Correlation of Serological, Biochemical and Molecular Viral Markers with Histological Parameters in Chronic Hepatitis B for Assessment of Response to Therapy

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ABSTRACT

Introduction: Hepatitis B virus (HBV) infects more than 350 million people worldwide. The presence of continuing viral replication correlates with continuing disease activity and is associated with Hepatitis B e antigen (HBeAg) and hepatitis B virus DNA (HBV-DNA) in serum. Subsequently, the patient may undergo a spontaneous or therapy induced remission, which is accompanied by loss of HBV-DNA and HBeAg. This prospective study was undertaken to correlate all the above parameters so as to have an insight to monitoring of therapy in chronic hepatitis B (CHB).

Material and methods: 66 patients of CHB were enrolled and were followed up for 24 months. Blood samples were collected for aspartate aminotransferase (AST), alanine aminotransferase (ALT), hepatitis B surface antigen (HBsAg), Anti-hepatitis B core antigen (HBcAg), HBeAg and HBV-DNA. Liver biopsies were done in all individuals. Immunohistochemical staining for HBsAg and HBcAg were done where indicated. The statistical analysis was done using SPSS (Statistical Package for Social Sciences) Version 15.0 statistical Analysis Software.

Results: There was a statistically significant improvement in the biochemical, serological and virological profile of the patients after therapy. However, the necroinflammatory activity showed improvement but was not statistically significant. Immunohistochemistry showed good correlation with viral load.

Conclusion: HBV-DNA is the most reliable marker to assess seroconversion followed by HBeAg. IgM-anti HBc positivity during or post-therapy denotes continuing necroinflammatory activity. Histology shows improvement but was not significant in our study.

Keywords: Chronic hepatitis B, therapy, serological and viral markers, histology

INTRODUCTION

Serendipity led to the identification of Australia antigen, now known as hepatitis B surface antigen (HBsAg).[1] Hepatitis B Virus (HBV) is a major health problem in Asia and sub-Saharan Africa.[2,3] Progression to long term infection occurs in 15-40% of cases resulting in chronic hepatitis B (CHB).[4,5]

The natural history of CHB includes a Hepatitis B e Antigen (HBeAg) positive, immune tolerant phase which progresses to a HBeAg-positive immune-reactive phase, HBeAg-negative inactive HBV carrier state, HBeAg-negative CHB phase and HBsAg negative phase (occult infection).[6] The HBV can be detected serologically by HBV DNA in the serum.[7] Histopathological changes include necroinflammatory activity and fibrosis, which can be correlated with various parameters.[8]

Suppression of viral replication is critical to reduce the risk of complications from HBV. In a large-scale, long-term follow up study of chronic HBV infection, elevated serum HBV DNA levels were found to be the strongest single risk factor for progression to cirrhosis.[9] Periodic serological and viral markers studies are required during antiviral therapy to assess treatment response. As complications occur after decades of infection and often long after treatment has been initiated, various surrogate markers are used to ascertain treatment benefit.[10] These include serum aminotransferase levels, HBeAg or anti-HBe, HBsAg or anti-HBs, serum HBV DNA level and liver histology. Numerous definitions have been used to assess response to antiviral therapy such as biochemical response, virological response, histological response and complete response (biochemical and virological response with loss of HBsAg).[11,12] Recent studies favor using durable HBV DNA suppression as the primary measure of therapeutic success.[9,13]

The aims and objectives of this study were:

1. To determine changes in serum aspartate aminotransferase (AST) and ALT levels and serological profile (HBsAg, Anti hepatitis B core (HBc) IgM and HBeAg) in CHB with therapy.
2. Quantitative determination of HBV DNA in plasma and its correlation with serology with therapy.
3. Determination of histology in liver biopsies in all cases and correlation of above parameters with immunohistochemical detection of HBsAg and HBcAg in the biopsies from patients with CHB.

MATERIAL AND METHODS

Patient selection: In this prospective study, 66 cases of CHB were followed for 24 months. Sample size was determined by the number of patients who were followed for a period of two years and for whom almost complete data was available. History of any concomitant illness was taken into consideration but was not an exclusion criterion. Informed consent and Institutional ethical clearance was obtained. Patients were treated with lamivudine with or without peg-interferon. All the patients were

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were evaluated for:

Biochemical parameters: AST and ALT were measured using ERBA kits in opERA system (BAYER) in accordance with principle based on International federation of clinical chemistry. Quality control measures were strictly followed.

