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Original Article

Occult hepatitis B in mexican patients with HIV, an analysis using nested polymerase chain reaction

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Abstract

Hepatitis B virus infection (HBV) with undetectable levels of HBsAg, has been named occult HBV infection and observed in immunosuppressed patients. The aim of this study was to determine the frequency of occult HBV infection in patients with HIV from the West of México, using a combination of serological markers and nPCR. Thirty eight HIV/AIDS patients, 32 men (84.2%) and 6 (5.8%) women, without liver damage related symptoms were studied. HBV coinfection was observed in 10 (26.3%) patients; while only 3 (7.9%) of them were positive to HBsAg. Thus, 7 (18.4%) occult HBV infected patients could be assessed in this population. One (10%) patient with occult HBV infection was positive to anti-HBs, in spite of the reinfection protection attributed to this serological marker. Anti-HBc was detected only in 2 (20%) patients with occult HBV infection. No significant association could be established between occult HBV infection and CD+4 cell count, biochemical, clinical parameters, AIDS stage, or any other risk factor. This study suggest that determination of HBV DNA utilizing highly sensitive techniques, as nPCR, should be performed to detect occult HBV infection, even in the absence of anti-HBc in HIV/AIDS patients, in order to have a reliable diagnosis, prevent HBV dissemination and acute exacerbation of chronic hepatitis B or even fulminant hepatitis. To our knowledge this is the first study of occult HBV infection in Mexican patients with HIV. However, further studies are necessary in order to determine HBV genotypes and its relationship with evolution and clinical manifestation of the disease.

Key words: Immunosilent HBV infection, co-infection HBV-HIV, lack of HBsAg detection, serologic markers, HBV DNA.

Introduction

Human hepatitis B virus (HBV) is a compact, partially double-stranded, enveloped, deoxyribonucleic acid (DNA) virus, member of the *Hepadnaviridae* family, of approximately 3,200 nt.[1] HBV infection is worldwide spread and is considered a major public health problem, with an estimation of 350 million people chronically infected.[2] The HBV replicates in the liver leading to hepatic dysfunction. Persistent HBV infection is associated with the development of chronic liver disease, including cirrhosis and hepatocellular carcinoma.[3,4]

Human immunodeficiency virus (HIV) and HBV share similar transmission routes. Up to 80% of HIV-infected patients have been exposed to HBV, and up to 10% exhibits chronic hepatitis.[5,6]

A carrier HBV chronic infection in HIV seropositive patients is usually diagnosed by circulating HBV surface antigen (HBsAg) and antibodies to HBV core antigen (anti-HBc).[7] Circulating HBsAg can be identified 30 to 60 days after risk factor exposure, maintaining a considerable level during up to six months in the case of acute hepatitis, and persistently in persons with chronic HBV.[3,7,8]

Serum HBsAg clearance and the appearance of antibodies against it (anti-HBs), along with serum aminotransferases normalization, have been generally asso-

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Abbreviations:
HBV, hepatitis B virus; DNA, deoxyribonucleic acid; HIV, human immunodeficiency virus; HBsAg, HBV surface antigen; anti-HBc, antibodies against HBV core antigen; anti-HBs, antibodies against HBsAg; AIDS, acquired immunodeficiency syndrome; HBeAg, HBV e antigen; anti-HBe, antibodies against HBeAg; PCR, polymerase chain reaction; nPCR, nested PCR; bp, base pairs; i.v., intravenous.

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ciated with spontaneous remission of acute or chronic HBV disease; meanwhile, anti-HBs confers protection against HBV reinfection.[3] Anti-HBc usually appears in the acute phase and keeps steady for a long time after virus elimination. The presence of anti-HBc alone, is often considered as evidence of past HBV infection.[3]

Negative HBsAg, together with or without other serological markers of previous infection in individuals infected with HBV, has been reported, showing that serological markers fail to diagnose HBV in these cases and that highly sensitive nucleic acid technology becomes essential. Moreover, serum HBV-DNA and liver isolation of the virus has been attained in these cases.[9,10] This mode of the disease has been referred as «occult HBV infection», since HBV-DNA is present even in the absence of HBsAg.[11,12] Low level replication of the HBV, or mutations at the determinant «a» of the S gene encoding aminoacid residues 99 to 169 of HBsAg, might be the underlying cause of this phenomenon.[13,14] Clinically, immunosuppression may explain the spontaneous HBV reactivation in individuals presenting occult HBV infection.[6,15-17] Both, prevalence and clinical meaning of occult HBV infection in HIV coinfected patients are poorly understood.

