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ORIGINAL ARTICLE

High HBcAg Expression in Hepatocytes of Chronic Hepatitis B Patients in Bangladesh

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ABSTRACT

The present study evaluates the intracellular expression of the hepatitis B Virus (HBV) core antigen in chronic hepatitis B (CHB) patients from Bangladesh in relation with serological, virological and histological variables. A cross-sectional study was carried out among 70 patients incidentally diagnosed as HBV chronic carriers who have undergone liver biopsy. Indirect immunofluorescence technique was used to study the intracellular expression of hepatitis B core antigen (HBcAg). The grade and cellular distribution were semiquantitatively scored according to the percentage of stained cells and their nuclear, cytoplasmic or mixed expression pattern respectively. A higher HBcAg grade was related to higher viral load, alanine aminotransferase levels and histological damage. Higher HBV DNA counts and HBcAg grade were found in hepatitis B e antigen (HBeAg)-positive patients.

Abbreviations: HBV: Hepatitis B virus; CHB: Chronic hepatitis B; HBcAg: Hepatitis B core antigen; HBeAg; Hepatitis B e antigen.

Keywords: Hepatitis B core antigen, Hepatocytes, Chronic hepatitis B, Bangladesh.

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INTRODUCTION

Hepatitis B is one of the most common infectious disease and the major cause of chronic hepatitis, cirrhosis and hepatocellular carcinoma (HCC) in Bangladesh. Over 350 million people are chronically infected with hepatitis B virus (HBV). One million related deaths are produced every year.[1-5]

The detection of hepatitis B core antigen (HBcAg) as an indicator of active viral replication is of great value in Chronic hepatitis B (CHB) patients, especially with Hepatitis B e antigen (HBeAg) mutant variants. It has been suggested that the shift of (HBcAg) detection from nucleus to cytoplasm may enhance the presentation of this target viral antigen and render the infected hepatocytes susceptible to immune attack by cytotoxic T cells.[6]

The characterization of HBcAg expression and cellular distribution patterns has the potentiality to provide additional value to biopsy studies, contributing to the classification of patients as 'true' inactive carriers as well as in the study of patient status after therapy.

The relationship of nuclear/cytoplasmic distribution of HBcAg and their association with serum HBV DNA level in Bangladeshi CHB patients has not been studied yet, in the present study, 70 serologically diagnosed CHB patients were enrolled to study the level and cellular distribution of the HBcAg in hepatocytes of Bangladeshi CHB patients, from both HBeAg-positive and -negative patients.

PATIENTS AND METHODS

Study Design

This cross-sectional study was carried out among 70 patients who were incidentally diagnosed as CHB patients and undergone liver biopsy as part of their routine clinical management. The study was conducted during the period of July 2009 to June 2010.

Subjects

Patients were selected from the Inpatient Department of Hepatology, Bangabandhu Sheikh Mujib Medical University (BSMMU) Hospital and laboratory works were performed at the Department of Virology. Patients who were included in this study had HBsAg positive for at least 6 months, serum HBeAg positive or negative, detectable serum HBV DNA levels, patients who had liver biopsy done as part of their routine clinical management, and patients who gave written consent. Patients with the history of significant alcohol consumption (i.e. >20 gm/day), severe renal disease, heart disease or malignancy, patients with detectable antibodies to human immunodeficiency virus (HIV) and hepatitis C virus (HCV), history of previous antiviral treatment and who did not give written consent were excluded from the study.

Methods

Sample Collection

Under all aseptic precaution a Tru-cut liver biopsy was done by a hepatologist. The samples were fixed in formalin and embedded in paraffin wax for routine histological study and for immunofluorescence staining.

Staining Procedure

Five micrometers of thick tissue sections from paraffin blocks were taken on albumin-coated slides and were incubated at 37ºC overnight for 16 hours. Then after deparaffinization, intrahepatic expression of HBcAg were studied by indirect immunofluorescence using rabbit anti-HBc (Dako, USA), as primary antibody followed by fluorescein isothiocyanate (FITC) conjugated secondary antibody (Polyclonal Swine Anti Rabbit Immunoglobulin/FITC, Dako, USA). All specimens were run with a known positive control. The amount of core antigen in hepatocytes were also semi-quantitatively scored according to the proportion of hepatocytes that stained positive on a 0 to 3+ scale (0%—absent, 1 to 10%—grade 1, 11 to 50%—grade 2, >50%—grade 3).[7] Intracellular localization of core antigen was labeled as nuclear, cytoplasmic or mixed type (mixed but predominantly nuclear and mixed but predominantly cytoplasmic).