Serological parameters: HBsAg, HBeAg and IgM-anti HBc were performed by enzyme immunoassay (Milano, Italy). Positive and negative controls were run simultaneously to check the validity of test.

Molecular viral marker: Extraction of DNA was done using AccuPrep Genomic DNA Extraction Kit (BIONEER) which is a column-based assay. Quantitative PCR assays were carried out using HBV RG Real-ArtTM reagents in Rotor-Gene 2000 instrument where fluorescence labeled oligonucleotide probe bind specifically to the PCR amplicate and fluorescence intensity during the course of Real time PCR enables verification as well as quantification of the accumulating product. Samples with more than 10^5 copies/ml were considered positive.

Histological evaluation: Specimens were fixed in 10% buffered formalin, processed by routine methods, embedded in paraffin and sections cut to 3-4 um in thickness. Sections were subjected to hematoxylin and eosin stain and reticulin stain. Scores were accorded as per modified Knodell-Ishaak scoring system.[14] Immunohistochemical (IHC) staining was done in selected cases for HBsAg and HBcAg with monoclonal antibodies (ready to use kit manufactured by SEROTEC (USA).
Interpretation of IHC staining:
(a) HBsAg: strong brown staining of cytoplasm or membranous or both pattern of staining.
(b) HBcAg: strong brown staining of nucleus, cytoplasm or mixed pattern.

STATISTICAL ANALYSIS

The statistical analysis was done using SPSS (Statistical Package for Social Sciences) Version 15.0 statistical Analysis Software. The values were represented in Number (%) and Mean±SD (Standard deviation). Wilcoxan assigned rank test was used to test the significance of two means. The level of significance "p" value was considered statistically significant if <0.05.

RESULTS

In this study, all the patients were males. 45% were in age group 20-30 years, 42 % in 31-40 years, 10.6% in 41-50 years and 2.4 % in 51-60 years. Mean age was 33.5 ($\pm$ 6.75 SD) years.

Biochemical Profile
A. **AST profile**: Before treatment, mean AST value was 73.83 $\pm$ 89.37 SD IU/L (Range= 15 to 494 IU/L) which reduced to a mean of 42.43 $\pm$ 25.29 SD IU/L (Range= 17-139 IU/L). AST profile is summarized in Table-1. Amongst these 32 cases had normalized, 12 remained static, 16

cases improved but did not normalize whereas 06 cases worsened. This reduction in the post-treatment AST levels shows statistical significance (p value = 0.0042).

B. **ALT profile:** The mean value of ALT was 122.8 ±159.6 SD IU/L (Range= 15 to 696 IU/L) which reduced to 62.27 $\pm$ 66.6 SD IU/L (Range= 17-409 IU/L). ALT profile is summarized in table-1. As compared to pre-treatment levels- 25 cases had normalized, 11 remained static, 12 cases had worsened and 18 cases improved but did not normalize. The reduction in the post-treatment value exhibits statistically significant reduction   (p value = 0.0018).

Serological profile
A. **HBsAg:** As per patient selection criteria, all cases were HBsAg positive. Post therapy, 16 cases (24.2%) became HBsAg negative whereas 50 cases (75.8%) continued to remain positive. Statistical correlation by Wilcoxan assigned ranks test shows a Z value of – 4.0 and p value of 0.002.

B. **HBeAg:** In this study, 42 cases (63.6%) were positive whereas 24 (36.4%) cases were HBeAg negative. After therapy out of 42 HBeAg positive cases, 35 (83.3%) cases became HBeAg negative, whereas 07(16.7%) cases remained HBeAg positive. Those cases, which were HBeAg negative continued to remain negative post-treatment. Statistical correlation by Wilcoxan assigned ranks test shows a Z value of – 5.96 and p value of 0.000.

