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Research Article

Utilization of Lesser Liver Tissue in Surgically Resected Specimens for Occult Hepatitis B Infection

Kumar R[1], Singh V[2] and Vaiphei K[1]*

[1]Department of Histopathology, Post Graduate Institute of Medical Education and Research, India

[2]Department of Hepatology, Post Graduate Institute of Medical Education and Research, India

***Corresponding author:** Vaiphei K, Department of Histopathology, Fifth Floor, Research/Anand Block A, Post Graduate Institute of Medical Education and Research, Sector 12, Chandigarh-160012, India

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Abstract

Context: Hepatitis B Virus (HBV) is one important cause for chronic liver disease. Occult HBV is a poorly recognized entity with varied prevalence all over the world.

Aims: To evaluate the histological features of liver in occult hepatitis B viral infection and to correlate with hepatitis B virus surface, core and X antigen expressions.

Settings and Design: To study liver histology in occult HBV infection and analyze patterns of different HBV proteins and HBV gene expressions by Immunohistochemistry (IHC) and nested Polymerase Chain Reaction (PCR) respectively.

Methods and Material: Liver tissue was obtained in fifty patients who were serology negative for HBsAg and were detected to be positive for HBsAg and/or HBcAg in paraffin liver sections by IHC.

Statistical Analysis Used: Data were expressed in percentages, the mean and standard deviation were calculated. Chi square test was applied for categorical data.

Results: Of the fifty cases, IHC showed cytoplasmic positivity for HBsAg in 22(44%) and HBcAg in 39(78%) cases. Nine (18%) cases showed both cytoplasmic and nuclear positivity for HBcAg. All biopsies showed cytoplasmic positivity for HBxAg on IHC. Forty seven biopsies (94%) were positive for HBV DNA by nested PCR. Eighteen biopsies (36%) showed features of chronic hepatitis, 6(12%) extrahepatic portal venous obstruction, 10(20%) metastasis, 8(16%) normal morphology, 2(4%) each with cholangitis, granuloma and Budd Chiari Syndrome, one each with non cirrhotic portal venous obstruction and veno-occlusive disease.

Conclusion: Occult hepatitis B virus infection is common among individuals who undergo routine surgical procedure and liver tissue being the best sample to document.

Keywords: Polymerase chain reaction; Immunohistochemistry; Occult hepatitis B

Introduction

Hepatitis B Virus (HBV) is a major causative factor for liver disease. HBV transmission can occur by various routes including transfusion products, needle contamination, sharing of needle or razors, sexual contact and vertically from mother to child [1]. It is estimated that about one third of population worldwide has been infected with HBV, 6% of whom, are chronic carriers [1]. Over 600000 people die each year from the sequels of HBV infection. Occult HBV (OHB) infection (OBI), a poorly recognized entity, has been defined as the presence of HBV DNA in liver or in serum, in the absence of detectable HBsAg in serum by the current detectable assay [2]. Prevalence of OBI is variable and depends on endemic disease level, different assay technique utilized and population in reference [3]. Hence, we analyzed the patterns of HBV associated proteins by IHC and correlated with HBV DNA by nested PCR using liver tissue DNA extracts in patients who were serology negative for HBsAg.

Subjects and Methods

Materials

Patients included in the study were those who showed positive staining for HBV surface and or core antigens in paraffin sections of liver by IHC. These were the patients who underwent surgery for conditions unrelated to primary liver disease. Liver tissue were removed either as a part of the surgical procedures or wedge biopsy to exclude primary liver disease.

Methodology

Informed consents were obtained from all recruited patients for the study. Detail clinical history and routine investigations were recorded according to a pre-made pro-forma. Patients were followed-up at the Hepatology Clinics for further assessment of the clinical parameters, Liver Function Test (LFT) and hepatotrophic viruses serology. Paraffin blocks were used for the study.

