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DETECTION OF HEPATITIS B SURFACE ANTIGEN USING ELISA AND REAL TIME PCR

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ABSTRACT

India has one of the largest pools of hepatitis B-infected patients. The presence of HBs Ag in serum or plasma is an indication of active Hepatitis B infection either acute or chronic. Quantitative detection of Hepatitis B virus (HBV) DNA in serum by real time polymerase chain reaction (PCR) assay emerged as a gold standard in guided anti viral therapy. Evidences are suggestive of association of HBV genotype in liver dysfunction. We evaluated the performance as well as usefulness of both ELISA and PCR for detection of the major blood borne pathogen, HBV in southern India. First ALT and AST enzymes level was determined to check liver function. Serum of patients with chronic hepatitis B (HBsAg positive) and healthy individuals were tested both quantitatively and qualitatively. 4.5% of participants were HBsAg Positive by ELISA, while 7.5% of participants showed HBV DNA in real time PCR.

KEYWORDS: HBV, Diagnosis, ELISA, Real Time PCR

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INTRODUCTION

Viral hepatitis is a systemic disease primarily involving the liver and most cases of acute viral hepatitis are caused by Hepatitis A, B and C virus. Chronic Hepatitis B virus is one of the most serious and prevalent health problems with more than 300 million chronic carriers affecting[1]. Approximately 25% of persons who become chronically infected during childhood and 15% of those who become chronically infected after childhood die from cirrhosis or liver cancer[2]. This virus stands as one of the most common human pathogens, thereby leading to significant public health problems. In recent studies conducted in Asia and Northern America, it was estimated that the lifetime risk of developing HCC increases by 25–37 times in HBsAg carriers as compared to the non infected populations[3]. The course of HBV infection depends on the immune system, age at infection and host genetic factors, and genetic variability of the virus.India has one of the largest pools of hepatitis B-infected patients[4, 5]. Substantial improvement in the understanding of hepatitis B virology and immunology during past decades has led to new insights into the natural history of such infection. The presence of HBs Ag in serum or plasma is an indication of active Hepatitis B infection either acute or chronic. Past exposure to hepatitis B virus is mostly determined by testing for specific antibodies using an approved enzyme immunoassay (EIA). The presence of antibody determines the infection with virus but does not indicate whether the infection is acute, chronic or resolved[6]. The recommendations for the screening of Hepatitis B also call for testing the serum or plasma specimens by ELISA tests[7]. Present study deals with the evaluation of performance as well as usefulness of both ELISA and PCR assay for detection of the major blood borne pathogen, HBV using sera.

MATERIALS AND METHODS

Study population

The study was carried out at the Indian Academy Degree college, Bangalore University. As a routine, our laboratory follows the World Health Organization (WHO) testing strategies for HBV test and this is documented. Majorities were of age group 31-40 yrs (65.3%). Males (92.7%) outnumbered females (7.3%) were HBsAg carriers.

ELISA (enzyme linked immunosorbent assay)

Serum samples from patients were labeled with a laboratory identification serial number and stored at -20°C in separate aliquots. All the conditions for the storage of the kits were strictly followed. The sera were thawed on the day of doing the tests and tested for HBVAg using enzyme linked immunosorbent assay kits as per manufacturer's instructions. All serum samples were tested in duplicate. **Sample Preparation a) Serum:** Blood was drawn using standard venipuncture techniques and were allowed to clot for one hour at room temperature and then centrifuged for 10 minutes (4°C) and serum was extracted. **b) Plasma:** Blood was drawn using standard venipuncture techniques and plasma was collected using sodium citrate, EDTA, or heparin as an anticoagulant. Plasma was quickly separated with less than 30 minutes on ice to ensure optimal recovery and minimal platelet contamination. Centrifuged for 10 minutes (4°C) to remove any particulate. This HBsAg ELISA Kit is not affected by haemolysis of specimens. No adverse effects have been noted in the presence of anti-coagulants, sodium citrate, EDTA, or heparin. Serum or plasma samples were stored at -20°C to avoid loss of bioactivity and contamination.

HBV Real-TM Quantification

It is a Real-Time test for Quantitative detection of Hepatitis B Virus in human plasma and simultaneous detection of a HBV-specific Internal Control (IC), by dual color detection. HBV DNA was extracted from plasma, amplified using Real Time Amplification and detected using fluorescent reporter dye probes specific for HBV or HBV IC. PCR reactions were carried out

in a thermal cycler (Nyxtechnik USA) with 5U *Taq* DNA polymerase (Fermentas USA).