C. **IgM anti-HBc:** In 52 cases, where IgM anti-HBc was carried out, 19 (36.5%) were positive and 33 (63.5%) were negative. Of the 19 cases, which were IgM- anti-HBc positive, 10 (52.7%) became negative whereas 09 (47.3%) cases continued to remain positive. Out of 33 cases which were IgM anti-HBc negative, 31 (94%) continued to remain negative whereas 02 (6.0%) became positive. Statistical correlation by Wilcoxan assigned ranks test shows a Z value of – 4.79 and p value of 0.001.

D. **Molecular viral marker:** Out of 66 cases, HBV-DNA was positive in 61 (92.4%) cases and only 05 (7.6%) cases were negative. Among HBV-DNA positive cases, the mean viral load was 701,239, 942. 5-copies/ ml. After therapy, 38 (62.3%) became negative whereas 23 (37.7%) remained positive. Amongst 05 cases, which were HBV-DNA negative, 01 (20%) case became positive, rest 04 (80%) remained negative. Statistical correlation by Wilcoxan assigned ranks test shows a Z value of – 5.86 and p value of 0.000. Post therapy, the mean HBV-DNA level reduced to 234,644,954.5-copies/ ml in HBV-DNA positive cases. The serological and molecular viral marker profile is summarized in table-2.

Histological profile
The scoring was done as per modified Knodell-Ishaak (KI)

	Pre-treatment profile				Post-Treatment Profile			
	upto 40	**41-80**	**81-160**	**> 160**	**0-40**	**41-80**	**81-160**	**>160**
AST (normal upto 40 IU/L) n=66	32	20	06	08	40	14	07	05
ALT (normal upto 40 IU/L) n=66	22	20	11	13	36	15	08	07
Table-1: AST and ALT profile								

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system, 34 (51.5%) cases were in the subgroup 0-4, 21 (31.2%) in the subgroup 0-8 and 11 (16.6%) in the subgroup more than 8. Amongst the category with KI score of 0-4, post therapy liver biopsy was done in 13 cases of which six cases improved, 04 cases remained the same whereas 03 cases worsened. Amongst the category with KI score 5-8; post therapy liver biopsy was done in 07 cases of which 06 cases improved whereas 01 case worsened. In the category with KI score of more than 8, post therapy liver biopsy was done in 05 cases of which 04 cases improved whereas 01 case worsened. Thus, overall 16 (64%) cases improved, 05 (20%) cases worsened whereas 04 (16%) remained static (table-3). Statistical correlation by Wilcoxan assigned ranks test shows a Z value of – 1.595 and p value of 0.111.

Immunohistochemistry profile
1. HBsAg: Two patterns of staining were noted - Cytoplasmic (Figure-1A) and cytoplasmic + Membranous (Figure-1B). Cytoplasmic positivity was seen in 23 (48.9%) of cases. The mean KI score in these cases was 3.6/22. Cytoplasmic and Membranous positivity was seen 20 (42.6%) cases with mean KI score of 5.3/ 22. These cases also had high viral load with mean HBV-DNA levels of 902,947,705.7-copies/ml. Negative staining for HBsAg was seen in 4 (8.5%) cases.

2. **HBcAg: Three patterns of staining were noted**
    (a) Nuclear staining (figure-1C): This pattern was seen in 13 (50%) cases that were HBeAg positive. HBeAg negative cases did not show this pattern.
    (b) Nuclear and cytoplasmic pattern (figure-1D): This pattern was observed in 10 (37.5%) HBeAg positive cases and was associated with high levels of HBV-DNA (mean=1,349,657,212.7 copies/ml). These cases had a mean KI score of 8/22. This pattern was not seen in HBeAg negative cases.
    (c) Only cytoplasmic pattern of staining was seen in 02 (7.6%) HBeAg negative cases. Mean KI score was 4.3/22. This pattern was not seen in HBeAg positive cases. 03 (12.5%) HBeAg positive cases and 19 (92.4%) HBeAg negative cases did not stain for HBcAg.

