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# Immunostaining for Hepatitis B Viral Antigens in Liver:

# Association with Clinical, Biochemical and Virologic Features of Disease

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Short title: Correlates of Staining for Hepatitis B Antigens

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**Abstract**

Background and Aims: Staining for hepatitis B viral antigens is often done in liver biopsies from patients with chronic hepatitis B but its correlates with clinical phenotypes are not well described.

Methods: Biopsies were collected from a large cohort of adults and children with chronic hepatitis B viral infection through the Hepatitis B Research Network. Immunohistochemical staining of sections was done for hepatitis B surface antigen (HBsAg) and hepatitis B core antigen (HBcAg) and then centrally read by the pathology committee. The degree of liver injury and pattern of staining were then correlated with clinical characteristics, including the clinical phenotype of hepatitis B.

Results: Biopsies from 467 subjects were studied, including 46 from children. Immunostaining for HBsAg was positive in 417 (90%) with scattered hepatocyte staining being the most common pattern. HBsAg staining correlated best with serum levels of HBsAg and HBV DNA; the absence of HBsAg staining was often a prelude to loss of HBsAg from serum. HBcAg staining was positive in 225 (49%) and, while cytoplasmic staining was more frequent than nuclear staining, both nuclear and cytoplasmic positivity were often seen in the same specimen. Staining for HBcAg correlated both with level of viremia and liver injury. No biopsies from inactive carriers had stainable HBcAg while 91% of the biopsies from those with HBeAg positive chronic hepatitis B stained positively for HBcAg.



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Conclusion: Immunostaining for HBV antigens may yield helpful insights into liver disease pathogenesis but appears to add little to commonly used serological and biochemical blood tests.

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**Lay Summary**

Liver biopsy remains a useful tool in assessment and management of patients with chronic hepatitis B and is often done to assess the severity or stage of liver disease. This study looks at the value of the special stains often done for viral antigens and finds that they correlate very well with biomarkers found in serum, suggesting that these special staining methods, although useful in histologic evaluation and diagnosis, do not add much to the overall assessment of the patient with known HBV infection.



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**Introduction**

Immunohistochemical staining for hepatitis B viral antigens has long been used as a part of the assessment of liver biopsies from individuals with chronic hepatitis B viral (HBV) infection (1). Both hepatitis B surface antigen (HBsAg) and hepatitis B core antigen (HBcAg) can be readily recognized within liver tissue using immunohistochemical methods, including immunoperoxidase staining (2). The role of immunohistochemistry in clinical assessment and classification of patients with chronic HBV infection remains uncertain (3-7); one factor responsible for this uncertainty is the bias associated with obtaining a liver biopsy. Individuals with persistently normal serum aminotransferase levels are less likely to undergo liver biopsy compared to those with active liver disease. Furthermore, few case series of HBV infection include large numbers of individuals with associated histologic or immunohistochemical data.

We describe here the findings from a cohort of persons with chronic HBV infection undergoing liver biopsy at or around the time of detailed clinical, biochemical and virologic evaluation. These subjects were enrolled in prospective studies conducted by the Hepatitis B Research Network (HBRN) which included adults and children as well as persons from diverse racial and ethnic backgrounds, living in North America (8). The biopsies were all stained in a single, central laboratory and interpreted by consensus of a working group of experienced liver pathologists blinded to clinical, biochemical and virologic results.



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**Patients and Methods**

***Patient Cohort***: The HBRN is an NIH-funded clinical research network dedicated to studying HBV infection in North America (8) and consists of a consortium of 11 medical centers in the United States and Canada with a central data coordinating center. Subjects with HBV infection were enrolled into a prospective natural history study (9) or into one of three treatment studies, two in adults and one in children (10-12). For this substudy, subjects were included if they had chronic HBV infection defined by the presence of HBsAg or HBV DNA for at least 6 months and a liver biopsy specimen available for review. Subjects were excluded if they had acute HBV infection or HIV coinfection at enrollment or if they had been on antiviral treatment for hepatitis B within 4 weeks prior to the liver biopsy being done. Other reasons for exclusion of subjects were pregnancy, history of hepatic decompensation, HCC, history of solid organ transplantation, bone marrow transplantation or the use of chronic immunosuppressive therapy. Biopsies were excluded from this analysis if the sample was thought to be inadequate by the pathologist (D.E.K.) or if relevant clinical laboratory values were not available within 24 weeks of the biopsy date.

Subjects with any other concomitant liver disease, including hemochromatosis or hepatitis C or D were excluded. The presence of non-alcoholic fatty liver disease (NAFLD) with steatosis and/or mild to moderate steatohepatitis was considered acceptable for enrollment in the treatment trials but NAFLD with severe steatohepatitis was exclusionary, but the baseline



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liver biopsy done to determine this was included in this analysis of the immunohistochemical staining. Any further details of the inclusion and exclusion criteria are provided in the publications describing the results from the Cohort Study (9) and the various treatment trials (references 10-12) and on ClinicalTrials.gov (NCT01263587, NCT01369212 and NCT01263600).

Subjects underwent an initial evaluation and were then seen regularly in follow up as part of a series of planned, prospective studies, as described above. Liver biopsy was not a requirement for entry into the natural history study but was required for a treatment trial of patients with immune active chronic hepatitis B with either tenofovir alone or the combination of tenofovir and peginterferon alfa-2a. Liver biopsies were collected from other subjects enrolled in the natural history study who had biopsies done for routine clinical care, based on usual clinical practice.

