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Immunostochimistry as a Tool for Chronic Hepatitis Diagnosis

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1. Introduction

Detection and localization of hepatitis viruses in liver tissue are vital for diagnostic purposes and clinical management of infected patients, as well as for the elucidation of viropathological mechanisms. The fragility of RNA and/or the low levels of viral expression in infected tissues are a constant limitation in molecular assays for viral characterization. Viral antigen detection, by immunochemistry, in liver biopsies is an attractive option for precise localization and quantification of viral proteins with direct access to histological patterns. We will describe studies using immunohistochemical methods effective on fixed, archived specimens, including liver biopsies and surgical resection samples.

1.1 Chronic hepatitis B

Natural course of type B hepatitis is now well established and the different phases of the disease are clearly defined. Briefly, after contamination early in life, there is a first phase of *immune tolerance*. During this phase, the virus is highly replicative, HBV DNA, HBs and HBe antigen are at high level in the serum, but clinical, biological, and histological signs are more or less normal. These patients are highly contagious, and most of them are going to be HBV carriers for the rest of their life. This model is frequent in highly endemic countries where a high rate of mother-to-baby transmission exists (especially in Asia); This phase is absent when people are infected as adults, their immune system being mature. They present an acute hepatitis and only a few of them (10%) will remain carrier with chronic hepatitis B. The phase of tolerance lasts for about 15 to 20 years. Nowadays very few people are having liver biopsy during that period. When it was done in the 70's/80's HBV antigens immunostaining were quite impressive: HBs antigen was present in a diffuse aspect grade associated with a strong expression for HBc antigen on more than 50% of nuclei (Table 1).

During the second phase of *the immune clearance phase*, hepatitis activity increase with fluctuating high level of ALT, necrosis, inflammatory cells infiltrates, fibrosis and decrease HBV viraemia. This period of high activity is associated sometimes with severe hepatic failure. At the end of this phase occurs HBe seroconversion which is often associated with clinical remission. The expression of HBV antigens detected by immunostaining is varying according

to the time from infection when the biopsy was performed. The large number of HBs diffuse positive hepatocytes is decreasing with time. Many small clusters of cytoplasmic or sub membranous cells are gathering in the liver tissue the nuclear expression of HBc antigen is progressively changed in cytoplasmic expression and finally HBc antigen is undetectable. This stage corresponds to a very low viral replication (HBV DNA from 1000 UI/ml to null.).

Phases of natural history	HBs diffuse	HBs cluster			HBc nuclear	HBc cytoplasmic
		membranous	Sub membranous	cytoplasmic		
Immune tolerance	++ to +++	unusual	unusual	unusual	very frequent	unusual
Immune clearance	0 to +++	frequent	emerging	rare	frequent	emerging
Inactive phase early period	0 to +	rare	frequent	frequent	unusual	frequent
Inactive phase late period	0 to +	absent	frequent	Very frequent	absent	rare or absent
Reactivation	+ to +++	frequent	frequent	frequent	frequent	frequent

Table 1. Summary of the aspects and patterns of HBV antigen along the natural course of chronic type B hepatitis.

During the third phase called the inactive carrier phase, clinical remission may occur earlier in life if infection had occurred in adulthood ; Usually liver biopsy is done at that period to graduate residual inflammatory activity and over all staging the fibrosis. HBs Ag can be detected by immunostaining :

- on very few hepatocytes; this can be seen after a very long carriage of HBV; it is correlated with a low level of HBs antigen in the serum.
- on very large clusters of cytoplasmic pattern including almost all the hepatocytes. These patients have a very high level of HBs antigen in serum, exclusively made of spherical and tubular empty particles.

HBc antigen is usually undetectable. In very low replicative patients some cytoplamic expression can be detected in rare hepatocytes.

During this inactive phase, reactivations may occur. If liver biopsy is done during this period, expression of viral antigens is similar to the clearance period, associated in many cases with intense expression of HBs antigen in the diffuse or cluster like pattern. If the patient is not treated for this reactivation, HBs antigen will decrease and HBc antigen will appear in some nuclei.

1.2 Liver transplantation

In the vast majority of the cases, histological examination is able to explain if a flare of activity (cytolysis) is associated with graft rejection or re-infection of the graft by HBV. In

case of re-infection, diffuse HBs antigen is weakly present in its cytoplasmic pattern and nuclear HBc is detected.

1.3 Occult HBV hepatitis

In some cases of occult hepatitis infection (HBV DNA detectable in the liver and possibly in the serum in the absence of the landmark of HBV infection, the HBsAg in the serum).

In those cases, few hepatocytes may be weakly positive for either HBs or HBc antigen. It can be helpful for the diagnosis of these very peculiar forms of HBV infections.

1.4 Hepatitis C hepatitis

The Hepatitis C virus (HCV) genome is a single stranded RNA molecule of about 9500 bp. This RNA encodes for a large poly-protein that is processed by host and virus proteases into several structural and non structural viral proteins (Envelope, Capsid and NS2a/b NS3a/b NS4a/b NS5a/b respectively). The detection of HCV replicative intermediates or virus antigen may be helpful for diagnosis or clinical management of patients with HCV infection and it is of crucial importance for monitoring patients prior and post- HCV-related liver transplantation. In a general point of view, HCV replication level seems to be relatively low in infected liver, hampering the detection of HCV particles directly in the liver (Shimizu *et al.*, 1996, Negro, 1998). Detection methods based on HCV RNA amplification as *in situ* PCR or *in situ* hybridization are not completely efficient for HCV detection and can lead to conflicting results concerning viral particles localization (Lau *et al.*, 1996). However they remain an interesting tool when used in complement of classical method (Biagini *et al.*, 2001, Comar *et al.*, 2006). Limitation of these methods is probably due to RNA rapid degradation in tissues and difficulty to design efficient probes to overcome high variability of HCV genomes.

Detection of HCV antigens by immunochemistry in liver biopsies constitutes therefore an interesting option that allows both localization and quantification of viral proteins (Galy *et al.*, 2006).