DISCUSSION
Focus of hepatitis B research is development of more effective therapies aimed at inhibiting HBV-DNA synthesis and in eliminating ccc DNA.[1,10]

Biochemical profile
In this study, best response was seen in cases with pre-treatment ALT more than 160 IU/L where out of 13, 06 (46.2%) cases normalized and 07 (53.8%) improved but did not normalize. In this subgroup, 07 cases were HBeAg positive before therapy, all became HBeAg negative with therapy. ALT levels were higher in HBeAg positive cases compared to negative cases with higher necroinflammatory score but they responded well to treatment. Normalization of the ALT level during therapy is thought to reflect improvement in necroinflammation. Serial measurement of ALT levels may also reveal emergence of antiviral resistance.[15,16] Assessing treatment response using serum ALT has limited predictive value as in early trials of lamivudine

	Pretreatment profile	Post-treatment profile	
		Positive	Negative
HBV-DNA n=66	Positive	61	23	38
	Negative	05	01	04
HBeAg n=66	Positive	42	07	35
	Negative	24	00	24
IgM anti-HBc n=52	Positive	19	09	10
	Negative	33	02	31
HBsAg n=66	All Positive		52	14

Table-2: Serological and molecular viral marker profile.

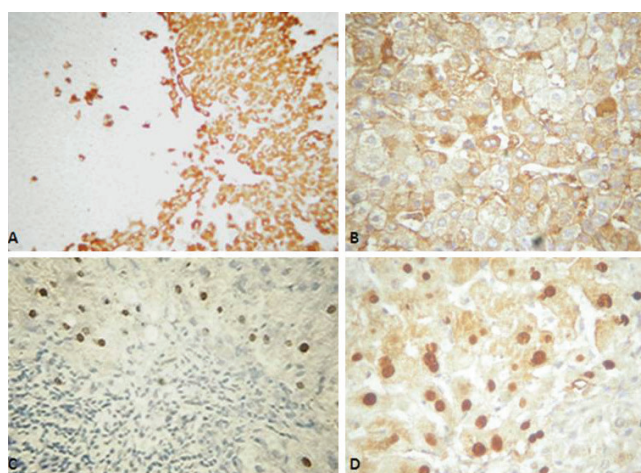| n=25	Improved (≥2 pts)	Same	Worsened
0-4	06	04	03
5-8	06	nil	01
>8	04	nil	01

Table-3: Histological response

Figure-1: Cytoplasmic (A) and cytoplasmic + membranous (B) pattern of HBsAg immunohistochemistry staining. Nuclear (C) and nuclear + cytoplasmic (D) pattern of HBcAg immunohistochemistry staining.

therapy not all patients who achieved biochemical response (70%) had improvement in necroinflammatory score (56%).[17,18] In a recent study of more than thousand CHB patients, significant number of patients with persistently normal ALT levels (<40 IU/L) had significant fibrosis or inflammation on biopsy.[19] The decrease in response rates with time can occur due to accumulation of YMDD mutants (Substitution of Isoleucine for Methionine at position 552) a virological breakthrough, which are always persistent.[20]

Serological profile
In our study, HBsAg seroconversion was seen in 16 cases. HBsAg seroconversion is most durable treatment endpoint but correlates poorly with therapy.[10] It occurs in less than 2% of patients taking nucleoside analogues for one year and 3 to 8% of patients receiving interferon or peg interferon.[11] As HBsAg seroconversion is more durable than HBV DNA suppression alone treatment cessation is possible only after this has been achieved.

In our study, HBeAg seroconversion was seen in 35 (83.3%) out of 42 cases. HBeAg has been advocated as an indicator of active underlying liver disease and high degree of infectivity. In contrast, the clearance of HBeAg from sera is associated

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with reduction in viral replication and normalization of transaminases.[16,21] Long term lamivudine therapy even after HBeAg seroconversion has shown additional benefit where relapse rate following cessation of therapy were 13% at one year and 16% at two years, suggesting that long-term therapy might increase the durability of response.[22]

Cases, which were HBeAg negative before therapy, continued to remain so in our study. None of the large prospective studies have reported any case becoming HBeAg positive with therapy who were negative before treatment.[10,23]

IgM anti-HBc is an indirect marker for acute phase of hepatitis and in the "window period", it is the only marker available for detection of HBV infection.[1,24] In our study, we found that among IgM anti-HBc positive cases, 09 (47.3%) continued to remain positive. Among IgM anti-HBc negative cases, 02 (6%) became positive. Thus, total 11 cases were positive after therapy. Out of these, liver biopsy was done in 03 cases, all of whom showed worsening of KI score compared to pre-treatment KI score.