The PCR was carried out using sense and antisense primers and the product was analyzed on 2 % agarose gel.

RESULTS

Alanine aminotranferease (ALT) and aspartate aminotransferase (AST) are enzymes located in liver cells that leak out into the general circulation when liver cells are injured. The observed level of ALT and AST was shown in Figures 1-4.

Figure 1

ALT values of the serum from the patients. The values are triplicates of the samples.

S.No	Marker	Age	Sex	Level* U/L	Clinical Significance
1	ALT	28	M	33	
2	ALT	48	F	64	HBV
3	ALT	45	F	30	
4	ALT	35	M	86	HBV
5	ALT	43	M	42	
6	ALT	22	M	35	
7	ALT	18	F	44	
8	ALT	52	F	40	
9	ALT	37	M	57	HBV
10	ALT	26	F	39	

Figure 2

Graph showing the ALT values of different age groups. Age group of 31-35 is found to be significant. All the values are means of the triplicates.

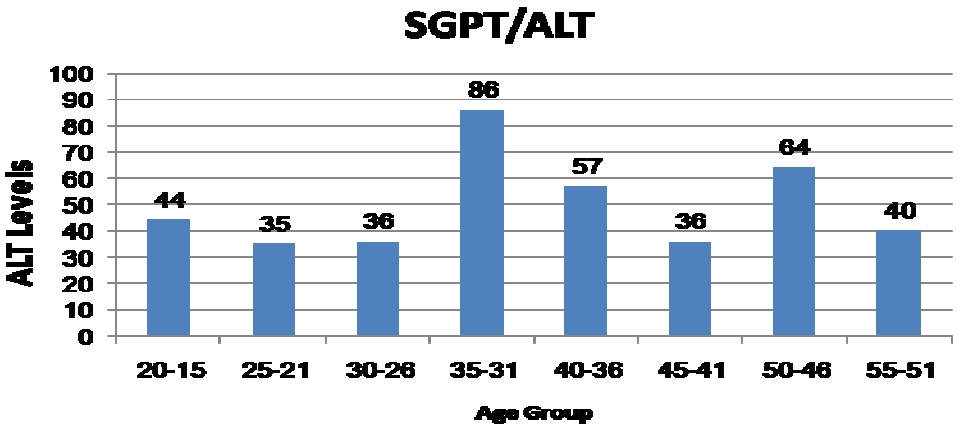


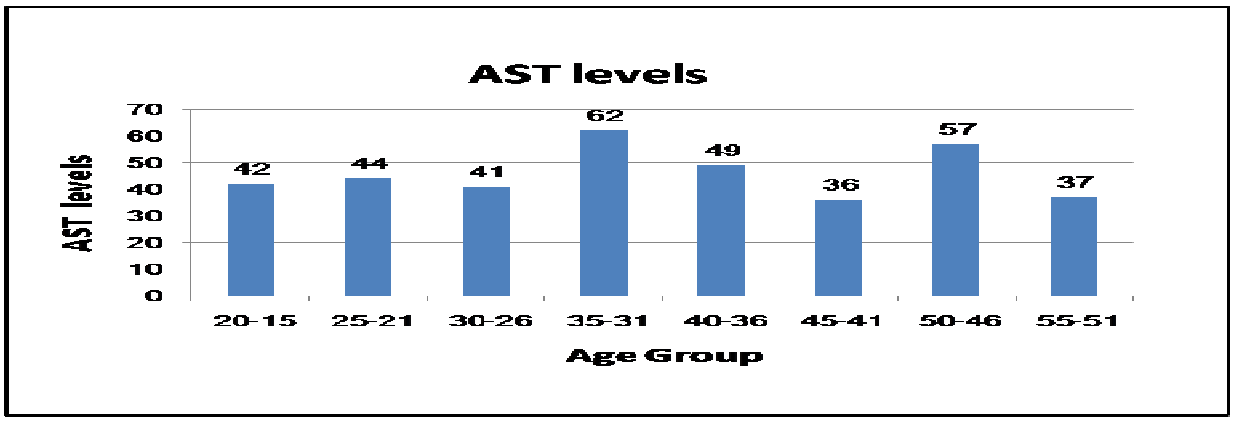
Figure 3

AST values of the serum from the patients.The values are triplicates of the samples.