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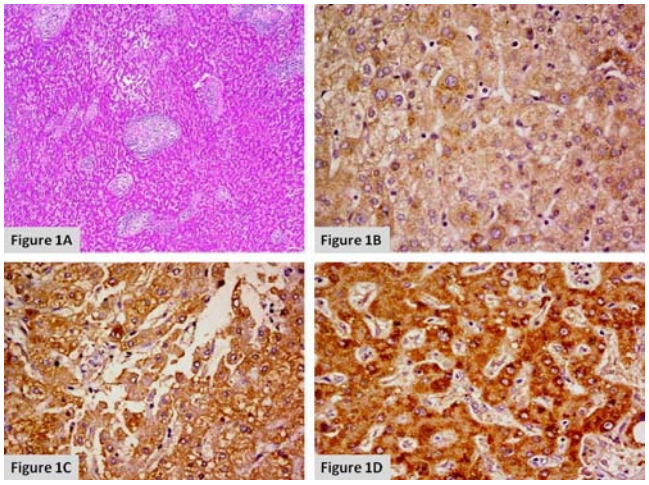

Figure 1: A: Low power photomicrograph of liver showing expanded portal tract, mild to moderate inflammation, interface hepatitis and lobular inflammation (H&E, x100).
B: Medium power photomicrograph showing diffuse cytoplasmic HBsAg positivity with 1+ intensity. (PAP, x200).
C: Medium power photomicrograph showing diffuse cytoplasmic HBsAg positivity with 2+intensity. (PAP, x200).
D: Medium power photomicrograph showing diffuse cytoplasmic HBsAg positivity with 3+intensity. (PAP, x200).

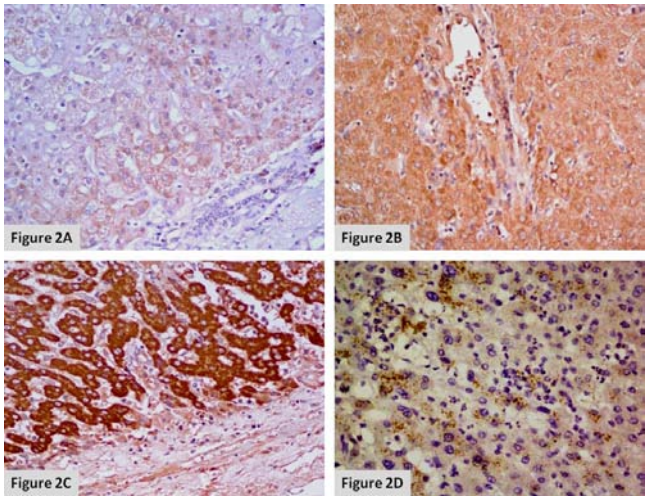

Figure 2: A: Medium power photomicrograph of liver showing cytoplasmic HBcAg positivity with 1+intensity. (PAP, x200).
B: Medium power photomicrograph showing diffuse cytoplasmic HBcAg positivity with 2+intensity. (PAP, x200).
C: Medium power photomicrograph showing diffuse cytoplasmic HBcAg positivity with 3+intensity. (PAP, x200).
D: Medium power photomicrograph showing granular HBsAg cytoplasmic positivity. (PAP, x200).

Histological examination of the liver was done on H&E, reticulin and Masson's trichrom stained sections. Morphological changes in liver were graded and staged according to Ishak et al [4]. IHC was carried out on two micron thick paraffin sections by peroxidase anti-peroxidase method. Primary antibodies for anti-Hepatitis B surface (DAKO, product no M3506, dilution 1:100), core (DAKO, product no B0586, dilution 1:700) and X antigens (SIGMA, product no HPA006812, dilution 1:50) were used. Dako's EnvisionTM+ detection system was used. IHC was carried out along with negative control without adding the primary antibody and positive control of known positive cases.

Interpretations

1000 hepatocytes were counted in the areas where there was maximum concentration of positively stained hepatocytes were visible, also noting the location of the positively stained hepatocytes in context to Rappaport's liver acini. Positively stained hepatocytes were calculated and expressed in percentages. Cytoplasmic or/and nuclear positive, cytoplasmic positivity documented as one of the followings i.e. granular/diffuse. Percentages of cells showing dual positivity were recorded. Intensity of the staining was graded subjectively as+, ++ and +++ (Figures 1 & 2). DNA for the nested PCR was obtained from paraffin sections using commercially available DNA extraction kit (Qiagen, Germany) according to the manufacturer's instruction. Primers used were - outer primers F5'-AAGGTCTTACATAAGAGGAC-3' R 5'-CTAACATTGAGATTCCCGAGATTGAGA-3'. Inner primers F5'-GGCTGTAGGCATAAATTGGTCTG-3' R5'-TTGCCTGAGTGCAGTATGGT-3'.