2. Methods

2.1 Hepatitis B antigens

Slides are deparaffinized in methylcyclohexane (two baths for 10 minutes) then re-hydrated through 4 baths of ethanol and soaked for half an hour in PBS pH 7.2; and endogenous peroxidase blocked by H₂O₂.

Slides are then incubated in a humid chamber for 30 minutes at room temperature with diluted anti-HBs antibody, and then rinsed 4 times in PBS pH 7.2. A second incubation in same conditions is made with the secondary antibody (anti rabbit or mouse immunoglobulin, according to the primary antibody) labelled with peroxydase. Commercial kit with DAB substrate (Dako USA) are used to develop the final reaction.

Counterstaining with Harris haematoxylin is added on slides for HBs antigen search. Usually no counterstaining is performed on slides tested for HBc antigen as it might interact with the nuclear expression of this viral antigen.

Reagents:

- Anti-Hbs monoclonal mouse antibody Dako USA (clone 3E7) or Argene F(ref:11-086)
- Polyclonal rabbit Anti -Hbc ready to use Dako USA
- Antibody diluent Dako USA
- Envision dual link kit Dako USA
- Hematoxylin Biomedica USA

2.2 Hepatitis C antigen

First tissue sections are deparaffinized in xylene for 10 minutes (twice). Then Rehydrate in graded ethanol concentrations (100% and 95% for 5 min each) and proceed immediately to Block endogenous peroxidase activities with incubation in methanol (0.3% hydrogen peroxide) for 30 minutes at room temperature. The unmasking procedure includes a microwave treatment. The slides are placed in microwave for 15min in antigen unmasking solution (Vector H3300 - Vector Laboratories, Burlingame, CA, USA). Keep slides to cool down for 30 min in the same unmasking solution. Incubate with PBS solution (5% skimmed milk and 0,1% BSA) for 1 hour at room temperature. Sections are incubated overnight with the primary mAb (For D_{4.12.9}: concentration 0.2µg/ml) at 4°C. Sections without primary antibody can be used as controls. Wash in PBS (3x5 min). Incubate 30 min with Secondary antibody at 5µl/ml (Elite Kit- Vector Laboratories, Burlingame, CA, USA) in PBS solution (0,1% BSA). Wash again in PBS (3x5 min). Amplify signal at 37°C for 45min with VECTASTAIN ABC Kit (Vector Laboratories, Burlingame, CA, USA) - Use 0,1%BSA PBS solution. Wash in PBS (3x5 min).Incubate tissue sections with the DAB substrate (Vector kit SK 4100, Vector Laboratories, Burlingame, CA, USA) at room temperature for 5 minutes.Wash in PBS (3x5 min). Counterstain the sections with Mayer's haematoxylin. Dehydrate through a successive ethanol baths (95% Ethanol, absolute ethanol and xylene -5 min in each solution). Mount slides using standard microscope cover glass and mounting medium.

2.3 Hepatitis delta antigen

Direct immunostaining in fluorescence may be used for delta antigen. Once deparaffinized as above, slides are at first incubated for 30 minutes with a solution of proteinase K(21 mg/ml in Tris EDTA buffer) then rinsed in PBS for 15 minutes . Incubation with the FITC labelled anti-delta antibody is performed in humid chamber for half an hour. Slides are rinsed in two baths of PBS for 15 minutes and mounted.

Reagents:

- Proteinase K recombinant Roche USA
- Tris EDTA buffer Sigma Aldrich USA
- Polyclonal rabbit anti delta antibody labelled with FITC is kindly provided by Dr Alan Kay INSERM unit 1052, Lyon, France.

3. Examples of viral hepatitis antigens detection

3.1 Hepatitis B

3.1.1 Cytological patterns of HBs antigen

Three different patterns are seen on liver biopsies:

- i. *Cytoplasmic* : this is the most common pattern , consisting in a large vacuole made of the Golgi reticulum full of viral envelopes. In light microscopy it is usually called "ground glass" hepatocytes. The intensity of the staining is closely correlated with the amount of viral envelope in the cell (Figure 1).
- ii. *Submembranous* : This pattern is not frequent ; it is associated with a strong production of viral envelope and might be a former stage of the cytoplasmic pattern (Figure 2).
- iii. *Membranous*: This is a less common pattern. It is often seen on a few hepatocytes; it is mostly associated with strong viral expression and is rather present in active periportal zone. Staining underlines the cell membrane in a perfect straight line. (Figure 3)

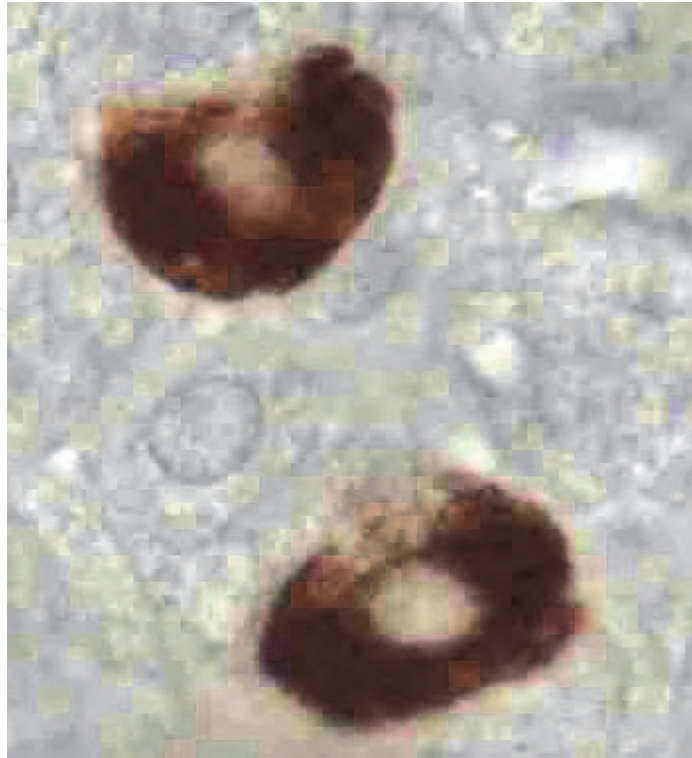


Fig. 1. Two typical ground glass hepatocytes stained with immunoperoxidase for HBs antigen.(magnification x 600)