***Histologic Evaluation:*** The first available liver biopsy on each subject that was deemed adequate in size and quality was used for this study. Biopsies taken less than 4 weeks after antiviral treatment were excluded in this analysis. If more than one biopsy were available, the earliest one was used provided there were clinical and serological data available within 24 weeks. For subjects with only one liver biopsy specimen, the clinical data used were the closest within a 24 week window around the time of the biopsy.

Unstained sections from liver biopsies were sent to a central pathology laboratory based at the University of Pittsburgh for routine and special staining. A section from each biopsy was



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stained respectively with hematoxylin and eosin, Masson Trichrome and for HBsAg and HBcAg by immunoperoxidase staining. Biopsies were then read centrally by the HBRN pathology committee who scored histological features including steatosis, necroinflammatory activity and fibrosis by consensus using the Ishak histological activity index (HAI) system (13,14). Histologic readings were done in a blinded fashion. Immunohistochemical staining was also evaluated in a systematic fashion, with estimates of the proportion of hepatocytes staining positively as well as the pattern of staining. Patterns of HBsAg were recorded as membranous, inclusion-like, or granular, which could be as scattered single cells or contiguous stained regions. These patterns could be present either alone or in combinations. HBcAg was recorded as nuclear, cytoplasmic or both (Supplementary Figure 1). The proportion of hepatocytes staining positively was estimated as: none, <10%, 10-50%, 50-90%, or >90%, but for purposes of simplicity in the current analysis they were grouped in three categories as: none, <10% or ≥10%.

***Serum Biochemical, Serologic and Virologic Testing:*** Serological testing for markers of HBV infection was done centrally, as previously described (15). Briefly, HBV DNA levels were determined using a real time PCR assay (COBAS Ampliprep/COBAS TaqMan HBV Test, v2.0; Roche Molecular Diagnostics, Branchburg, NJ). HBV genotype was determined by mass spectrometry at the Molecular Epidemiology and Bioinformatics Laboratory in the Division of Viral Hepatitis at the Centers for Disease Control and Prevention (CDC) with mass spectrometry (16) while quantitative HBeAg and HBsAg levels were measured by Elecsys HBeAg II Quant and



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Elecsys HBsAg II Quant assay, respectively (Roche Molecular Systems, Inc) (17,18). The lowest detectable value for HBV DNA was 10 IU/mL, for HBeAg was 0.3 IU/mL and for HBsAg was 0.05 IU/mL; the lowest quantifiable value for HBV DNA was 20 IU/mL. If not available from the central laboratory, local data were used. Serum biochemical testing including serum aminotransferase and bilirubin levels was done at local laboratories.

***Clinical Phenotypes:*** Clinical phenotype was determined based upon HBeAg status, serum HBV DNA concentrations, and ALT levels, categorizing patients as having chronic hepatitis B (HBeAg positive or negative), immune tolerant disease, the inactive carrier state or as one of three indeterminant categories as previously described (19,20) (see Table 1). Briefly, the indeterminant A group were seropositive for HBeAg but had low levels of HBV DNA in serum. The indeterminant B phenotype was defined by lack of HBeAg and low levels of HBV DNA but raised ALT values (>30 U/L for males and >20 U/L for females) while the indeterminant C cohort comprised individuals who lacked HBeAg, had normal ALT levels but moderate-to-high concentrations of HBV DNA (19,20).

***Informed Consent:*** All details of the protocols for the cohort study and 3 clinical trials were approved by the Institutional Review Boards at each site as well as by a central Data Safety and Monitoring Board chosen by the NIDDK to monitor the study. All subjects gave written, informed consent for participation including consent to have sections of liver biopsies



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done as a part of routine patient care to be sent to the central repository. In the case of minors, consent was given by their parents or legal guardians, and where possible the assent of the child was also obtained.

**Statistical methods**

Continuous variables across grades of HBsAg or HBcAg staining were compared using non-parametric analysis of variance (Kruskal-Wallis test) for continuous variables, while categorical variables were compared by using Chi-square test or the Fisher's exact version (in the case of small cell frequencies). Patterns and semiquantitative measures of HBsAg and HBcAg staining across clinical phenotypes were compared using exact Chi-square test. The rates of HBsAg loss (and the same for HBeAg loss among HBeAg positive subjects) were calculated by taking the length of follow-up time into account using a Poisson rate approach. These rates were compared across groups using Wald test or using Fisher's exact test, as appropriate. The results are presented as estimated rates, 95% confidence intervals and p-values. All the analyses were conducted using statistical software package SAS (version 9.4, SAS Institutes, Inc.)

**Results**



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Of 2,491 participants in the HBRN Cohort study or the immune active treatment trial, 467 (19%) had liver biopsy data available and were included in this analysis. The cohort comprised 421 adults (including 194 upon entry into a treatment study [reference 12]) and 46 children (<18 years at the time of biopsy) and their demographic characteristics are shown in Table 1. Most patients were Asian (81%) while Whites (10%) and Blacks (9%) were in the minority.

The most frequent clinical phenotype was chronic hepatitis B (281, 72%), either HBeAg positive (149, 38%) or negative (132, 34%), largely because these subjects were required to have liver biopsies for enrollment in the immune active trial. Nevertheless, the cohort included a proportion of subjects with immune tolerant hepatitis B (7, 2%) and some inactive carriers (18, 5%), groups who are not typically biopsied. The group of indeterminant phenotypes was relatively large (82, 21%) with indeterminant category B (HBeAg negative, low HBV DNA but elevated ALT levels) being most frequently represented (65, 17%).