The product was 815 base pair in first round and 295 base pair in second round. They were used at a final concentration of 0.2 µM each. Taq DNA polymerase enzyme 5 Unit/µl, and dNTPs mix, 10 mM each were purchased from Fermentas Life Sciences. Reaction was carried out in 25 ml of reaction mixture (10X Taq buffer, 2.5 mM $MgCl_2$, 0.2 µM primer 1, 0.2 µM primer 2, 0.2 mM dNTPs, 0.5 Unit Taq polymerase, and 100 ng DNA template). Master Mix was prepared using above mentioned reagents (except template DNA), briefly mixed with the help of cyclo mixer and briefly centrifuged for 3 to 4 seconds in a mini spin before adding it in to a 0.2 ml PCR tubes. Reaction mixture was prepared on ice. Extreme care was taken to avoid contaminations during the process of DNA isolation, PCR and post PCR experiments in a specified area and followed the recommended standard precautions.

Thermo profile and cycling conditions Initial denaturation step : 5 minutes at 94°C Denaturation step : 30 seconds at 94°C Primer annealing step : 30 seconds at 53 °C 35 cycles Polymerization step : 30 seconds at 72°C Final extension step : 10 minutes at 72°C Standardized conditions for both outer and inner primers pairs were same. PCR conditions were standardized using Nexus Gradient Master cycler with DNA extracted from known HBV positive samples.

Results

Mean age was 40.66 (±19.46) years (range= 1-74 years) with equal number of males and females. Mean age for females and males were 41.7±20.37 and 42.9±20.33 years respectively. Clinical diagnoses, histological features of the liver and type of the specimens examined, are shown in Table 1. All the fifty enrolled patients were negative for serology hepatitis C virus.

Liver histology

8(16%) biopsies showed normal morphology, 18(36%) biopsies showed features of chronic hepatitis with moderately heavy chronic inflammatory cell infiltration of portal tracts, interface hepatitis, lobular inflammation, focal steatosis, intra hepatocytic and canalicular cholestasis. Liver biopsies in NCPF cases showed relatively well maintained lobular architecture, the portal tracts were

Table 1: Distribution of histological diagnoses, specimen type and clinical diagnosis.

Histological diagnosis	Specimen type	Clinical diagnosis	Number (%)
Chr hepatitis (04), Metastasis (04), N(02), Granuloma (01)	Ext. cholecystectomy(08), Lobe resection (03)	Ca GB	11 (22%)
Chr hepatitis (03) N (02)	Extended cholecystectomy(01), Wedge biopsy(4)	Chr cholecystitis	05 (10%)
EHPVO (06)	Wedge biopsy	EHPVO	06 (12%)
Chr hepatitis (01), Metastasis (06)	Wedge biopsy	Liver secondary	07 (14%)
Chr hepatitis(02), N (01)	Wedge biopsy(02), Lobe resection(01)	Choledochal cyst	03 (06%)
BCS (02),VOD(01)	Wedge biopsy	BCS	03 (06%)
Chr hepatitis(02), N (01)	Partial hepatectomy	Hemangioma	03 (06%)
Chr hepatitis (02), N (01)	Wedge biopsy	Biliary stricture	03 (06%)
N(01), cholangitis (01)	Partial hepatectomy	Cholangiocarcinoma	02 (04%)
Chr hepatitis, NCPF	Core biopsy, Wedge biopsy	PH	02 (04%)
Chr hepatitis	Wedge biopsy	Perforation peritonitis	01 (02%)
Chr hepatitis	Lobe resection	Liver injury	01 (02%)
Granuloma	Wedge biopsy	Tuberculosis	01 (02%)
Chr hepatitis	Wedge biopsy	Pseudo-aneurysm of aorta	01 (02 %)
cholangitis	Extended cholecystectomy	Cholangitis	01 (02%)
Total			

BCS: Budd Chiari Syndrome; Ca: carcinoma; Chr: Chronic; EHPVO: Extra Hepatic Portal Vein Obstruction; GB: Gall Bladder; NCPF: Non Cirrhotic Portal Fibrosis; N: Normal Morphology; PH: Portal Hypertension; VOD: Veno Occlusive Disease

Table 2: Distribution of patients with respect to HBsAg and HBcAg on immunohistochemistry.