S. No	Marker	Age	Sex	Level* U/L	Clinical Significance	
1	AST	28	M	37		
2	AST	48	F	57	HBV	
3	AST	45	F	33		
4	AST	35	M	62	HBV	
5	AST	43	M	39		Normal: <46
6	AST	22	M	44		
7	AST	18	F	42		
8	AST	52	F	37		
9	AST	37	M	49	HBV	
10	AST	26	F	45		

Figure 4

Graph showing the AST values of different age groups. Age group of 31-35 is found to be significant followed by the age group 46-50. All the values are means of the triplicates.



Infection with HBV results in the appearance of a number of serological markers and one of the first of such markers is Hepatitis B surface antigen (HBsAg). In current study, samples were tested for evaluation of hepatitis B surface antigen (HBsAg) by ELISA and the results were summarized in Figure 5 and 6. Positive: P/N value is equal to or greater than 2.1. Negative: P/N value is less than 2.1. P/N value = OD value of specimen/Average OD value of Negative Control. If the OD value of the negative control is less than 0.05, it was reported as 0.05. If it is more than 0.05, it was reported as the actual OD value measured. It is evident that HBsAg gives uniform results by identifying all panel samples correctly with good performance (100% sensitivity, specificity, PPV, NPV and efficiency). A student's *t* test also used to assess the statistical variability in efficiency values due to 'lots' and this statistical analysis decipher that the efficiency of the ELISA kit didn't vary significantly ($p > 0.05$) among the lots.

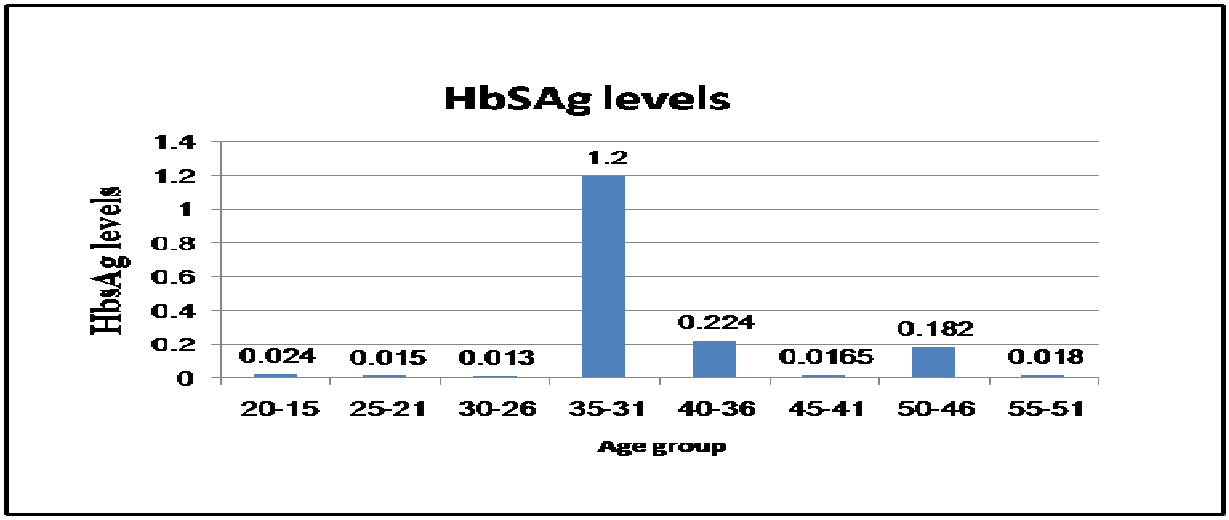
Figure 5

HbsAg values of the serum from the patients.The values are triplicates of the samples.

S. No	Marker	Age	Sex	Level* OD	Clinical Significance
1	HbsAg	28	M	0.015	
2	HbsAg	48	F	0.182	Reactive
3	HbsAg	45	F	0.011	
4	HbsAg	35	M	1.2	Reactive
5	HbsAg	43	M	0.022	
6	HbsAg	22	M	0.015	
7	HbsAg	18	F	0.024	
8	HbsAg	52	F	0.018	
9	HbsAg	37	M	0.224	Reactive
10	HbsAg	26	F	0.011	

Figure 6

Graph showing the HbsAg values of different age groups. Age group of 31-35 is found to be significant. All the values are means of the triplicates.



The same samples were tested for evaluation of hepatitis B surface antigen (HBsAg) by real time PCR and the results were summarized in Figure 7 and 8. The Ct values confirms the gene amplification and shows that the experimental samples were positive for HBV infection

Figure 7
Real time PCR graph.