The virologic characteristics of the group are summarized in Table 2: 57% were seropositive for HBeAg, 88% had detectable levels of HBV DNA with levels ranging widely (from <20 IU/mL to greater than $1.7x10^8$ IU/mL). The HBV genotype could be determined in 414 subjects, the most common being genotypes B (40%) and C (36%). Sequencing demonstrated viral variants affecting viral replication in a substantial proportion including 33% with the



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G1886A mutation in the pre-core region and 45% with one of two common variants in the basal core promoter.

Histologic and immunohistochemical features among the 467 patients with liver histology are summarized in Table 3. Few subjects had very severe liver disease histologically – thus only 6% had cirrhosis (Ishak fibrosis stage 5 or 6) and only 4% had HAI of 13 or more (Table 3). In fact, the majority had minimal (57%) or no fibrosis (22%) and 43% had minimal necroinflammatory activity (HAI <5).

HBsAg staining

Immunostaining revealed HBsAg positivity in 90% of biopsies, most commonly (52%) affecting at least 10% of hepatocytes. Clinical, biochemical and virologic features of patients with different proportions of HBsAg positive cells in liver are shown in Table 4. Patients without HBsAg immunostaining in liver tended to be older and to have lower levels of serum HBsAg and HBV DNA. The pattern of HBsAg staining was often mixed. The most frequent pattern was hepatocytes with granular cytoplasmic staining (94%) and because this pattern was ubiquitous, it was not further analyzed. Inclusion-like staining, as is typical of ground glass hepatocytes, was present in 72%, contiguous granular staining in 61%, and membranous staining in 49%.



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The frequency and pattern of HBsAg staining did not clearly separate the various clinical phenotypes of HBV infection (Table 5) although HBeAg positive chronic hepatitis B most frequently had membranous staining (70% vs 37% all other phenotypes) and inactive HBsAg carriers most frequently had no HBsAg staining (28% vs 7% all other phenotypes).

HBcAg staining

Immunostaining revealed HBcAg positivity in 49% of biopsies. Of these, HBcAg was noted in <10% of hepatocytes in 54%, while only 4% had >90% HBcAg staining. Two distinct patterns of HBcAg staining were noted – cytoplasmic staining (78%) and nuclear staining (85%) with both being present in 63% of the biopsies where it was present.

Selected clinical, biochemical and virologic associations with HBcAg staining are shown in Table 6. There were significant associations of HBcAg staining with lower age, higher serum HBV DNA, higher HBsAg and higher HBeAg as well as higher ALT levels (see Table 6 and Figure 1). The presence of either the G1896A pre-core variant or a basal core promoter variant was associated with a lower frequency of HBcAg staining positivity. Seropositivity for HBeAg was strongly associated with high levels of HBcAg staining (see Table 6). This correlation was consistent for all HBV genotypes (Supplementary Figure 1). Analyses of HBcAg staining frequency and pattern in relation to clinical phenotypes are shown in Table 7. HBcAg staining was present in most patients with HBeAg positive chronic hepatitis B (91%), frequently in >10%



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of hepatocytes (51%). In contrast, HBcAg staining was not detected in any of the inactive carriers and was infrequent in patients with indeterminant phenotypes (11% to 29%) and in HBeAg negative chronic hepatitis B (28%). When detected in HBeAg negative subjects, HBcAg staining was detected in ≥ 10% of cells in only 3%. Interestingly, of seven subjects with "immune tolerant" HBV infection (HBeAg with high HBV DNA but normal serum aminotransferase levels) only four had detectable HBcAg and the staining was above 10% in all four. HBcAg staining was not correlated with HBV genotype (see Supplementary Figure 2).

Immunohistochemical associations with loss of HBsAg from serum

Of the 307 participants who were seropositive for HBsAg at the time of biopsy and were followed with subsequent serum HBsAg testing, 22 went on to lose HBsAg from serum (in 15 of these HBsAg loss occurred at some point in time after initiation of antiviral therapy, in most cases in the setting of a prospective, randomized controlled trial [12]), a mean of 3.4 years after the biopsy (range 0.6 to 6.3 years). The overall calculated rate of loss of HBsAg was 1.5 per 100 patient years (95% C.I. 1.0 to 2.3). Interestingly, the frequency of HBsAg loss was significantly higher among those where HBsAg was not stainable in the liver compared to those where it was detected (5.2 [95% C.I. 2.3-11.4] versus 1.2 [95% CI 0.7-1.9] per 100 person-years, p value 0.002).



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Immunohistochemical associations with loss of HBeAg from serum

Of the 139 subjects who were seropositive for HBeAg at the time of their liver biopsy and were followed with subsequent HBeAg testing, 72 went on to lose HBeAg, (in 66 of these HBeAg loss occurred at some point in time after initiation of antiviral therapy, in most cases in the setting of a prospective, randomized controlled trial [12]), a mean of 2.1 years (range 0.1 to 5.3 years) after the liver biopsy. The frequency of HBeAg loss was statistically significantly higher among those where HBcAg was not stainable in the liver compared to those where it was present (31.3 [95% C.I. 17.3-56.4] versus 16.1 [95% CI 12.5-20.6] per 100 person-years, p value 0.04).

Immunohistochemical associations with the presence of hepatic steatosis

Significant hepatic steatosis (>5% of hepatocytes) was noted in 126 of 461 biopsy specimens. Of those with steatosis, 110 (87%) had demonstrable HBsAg staining while, of those without significant steatosis, 307 (92%) had demonstrable HBsAg staining. There were no differences noted in the HBsAg staining pattern in those with and without steatosis. Similarly, of those with steatosis, 53 (42%) had demonstrable HBcAg staining while of those without significant steatosis 174 (52%) had demonstrable HBcAg staining. There were no differences noted in the HBcAg staining pattern in those with and without steatosis.