The aim of this study was to determine the frequency of occult HBV infection in a group of HIV serum-positive patients or patients presenting acquired immunodeficiency syndrome (AIDS) of the west of México, utilizing a combination of serological markers and highly sensitive HBV-DNA detection.

Experimental procedures

Subjects

Voluntary HIV/AIDS patients (32 men, 6 women; age 20-70 y), were recruited in a transverse study from January to December 2002, at the Civil Hospital of Guadalajara. Clinical history, including social-demographic characteristics, intravenous drugs abuse, tattooing, surgeries, blood transfusions, and sexual lifestyle, was carried out.

Ethical considerations

Informed consent was obtained from each patient, and the study protocol conformed to the ethical guidelines of the hospital, according to the 2000 Declaration of Edimburg.[18]

Clinical specimens

Peripheral blood samples (7 mL) were collected from all 38 patients, in a plain vacutainer tube, after a 12 hour fasting period. Serum was obtained, poured into two different tubes and stored at –70°C after centrifugation, until the extraction of the HBV-DNA or the performance of biochemical and serological analysis. Also, a fresh citrated sample was taken to carry out prothrombin time determination.

Biochemical determinations and CD4+ T-lymphocyte count.

Alanine-aminotransferase, aspartate-aminotransferase, serum albumin, total bilirubin, urea, creatinine, prothrombin time, and CD4+ T-lymphocyte count, were determined in all samples according to standard procedures.

Serological tests

HBsAg, anti-HBs, IgG anti-HBc, IgM anti-HBc, HBV «e» antigen (HBeAg), and anti-HBe, were determined using Axsym assays (Abbot laboratories, North Chicago, USA). Serological protocol to confirm HIV infection was followed in every patient.

HBV-DNA determination by polymerase chain reaction (PCR).

HBV-DNA was extracted from 100 µl serum samples, according to the phenol-chloroform method after treatment with proteinase K, as reported elsewhere.[19] The resulting pellet was dissolved in nuclease-free water and stored at -70°C, until amplification of the viral genome.

Primers DS7 and DS8: 5'TCCTGCTGGTGGCTCCAGTT3' and 5'CAAACGGGCAACATACCTTG3', respectively, were utilized for PCR. Primers MS1 and MS2: 5'GGACCCCTGCTCGTGTTACA3' and 5'CAGGATGAA GAGGAA(T/G)ATGA3', respectively, were utilized for nested PCR (nPCR). Both pairs of primers were targeted to amplify specific sites of the HBV-S gene, rendering 415 base pairs (bp) and 234 bp fragments for PCR and nPCR, correspondingly, following the procedure previously reported.[20]

Briefly, 5 µL of HBV DNA were added to 45 µL of reaction mixture, containing 1x PCR buffer, 0.5 µM of each primer, 75 µM dNTP's (dATP, dGTP, dCTP and dUTP), 1.5 mM $MgCl_2$, 5 U uracil DNA-glycosylase and 5 U Taq polymerase (Invitrogen). Both PCR and nPCR were performed in a DNA thermal cycler (model 480; Perkin-Elmer, Norwalk, Conn, USA), using mineral oil to avoid evaporation, and the following analysis conditions: an initial denaturation step at 94ºC during 5 min, 40 cycles of denaturation at 94°C for 60 sec, annealing at 60ºC during 60 sec, and extension at 72°C for 60 sec, and a final elongation step of 72ºC for 10 min. For nPCR, 25 cycles were carried out. Uracil DNA-glycosylase and negative controls were included to discard cross-contamination. Specificity of the PCR assay was ensured by the inclusion of a positive control which consisted of the cloned complete HBV-DNA genome. Positive samples were considered only after at least two positive independent assays were observed.

The analytical sensitivity of our house nPCR to detect HBV-DNA was 10 copies per ml of serum; calculation was based on dilutions of cloned HBV-DNA genome in serum samples negative to HBV-DNA.

Amplification products were visualized on a 2% agarose gel electrophoresis, stained with ethidium bromide.

Statistical analysis

Chi-square was performed to establish the association between HBV DNA and clinical symptoms; also, between HBV-DNA and risk factors. Probability values under 0.05 were considered statistically significant.

Results

A total of 38 patients were studied, mean age 36.5 years old (ranged 22–70 years). Thirty two patients (84.2%) were men, and 6 (5.8%) women.