Pattern of positivity	HBsAg Intensity			Total	HBcAg Intensity			Total
	+	++	+++		+	++	+++	
Nuclear and cytoplasmic	-	-	-	__	__	05	04	09 (18%)
Granular cytoplasmic	02	04	01	07 (14%)	02	26	02	30 (60%)
Diffuse cytoplasmic	013	02	_	15 (30%)	04	04	01	19 (18%)
Total	15 (30%)	06 (12%)	01 (02%)	22 (44%)	06 (12%)	35 (70%)	07 (14%)	48 (96%)

Table 3: Distribution of patients based on immunohistochemistry, clinical and histological diagnosis.

	HBxAg +ive (%)	Liver histology	Clinical diagnosis
HBsAg +ive	22 (44%)	Ch hepatitis (07), normal (04), metastasis (05), EHPVO(04), BCS(01), Granuloma(01)	Ca GB (05), EHPVO (04), Metastasis (04), Cholangio Ca (01), Choledochal cysts (03), ch cholecystitis (02), biliary stricture (01), BCS (01), perforation peritonitis (01)
HBsAg -ive	28 (56%)	Ch hepatitis (11), normal (04), metastasis (05), EHPVO(02), BCS (01), Granuloma(01), Ascending cholangitis(02), NCPF(01), VOD(01)	Ca GB (06), EHPVO (02), Metastasis (03), Cholangio Ca (01), ch cholecystitis (03), biliary stricture (02), BCS (02), TB (01), liver injury (01), hemangioma (03), pseudoaneurism (01), cholangitis (01), PH (02)
HBcAg cytoplasmic +ive	39 (78%)	Ch hepatitis (13), normal (08), metastasis (07), EHPVO(05), BCS(01), Granuloma(01), Ascending cholangitis(02), NCPF(01),VOD(01)	Ca GB (09),Ch cholecystitis (06), EHPVO (05),Metastasis (04),Choledochal cyst (01), Pseudoaneurysm of aorta (01), Hemangioma (01), Biliary stricture (02), Cholangitis (01), PH (02), Perforation peritonitis (01),Liver injury (01), BCS (02), Cholangio Ca(02)
HBcAg cytoplasmic -ive	02(3.8%)	Ch hepatitis (01), granuloma (01)	Ca GB (01), TB (01)
HBcAg nuclear +ive	09(18%)	Ch hepatitis (04), metastasis (03), EHPVO (01), BCS(01)	Ca GB (01), EHPVO(01), metastasis (02), choledochal cyst (01), biliary stricture (01), BCS (01), hemangioma (02)

BCS: Budd Chiari Syndrome; Ca: Carcinoma; Ch: Chronic; EHPVO: Extrahepatic Portal Venous Obstruction; GB: Gall Bladder; NCPF: Non Cirrhotic Portal Fibrosis; PH: Portal Hypertension; TB: Tuberculosis; VOD: Veno-Occlusive Disease

mildly expanded by fibrosis with multiple dilated thin wall veins. Biopsies from EHPVO patients showed increased haemosiderin pigment deposition within kupffer cells and regenerating hepatocytes. The single case of abdominal tuberculosis showed epithelioid cell granulomas within portal tracts. Stain for acid fast organism however was negative. Liver biopsy in the patient with clinical diagnosis of choledochal cyst showed the effects of extraheptic biliary tract obstruction and the periportal hepatocytes showed evidence of giant cell transformation. The IHC staining patterns and clinical parameters are shown in (Tables 2 & 3). HBxAg staining showed diffuse finely granular cytoplasmic positivity in 14(28%) biopsies and coarse granular cytoplasmic positivity in 36 (72%) cases.

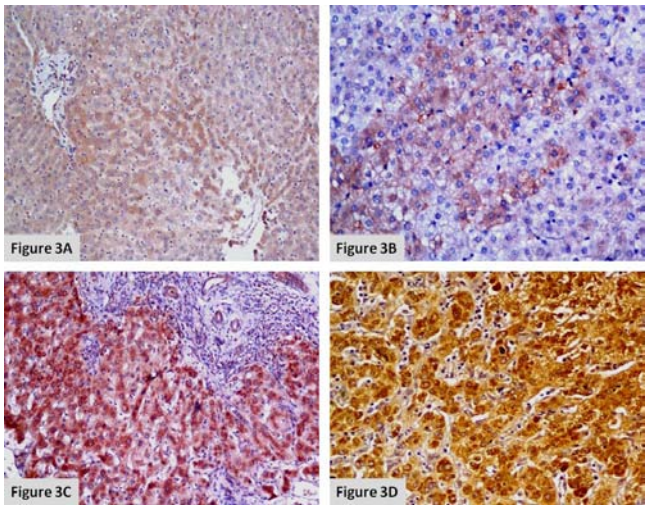

Figure 3: A: Medium power photomicrograph showing diffuse cytoplasmic HBxAg positivity with 1+ intensity. (PAP, x200).
B: Medium power photomicrograph showing diffuse cytoplasmic HBxAg positivity with 2+intensity. (PAP, x200). C: Medium power photomicrograph showing diffuse cytoplasmic HBxAg positivity with 3+intensity. (PAP, x200). D: Medium power photomicrograph showing diffuse cytoplasmic and nuclear HBcAg positivity. (PAP, x200).

