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SEROLOGICAL AND MOLECULAR STUDY OF HEPATITIS A, B, C INFECTIONS IN CHRONIC LIVER DISEASE IN A TERTIARY CARE HOSPITAL

Microbiology

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ABSTRACT

Background: Hepatitis B and C viruses are the major causes of chronic liver disease worldwide. **Aims & objectives:** To assay the HBV serological markers & quantitate viral load and compare these with clinical profile. **Materials and methods:** 281 serum samples were collected and subjected to HB s Ag ELISA . Then 88 randomly selected samples were assayed for other serological markers. Quantification of viremia was done by realtime PCR. **Results and Analysis:** Among 281 CLD patients 209 were males and 72 were females. HBV infection was more common in >50 yrs age group. Among 75 HBsAg positive cases, 9 patients had viral load in the range of 10^{4}-10^{5} copies / microlitre. 7 HBsAg negative patients had viral load > 10^{3}copies/ microlitre. **Conclusion:** The usefulness of HBV load assay in the follow up of patients on antiviral treatment was evident.

KEYWORDS

Chronic Liver Disease, Hepatitis, ELISA, HBV Load, Realtime PCR

INTRODUCTION:

Chronic liver disease is a major cause of morbidity and mortality.The disease becomes life threatening when cirrhosis develops. Established cirrhosis has 10 year mortality of 34-66% and it depends on the cause.(1)

Liver, the largest internal organ of our body carries out thousands of biochemical functions, the important one is bile production. Liver disease may be acute or chronic. Chronic hepatitis is chronic inflammatory reaction in the liver that continues without improvement for atleast 6 months. The causes of chronic liver diseases (CLD) are several. Chronic alcohol intake is an important preventable cause accounting for 10 to 15% of CLD. The other major causes are

1. Hepatitis viruses Hepatitis B, C viruses, Dual infection with HBV,HDV
2. Hepatotrophic viruses - Varicella zoster virus, yellow fever virus, cytomegalo virus, Ebstein Barr virus

So this study is undertaken to understand the profile of chronic liver disease in relation to HBV in our hospital. A molecular study of HBV was carried out to see the correlation between the hepatitis B viral load and severity of chronic liver disease and antiviral treatment.

MATERIALS AND METHODS:

MATERIALS

This study was conducted in the Institute of Surgical Gastroenterology and liver transplant Govt Stanley medical college and Hospital, Chennai.

Study period: January 2010-October 2010

Instituitional Ethical Committee Approval Obtained:

Patients diagnosed as Chronic liver disease due to any cause, compensated and decompensated liver disease, cirrhosis liver and hepatocellular carcinoma attending Medical and Surgical Gastroenterology outpatients department, wards as well as from other medical, surgical, obstetrics and gynaecological wards were included in the study.

The study population comprised of 281 patients with chronic liver disease and GIT diseases.

Meticulous care was taken in eliciting the history of the patients with special emphasis on H/O jaundice, GI bleed, chronic drug intake of hepatotoxic drugs, H/O blood transfusion, surgery in the past etc.

The serum samples were collected after getting proper informed consent and following universal barrier precautions.

METHODS:

Collection of Samples:

About 7 ml of blood was collected from cubital vein with aseptic precautions using sterile disposable syringe with 23 G needle. Blood was dispensed in sterile test tubes without anticoagulant. The serum samples were obtained by centrifugation of plain blood sample at 1500 rpm for 10 minutes at 4°C. Separated sera were then aliquoted in eppendorf tubes.

All the serum samples were tested for HBsAg ,Anti HCV,Anti HIV by ELISA tests

The serum samples were stored in deep freezer at -35°C in duplicate for further serological and molecular study.

Selection of study samples:

Of this study samples of patients with chronic liver diseases 88 samples were randomly selected for the battery of serological and molecular investigations as described below.

With one set of samples ELISA tests were performed for the detection of Anti HBc Ig Total, Anti HB c IgM , Anti HBs , Anti HBe and HBeAg the other set of samples were used for realtime PCR HBV DNA quantification Assay by Standard curve method.