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The presence of hepatic steatosis was of particular interest in subjects with the Indeterminant B phenotype (characterized by absence of HBeAg and low levels of HBV DNA in serum, but raised ALT values). Of 82 subjects with an Indeterminant phenotype, 30 (37%) had some degree of steatosis. Of 65 subjects specifically with Indeterminant B phenotype, 22 (42%) had some degree of steatosis. Using an exact test, there was no statistically significant difference in the presence or absence of steatosis across the Indeterminant phenotypes (p=0.23). With regard to the degree of hepatic steatosis among those with Indeterminant B, it was present in up to one third of hepatocytes in 41%, between one and two thirds in 48% and in greater two thirds in 11%. Again, these proportions were not statistically significantly different compared to other indeterminate phenotypes (p=0.35).

**Discussion**

This study cohort was from North America, was racially and ethnically diverse and included both adults and children. Overall, the liver disease in this cohort was relatively mild but it did encompass the range of disease activity and stages from active liver disease to inactive carriers, no fibrosis to cirrhosis, low to high levels of virus, normal to markedly raised ALT levels.

This analysis found that liver biopsies from most subjects stained positively for HBsAg (90%) while only about half stained positively for HBcAg. Inactive carriers were the group least



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likely to stain positively for HBsAg, and those who stained negatively for HBsAg were more likely to go on to clear HBsAg from serum. This indicates that although HBsAg staining is almost universal, the lack of staining is a favorable feature consistent with inactive carrier status and greater likelihood of clearing HBsAg from serum. HBcAg staining was well correlated with levels of viremia and the highest degrees of staining were largely found in patients with high levels of circulating viremia – for example patients with HBeAg positive chronic hepatitis B almost all had positive HBcAg staining in the liver. Among inactive carriers, all of whom (by definition) had less than 1000 IU/mL of HBV DNA in serum, none had detectable HBcAg staining in liver.

Interestingly HBcAg staining was positively associated with serum ALT levels, whereas HBsAg staining was not. This difference is supportive of the hypothesis that liver injury in chronic hepatitis B is mediated by cytotoxic T cell injury directed against hepatocytes expressing HBcAg (21, 22) and is supported by the finding of a lack of correlation between HBsAg staining and liver injury assessed by either HAI or fibrosis but a positive (albeit weak) correlation between HBcAg staining and HAI and fibrosis. Viral variants affecting the pre-core region of the genome or the basal core promoter, when present, were associated with lesser degrees of expression of intra-hepatic HBcAg, consistent with current thinking that these variants replicate less efficiently and are generally associated with lower levels of viremia.

In addition to the presence or absence of staining for viral antigens, the pattern of that expression was also examined and while there were some rough correlations identified, in this



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large and diverse cohort, sufficient variability was found to negate some of the prevailing hypotheses about the significance of various patterns of staining. For example, Chu and Liaw (23) found that membranous staining of HBsAg on the hepatocyte correlated with serum HBV DNA levels and could be recognized as a marker of active hepatitis B virus replication. It has also been suggested that membranous staining of HBsAg might possibly correlate with binding of circulating HBV virions on the cell surface rather than expression of viral proteins on the cell membrane of infected cells. However, in this cohort, membranous staining was present in about half of cases staining positively for HBsAg and although this pattern of staining was associated with the presence of HBeAg, it was also noted in substantial proportions of cases with lower levels of viral replication using serum markers (see Tables 3 and 4). The inclusion type of staining which is more or less the equivalent of ground glass hepatocytes was least frequent among inactive carriers. Ground glass hepatocytes represent a histological hallmark of chronic hepatitis B virus infection and contains surface antigens in the endoplasmic reticulum (24) which stain positively for HBsAg with an inclusion-like pattern and it has been suggested that this may reflect pre-S1 mutations with accumulation of HBsAg in the ER, giving the ground glass appearance (24). However, the HBV pre-S sequences of viral isolates from subjects in this study have not been evaluated.

In this cohort, several viral and host factors were found not to be related to the staining pattern for either HBsAg or HbcAg. Thus, HBV genotype did not seem to predict the various



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patterns of HBV antigen expression. The presence of steatosis noted on liver biopsy might have been thought to affect or impair the ability to interpret immunohistochemical staining for viral antigens, but it did not. Although viral variants (mutants) were associated with lower levels of HbcAg staining, probably indirectly through viral replicative efficiency and levels of viremia, this association was not found for HbsAg.

**Summary and Conclusions**

In this large study, HbsAg immunostaining was noted to be positive in most subjects. Its presence and the degree of staining were associated with higher serum levels of HbsAg and HBV DNA but not with disease activity. The absence of HbsAg staining was most common amongst inactive HbsAg carriers and was linked to a higher subsequent loss of HbsAg from serum. In contrast, while HbcAg staining was less frequent than HbsAg, its presence correlated well with higher levels of viremia and measures of worse liver injury. None of the inactive HbsAg carriers had positive liver staining for HbcAg, while almost all of those with HbeAg positive chronic hepatitis B did have positive HbcAg staining.

We conclude that while immunostaining for HBV antigens may yield insights into liver disease pathogenesis, it appears to provide little additional clinical information that cannot be gained through less invasive biochemical, serologic and virologic tests of serum.