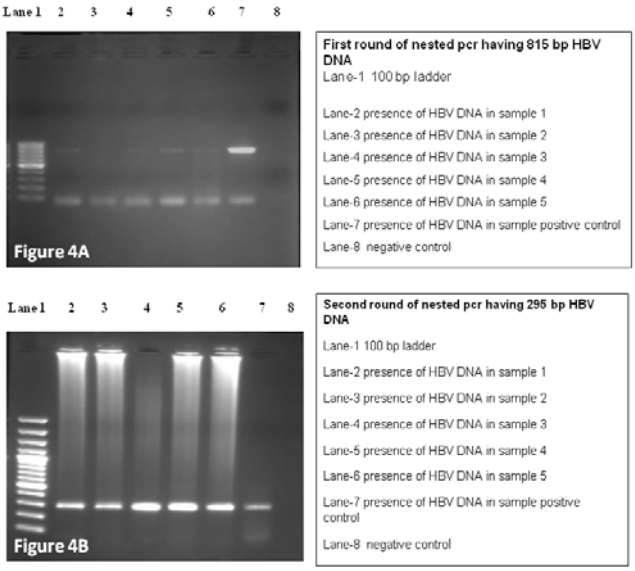

Figure 4: A: Photograph to show the positive bands on gel electrophoresis plate after the 1st round of PCR.
B: Photograph to show the positive bands on gel electrophoresis plate after the second round of PCR.

Polymerase chain reaction

Five to six of 10µ thick paraffin sections weighing about 40 to 50 mg of liver tissue were used for DNA extraction using standard protocol. Nested PCR was performed in all 50 biopsies, 47(94%) were positive and 3(06%) were negative. All PCRs were performed in duplicate. Intensity of bands in first round of PCR was less intense compared to second round PCR product (Figures 3 & 4). Cases where product was not visible in first round, the second round PCR yielded the expected product. A case was documented to be negative PCR only if no product was visible after the second round of PCR, even in the second set of nested PCR process. Positive and negative controls were run along with each process. Final product was run in 1.5% agrose gel along with 100bp DNA ladder and was visualized in ultraviolet light. Final product of 295 bp length was considered positive (Figure 4).

Twenty five prospective patients were followed up in OPD for a period of six months. Liver function tests, routine hemogram and other biochemical parameters carried out, which were found to be within normal limits. Serology marker for HBV i.e. anti-HBe, HBeAg, total anti HBc and anti-HBc IgM were re-tested. One patient turned out to be positive for total anti-HBc antibody and negative for anti-HBc IgM. In 18 patients, quantitative serum HBV DNA was tested and all were found to be negative.

Discussion

We analysed pathological spectrum of liver in occult hepatitis B in 50 patients who underwent hospitalization for diseases not related to HBV. In the previous reported studies had utilized more easily available sample like blood to exclude or document OBI [5]. Getting liver tissue in clinically asymptomatic OBI individual is not ethically justified. Any age group is not immune to HBV infection and the present study included wide range including one year old child. Most of the patients included in this study were from region known for high prevalence of HBV infection [6]. History of multiple blood transfusions was present in two patients with EHPVO and intravenous medication in one patient each of diabetes mellitus and Ca GB. None of the patients gave history of HBV infection in the past or at the time of enrolment for the present study. Possible routes of transmission of HBV that may go unnoticed or undocumented may be minor cut, dental manipulation, tattooing, needle stick injury, mucous membrane splash, piercing, sharing of razors and toothbrush [7-9]. Children get infection mainly by vertical transmission [10]. Histological evidence of chronic hepatitis was lower than the report by Chemin et al. [11] Low number of chronic hepatitis in the present study may be related to the patient selection whereas Chemin et al. [11] selected patients who were already diagnosed as chronic hepatitis on biopsy. Few studies explains negative serum markers but positive IHC on liver tissue. Escape mutation is one mechanisms which leads to decreased reactivity in HbsAg detection assays [12]. Humoral and cellular immune response on HBV envelop proteins are major mechanisms in generating OBI [13].

