0.01 |
| Anti-HBs positivity (n,%);                        | 86 (52.1%)            | 24 (11.4%)            | < 0.01 |
| HBV DNA positivity (n, %)                         | 3 (1.8%)              | 18 (8.5%)             | < 0.01 |

HCV: hepatitis C virus; SD: standard deviation; n: number; ALT: alanine aminotransferase; AST: aspartate aminotransferase; IU/L: international unit per liter; anti-HBc: hepatitis B core antibody; anti-HBs: hepatitis B surface antibody; HBV DNA: hepatitis B virus deoxyribonucleic acid.
TABLE 2 - Demographic and laboratory data for the total anti-HBc-positive cases.

<table>
<thead>
<tr>
<th></th>
<th>Total anti-HBc-positive cases (n=81)</th>
<th>Total anti-HBc-positive cases (n=53)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years), mean (SD)</td>
<td>45.9 (12.1)</td>
<td>47.5 (8.0)</td>
<td>0.24</td>
</tr>
<tr>
<td>Sex (n, %)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>male</td>
<td>45 (55.6%)</td>
<td>36 (68.0%)</td>
<td>0.21</td>
</tr>
<tr>
<td>female</td>
<td>36 (44.4%)</td>
<td>17 (32.0%)</td>
<td></td>
</tr>
<tr>
<td>ALT in IU/L, mean (SD)</td>
<td>20.3 (9.7)</td>
<td>54.3 (43.4)</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>AST in IU/L, mean (SD)</td>
<td>21.9 (10.8)</td>
<td>50.5 (34.0)</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Anti-HBs positivity</td>
<td>51 (63.0%)</td>
<td>10 (18.9%)</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>HBV DNA positivity (n, %)</td>
<td>2 (2.5%)</td>
<td>18 (34.0%)</td>
<td>&lt; 0.01</td>
</tr>
</tbody>
</table>

**anti-HBc**: hepatitis B core antibody; **HD**: regular hemodialysis; **HCV**: hepatitis C virus; **SD**: standard deviation; **n**: number; **ALT**: alanine aminotransferase; **AST**: aspartate aminotransferase; **IU/L**: international unit per liter (IU/L); **anti-HBs**: hepatitis B surface antibody; **HBV DNA**: hepatitis B virus deoxyribonucleic acid.

TABLE 3 - Characterization of HBV DNA-positive cases in the two study groups.

<table>
<thead>
<tr>
<th></th>
<th>HBV DNA-positive cases (n=3)</th>
<th>HBV DNA-positive cases (n=18)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years), mean (SD)/(range)</td>
<td>47.6 (7.37)/(42-56)</td>
<td>44.3 (8.9)/(26-56)</td>
<td>0.49</td>
</tr>
<tr>
<td>Sex (n, %)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>male</td>
<td>1 (33.3%)</td>
<td>13 (22.2%)</td>
<td>0.51</td>
</tr>
<tr>
<td>female</td>
<td>2 (66.7%)</td>
<td>5 (77.8%)</td>
<td></td>
</tr>
<tr>
<td>ALT in IU/L, mean (SD)/range</td>
<td>19.3 (18.0)/(7-40)</td>
<td>39.0 (21.2)/(10-81)</td>
<td>0.11</td>
</tr>
<tr>
<td>AST in IU/L, mean (SD)/range</td>
<td>17 (6.0)/(10-21)</td>
<td>21.5 (11.3)/(8-49)</td>
<td>0.47</td>
</tr>
<tr>
<td>Anti-HBs positivity (n, %)</td>
<td>0 (0.0%)</td>
<td>10 (55.5%)</td>
<td>0.22</td>
</tr>
<tr>
<td>Total anti-HBc positivity (n, %)</td>
<td>2 (66.7%)</td>
<td>18 (100.0%)</td>
<td>0.14</td>
</tr>
</tbody>
</table>

**HBV DNA**: hepatitis B virus deoxyribonucleic acid; **HD**: regular hemodialysis; **HCV**: hepatitis C virus; **SD**: standard deviation; **n**: number; **ALT**: alanine aminotransferase; **AST**: aspartate aminotransferase; **IU/L**: international unit per liter (IU/L); **anti-HBs**: hepatitis B surface antibody; **anti-HBc**: hepatitis B core antibody.

FIGURE 1 - Agarose gel electrophoresis of the nested PCR used to detect HBV DNA. Lanes: M, molecular weight marker (100bp DNA ladder); 1 and 8, negative and positive controls, respectively; 2, 5, 7 and 9-15, negative HBV DNA clinical samples; 3, 4 and 6, clinical samples positive for HBV-DNA. **PCR**: polymerase chain reaction; **HBV DNA**: hepatitis B virus deoxyribonucleic acid.
HBV infection is a major health concern due to its widespread prevalence, with 350,000 million infected individuals worldwide\(^\text{28}\). HBV infection leads to major complications, including fulminating hepatitis, chronic liver disease and HCC, which carry the significant burden of high health costs. OBI is a variant of HBV infection that occurs when patients show no HBsAg in the serum but persistent HBV DNA in the liver tissue with or without detection of the HBV genome in the serum. Patients with ESRD on HD and chronic HCV-infected patients are highly susceptible to acquiring HBV infection. Our study was designed to measure the magnitude of this infection in these two groups of patients in a large district in our country. The current study showed marked variations in patients' ages, because we included variable age groups (from children to elderly patients with a range of 12 to 76 years in the HD group and from 17 to 56 years in the HCV group). This approach enabled a more accurate assessment of OBI in our patients, as these samples were representative of the age groups in the general population.