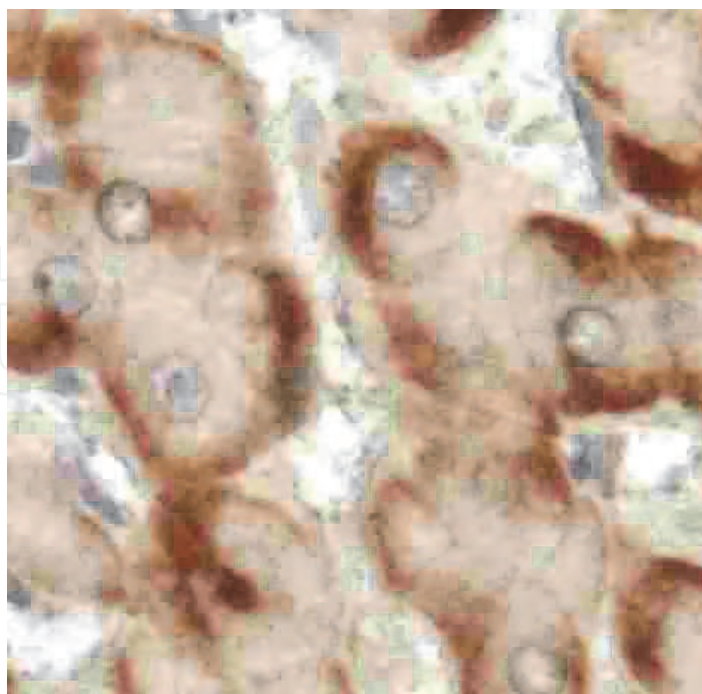


Fig. 2. Submembranous expression of HBs antigen in hepatocytes (immunoperoxidase magnification x 600).

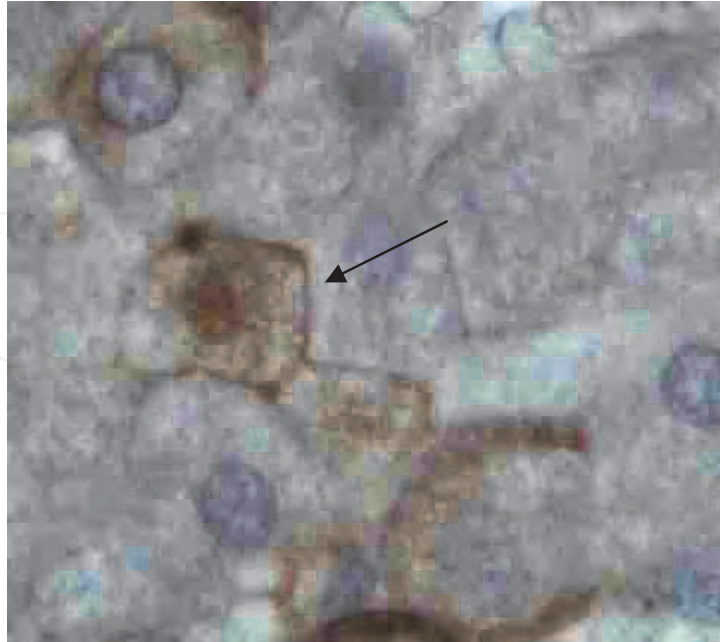


Fig. 3. Membranous expression of HBs antigen: arrow shows the perfectly straight pattern of the cell membrane (immunoperoxidase magnification x 600).

3.1.2 Cytological patterns of HBc antigen

- i. *Nuclear*: This is the most common expression pattern of this antigen in liver cells (Figure 4).
- ii. *Cytoplasmic*: The design is different from the HBs Antigen one as it is not gathered in a part of the cell but more or less diffuse in the whole cytoplasmic area (Figure 5).
- iii. In many cases, cells can express both of these patterns (Figure 6).

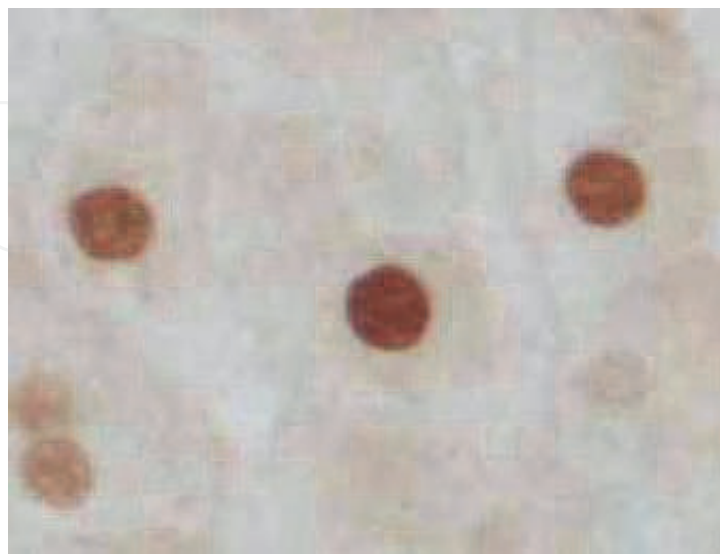


Fig. 4. Nuclear expression of HBc antigen. Note the large scale of intensity of the staining (immunoperoxidase, magnification x 600).