ELISA tests for detection of HBs Ag, anti HBs , anti HBcore total, anti HBcore Ig M, HBeAg, HBe Ab were done. The kits used were Erbalisa HBsAg, Monolisa Anti HBcore Plus, DRG anti HBcore IG M, Immunolisa Anti HBs and Immunolisa Anti HB e .

Real time PCR methodology for HBV DNA QUANTIFICATION:

Instrument used: Applied Biosystems 7500 Fast

Method: Absolute quantification by Standard curve method

Kit for D.N.A. Extraction: QIAGEN DNA Blood mini kit Nucleic acid extraction:

HBV DNA was extracted from serum samples using the QIAamp Minelute Virus spin kit (Qiagen, GmbH, Hilden, Germany). A sample volume of 200 microlitre was used for the extraction as per manufacturers instruction. Nucleic acid was eluted in 200 microlitre of elution buffer provided by the manufacturer.

Amplification:

The probe for the detection of HBV DNA was labelled with reporter dye (ROX, FAM) at the 5' end and with black hole quencher at the 3' end (Operon Biotechnologies, GmbH, Cologne, Germany).The primer and probe sequences are shown in table 1. (Genotype A) The specificity of the primers and probe were checked using the BLAST search. This search indicated that the primer and probe set had the capacity to detect all the 8 HBV genotypes.

Primers used in this study are to amplify the S region of the HBV genome.

Primers and probes used for HBV PCR Real time Assay		
Region	**Primer sequences**	**Target Sequence**
S gene of surface Antigen	CTG CTG CTA TGC CTC ATC TTC TTA T ATC TTC TTA T	Locus X75666 Accession 681 bp genotype A (workshop no 89 – 14th Nov 1993
	ACT GGT TGT TGT TGA TCC TGG AAT T	
HBV Probe	ACG GGC AAC ATA CCTT	

Eurohep W.H.O. HBV DNA standard: from National Institute of biological standards and controls, NIBSC 97/746
European study group on viral hepatitis
German reference centre for viral hepatitis
Genotype A, HBsAg subtype adw2 contains 10 million HBV DNA copies/ micro litre This standard was run in triplicate with dilution of it serially ie, 10^6 ,10^5 , 10^4 , 10^3 HB V DNA copies per microlitre. The amplification and detection of HBV DNA was performed with ABI 7500 fast equipment by Standard curve method.

RESULTS AND ANALYSIS

281 Patients with chronic liver diseases were analysed in our study. 209 were males and 72 were females. The age ranged from newborn to 91 years. 125 patients were positive for HBsAg.

HBV infection was more common in < 50 yrs. The most common clinical presentation in HBV infection is cirrhosis followed by hepatocellular carcinoma.

88 samples among the 281 patients were subjected to Real Time PCR for HBV DNA, HBV viral markers - Anti HB core total & IgM, HBeAg and Anti HBe, Anti Hbs.

Among the 75 HBsAg positive patients with CLD, 9 patients had viral load in the range of 10^4– 10^5 copies per microlitre, 18 patients had viral load in the range of 10^3– 10^4 copies per microlitre. 8 patients had viral load in the range of 10^2– 10^3. 21 patients had viral load between 0-100 copies per microlitre.

Cirrhosis and Hepatocellular Carcinoma were associated with high viral load. High viremia was associated with all ALT levels ranging between normal and very high. The 2 patients with very high levels of >500 IU/ L had the high viral load.