Other Methods

The biochemical [alanine aminotransferase (SGPT)], virological (HBV DNA), serological [Serum hepatitis B surface antigen (HBsAg)] and hepatitis B e antigen (HBeAg) tests were conducted at Lab Aid Hospital, Dhaka, by conventional methods. Liver histology was seen by routine H&E staining under light microscope.

STATISTICAL ANALYSIS

Statistical analysis was done by Prism Software, version 4. Results were expressed as percentage and mean error. For comparison between two groups, unpaired Student t-test was conducted, one-way ANOVA with Newman-Kewls post test and Dunn's multiple comparison test or Kruskal-Wallis tests were performed in multiple group comparison. Ninety-five percent confidence interval was used to detect the significant level and 99% to demonstrate very significant level.

RESULTS

The present study was carried out in 70 consecutive chronic hepatitis B patients (positive HBsAg in blood for more than 6 months) and proposed for biopsy at Farabi Hospital, Dhaka, Bangladesh. A total of 58 patients (84%) were males and the mean age of the study population was 30.51 years, where 23 (32.9%) were ≤ 25 years old, 30 (42.9%) were between 25 and 35 years and the remaining 24% were older than 35 years. Among 70 cases, eight (11.43%) were HBeAg positive and 62 (88.57%) were HBeAg negative (Table 1).

Table 1: Demographic characteristics of the patients enrolled in the study

	Frequency	*Percentage*
Age		
≤25	23	32.9
26-35	30	42.9
36-45	12	17.1
>45	5	7.1
Sex		
Male	58	82.9
Female	12	17.1
HBeAg		
Pos.	8	12.9
Neg.	62	87.1

HBcAg Detection in Hepatocytes According to DNA Levels

A total of 55 out of 62 HBeAg-negative cases (88.7%) were positive for core antigen in hepatocytes, 37 among these 55 patients (67.3%) had lower HBV DNA levels ($<10^5$ copies/ml). All eight (100%) HBeAg-positive cases were also positive for core antigen and had higher circulating DNA counts.

HBeAg-negative patients having HBcAg expression in hepatocytes had an increase in viral load associated with the higher expression of HBcAg. Kruskal-Wallis and Dunn's multiple comparison test showed very significant differences between grades 3 and 1 of HBcAg expression in relation to HBV DNA ($p < 0.01$) (Fig. 1). However, most (7 out of 8) HBeAg-positive patients were classified as grade 3 with a mean HBV DNA of $1.2 \times 10^{11} \pm 3.3 \times 10^{11}$ copies/ml, only one patient was classified as grade 1 with a comparatively lower viral load (data not shown).

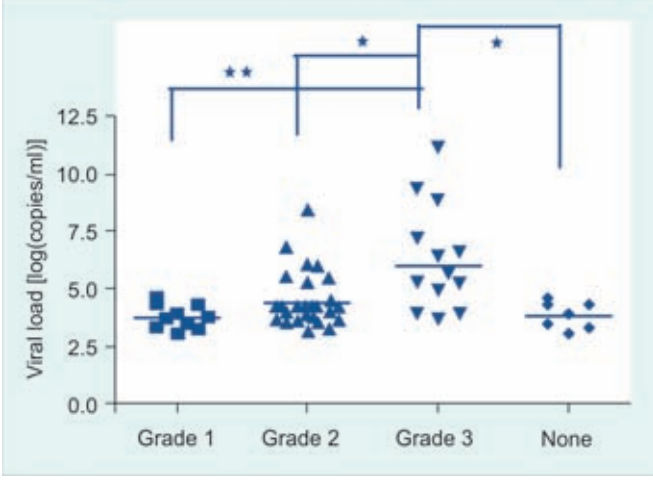


Fig. 1: Relation between the HBV DNA load and the HBcAg grade among HBeAg-negative patients. **: $p < 0.001$; *: $p < 0.05$, Kruskal-Wallis and Dunn's multiple comparison test

HBcAg Detection According to SGPT Levels

There was an increase trend of HBcAg expression in HBeAg negative cases found along with the increasing SGPT (Fig. 2). A statistically significant difference was found between the grades 1 and 3 groups ($p > 0.05$; unpaired student t-test with Welch's correction), however, most HBeAg-positive cases had the higher grade of HBcAg and the mean ± SD of ALT was 64.43 ± 40.76 IU/l (data not shown).