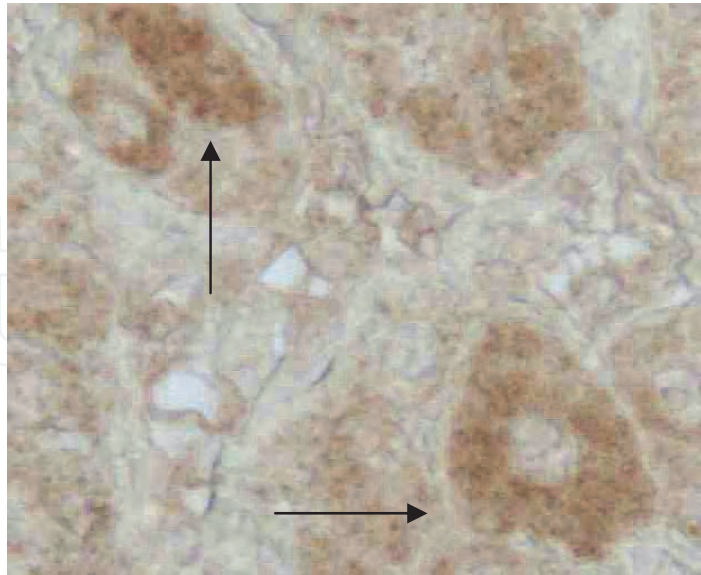


Fig. 5. Cytoplasmic expression of HBc antigen (immunoperoxidase, magnification x 600).

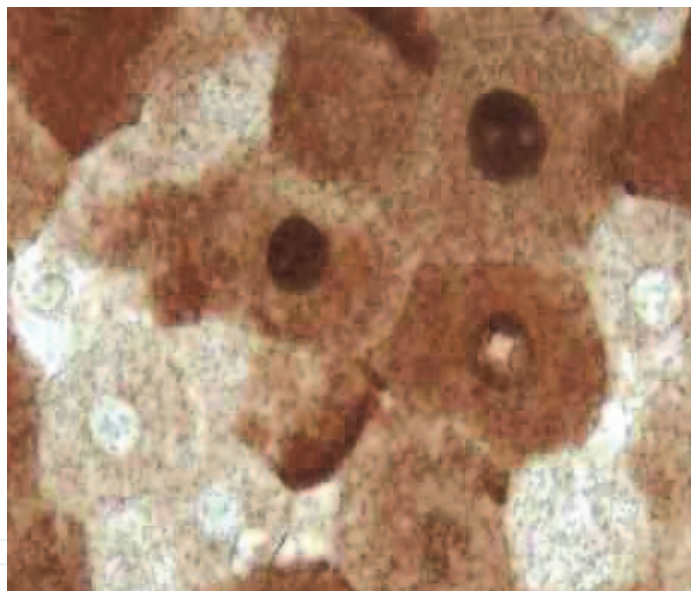


Fig. 6. Double expression of HBc antigen: cytoplasmic and nuclear, sometimes in the same hepatocyte (immunoperoxidase magnification x 600).

3.1.3 Antigen expression in liver tissue

The antigen expression in liver tissue is important to observe as it correlates to the stage of replication and natural history of the disease.

HBs antigen

Two main patterns can be observed:

- i. *Diffuse or dotted*: Positive liver cells are non confluent, diffuse in the lobule. It can be useful to estimate the number of positive cells in the biopsy. This can be done semi quantitatively as an amount grade +, ++, +++ (Figures 7, 8, 9).

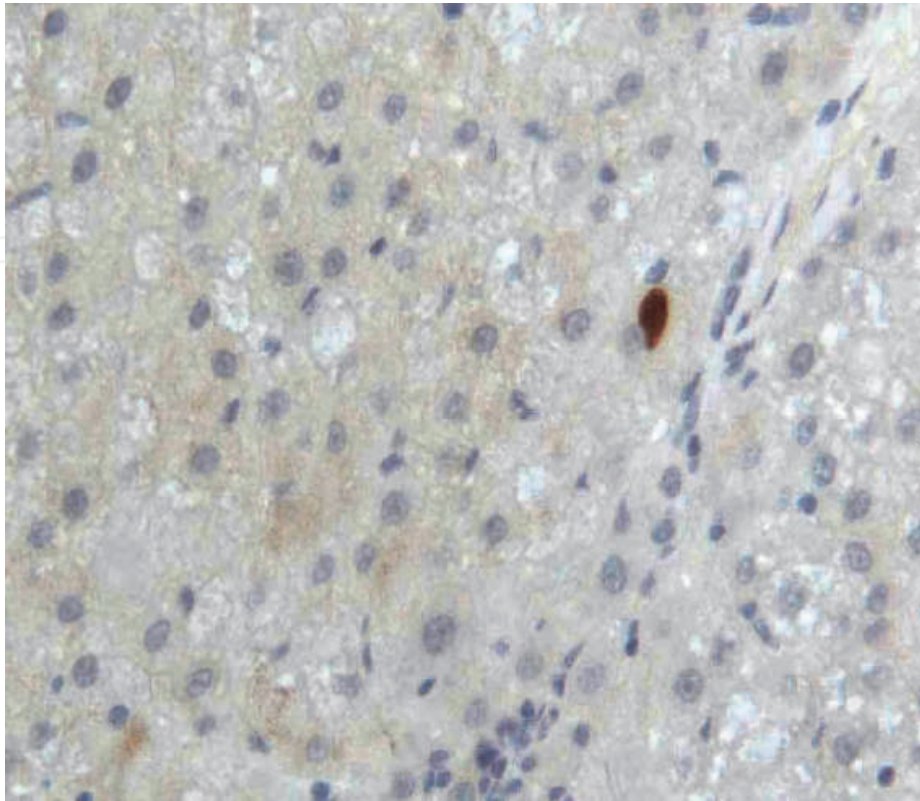


Fig. 7. Diffuse expression of cytoplasmic HBs antigen: amount grade: + , the only strong positive cell out of a 15 mm length needle biopsy (immunoperoxidase magnification x 100).