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Table 1: Baseline characteristics of 467 subjects with chronic HBV infection and a liver biopsy

| Characteristic | Number Tested | Mean (range) or number (percent) |
|---|---|---|
| Age (years) | 467 | 39 (2-77) |
| | | |
| Sex (male) | 467 | 284 (61%) |
| | | |
| Race | 453 | |
| White | | 49 (10%) |
| Black | | 39 (9%) |
| Asian | | 365 (81%) |
| | | |
| Clinical Phenotype[1] | 388 | |
| Immune tolerant | | 7 (2%) |
| HbeAg pos Chronic Hepatitis B | | 149 (38%) |
| HbeAg neg Chronic Hepatitis B | | 132 (34%) |
| Inactive carrier | | 18 (5%) |
| Indeterminant A[*] | | 7 (2%) |
| Indeterminant B | | 65 (17%) |
| Indeterminant C | | 10 (2%) |

[*]The indeterminant A group comprised subjects who were HBeAg positive but had HBV DNA levels below $10^5$ IU/mL. The indeterminant B phenotype included HBeAg negative participants with low levels of HBV DNA ($\leq 10^4$ IU/mL). The indeterminant C cohort comprised HBeAg negative individuals with normal ALT levels (<30 U/L for male and < 20 U/L for female) but moderate-to-high concentrations of HBV DNA (see reference 19).



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Table 2: Virologic and Serum Biochemical characteristics

| Characteristic | Number tested | Median/Mean (range) or number (percent) |
|---|---|---|
| Median HBV DNA ($log_{10}$ IU/mL) | 410 | 6.0 (<1.3->8.2) |
| HBeAg positive | | 228 (57%) |
| HBeAg negative | | 175 (43%) |
| Median HBeAg (IU/mL) | 146 | 980 (0.4-8180) |
| Median HBsAg (IU/mL) | 263 | 3441 (0.4-325,300) |
| HBV genotype | 414 | |
| A | | 59 (14%) |
| B | | 164 (40%) |
| C | | 151 (36%) |
| Other | | 40 (10%) |
| Viral variants | | |
| Basal core promoter (A1762T or G1764A) | 262 | 118 (45%) |
| Pre-core (G1896A) | 271 | 89 (33%) |
| Median ALT (U/L) | 386 | 61 (10-718) |
| ALT elevated (> 30 U/L for male, >20 U/L for female) | 386 | 348 (90%) |
| Median AST (U/L) | 380 | 42 (11-678) |
| AST elevated | | 203 (53%) |
| Total bilirubin (mg/dL) | 376 | 0.7 (0.1-3.6) |

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Table 3: Histologic and Immunohistochemical Characteristics

| Characteristic | No. | Mean (range) or number | Percentage |
|---|---|---|---|
| Histological activity index | 467 | 5.6 (0-17) | |
| 0-<5 | | 201 | 43% |
| 5-<9 | | 192 | 41% |
| 9-<13 | | 56 | 12% |
| >=13 | | 18 | 4% |
| Mean Fibrosis Score | 467 | 1.6 (0-6) | |
| 0 | | 102 | 22% |
| 1-2 | | 265 | 57% |
| 3-4 | | 69 | 15% |
| 5-6 | | 31 | 6% |
| HBsAg positive staining | 461 | 417 | 90% |
| HBsAg staining pattern* | 417 | | |
| Inclusion-like | | 301 | 72% |
| Scattered hepatocytes | | 391 | 94% |
| Contiguous regions | | 256 | 61% |
| Membranous | | 204 | 49% |
| HBcAg positive staining | 459 | 227 | 49% |
| HBcAg staining pattern | 227 | | |
| Nuclear only | | 49 | 22% |
| Nuclear > Cytoplasmic | | 49 | 22% |
| Cytoplasmic > Nuclear | | 94 | 41% |
| Cytoplasmic only | | 35 | 15% |

*An individual could belong to more than one category.

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Table 4: Selected associations with HBsAg immunohistochemical staining

| Characteristic | 0% (n=44) | <10% (n=177) | >=10% (n=240) | P value |
|---|---|---|---|---|
| Mean age (range) | 45 (4-77) | 40 (2-74) | 38 (3-74) | 0.004 |
| Median $Log_{10}$ HBV DNA (range) | 4.0 (<1.3->8.2) | 5.7 (1.3->8.2) | 6.3 (1.6->8.2) | <0.001 |
| Median qHBsAg (range) | 349 (0.5-91940) | 3690 (0.4-325,300) | 5495 (98-222,500) | <0.001 |
| Median qHBeAg (range) | 355 (3.3-4444) | 1096 (1.3-8179) | 953 (0.4-7950) | 0.46 |
| G1896A Pre-Core variant | 5 (26%) | 34 (34%) | 49 (33%) | 0.81 |
| A1762T or G1764A Basal Core Promoter variant | 11 (61%) | 44 (44%) | 63 (45%) | 0.39 |
| HBeAg Positive (No. and percent) | 6 (20%) | 64 (42%) | 103 (48%) | 0.02 |
| Median ALT (range) | 44 (14-242) | 60 (11-718) | 62 (10-539) | 0.22 |
| Median HAI (range) | 4 (1-14) | 5 (1-17) | 5 (0-16) | 0.04 |
| HAI >13 | 1 (2%) | 5 (3%) | 6 (3%) | 0.97 |
| Median Fibrosis (range) | 1 (0-6) | 1 (0-6) | 1 (0-6) | 0.49 |
| Fibrosis score 0 | 12 (27%) | 36 (20%) | 52 (22%) | 0.13 |
| Fibrosis score 1-2 | 25 (57%) | 104 (59%) | 132 (55%) | |
| Fibrosis score 3-4 | 3 (7%) | 21 (12%) | 45 (19%) | |
| Fibrosis score 5-6 | 4 (9%) | 16 (9%) | 11 (5%) | |