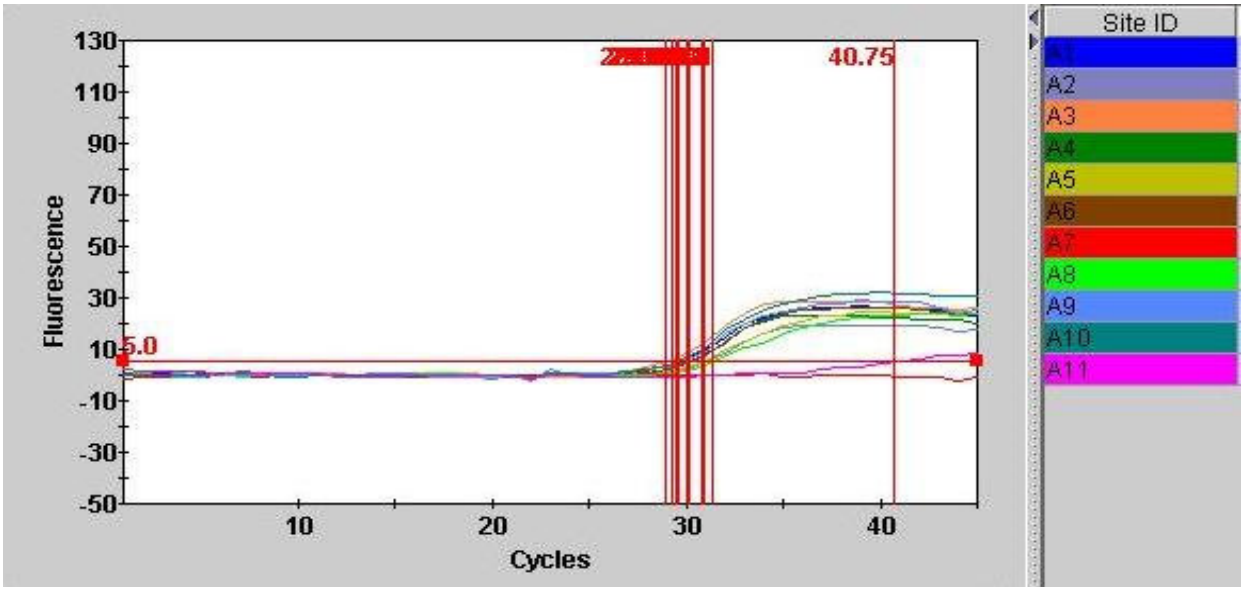


Figure 8
Table showing comparative values of the ELISA readings and Real time PCR Ct values of the HbSAg levels.

Age group	HbSAg value	Ct value	Copies
15-20	0.024	Nil	
21-25	0.015	38	868
26-30	0.011	Nil	
	0.015		
31-35	1.2	28	112868
36-40	0.224	37	4638
41-45	0.022	Nil	
	0.011		
46-50	0.182	36	7535
51-55	0.018	Nil	

DISCUSSION

Confirmation of diagnosis in hepatitis B viral infection and assessment of prognosis is based on wide array of advanced immunological, molecular and histological assays. The immunological techniques include second generation ELISA, 3rd generation EIA and RIBA.. Conventional enzyme linked immunosorbent assay (ELISA) is most referred screening technique[8] and possess an accuracy of about 99.9% with improved sensitivity[9, 10]. Comparatively, PCR tests are easier and quicker. Serum of patients with chronic hepatitis B (HBsAg positive) and healthy individuals of control group who were selected from the healthy people in community were tested both quantitatively and qualitatively. Our result shows significant ELISA values in the age group of 31-35. The Real time PCR confirms of the values of the ELISA. The Ct value of the gene amplified showed significant values in the age group 31-35, which confirms the readings of the ELISA. There was amplification at 28th cycle with the gene copies reaching upto approximately 112868. The HbSAg gene also showed amplification in the age groups 21-25, 36-40 and 46-50. The Ct values confirm the gene amplification and shows that the experimental samples were positive for HBV infection. These

are the confirmatory readings for the ELISA. Two individuals from the control group were HBsAg positive with ELISA. PCR and Real Time PCR were applied for all individuals in case and control group. Three of the samples in control group showed HBV DNA in their sera while their ELISA was negative. In other words 4.5% of participants in control group were HBsAg Positive by ELISA, while 7.5% of participants in this group had HBV DNA by PCR and real time PCR. In another study from India the seropositivity rate for Hepatitis B surface antigen was found 2.0% in hospital-based population[11]. From our results it can be concluded that 7% of individuals in control group were the chronic carriers of HBV while ELISA could detect just 2.8% of them only.

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