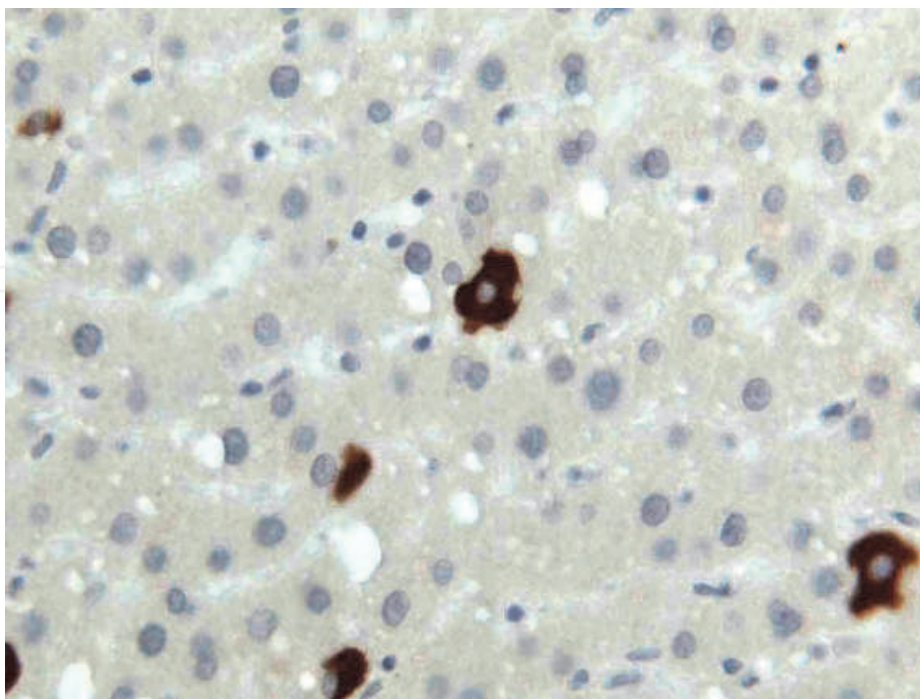


Fig. 8. Diffuse expression of cytoplasmic HBs antigen in strongly stained hepatocytes, amount grade: ++ (immunoperoxidase magnification x 100)

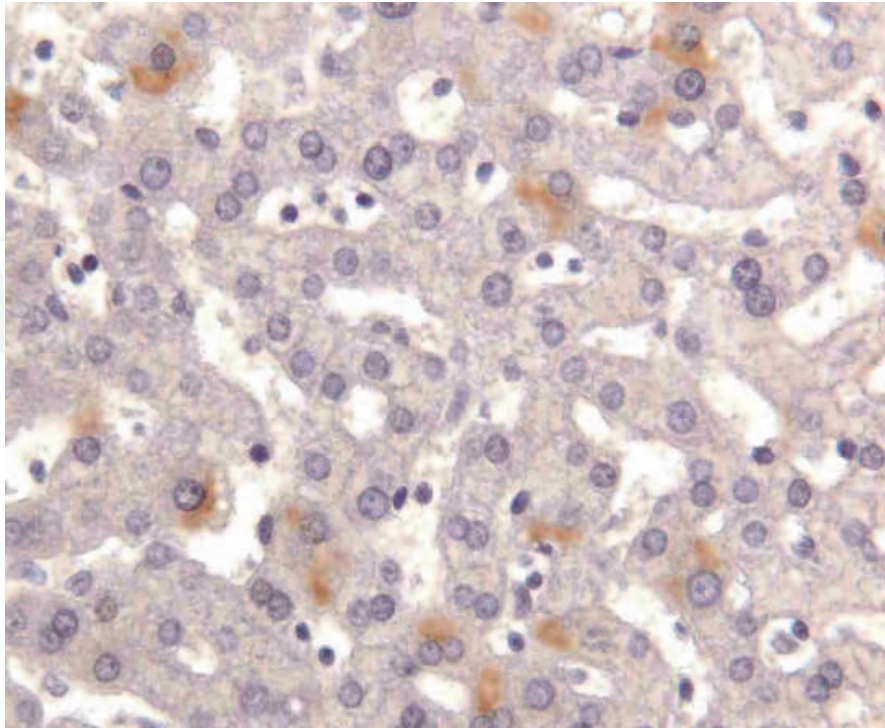


Fig. 9. Diffuse expression of cytoplasmic HBs antigen, light staining, amount grade +++ (immunoperoxidase, magnification x 100)

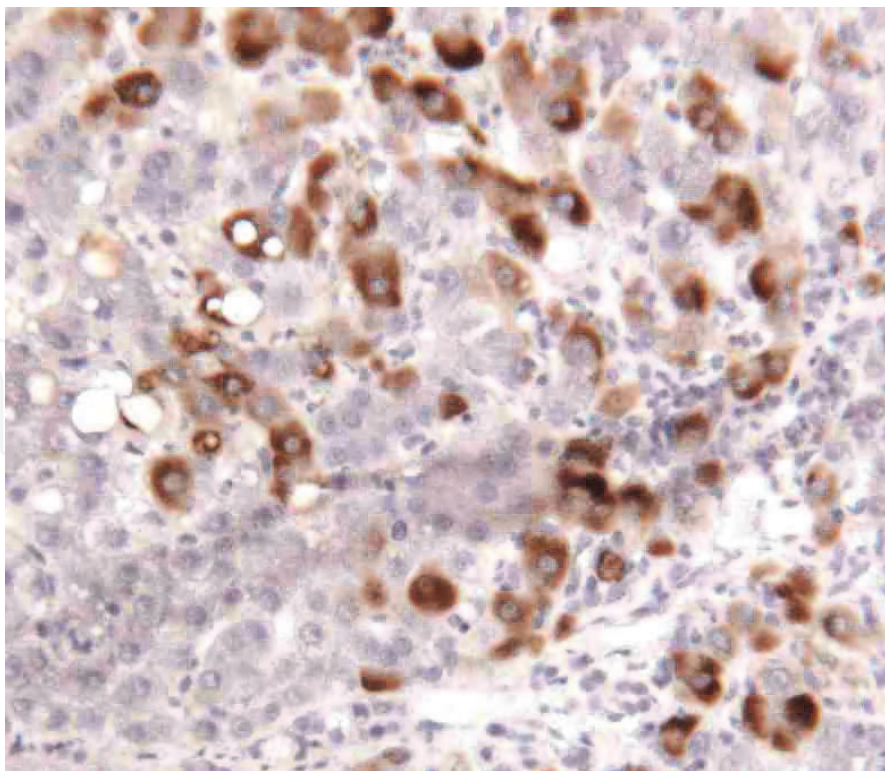


Fig. 10. Diffuse expression of cytoplasmic HBs antigen, strong staining, amount grade +++ (immunoperoxidase, magnification x 75).