CLINICAL CATEGORIES WITH RESPECT TO VIRAL HEPATITIS

	HBsAg positive	**HBsAg negative**
Cirrhosis	81	39
Primary Hepato Cellular Carcinoma	17	22
Primary cholangio carcinoma	1	-
Secondaries liver	1	6
Cholecystitis	4	21
Non liver related GI disease	21	68
Total	125	156

HBV DNA LOAD CORRELATION WITH ALT RANGE

Viral load CPM	**Alanine Amino Transferase (ALT) units per liter**							
	<37	**37-80**	**81-120**	**121-160**	**161-200**	**201-250**	**251-500**	**500- 1000 1000**
>1L	2	3	2	-	-	-	-	-
10001-1L	1	5	--		1			2
1001-10000	7	6	4	-	-	1		_
101-1000	3	3	1	_	_	1		-
11-100	4	4	3	_	1	-		_
1-10	6	1	1	_	_	1	-	_
UD	15	7	1	1	_	_	1	
88	38	29	12	1	2	3	1	2

High viremia was associated with all ALT levels ranging between normal and very high. The 2 patients with very high levels of >500 IU/L had also a high viral load.

DISCUSSION

Among a study population of 281 with Chronic Liver Disease, 75 of them were HbsAg Positive. **Primers used in this study** are to amplify the S region of the HBV genome.

Primers and probes used for HBV PCR Real time Assay		
Region	**Primer sequences**	**Target Sequence**
S gene of surface Antigen	CTG CTG CTA TGC CTC ATC TTC TTA T ATC TTC TTA T	Locus X75666 Accession 681 bp genotype A (workshop no 89 – 14th Nov 1993)
	ACT GGT TGT TGT TGA TCC TGG AAT T	
HBV Probe	ACG GGC AAC ATA CCTT	

The present study at SGE shows the HBV DNA PCR positivity among the study group to be 47.72% reflecting the active replication of HBV among this group. The remaining 52.27% of study group showed <200 copies HBV DNA per microlitre and seem to be in inactive carrier state / or had spontaneously cleared the viremia as spontaneous viral clearance is 15-45%. Fifty eight (77.33%) of the 75 HbsAg positive samples were viremic. 7 patients had a high viral load in the range of 1-10 lakhs HBV DNA copies per microlitre . All these patients were HBsAg positive. But in case of low viral load HBsAg seropositivity was found only in 76.5% . Rodrigues et al [9] in their study observed that 166 (63.11%) of 263 HBsAg positive samples were PCR positive.

Among the HbsAg negative patients, 7 patients (53.84%) also showed viremia in this present study. Of these 7 patients, 6 patients also had anti Hbe. Fabien Zoulim etal[10] in their study conclude that in patients with anti HBe seroconversion serum HbsAg concentration decreased at the time of anti Hbe seroconversiion.. Rodrigues et al [9] found that 2/32 (6.25%) patients without HbsAg were HBV DNA positive on PCR.

Five HbsAg positive patients, in the present study were on treatment with Lamivudin 100 mg twice a day for four months. Three among these had cleared the virus to undetectable levels, and all of them had normal ALT levels. The other two patients on antiviral treatment had a reduced viral load of less than 100 copies per microlitre of whom one had normal while the other a mildly elevated level of ALT level (115 IU/L). The usefulness of HBV viral load assay in the follow up of patients on anti-viral treatment is evident in this study.

Only 7 0f the HBV DNA positive patients had HBe antigen in the blood, and the remaining were HBeAg negative. The presence of HBV DNA in HbeAg negative individuals could be due to low viremia in non-replicative HBV disease or due to the presence of a pre-core/core stop codon mutation leading to HBeAg negative states[5] while on the other hand only 7 of 11 HBeAg positive patients had viremia. Hitherto HBeAg was relied upon as a parameter to indicate infectivity or response to treatment.

Our study shows that the HBeAg assay cannot substitute DNA Assay in this regard and antiviral treatment monitored by viral load assays may have to be continued even after HBeAg seroconversion. James Fung et al 13 [9] also concurs with this observation , stating that antiviral therapy could be stopped after HBe seroconversion when virus becomes undetectable and liver enzymes remain normal.