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Table 5: Association between clinical phenotype and various patterns of immunohistochemical staining for HBsAg

| HBsAg staining pattern | Immune Tolerant (n=7) | HBeAg Positive Chronic Hepatitis B (n=147) | HBeAg Negative Chronic Hepatitis B (n=130) | Inactive Carrier (n=17) | Indeterminant A (n=7) | Indeterminant B (n=64) | Indeterminant C (n=10) | P Values |
|---|---|---|---|---|---|---|---|---|
| None | 0 (0%) | 6 (4%) | 7 (5%) | 5 (28%) | 0 (0%) | 11 (17%) | 2 (20%) | <0.001 |
| Membranous | 4 (57%) | 99 (70%) | 47 (38%) | 3 (23%) | 3 (43%) | 18 (34%) | 3 (38%) | <0.001 |
| Inclusion-like | 5 (71%) | 114 (81%) | 88 (72%) | 6 (46%) | 6 (86%) | 33 (62%) | 6 (75%) | 0.04 |
| Contiguous Hepatocyte | 0 - | 73 (52%) | 99 (81%) | 8 (62%) | 5 (71%) | 33 (62%) | 6 (75%) | <0.001 |



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Table 6: Selected associations with HBcAg immunohistochemical staining

| Characteristic | 0% (n=232) | <10% (n=123) | >=10% (n=104) | P value |
|---|---|---|---|---|
| Mean age (range) | 44 (2-77) | 37 (3-74) | 32 (3-74) | <0.001 |
| Median $Log_{10}$ HBV DNA (range) | 4.4 (<1.3– >8.2) | 6.6 (1.6->8.2) | 8.2 (4.8->8.2) | <0.001 |
| Median qHBsAg (range) | 1802 (0.4-325,300) | 2907 (1.1-194,700) | 39,390 (51-256,400) | <0.001 |
| Median qHBeAg (range) | 352 (1.3-2411) | 351 (0.4-6177) | 1486 (27-8179) | <0.001 |
| G1896A Pre-Core variant | 58 (47%) | 21 (30%) | 9 (12%) | <0.001 |
| A1762T or G1764A Basal Core Promoter variant | 67 (57%) | 32 (46%) | 19 (26%) | <0.001 |
| HBeAg status Positive (No. and percent) | 21 (11%) | 66 (60%) | 86 (97%) | <0.001 |
| Median ALT (range) | 46 (10-718) | 72 (14-631) | 84 (21-549) | <0.001 |
| Median HAI | 4 (0-17) | 6 (1-16) | 6 (1-16) | <0.001 |
| HAI >13 | 3 (1.3%) | 5 (4.1%) | 4 (3.9%) | 0.20 |
| Median Fibrosis score | 1 (0-6) | 2 (0-6) | 2 (0-6) | <0.001 |
| Fibrosis 0 | 75 (32%) | 12 (10%) | 13 (13%) | <0.001 |
| Fibrosis 1-2 | 112 (48%) | 78 (63%) | 70 (67%) | |
| Fibrosis 3-4 | 30 (13%) | 22 (18%) | 16 (15%) | |
| Fibrosis 5-6 | 15 (7%) | 11 (9%) | 5 (5%) | |



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Table 7: Association between clinical phenotype and immunohistochemical staining for HBcAg (p Value <0.001)

| HBcAg staining | Immune Tolerant (n=7) | HBeAg positive Chronic Hepatitis B (n=147) | HBeAg negatve Chronic Hepatitis B (n=130) | Inactive Carrier (n=17) | Indeterminant A (n=7) | Indeterminant B (n=64) | Indeterminant C (n=10) |
|---|---|---|---|---|---|---|---|
| None | 3 (43%) | 13 (9%) | 93 (72%) | 17 (100%) | 5 (71%) | 57 (89%) | 8 (80%) |
| <10% | 0 - | 59 (40%) | 33 (25%) | 0 - | 1 (14%) | 7 (11%) | 2 (20%) |
| >=10% | 4 (57%) | 75 (51%) | 4 (3%) | 0 - | 1 (14%) | 0 - | 0 - |
| | n=4 | n=134 | n=37 | n=0 | n=2 | n=7 | n=2 |
| Nuclear only | 1 (25%) | 22 (16%) | 11 (30%) | 0 | 1 (50%) | 3 (43%) | 1 (50%) |
| Cytoplasmic only | 0 | 14 (10%) | 8 (22%) | 0 | 0 | 4 (57%) | 0 |
| Both | 3 (75%) | 98 (73%) | 18 (49%) | 0 | 1 (50%) | 0 | 1 (50%) |