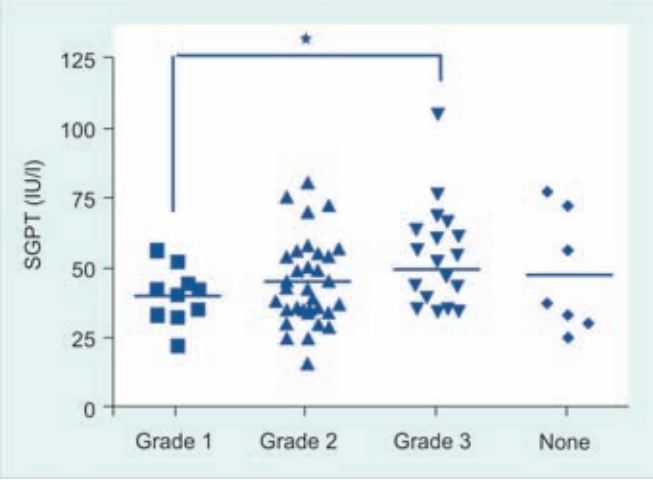


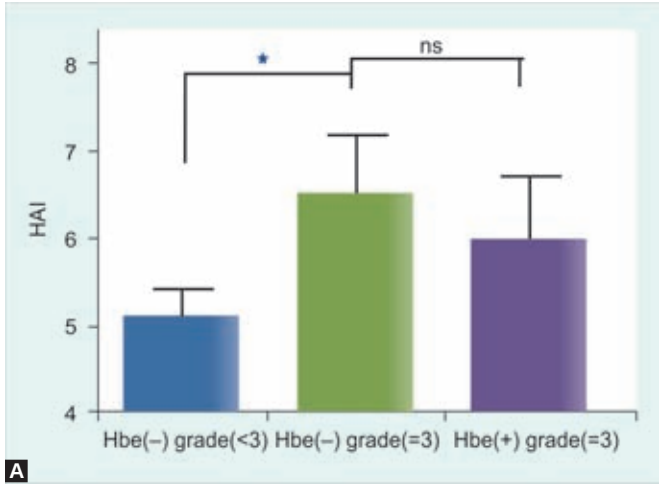
Fig. 2: Relation between the ALT levels and the HBcAg grade among HBeAg-negative patients. *: $p < 0.05$, Unpaired student t-test with Welch's correction

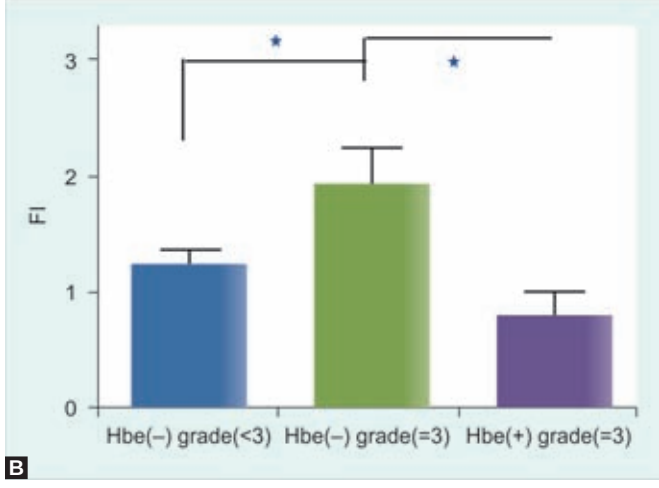
HBcAg Grade of Detection According to Histological Damage

In general, the histology of subjects was graded as minimal, mild, moderate and severe according to Knodel index. A total of three (37.5%) of both HBeAg-positive and core antigen positive patients showed minimal chronic hepatitis, while five (62.5%) patients showed mild hepatitis. Among HBeAg negative and core positive patients, 22 (40%) showed minimal, 25 (45.45%) mild, seven (12.73%) moderate and one (1.82%) showed severe hepatitis (Table 2).

To study the relation between HBcAg detection in hepatocytes with the severity of the liver lesion, patients were classified according to the level of detection in two groups, one with grade 3 of HBcAg intracellular expression and the second group including all patients under the level of 3 (grades 0-2).