- ii. *Confluent or cluster-like pattern*: Positive cells are closely gathered inside the lobule can be detected on contiguous lobules (Figures 11, 12, 13). Sometimes, this pattern can be associated with the diffuse pattern on the external zone of the cluster (around the cluster). The distribution of these clusters of positive cells is not homogenous in the whole liver. False negative of the immunochemistry staining is possible if the needle doesn't reach a positive zone (See Figure 12) with a strong contrast between positive and negative area on a surgical biopsy);

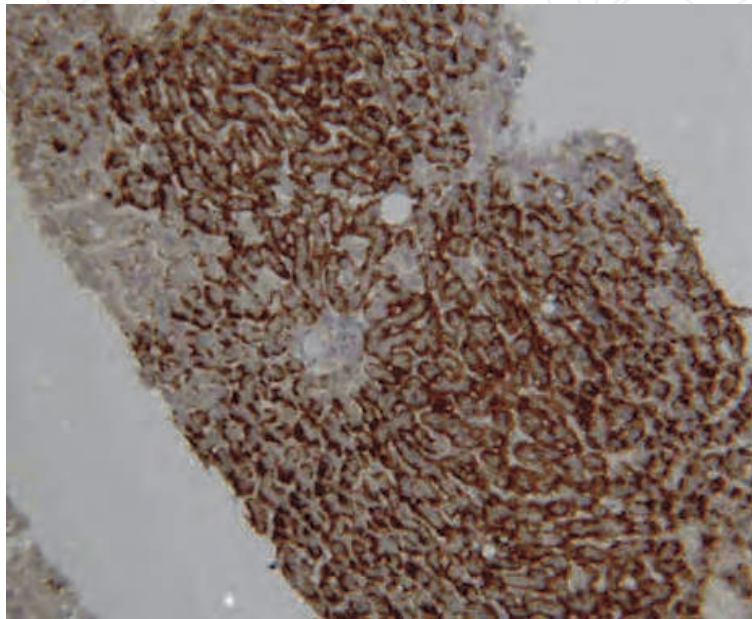


Fig. 11. Large cluster of sub membranous HBs antigen positive hepatocytes (immunoperoxidase, magnification x 40).

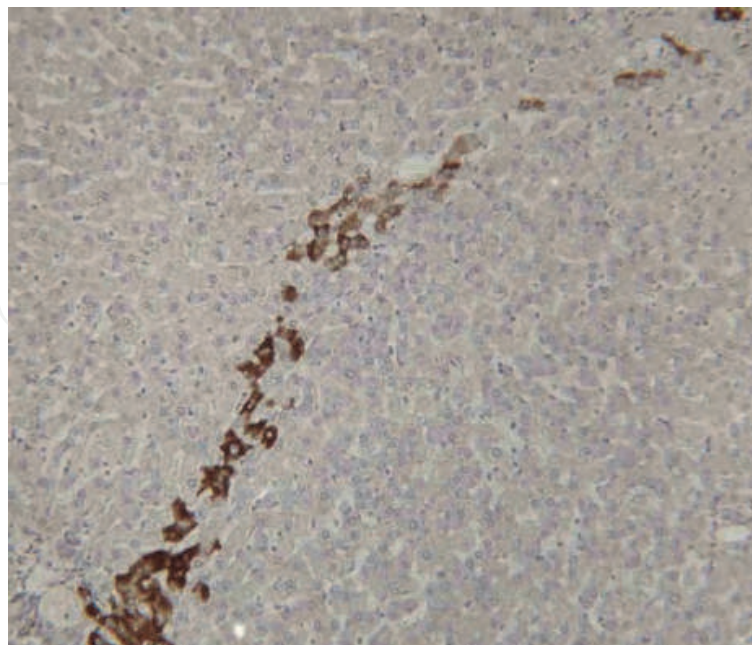


Fig. 12. Cluster of HBs antigen positive cells crossing a lobule from portal tract to Centrolobular vein. (immunoperoxidase, magnification x 75

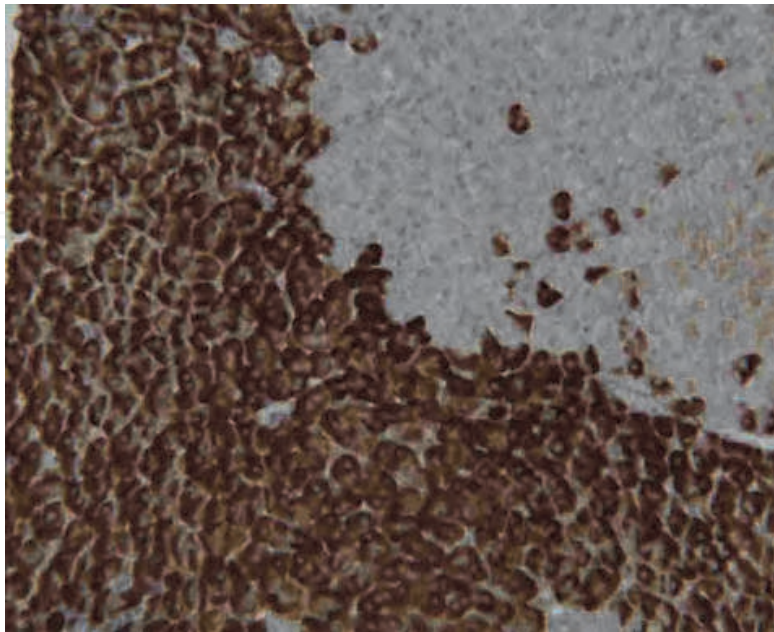


Fig. 13. Large cluster of HBs antigen positive hepatocytes with a cytoplasmic pattern. Note the strong contrast between positive and a very weak positive area (immunoperoxidase, magnification x 40).