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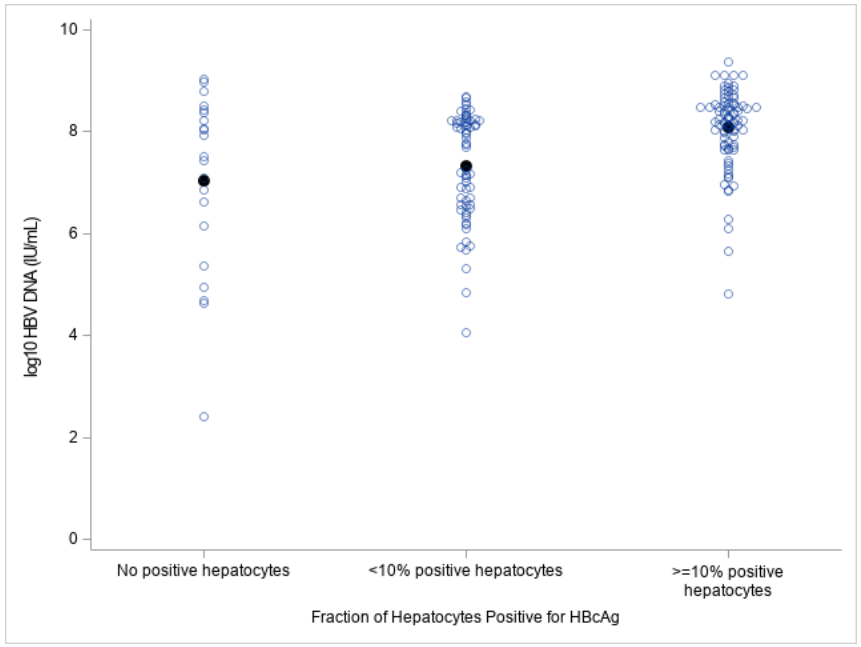
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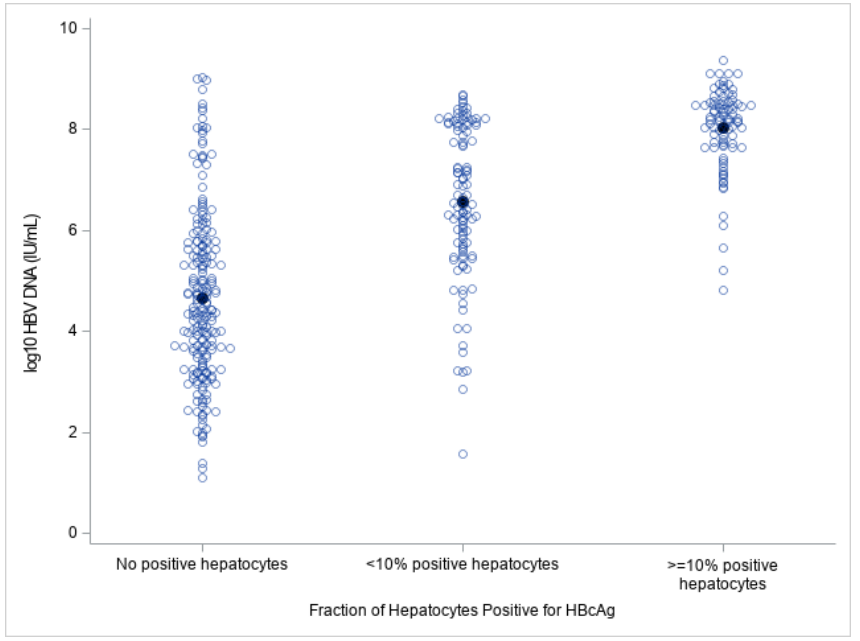
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**Figure 1: Serum HBV DNA by HBcAg staining positivity (Panel A (left) HBeAg positive. Panel B (right) HBeAg negative**




HBV DNA by HBcAg staining positivity among HBeAg positive: p-value<0.001

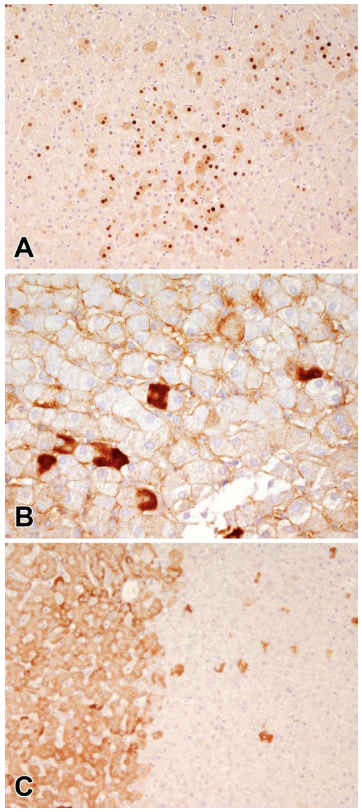



HBV DNA by HBcAg staining positivity among HBeAg negative: p-value<0.001



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**Supplementary Figure 1: Examples of immunostaining patterns.**

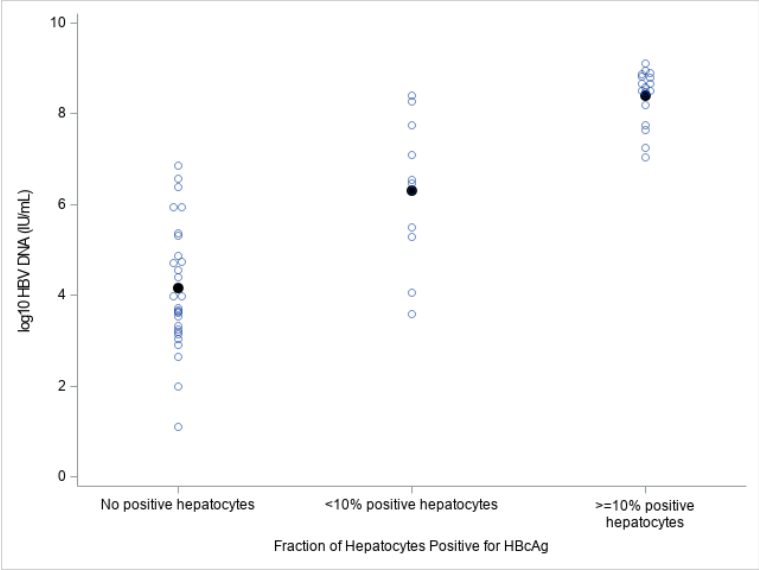
A. Hepatitis B core antigen stain showing hepatocytes with nuclear and cytoplasmic staining. Some cells only show nuclear or cytoplasmic staining (200x).
B. Hepatitis B surface antigen staining showing inclusion-like and membranous staining patterns (400x).
C. Hepatitis B surface antigen staining showing a contiguous region of cytoplasmic staining as well as individual scattered positive cells with granular cytoplasmic staining. (200x).