A 415 bp and a 234 bp fragments were obtained in PCR and nPCR, correspondingly. Detection of HBV-DNA by PCR was positive in 10 cases (26.31%). Clinical symptoms related with liver damage in patients positive and negative to HBV-DNA, were evaluated, but no statistical difference was observed between both groups. Five (50%) patients positive to HBV-DNA exhibited symptoms related with HIV/AIDS immunosuppression; meanwhile, the remaining 5 (50%) HBV-DNA positive patients kept asymptomatic. Acute liver disease related with the antiretroviral treatment developed in one HBV-DNA negative patient and remission after treatment modification was attained *(Table I)*.

Statistically significant difference ($p < 0.05$) was observed when HBV-DNA presence in HBsAg seropositive *vs* seronegative patients, were compared. Three patients (7.9%) exhibiting both HBV-DNA and HBsAg and 7 patients (18.4%) negative to HBsAg, but positive to HBV-DNA, were observed. Thus, according to HBsAg negativity criteria, and positivity to HBV-DNA, 18.4% occult HBV infection was detected in the population studied *(Table II)*.

Table III shows other serological markers related to HBV infection, such as anti-HBs, IgG anti-HBc, IgM anti-HBc, HBeAg, and anti-HBe in patients positive to HBV-DNA. Positivity for at least one of these serological tests was observed in 5 (50%) out of 10 patients. Interestingly, anti-HBs together with IgG anti-HBc and absence of HBsAg, was positive in 1 (10%) patient.

Mean CD4+ cells count was 169 cells/mm^3 (0–567 cells/mm^3) and 211 cells/mm^3 (2-942 cells/mm^3) in HBV-DNA positive and negative patients, respectively. Statistically significant difference could not be appreciated between both groups. Also, no statistical difference was observed in aminotransferases, serum albumin, total bilirubin, urea, creatinine, or prothrombin time between patients coinfected and not coinfected with HBV (data not shown).

Sex, schooling years, working condition, number of sexual partners, sexual preferences, AIDS stage, use of intravenous (i.v.) drugs, blood transfusions, tattooing and time elapsed since HIV diagnosis, were variables not significantly associated with HBV coinfection *(Table IV)*.

Table I. Clinical symptoms in HIV/AIDS patients positive or negative to HBV-DNA.

	Number of patients (%)		
Variable	HBV PCR (+)	HBV PCR (-)	Total
Number of patients	10 (26.3)	28 (73.7)	38 (100)
Fever	1 (10.0)	1 (3.6)	2 (5.3)
Tiredness	1 (10.0)	1 (3.6)	2 (5.3)
Liver damage	0	1 (3.6)	1 (2.6)
Diarrhea	2 (20.0)	3 (10.7)	5 (13.2)
Oral candidiasis	0	1 (3.6)	1 (2.6)
Neurocryptococcosis	1 (10.0)	2 (7.0)	3 (7.9)
Asymptomatic	5 (50.0)	19 (67.9)	24 (63.1)

Table II. HBV DNA detection in HBsAg seropositive and seronegative patients.

	Number of patients (%)		
HBsAg	HBV PCR (+)	HBV PCR (-)	*p*
Positive	3 (7.9)	0	< 0.05
Negative	7 (18.4)	28 (73.7)	< 0.05

Table III. Patients infected with HIV showing «occult HBV infection», according to serological and molecular markers.

Patient	HBsAg	Anti-HBs	IgG Anti-HBc	IgM Anti-HBc	HBeAg	Anti-HBe	HBV-DNA	Occult HBV infection
1	-	-	-	-	-	-	+	Yes
2	-	-	-	-	-	-	+	Yes
3	+	-	-	-	+	-	+	No
4	-	-	-	-	-	-	+	Yes
5	-	-	-	-	-	-	+	Yes
6	-	-	-	-	-	-	+	Yes
7	-	-	+	-	-	-	+	Yes
8	-	+	+	-	-	-	+	Yes
9	+	-	+	+	+	-	+	No
10	+	-	-	-	-	-	+	No

Table IV. Risk factors associated to coinfection with HBV in HIV-seropositive patients.