Sub membranous and membranous pattern are always expressed in the liver tissue as a cluster like pattern (Figure 14)

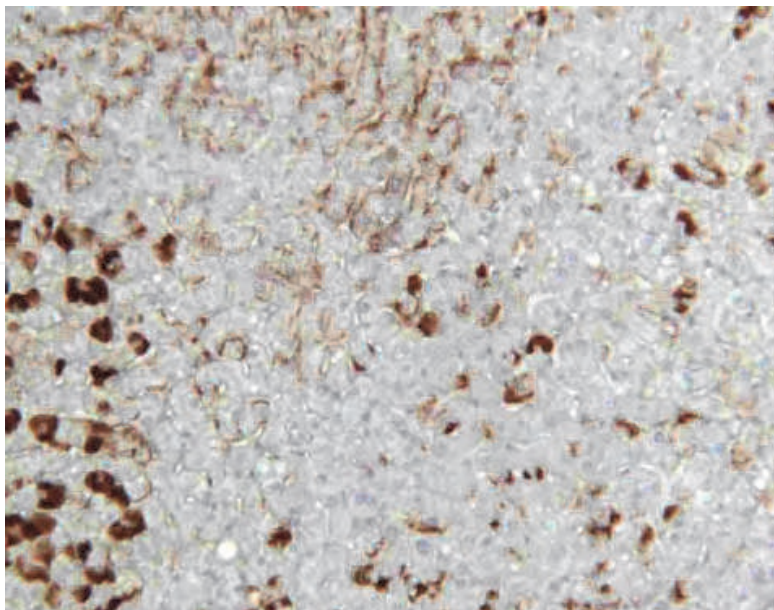


Fig. 14. Presence of three patterns of HBs antigen:
top : sub membranous, centre: membranous and left: cytoplasmic. (immunoperoxidase, magnification x 40)

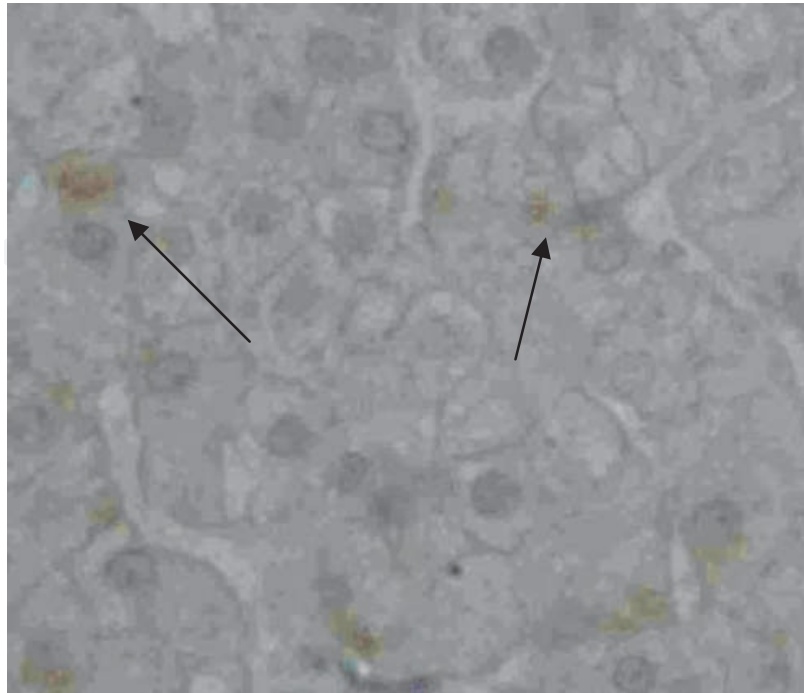


Fig. 15. Very weak expression of HBs antigen in a liver biopsy of an occult carrier of HBV (immunoperoxidase, magnification $\times 400$).

HBc antigen

As the presence of HBc antigen in the liver is correlated with the amount of infective whole Dane particles, quantification is possible. For the nuclear pattern it is usual to calculate the percentage of positive cells in the biopsy.

Five classes of percentage are described: less than 1%, from 1 to 5%, from 5 to 20%, from 20% to 50% and over 50%.

The cytoplasmic pattern is more difficult to quantify as it might be very weakly expressed in some cells. We usually describe 4 classes: rare positive cells, positive +, positive ++, positive +++.

3.2 Hepatitis C

In our personal experience (Galy *et al.*, 2006), the staining location for Anti-E2 immunostaining is mainly cytoplasmic with occasional perimembranous staining (including cytoplasmic and nuclear membrane). Staining pattern is mainly coarse granular with microvesicular pattern (Figure 16). HCV staining patterns appears to differ slightly according to the pathological status of the liver tissue. We observed a very strong staining of hepatocyte membrane, cytoplasm and perinuclear regions in liver from patient with active HCV-related cirrhosis (Intense plasma and nuclear membrane staining was observed in cases with high inflammatory activity). In non-cirrhotic and non-tumoral tissues Anti-E2 staining intensity increases with hepatitis fibrosis state. In HCV-related tumors, staining was exclusively detected within regeneration nodules and confined to hepatocytes whose morphology remains unchanged. Staining pattern appeared with two distinct patterns: trabecular throughout the hepatic parenchyma or concerning isolated cells.

