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RESEARCH ARTICLE

SCREENING FOR OCCULT HEPATITIS B. INFECTION AMONG CHRONIC LIVER DISEASE PATIENTS ATTENDING TERTIARY CARE HOSPITAL

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Abstract

Background and objectives- Occult Hepatitis B Virus infection (OBI)is defined as a condition when HBsAg virus infection is undetectable in serum, despite the presence of HBV DNA in liver or blood. This study was done to screen for occult hepatitis B infection among patients with CLD (chronic liver disease) attending gastroenterology OPD of tertiary care hospital.

Materials and methods- Observational Cross-sectional study conducted for period of one year.All patients with history of chronic liver disease were tested for HBsAg by ELISA. All HBsAg negative patients were tested for total antiHBc antibody by ELISA and HBV DNA by real time PCR.

Results- Prevalence of OBI(confirmed OBI) is 2.96% with isolated anti HBc positivity (probable OBI)being 7.24% in this study. Prevalence of seropositive OBI was 1.93% and prevalence of seronegative OBI was 0.96%. Fair agreement was observed between Total antibody against core antigen by ELISA and RTPCR for the diagnosis of OBI(k value=0.354).

Conclusion It is recommended that Total Anti HBc by ELISA may be used as a surrogate marker for diagnosis of OBI in HBsAg negative patients with CLD and for confirmation HBV DNA PCR should be done if molecular facility is available.

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Introduction:-

Hepatitis B Virus (HBV) infection is a major public health problem which affects approximately 250 million people globally.[1] Occult Hepatitis B Virus Infection (OBI) is a condition in which the HBsAg virus is undetectable in serum despite the presence of HBV DNA in the liver or blood.[2] Occult hepatitis B infection is basically the persistence of HBV in the liver which appears to be a heterogenous entity to the presence of key parameters - Past HBsAg positive chronic Hepatitis B infection , detectable anti-HBc (+/- anti-HBs) antibodies, mutations in specific regions of the HBs Ag coding gene, other conditions such as liver disease, HIV, hepatitis C virus coinfection or immunosuppressive therapies. Based on the sensitivity of HBV DNA detection assays, sample size, and real-time PCR detection of HBV DNA in liver tissue and serum, OBI prevalence differs by location. HBV carriers who are negative for HBsAg but have low amounts of HBV-DNA in serum or liver tissue can now be identified thanks to the development of highly selective polymerase chain reaction techniques. In order to avoid the spread of OBI, it is necessary to evaluate occult HBV infection. HBV DNA testing should be implemented in blood donors, chronic liver disease patients, and immunosuppressive patients, as HBV DNA has a lot of clinical significance in particular clinical circumstances.[3]

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In 1985, HBV DNA was discovered in patients with chronic liver disease (CLD) who were negative for hepatitis B surface antigen (HBsAg). Until the mid-1990s, this finding was divisive, with approximately equal numbers of research confirming or refuting the original finding. Such discrepancies were because of the differences in epidemiology and performance of the methods utilized for HBV DNA detection. Occult HBV infection (OBI) was emphasised in a significant number of research, confirming the existence and scientific significance of this new aspect of this "old" viral infection.[4] In 2008, an international workshop in Taormina, Italy, where a group led by G Raimondo defined OBI as the presence of HBV DNA in the liver (with detectable or undetectable HBV DNA in the serum) of persons who tested HBsAg negative using currently available detection methods tests.[5] Prevalence of OBI is generally underestimated because prevalence in different areas and individuals seems to be higher among patients at high risk for HBV/HCV infection and with liver disease than among individuals at low risk of infection and those without liver disease . [6]

HBV nucleic acid amplification testing (NAT),which is a PCR technique is highly sensitive and specific and it has detection limits of 10 copies HBV DNA per response, became the gold standard for diagnosing OBI. Anti-HBc should only be used as a less-than-ideal surrogate marker for probable detection of seropositive OBI people in cases of blood, tissue, or organ donation, and when immunosuppressive medication is required, if testing by highly sensitive HBV DNA is not possible.[5] The presence of anti-HBc antibody in serum is a crucial factor in OBI detection. There is a requirement for evaluation of Occult HBV infection in order to prevent the spread of Occult Hepatitis B.

This study was done with the aim to assess the prevalence of OBI in a tertiary care hospital, its clinico epidemiological profile and role of anti HBc as surrogate marker of OBI and diagnosis of OBI by PCR.

Materials and Methods:-

The Observational Cross-sectional study was conducted at Microbiology and Gastroenterology Department of Govind Ballabh Pant Institute of Postgraduate Medical Education and Research New Delhi over one year amongst CLD ,cirrhosis patients attending Gastroenterology OPD . The study was conducted after approval from the Institutional Ethical Committee.

Inclusion Criteria:

All HBsAg negative adult patients (18 -70years) with CLD and cirrhosis.

Exclusion Criteria

1. All patients less than 18 yrs age.
2. All those patients who were not willing to take part in the study.

All patients were subjected to the following:

1) Informed consent was obtained from all patients before participation in the study.