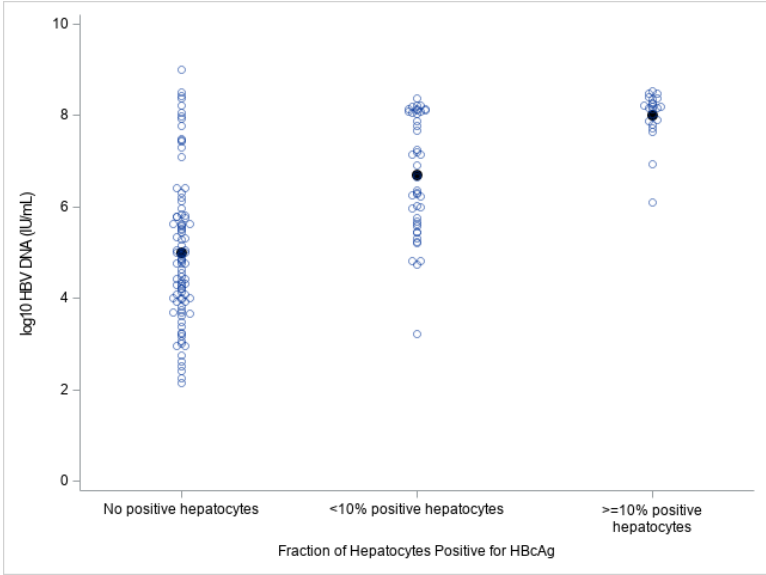
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**Supplementary Figure 2: Serum HBV DNA and HBcAg staining positivity by HBV genotype (panel A, genotype A; panel B, genotype B, panel C, genotype C, panel D, other genotypes)**




HBV DNA by HBcAg staining positivity among genotype A: p-value<0.001

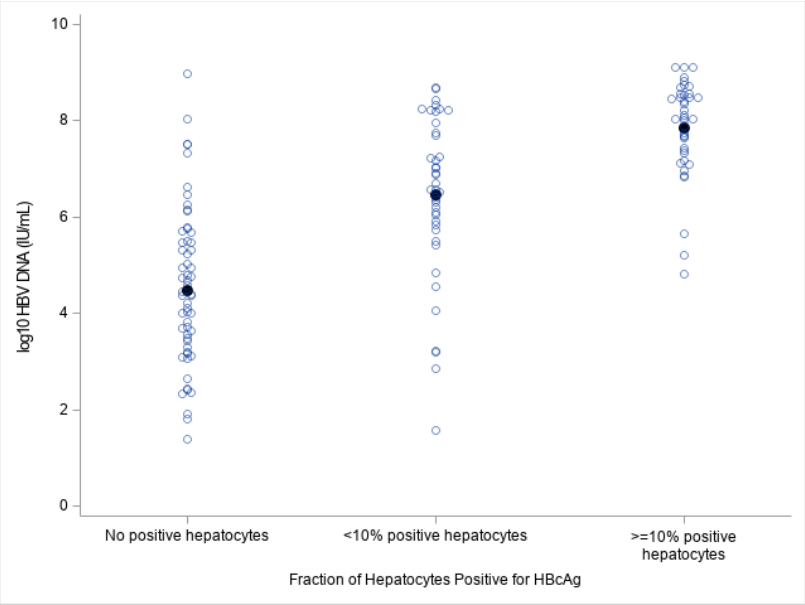



HBV DNA by HBcAg staining positivity among genotype B: p-value<0.001

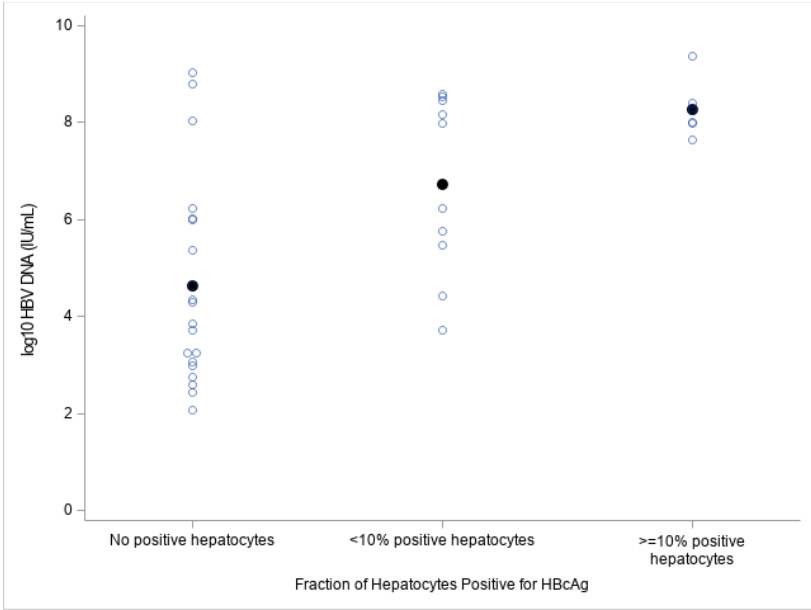


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HBV DNA by HBcAg staining positivity among genotype C: p-value<0.001




HBV DNA by HBcAg staining positivity among other genotypes : p-value=0.002



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