Variable	Number of subjects (%)		Total	*P*	•²
	HBV PCR (+)	HBV PCR (-)			
Number of patients	10 (26.3)	28 (73.7)	38 (100)		
Sex:				0.5	0.18
Men	8 (80.0)	24 (85.7)	32 (84.2)		
Women	2 (20.0)	4 (14.3)	6 (15.8)		
Schooling years:				0.6	1.01
6 - 9	6 (60.0)	15 (53.6)	21 (55.3)		
10 – 12	3 (30.0)	6 (21.4)	9 (23.7)		
• 3	1 (10.0)	7 (25.0)	8 (21.0)		
Civil status:				0.7	1.62
Single	9 (90.0)	22 (78.7)	31 (81.6)		
Married	1 (10.0)	2 (7.1)	3 (7.8)		
Widower	0	2 (7.1)	2 (5.3)		
Divorced	0	2 (7.1)	2 (5.3)		
Working condition:				0.9	1.16
Unemployed	1 (10.0)	2 (7.1)	3 (7.9)		
Prostitution	1 (10.0)	1 (3.6)	2 (5.3)		
Technician	0	1 (3.6)	1 (2.6)		
Professional	1 (10.0)	2 (7.1)	3 (7.9)		
Laborer	7 (70.0)	22 (78.6)	29 (76.3)		
No. of sexual partners:				0.9	0.38
One	1 (10.0)	4 (14.3)	5 (13.2)		
2-9	4 (40.0)	13 (46.4)	17 (44.7)		
10-19	1 (10.0)	2 (7.1)	3 (7.9)		
• 20	4 (40.0)	9 (32.2)	13 (34.2)		
Sexual preference:				0.1	5.21
Heterosexual	3 (30.0)	9 (32.1)	12 (31.6)		
Homosexual	7 (70.0)	10 (35.7)	17 (44.7)		
Bi-sexual	0	9 (32.1)	9 (23.7)		
AIDS stage:				0.6	1.71
A1	1 (10.0)	3 (10.7)	4 (10.5)		
A2	3 (30.0)	8 (28.6)	11 (28.9)		
A3	1 (10.0)	8 (28.6)	9 (23.7)		
C3	5 (50.0)	9 (32.1)	14 (36.8)		
User of i.v. drugs:				0.5	0.51
Yes	1 (10.0)	1 (3.6)	2 (5.3)		
No	9 (90.0)	27 (96.4)	36 (94.7)		
Blood transfusions:				0.3	1.01
Yes	1 (10.0)	7 (18.9)	8 (21.0)		
No	9 (90.0)	21 (81.1)	30 (79.0)		
Tattooing:				0.8	0.08
Yes	1 (10.0)	2 (7.1)	3 (7.9)		
No	9 (90.0)	26 (92.9)	35 (92.1)		
Years since HIV diagnosis:				0.9	0.13
• 5	8 (80.0)	21 (75.0)	29 (76.3)		
6-10	1 (10.0)	4 (14.3)	5 (13.1)		
• 11	1 (10.0)	3 (10.7)	4 (10.6)		

Discussion

To our knowledge this is the first study on occult HBV infection in HIV/AIDS Mexican patients. HBV infection with not detectable levels of HBsAg, is still a debating matter. However, prevalence and clinical relevance of occult HBV infection is increasing.[21] Negative HBsAg with positive HBV-DNA, has been reported mainly in some clinical situations, such as: chronic liver disease, alcoholism, hepatocellular carcinoma,[22-24] secondary chemotherapy or immunosupression-induced HBV reactivation,[25,26] blood transfusion[27] and stem cells donation.[28] Occult HBV infection is not restricted to highly endemic zones, but it has also been reported in Occidental countries.[29]

Currently, 90-95% of acute HBV cases are spontaneously resolved in otherwise healthy individuals, in association with loss of HBsAg and seroconversion to anti-HBs that conveys protection against reinfection.[3] Nevertheless, clearance rate of HBsAg is reduced in HIV infected patients, probably due to immunosuppression.[30,31]

Studies in different populations have shown that up to 80% of HIV-infected patients in the world have been ex-

posed to HBV, and up to 10% have chronic hepatitis B.[5,6,32] Coinfection with HIV and HBV is common because of shared modes of transmission.[33] Moreover, HIV and HBV can potentially infect the same type of cells and interact to directly influence each other replication rate, favoring transmission among high risk patients.[34] This clinical deleterious condition, could lead to immunosuppression worsening, where aminotransferases could keep normal or slightly increased, in spite of continuous HBV contact.[35] No biochemical or clinical alterations related with liver damage, in patients positive to HBV-DNA was observed in our study. Five patients positive to HBV-DNA (50%) presented signs of an opportunistic infection, secondary to immunosuppression induced by HIV, with no apparent symptoms related to viral liver disease; meanwhile, other 5 (50%) HBV-DNA positive patients remain totally asymptomatic.