The histological activity index (HAI) and fibrosis index (FI) were studied. HBeAg-negative patients with grade 3 HBcAg detection in hepatocytes had a significantly increased level of HAI and FI ($p < 0.05$, unpaired student t-test) compared with HBeAg-negative patients under the level of 3 (Figs 3A and B). In the case of HBeAg-positive patients, a similar comparison was not possible as, with one exception, all patients were classified as grade 3 according to their HBcAg levels in hepatocytes. When compared HAI





Figs 3A and B: Histological variables (mean ± SE) according to HBcAg grade. Two groups have been conformed, one group includes the histological data from grade 0-2 (less than 50% of the cells positive for HBcAg) and the second includes those with grade 3 (more than 50% of the cells positive for HBcAg). Data was splitted between HBeAg-positive and negative patients. (A) HAI according to grade; (B) FI according to grade. Comparison unpaired student t-test, *: ($p < 0.05$)

Table 2: Histology of subject (Knodel index) according to HBeAg status for patients with HBcAg detection in liver cells

HBeAg status	*Histology*				*Total*
	Minimal	*Mild*	*Moderate*	*Severe*	
Positive	3 (37.5)	5 (62.5)	—	—	8 (100.0)
Negative	22 (40.0)	25 (45.45)	7 (12.73)	1 (1.82)	55 (100.0)

of grade 3 HBeAg-positive and -negative groups, there was no significant differences ($p > 0.05$), however, HBeAg-negative cases had a superior FI compared to same grade HBeAg-positive patients.

HBcAg Cellular Distribution According to DNA Levels

Different cellular distributions were clearly identifiable from IIF staining of cells. Patterns of nuclear or cytoplasmic distributions were detected, in addition mixed patterns with predominant nuclear or cytoplasmic detection were also found.

Among HBeAg-negative cases, the level of HBV DNA according to cellular distribution is shown in Figs 4A and B. In general, patients with a nuclear distribution pattern (nuclear and mixed but predominantly nuclear) had the higher levels of HBV DNA ($p < 0.05$, Kruskal-Wallis and Dunn's multiple comparison test), compared to groups with cytoplasmic and mixed but predominantly cytoplasmic pattern (see Fig. 4A).

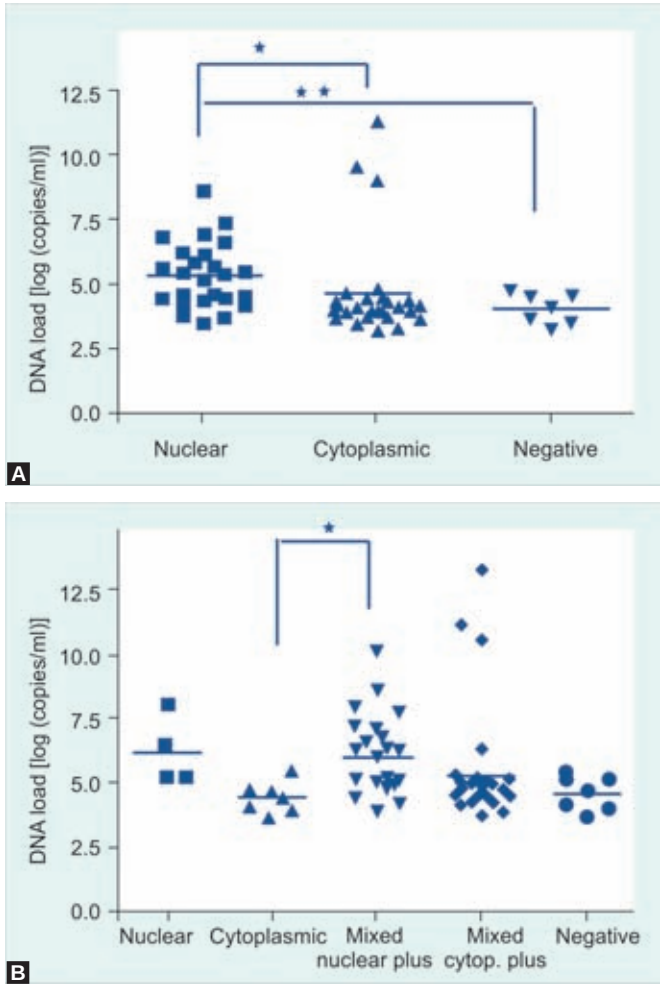
The analysis of individual patterns (Fig. 4B) made evident the high prevalence of mixed pattern distribution among HBeAg-negative patients in Bangladesh, accounting for the 78.2% of HBeAg-negative patients with detectable HBcAg in hepatocytes and 69.4% from all HBeAg-negative patients. From HBeAg-negative patients, a total of 51 patients (92.7%) evidenced cytoplasmic expression, both in mixed patterns or exclusively cytoplasmic distribution, representing the 82.3% of all HBeAg-negative patients.