Patterns of positive staining observed for HBsAg and HBcAg in liver tissue were comparable to those reported in chronic HBV carrier's patients [14]. In the present study we observed that hepatocytes expressing HBsAg were low compared to HBcAg and HBxAg. HBsAg cytoplasmic positivity was 44% similar to Uchida et al. [14] who reported in 50% of serologically silent HBV patients. Expression patterns and intensity of HBcAg and HBxAg were also examined. Synthesis of HBx protein occurs in the cytoplasm and the IHC showed cytoplasmic positivity. Although nuclear expression of HBxAg had been documented in previous studies cytoplasmic positivity was considered more significant and these observations were similar to the present study [15,16]. Granular cytoplasmic and diffuse cytoplasmic HBcAg expression were seen in 60 % and 38% of the biopsies respectively. The possible mechanism of cytoplasmic HBcAg expression is explained by nuclear to cytoplasmic shift of

HBcAg during cell division [17]. Viral DNA is converted into cccDNA in the nucleus which serves as a template for the transcription to pre-genomic RNA which is reverse transcribed to relaxed circular DNA [18,19]. In absence of any liver injury and hepatocyte proliferation, HBcAg is expected to be localized predominantly in the nucleus however the protein synthesis takes place in cytoplasm in the Endoplasmic Reticulum (ER) [20]; which explains the peri-nuclear cytoplasmic granular positivity. Once these proteins are released or eased out of ER and golgi apparatus to cytoplasm result in diffuse cytoplasmic positivity. Cytoplasmic expression of HBcAg correlated with histological activity in the present study, which had also been reported by other studies [20-23]. There were 10 cases showing nuclear positivity for HBcAg but none showed nuclear expression for HBxAg. In this study periportal hepatocytes (zone 1) showed stronger positivity for HBcAg and HBxAg, which possibly is associated with earlier viral exposure. The three patients negative for nested PCR, two showed cytoplasmic positivity for HBsAg and HBcAg, and all the three had shown HBxAg cytoplasmic positivity. We consider them to be related to infection by mutant virus and could not be detectable by the standard primers used for the nested PCR.

There was an interval of 6 months between the enrolment and the follow-up sample collection. One patient was turned out to be positive for serum anti-HBc IgG however, HBV DNA was not detectable by QCR in blood sample. A similar study from Israel [24] demonstrated 38% of HBsAg negative chronic liver disease patients had detectable serum HBV DNA by PCR and the positive rates for serological markers in these patients being 45%. We consider that replication of HBV may be very low in our patients related to suppression by host immune system, or related to presence of co-infection by another virus, as had been reported in the literature [25-27]. The patients in the present study need to be followed up for longer duration of time to look for development of any hepatitis related signs and symptoms, which were not obvious during the short span of follow-up in the present study. Honarkar et al. had studied cryptogenic liver cirrhosis patients and haves shown a more than 50% of occult hepatitis B, but none being positive for serology marker or history of any previous exposure to HBV. Uchida et al. [14] have demonstrated HBV DNA in liver in 88% patients (total 40 patients) with serology HBsAg negative status. In the present study HBV DNA was detected in 94% of the patients using liver tissue and all liver biopsies were positive for more than one of HBsAg, HBcAg and HbxAg on IHC. Literature review shows that occult hepatitis B are observed with either acute or resolving HBV infection or with chronic HBV infection, very often co-infected by HCV [28-33]. The mean concentration of extracted DNA by standard protocol was 31.1 (±18.23) ng/µl and ranged from 11 -136 ng/µl. We consider that the variation in the extracted DNA concentration following the same protocol might have been related to either viral load or integrity of DNA. We also observed that the amplification seen on the gel in nested PCR were good if the concentration of DNA was more than 20 ng/µl and better if it were more than 40 ng/µl.

Conclusion

Liver tissue is the best sample to document occult hepatitis B infection. Although IHC can be of help, highly sensitive and specific molecular techniques like nested PCR is more rewarding. Long term follow up is indicated to realize HBV related clinical symptom. The information extrapolated in the present study has epidemiological implication in healthy occult carriers, who are at risk of reactivation and as source for viral transmission.

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