Colleredo et al using semi-quantitative assessment of IgM anti-HBc showed that antibody titer below 0.2 has 75% predictive of a mild necroinflammatory activity and rules out severe activity (29% sensitivity and 91.6% specificity) whereas antibody titer between 0.2 to 0.5 and more than 0.5 was associated with moderate and severe necroinflammatory activity, respectively. They also concluded that although necroinflammatory activity correlates with IgM anti-HBc levels, stage of fibrosis was unrelated to IgM anti-HBc antibodies.[25] Quantitative hepatitis B core antibody level may be a novel biomarker for predicting treatment response in HBeAg-positive patients receiving therapy.[24]

Molecular viral marker profile

HBV-DNA is the hallmark of active viral replication as it has been found in the liver biopsies of cases which were HBsAg and HBeAg negative serologically. Molecular hybridization techniques have demonstrated HBV-DNA in liver biopsies in cases, which were anti-HBe positive and HBsAg negative.[26]

In our study, HBV-DNA seroconversion was seen in 38 (62.3%) HBV-DNA positive cases. In cases which were HBeAg and HBV-DNA positive before therapy, 23 (59%) cases became HBV-DNA negative whereas 16 (41%) cases remained HBV-DNA positive. Amongst cases, which were HBeAg negative but HBV-DNA positive before therapy, 15 (68.2%) cases became HBV-DNA negative whereas 07 (31.8%) cases continued to remain HBV-DNA positive. Thus overall, we found HBV-DNA seroconversion of 62.3%.

Serum HBV DNA level estimation at various time points during therapy play an important role in determining the course of therapy. Recent studies suggest that initial viral kinetics during therapy can predict the sustained virological response in CHB.[27]

Regarding cases, which became HBeAg negative but remained HBV-DNA positive are those which are associated with circulating HBV genomes harboring mutations in the precore promoter i.e. A to G substitution at position 1896.[28]

A major problem of anti-viral therapies is the emergence of drug resistance conferred by mutations in the YMDD motif of HBV-DNA reverse transcriptase. The prevalence of YMDD mutations increases with longer duration of antiviral therapies and this has been detected in 20% of immunocompetent patients per year of treatment.[20,28] In our study, HBV-DNA positivity post-therapy is

also probably due to emergence of mutant strains.

Histological profile

We used modified Knodell-Ishaak scoring system which includes interface hepatitis and bridging necrosis, lobular inflammation, portal inflammation and fibrosis. Histological response is defined as an improvement in the histology activity index (HAI) of 2 points or more or improvement in the fibrosis score.[10] Post therapy, the improvement was seen in interface hepatitis (figure-2 A,B), lobular inflammation (figure-2 C, 1D) as well as in portal inflammation (figure-3 A,B). Of the cases that improved, extent of fibrosis also improved in 07 (43.7%) cases (figure-3 C,D).

In HBeAg positive and HBV DNA positive cases, improvement was seen in 10 cases, worsening in 04 cases while 01 case remained static. Amongst the cases, which worsened histologically, 03 cases continued to remain HBV-DNA positive after therapy whilst HBeAg seroconversion was seen in all cases. 02 of the cases, which worsened after treatment, were found to be HIV positive. Other two cases, which worsened
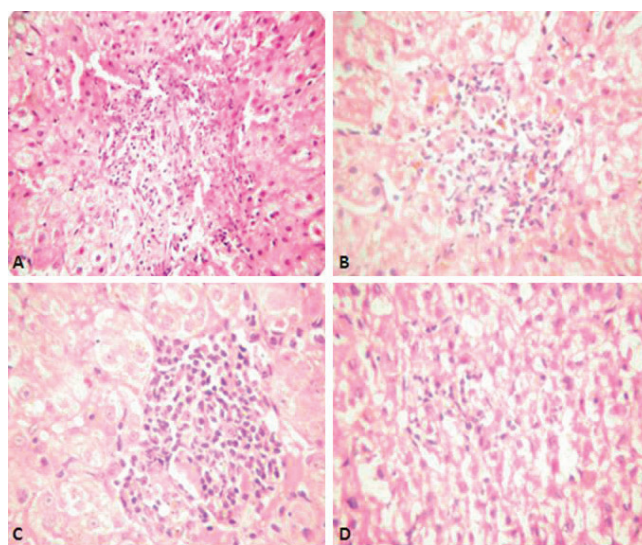