Hepatitis viruses especially hepatitis B and Hepatitis C are the predominant causes of chronic viral liver disease worldwide. The prevalence rate is high in Asia. India stands second next to China with regard to Hepatitis B. In our study Hepatitis B infection in chronic liver diseases is 44.48% . In the study done by R.Shantha et al [7] in 2002, the seroprevalence of HBV infection among chronic liver diseases in Chennai was 43.7% which is similar to that in our study. The study done by Dr.Manisha Jain and Dr.Anita Chakravarthy et al[12] in 2009 at Maulana Azad medical college, New Delhi where HBsAg positivity in chronic liver diseases was 36.36% . The Institute of Surgical Gastroenterology and Liver transplantation being a tertiary care centre has referrals from across the country and this may contribute to the higher rate.

Most of the HBV positive cases in the present study belong to the age group below 50 years , This is in similar to the finding of Salem A, Bin Selm who report HBV infections in the age group 46.5 +/- 12 years in Chronic Liver Disease patients.[13] Ganeshkumar et al [14] had observed in their study at Trichirappalli that most of the HBV positive cases belong to the age group 21-30 years. In the present study the HBsAg positivity among male patients with chronic liver diseases was higher

at 46.88% in comparison with 37.50% in female patients whereas Taher Salim Khan and Farhat Rizvi, Abbottabad report 73.43% and 26.56% repectively[13] Hence this shows that HBV related chronic liver diseases is more common in males than females.

The clinical significance of Anti HB c Total antibody positivity in chronic liver disease was studied. The presence of anti HBc Total in the absence of HBsAg which is usually indicative of a past self-limiting HBV infection was found in 5 (5.68%) out of 88 patients included in our study. Out of these 5 patients, 2 patients had hepatocellular carcinoma. This is similar to the observation by Manisha Jain et al in 2009 wherein 28 patients out of 77 CLD cases had anti HBc IgG in the absence of any other serological markers of HBV infection. [12]. Hence anti HBc IgG should be tested in all patients with Chronic liver disease and the presence of coinfection with HCV should be actively searched for in such patients.

There was significant correlation between the aminotransferase levels and the degree of liver damage. High viremia was associated with normal as well as all levels of increased serum ALT. But the 2 patients in the study group who had ALT levels > 500 IU/L had also a high viral load in the range of 1 lakh copies per microlitre.

In this present study out of 8 anti HBs positive samples HBVDNA positivity by PCR is 4.54% and this is different from the study done by Jin-Rong Zhao et al at Military Medical University, Xion, China where the same was reported as 6.9%.[15] This may be explained by the differences attributable due to presentation of cases in late phase of viremia where HBV DNA is still detectable and anti HBs also appears.
In this present study the antenatal HBsAg positive carrier woman is observed to be HBeAg negative and PCR negative and the baby delivered by labour naturale is also observed to be HB serological markers negative and PCR Negative. This is similar to the observation by Liz Highleyman who concludes in a case control study that transmission of HBV to infants was more likely in mother positive for HBeAg and high HBV DNA load even though the babies received HBV passive prophylaxis. [16]

SUMMARY & CONCLUSION

1. 281 Patients with chronic liver diseases ie, 209 males and 72 females, ranging in age from newborn to 91 years were analysed.
2. 125 (44.48%) were HBsAg positive.
3. Primers for amplification of S gene was used to quantify HBV D.N.A by Real Time PCR.
4. HBV infection was more common in < 50 yrs..
5. The most common clinical presentation in HBV infection is cirrhosis followed by hepato cellular carcinoma.
6. Cirrhosis and hepatocellular Carcinoma were associated with high viral load
7. HBV DNA PCR was positive in 47.72% reflecting the active replication of HBV. 52.27% showed <200 copies HBV DNA per microlitre and seem to be in inactive carrier state / or had spontaneously cleared the viremia.
8. High viremia was associated with all ALT levels ranging between normal and very high. The 2 patients with very high levels of >500 had the high viral load.
9. 27 HBsAg positive patients did not show viremia, while 7 out of 13 HBsAg negative patients had viremia , 7 with viral loads of > 10^3 copies per microlitre. Only seven of the HBV DNA positive patients had HBe antigen in the blood, and the remaining were negative. The study shows that the HBeAg assay cannot substitute DNA Assay in this regard and antiviral treatment monitored by viral load assays may have to be continued even after HBeAg seroconversion.

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