Our results showed significantly different ALT and AST levels between the study groups. The levels were more elevated in the HCV group, which may be attributed to hepatic dysfunction due to chronic hepatitis.

Anti-HBc constitutes the first antibody response to HBV infection, and its detection in the serum may denote acute or chronic infection. Patients remain anti-HBc-positive after recovery, and this antibody therefore serves as the only serologic marker for HBV during the window period of infection. As a result, this marker is considered one of the most valuable serological markers for the diagnosis of OBI\(^\text{27} (28) (29)\) and has been proposed as a surrogate screening marker for OBI diagnosis when viral DNA detection methods cannot be applied\(^\text{26}\). In contrast, the anti-HBs antibody appears late in infection after the disappearance of HBsAg and has a protective neutralizing effect. Anti-HBs is the only HBV serologic marker that can be detected after vaccination against this virus.

In the current study, the rates of detection of both anti-HBs and anti-HBc differed significantly between the study populations, and both markers were detected at higher levels in the HD group compared to the HCV group. This finding can be explained either by a true difference in the infection rate between groups or by immune unresponsiveness or a weak immune response to HBV antigens as a result of the suppression of replication and expression of this virus in HCV co-infected patients\(^\text{14} (15)\). Moreover, the positivity for both anti-HBc and anti-HBs could be explained by either recovery from previous acute or chronic HBV infection\(^\text{26}\) or OBI seropositivity in which HBsAg cannot be detected in the serum because it is cleared to an undetectable level\(^\text{30}\). Additionally, the increased number of anti-HBs-positive patients in the HD group may be a result of vaccination against HBV, which is routinely performed in HD patients in our region. As shown in Table 2, a considerable number of the anti-HBc-positive patients were negative for both anti-HBs and HBV DNA; this result may be due to either recovery from previous HBV infection or true occult HBV infection without detectable DNA in the serum. Thus, the rate of DNA detection may increase if liver biopsies are examined.

The detection of HBV DNA is considered the most definitive diagnostic tool for the detection of occult hepatitis infection. In our study, we used nested PCR because this method is simple and accurate, easy to interpret, can detect even very low amounts of viral DNA and has been previously applied on a wide scale for the detection, genotyping and phylogenetic analysis of HBV\(^\text{31} (32) (33) (34)\). Specifically, we targeted the S region of the HBV genome in patient serum samples; this region was selected as a target for amplification by PCR because it was found to be more sensitive for the detection of HBV DNA in serum\(^\text{35}\).

Our nested PCR demonstrated that the prevalence of OBI was 1.8% and 8.5% in the HD and HCV groups, respectively; the difference in these prevalence rates was statistically significant. Marked variation was found by comparing these rates with OBI prevalence rates reported in other related national or international studies\(^\text{25} (24) (35) (36) (37) (38) (39)\). This variation may be attributed to differences in the sample size of the study populations, demographics, immunologic status of the studied patients, endemcity of HBV infection, levels of viral DNA in the blood, sampling conditions and the types of diagnostic tool(s) used for diagnosis\(^\text{40}\). This comparison also revealed that the prevalence of OBI in our target HD patients was relatively low, possibly due to the application of safety precautions during the dialysis process in these units and the implementation of the routine vaccination program. Additionally, the low rate can be attributed to the use of serum samples to detect HBV DNA in the current study. The likelihood of detecting DNA is much lower in serum samples than in liver tissue, although the use of liver biopsies for the detection of OBI is difficult, especially in HD patients. Moreover, the higher prevalence rate of OBI in the chronic HCV group can be explained by the occurrence of coinfection with HBV, as both of these viruses share common routes of infection\(^\text{41}\).

Two of the three HBV DNA-positive patients in the HD group were seropositive for anti-HBc alone, while the third patient was seronegative. In contrast, all HBV DNA-positive cases in the HCV group were positive for anti-HBc, and more than half were positive for anti-HBs. This result is in accordance with the finding that OBI is more prevalent in seropositive patients who are positive for anti-HBc and/or anti-HBs\(^\text{25} (29) (37) (42) (43)\). The occurrence of occult infection despite the presence of neutralizing anti-HBs can result from mutations affecting the S region of the HBV genome\(^\text{39}\). However, the absence of all HBV serological markers (even anti-HBs) in some HBV DNA-positive patients strengthens the proposal that the detection of viral DNA is the gold standard for the diagnosis of OBI. Therefore, the presence of seronegative cases should be given consideration during the management of HD and HCV patients.

From the current study, we can conclude that OBI occurs in HD and chronic hepatitis C patients in our area at rates of 1.8% and 8.5%, respectively. Despite these relatively low rates, this infection is considered to be a real health problem in these debilitated patients and necessitates regular follow-up for the early detection of OBI using DNA detection methods.
CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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