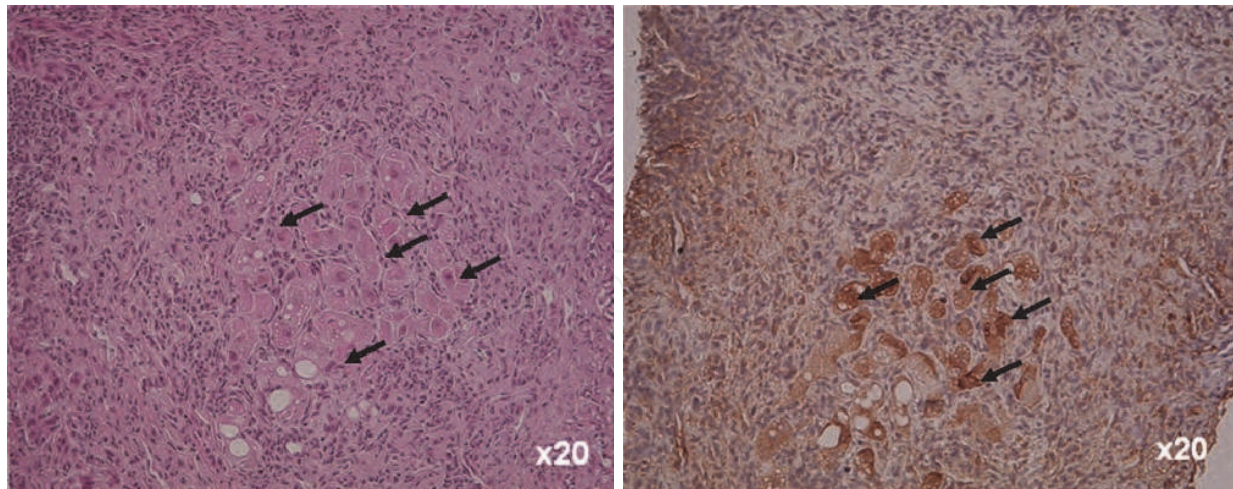


Fig. 16. Expression of HCV E2 antigen in a liver biopsy of cirrhotic liver of an HCV chronic carriers. Arrows: Few remaining hepatocytes strongly positive with anti-E2 antibody. Immunoperoxidase magnification X 200

3.3 Hepatitis delta

Antigen delta is expressed mainly in the nucleus of the hepatocyte and in rare cases can also be expressed in the cytoplasm; it depends on the stage of the replication and the production of antigen. The nuclear expression seems to be more correlated with a period of stoking of viral core. The result of the test is qualitative: negative or positive (Figure 17).

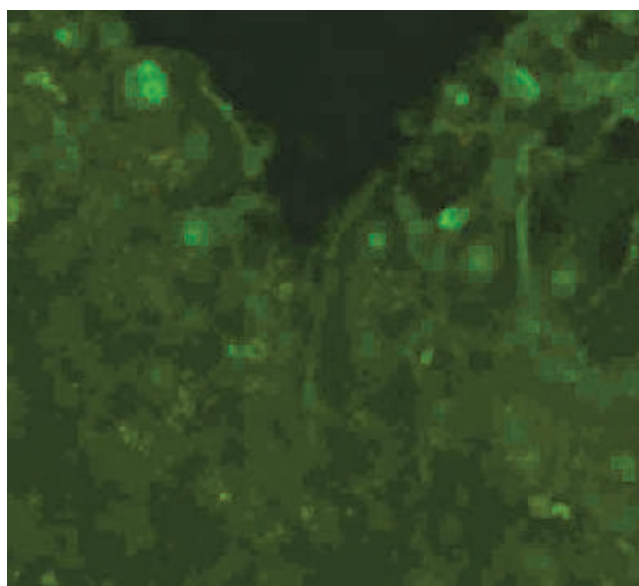


Fig. 17. Intranuclear expression of Delta (HDV) antigen. FITC labelling, magnification x 100

4. Conclusion

Concerning Hepatitis B virus (HBV) detection of HBs and HBc antigens are informative and provide different patterns providing elements in correlation with the natural history of HBV infection including viral load. In the specific case of occult HBV infection (HBV DNA

detected in the absence of HBsAg in the serum) it may help to confirm the diagnosis. For Hepatitis C virus (HCV) antigen detection it exists antibodies that are able to successfully detected HCV on paraffin-embedded sections from fresh as well as archived-frozen, material. It should be noted that HCV detection in archived serum may be problematic due to delicate extraction and stability of RNA and this is the same for hepatitis delta virus diagnosis (HDV). Thus, IHC represents a very stable diagnostic tool as compared to RNA-based approaches. We found important differences in the localization of HCV between tumor and non-involved, adjacent tissue in HCC cases.

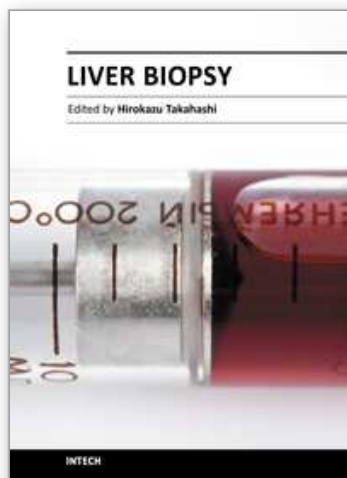
These protocols offer easy, precise and strong staining resolution with distinct patterns consistent with the liver pathology, irrespective of the hepatitis viruses examined. This approach provides applications for diagnosis as well as for exploratory pathological studies. Thus, IHC to detect viral hepatitis antigens may have a number of important applications for clinical diagnostic, research on the mechanisms of pathogenesis of viral hepatitis-containing lesions, and retrospective evaluation of the contribution of hepatitis viruses to liver diseases using archives of paraffin-embedded material not suitable for molecular analysis.

5. Acknowledgements

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Liver Biopsy

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Liver biopsy is recommended as the gold standard method to determine diagnosis, fibrosis staging, prognosis and therapeutic indications in patients with chronic liver disease. However, liver biopsy is an invasive procedure with a risk of complications which can be serious. This book provides the management of the complications in liver biopsy. Additionally, this book provides also the references for the new technology of liver biopsy including the non-invasive elastography, imaging methods and blood panels which could be the alternatives to liver biopsy. The non-invasive methods, especially the elastography, which is the new procedure in hot topics, which were frequently reported in these years. In this book, the professionals of elastography show the mechanism, availability and how to use this technology in a clinical field of elastography. The comprehension of elastography could be a great help for better dealing and for understanding of liver biopsy.

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