Serological methods to identify HBV infection, have been developed.[7,8] However, there is a group of chronic HBV infected patients detected only by highly sensitive PCR, this is occult HBV infection, presenting HBV-DNA, but negative HBsAg.[11]

Routinely, HBsAg is determined in every immunosuppressed patient attending to the Civil Hospital of Guadalajara to identify HBV infection. Nevertheless, in this study we observed a high percentage (70%) of occult HBV infection in HIV coinfected patients. Based on this serological marker, we could only identify 3 (30%) HBV infected HIV/AIDS patients, and 10 (100%) using nPCR. We detected 7 (18.4%) HBV DNA positive patients, negative to HBsAg in the 38 patients included in our study, which indicates that occult HBV infection is a common finding in this population.

Genotype and/or mutations can provoke important variability among HBV strains, which could explain HBsAg negative results. It has been reported that changes in the S and pre-S HBV genomic regions lead to alterations in HBsAg epitopes, rendering probably undetectable proteins by serological tests.[21,36,37] Nevertheless, we can not assume that mutations constitute the underlying reason for seronegativity, since we did not sequenced occult HBV infecting genomes found in our patients.

Lamivudine treatment withdrawal reactivation of HBV in association with anti-HBc alone, has been reported.[6] We could observe one (14.3%) occult HBV infected patient with positive IgG anti-HBc alone, but not associated with antiviral withdrawal.

Occult HBV infection has been reported with positive anti-HBc and anti-HBs,[16] as we could observe in 1 (14.3%) of our patients presenting the occult disease. Interestingly, one of our occult HBV-DNA positive patients presented anti-HBs, even when this marker has been related to reinfection protection.

Promiscuity has been associated with HIV and HBV coinfection, since HBV is more efficiently transmitted than HIV in men who have sex with men and in i.v. drug users.[32] However, no significant association with these risk factors, including, blood transfusion, tattooing, AIDS stage, and others, could be appreciated in this study.

It has been reported that 90% of HIV positive patients present markers of past or recent HBV infection;[35,38] meanwhile, in this study we observed at least one serological marker only in 5 (13.2%) out of 38 patients. The lack of serological markers could be explained by the low level of HBV-DNA, since our study utilized a highly sensitive PCR assay in order to be able to detect HBV-DNA. This suggests a low viral load which could affect HBV protein expression, rendering a poor humoral immune response and no detectable serological HBV antibodies.

The prevalence and clinical significance of occult HBV in HIV-infected patients has been controversial. In the United States, up to 10% of all HIV-infected patients have HIV-HBV coinfection.[39] A study in a Swiss HIV population of 57 HBsAg negative patients showed that 29.8% had positive HBV-DNA.[40] However, HBV-DNA could not be demonstrated in 85 HIV infected Spaniard patients negative to HBsAg.[26] We found HIV-HBV coinfection in 7 (18.4%) negative HBsAg patients. Our results indicating that a considerable number of HIV-HBV coinfected patients negative to HBsAg and positive to HBV-DNA, should alert clinicians to carefully interpret serological markers in this population.

The first European consensus conference on the treatment of chronic hepatitis B and C in HIV co-infected patients, establishes that if anti-HBc is present at the initial assessment, this may be indicative of occult HBV infection.[41] Other authors report that prevalence of anti-HBc serology in occult HBV infection related to clinical entity and geographic distribution may fluctuate from 9% to 80%.[42,43] In our study, anti-HBc was negative in 71.4% of our patients presenting occult HBV infection; showing that occult HBV infection could be present in HIV infected patients without serological evidence of past or present HBV infection, including negative anti-HBc, as well as reported by others.[43,44] Therefore, we suggest HBV-DNA determination utilizing highly sensitive techniques, as nPCR, should be performed to detect occult HBV infection, even in the absence of anti-HBc in HIV/AIDS patients, in order to have reliable diagnosis and prevent HBV dissemination.

Most cases of occult hepatitis B do not need antiviral therapy.[41] However, lifetime monitoring of the patients with occult HBV infection, in spite of the absence of liver damage clinical evidence, will be important in the opportune detection of active and progressive liver disease and could favor the decision to start anti-HBV therapy based on biochemical evidence of liver damage.[41-43]

Further prospective studies are therefore needed to improve our knowledge and understanding about the clinical meaning of occult HBV infection in HIV coinfected patients and to generate more reliable diagnosis strategies.

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