The individual group comparison also evidenced that most patients with nuclear or mixed but predominantly nuclear distribution pattern had higher levels of HBV DNA when compared to groups with cytoplasmic or mixed but predominantly cytoplasmic, the difference reached statistically significant levels ($p < 0.05$, Kruskal-Wallis test and Dunn's multiple comparison test) in the case of mixed predominantly nuclear distribution pattern, when compared to cytoplasmic distribution pattern (Fig. 4B).

In the case of HBeAg-positive patients, all patients showed a mixed pattern of distribution and a very high viral load. Taken together, HBeAg-positive and -negative patients, 72.9% of patients evidenced mixed distribution pattern, whereas cytoplasmic expression in both mixed or exclusively cytoplasmic pattern accounted for 84.3% from all patients.

HBcAg Cellular Distribution According to ALT Levels

The mean SGPT level found in HBeAg negative with mixed but predominantly cytoplasmic distribution pattern was



Figs 4A and B: Relation between the HBV DNA load and the HBcAg grade among HBeAg-negative patients. (A) Nuclear distribution pattern (nuclear and mixed but predominantly nuclear) *vs* cytoplasmic distribution pattern (cytoplasmic and mixed but predominantly cytoplasmic). (B) Analysis of the individual patterns. **: $p < 0.001$; *: $p < 0.05$, Kruskal-Wallis and Dunn's multiple comparison test

comparatively superior to the rest of the HBcAg distribution patterns. A significant difference ($p < 0.05$) was established between this group and the patients with a nuclear or cytoplasmic only patterns (Fig. 5).

Among HBeAg-positive patients with mixed but predominantly cytoplasmic distribution pattern ALT mean level reached 78.75 ± 51.49 IU/l while for HBeAg negative it was 52.05 ± 14.84 IU/l. The rest of the groups ranged from 36 to 45 IU/l. It is worth mentioning that HBeAg patients evidenced only showed mixed distribution patterns.

HBcAg Cellular Distribution According to Histological Damage

To evaluate the effect of HBcAg intracellular distribution on the HAI or FI, HBeAg-negative or -positive patients two groups were conformed, a group called nuclear included patients with nuclear or mixed but predominantly nuclear

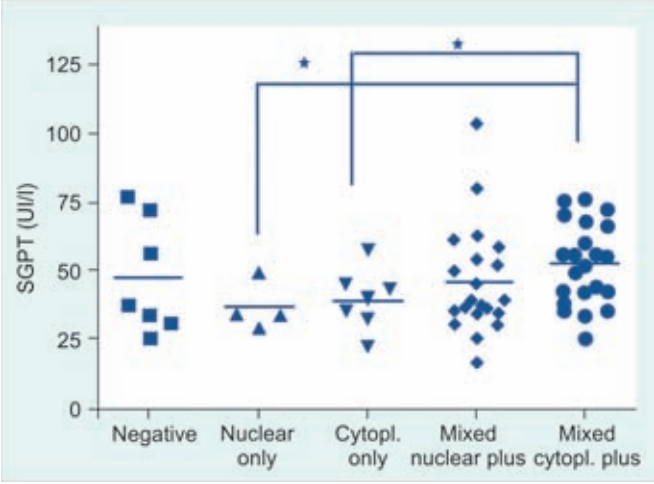


Fig. 5: Relation between the SGPT levels and the HBcAg cellular distribution among HBeAg negative patients. *: $p < 0.05$, Unpaired Student t-test

distribution and the second group, named cytoplasmic, included patients with cytoplasmic or mixed but predominantly cytoplasmic distributions. There was no difference in HAI or in FI between both groups. The analysis of the individual groups did not reveal any trend or significant difference in favor of any specific type of distribution (data not shown).