Figure-2: Improvement in interface hepatitis (A-pre-treatment; B-post treatment) and lobular inflammation (C-pre-treatment; D-post treatment) with therapy.
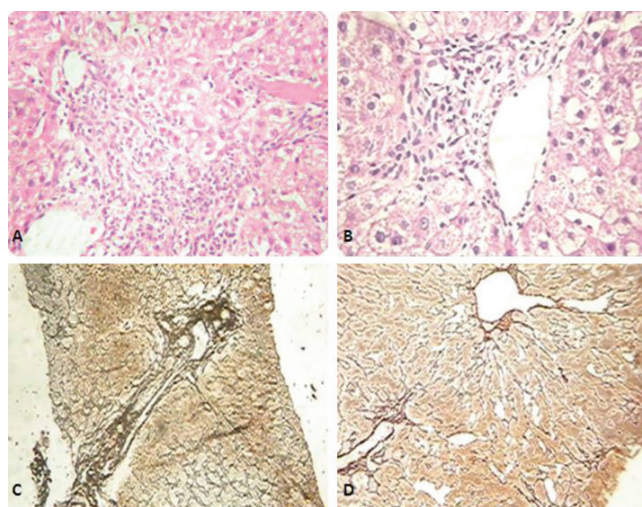

Figure-3: Improvement in portal inflammation (A-pre-treatment; B-post treatment) and fibrosis (C-pre-treatment; D-post treatment) with therapy.

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histologically, may be cases of some other chronic infection or reaction to drugs. The case, which became HBV-DNA negative with therapy but worsened histologically, might be harboring HBV-DNA mutants, which could not be detected during routine screening using conventional primers.

In cases, which were HBeAg negative but HBV DNA positive before treatment, improvement was seen in six cases, worsening in 01 case and 03 cases remained static. The case, which worsened, was HBV-DNA positive after therapy.

Immunohistochemistry profile

We found that cytoplasmic + membranous pattern for HBsAg and nuclear +cytoplasmic pattern for HBcAg was associated with high viremia and increased necroinflammatory activity.

03 HBeAg positive and 19 HBeAg negative cases did not show positive staining for HBcAg. This can be explained on the basis of sequencing analysis of integrated viral DNA which suggested that the HBsAg gene remains intact whereas the HBcAg gene gets either deleted or rearranged resulting in impaired synthesis of HBcAg in the liver with integrated HBV-DNA.[29]

Ramalho et al found significant correlation between intrahepatic HBcAg expression with HBeAg and HBV-DNA (p<0.001) with highest levels of HBV-DNA found in the cases with nuclear and cytoplasmic pattern of staining (mean= 10^6 viral genomes/ml). They also found significant link between HBV-DNA and membranous pattern of HBsAg staining (p=0.001).[30]

In our study, lack of HBcAg staining in HBeAg negative cases can be explained on the basis that the accumulation of viral nucleocapsid antigen in the cytoplasm is caused by defective maturation and/or secretion of this antigen. According to pathogenetic theory, the immune response to HBeAg (membrane bound nucleocapsid antigen) is responsible for liver damage, while the immune response to free HBcAg has no apparent antiviral effect since the nucleocapsid is always masked within the HBsAg envelope of the virion.[30] In HBeAg negative carriers with precore mutations, there is a less severe liver damage than those with wild type mutations.[31] Furthermore, the data suggests that HBcAg expression is not associated with integrated form of HBV-DNA and HBsAg staining can be seen in integrated as well as episomal forms of HBV-DNA.[29]

CONCLUSION

Based on above findings, we conclude that HBV-DNA seroconversion is the single most reliable marker for assessing therapy-induced response. HBeAg seroconversion is a good marker for predicting therapy response but is not as reliable as HBV-DNA seroconversion. Presence of IgM anti-HBc after therapy signifies continuing necroinflammatory activity. Pretreatment high ALT levels along with HBeAg positivity predict a successful therapeutic response. Histological profile shows improvement with therapy, although the overall histological response was not statistically significant in our study.

In IHC, membranous + cytoplasmic pattern of HBsAg and nuclear + cytoplasmic pattern of HBcAg signifies high viral load and marked necroinflammatory activity.

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