DISCUSSION

Present results of high HBcAg expression in hepatocytes from HBeAg-positive cases are in line with studies in Taiwan and Korea.[7,8] Among HBeAg-negative cases, a very high percentage of patients (88.7%) were HBcAg positive in hepatocytes compared to reports from Asia, were the HBcAg detection ranged from 59% in Korea[7] to 0% among HBeAg negative cases in India.[9] Present results were further confirmed by indirect immunoperoxidase in more than 90% of the samples to avoid any technique related variability.[10]

The increased grade of HBcAg expression in HBeAg negative patients in relation with the higher DNA levels confirmed the role of HBcAg detection in hepatocytes as a marker of active replication.[11] This particular variable—HBcAg grade—, could provide important information in HBeAg-negative patients as serum HBV DNA fluctuates in this group of patients and the decision to start treatment is often unclear.[12,13]

The increased grade of HBcAg expression in HBeAg negative cases found along with the increasing SGPT values also support the use of intracellular detection of HBcAg as a valuable marker of disease progression from the biochemical point of view. Frequent fluctuations in SGPT values have been detected in HBeAg-negative patients in relation to fluctuations in HBV DNA,[14] further complicating the scenery for diagnosis and treatment of HBeAg-negative patients and their differentiation from true inactive carriers.

The higher levels of necroinflammatory and FIs found for grade 3 HBeAg-negative patients confirm the role of the intracellular expression of HBcAg with liver damage, supporting DNA and SGPT results. The increased intracellular expression of HBcAg is logically related to the higher presentation of HBcAg epitopes on HLA molecules and consequently, to the potential increase in liver damage. The high percentage of HBeAg-negative patients expressing HBcAg in liver could explain the high levels of cirrhosis found in HBeAg-negative patients at very young ages in Bangladesh.[15-17] The HBeAg tolerogenic effect,[18,19] is the most rational explanation about the lower FI found among HBeAg positive with grade 3 HBcAg expression.

In general, our results provide a further support to previous studies showing that the detection of HBcAg in liver usually correlates with active viral replication, associated with variable degrees of inflammatory activity in the liver, while the absence of HBcAg in hepatocytes usually indicates low levels of viral replication, associated with little or no inflammatory activity.[11,20]

The presence of mixed pattern of cellular distribution in all HBeAg-positive patients is supported by the high viral loads as well as by the high count of cells stained for HBcAg –mostly grade 3 levels. This result is consistent with previous results, where most HBeAg-positive patients had high viral loads and mixed pattern of HBcAg intracellular expression.[7,21]

In HBeAg-negative patients, the most frequent cellular distribution found was the mixed pattern. The high frequency of HBcAg mixed pattern distribution found in hepatocytes is added to the higher grade of HBcAg expression in HBeAg-negative patients of Bangladesh. This is another issue that further differentiates the present study from previous reports. A recent publication from Korea found 51% of positive cells expression HBcAg while only 49% from those expressing HBcAg showed a mixed distribution pattern, for a 25% in the total HBeAg-negative population.[21]

The higher viral load was related to the nuclear distribution pattern (Fig. 4), in line with previous results from literature.[8,11,21]

The higher SGPT levels found in the group of patients with cytoplasmic and mixed but predominantly cytoplasmic expression of core antigen support the theory that cytoplasmic expression of HBV core antigen is related with higher biochemical and inflammatory activity of the liver than nuclear expression of core antigen. The present results confirm previous reports.[11]

As proposed by Chu et al,[8] almost all hepatocytes with nuclear expression of HBcAg are resting cells, whereas about half of the hepatocytes with cytoplasmic expression of HBcAg are proliferating cells. This finding indicated that the subcellular localization of HBcAg in hepatocytes was cell-cycle regulated and suggested that the predominant cytoplasmic localization of HBcAg in patients with active and ongoing hepatitis may be secondary to liver damage and regeneration.

The very high levels of mixed pattern found in CHB population in Bangladesh suggests that the patient's cells are in the status of differentiation and proliferation after histological damage, as it has been previously described.[11,20,22] The results of histology also demonstrated that HBeAg-negative patients have more advanced diseases than HBeAg-positive patients and this group requires careful evaluation.

It would be desirable to conduct evaluations with samples of HBeAg-negative patients from different viral genotypes and from different genetic backgrounds to understand the potential scope of the present results and discard interlaboratory differences.

For CHB patients treated with traditional or novel immunotherapeutic candidates containing HBcAg,[23,24] the levels of HBcAg in hepatocytes could be an important variable related to treatment efficacy and also in the measurement of the antiviral effect.

CONCLUSION

The present results confirm that HBcAg detection is an important marker of active viral replication in both HBeAg-positive and -negative CHB patients from Bangladesh. The frequency and grade of detection can be classified as high for HBeAg-negative patients from Bangladesh compared to their homologous in other countries in Asia. This technique may provide further support for treatment decision in HBeAg-negative patients and help in the characterization of the response to therapy.

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