2) History taking was done with special emphasis on history of alcohol intake , history of hepatitis, family history, previous treatment and use of immunosuppressive drugs.

3) Thorough clinical examination with special emphasis on local examination of liver and spleen and detection of ascites was done.The clinical history of the patients was recorded by filling a clinical proforma.

4)Laboratory investigations: Qualitative screening of serum sample from all study participants for HBsAg was done by ELISA (BIO-RAD MONOLISA HBsAg ULTRA kit) . For all HBsAg negative samples, Total anti HBc antibody ,Anti HBs by ELISA (DIA PRO DIAGNOSTICS BIOPROBE kit) was done and HBV DNA was done by real time PCR. DNA extraction (FAVORGEN BIOTECH CORP kit) from plasma was done and real time PCR for amplification using Geneproof kit for quantitative detection of HBV DNA. Liver function tests (LFT) was performed for all study participants.

Probable OBI was diagnosed as a condition when HBsAg is undetectable in serum and only Total antiHBc antibodies are positive by ELISA with no HBV DNA detected by real time PCR..

Confirmed OBI was diagnosed as a condition when HBsAg is undetectable in serum , total anti HBc(+/-) and anti HBs (+/-) by ELISA and HBV DNA by real time PCR is present in serum.

Seropositive OBI : Presence of HBV antibodies (+anti HBc / +_anti HB s) and HBV DNA .

Seronegative OBI : Total HBV antibodies (antiHBc/antiHBs) are negative and HBV DNA is present.

Statistical Analysis

The collected data was entered in Microsoft Excel and then was analysed and statistically evaluated using SPSS version 25. Qualitative data was expressed in percentage and difference between the proportions was tested by chi square test or fischer's exact test.P value less than 0.05 would be considered statisticaly significant.

Result:-

Total of 207 participants were included in the study. Out of total cases (n=207), patients were mostly in age group of 36-45years of age(46.4%) and only (1%) case was <65 years of age. Mean age was 42.53+-8.80 years. Out of total 207 patients , 182 male patients(87.9%) and 25 female patients(12.1%) were there in the study. LFT was normal (55.6%) in 115 patients and deranged in 92 patients(44.4%). Total antibody against core antigen (Anti HBc by ELISA) was positive in 15 patients (7.24%) (Table 1) out of total number of cases indicating probable OBI. HBV DNA PCR was positive in 6 cases (2.96%) indicating confirmed OBI. Out of 6, 4(1.93%) was prevalence of seropositive OBI and 2 (0.96%) was prevalence of seronegative OBI. Out of total OBI cases(n-6),5 patients were male and of age group 36-45 years(83.3%),1 patient was female in age group 26-35years(16.7%). Table 2 shows the OBI markers in all cases. Out of total 207, 15 samples were positive for Total antiHBc. Out of 15, only 4 were positive for HBV DNA . Out of the 4 (both Total antiHBc and HBV DNA positive cases), only one sample was positive for antiHBs also. Out of 192 samples that were negative for Total antiHBc, only 2 samples were positive for HBV DNA only. There was no statistically significant correlation (table 3) of demographic profile among OBI positive and negative cases . LFT mean values (table 4) of OBI seropositive cases were also comparatively more as compared to OBI seronegative cases with no statistical significance. P value was >0.05 which was statistically not significant,hence there was no correlation (table 5) between risk factors and OBI positive and negative cases. Kappa value of 0.354 (table 6) depicts fair agreement between Total antibody against core antigen by ELISA and RTPCR for the diagnosis of OBI. Among confirmed OBI cases by RT PCR, highest HBV DNA level was LOG 5.15IU/ml while lowest HBV DNA level was LOG2.9IU/ml. The correlation between DNA level with total antibody to HBV core antigen (anti HBc) is not statistically significant.

Table 1:- OBI prevalence by ELISA and RT-PCR (n=207).

<table>
<tr><td colspan="2">Test</td><td>No</td><td>%</td></tr>
<tr><td colspan="2">Anti HBc+ive by ELISA (probable OBI)</td><td>15</td><td>7.24%</td></tr>
<tr><td colspan="2">HBV DNA by RTPCR (confirmed OBI)</td><td rowspan="2">6</td><td rowspan="2">2.96%</td></tr>
<tr><td>Seropositive OBI(N=4) PREVALENCE-1.93%</td><td>Seronegative OBI(N=2) PREVALENCE-0.96%</td></tr>
</table>

Table 2:- OBI biomarkers in all cases.

HBsAg	AntiHBs	Total AntiHBc	HBV DNA	Interpretation	TOTAL CASES
-	-	+	+	Seropositive OBI	3
-	+	+	+	Seropositive OBI	1
-	-	-	+	Seronegative OBI	2
-	-	+	-	'HBc alone'	11
-	-	-	-	No OBI	190
Total					207

Table 3:- Comparison of demographic profile among OBI positive and OBI negative cases.

Gender	**OBI +ve**	**OBI -ve**	**p value**

Male	5 (83.3%)	177 (88.1%)	0.54
Female	1 (16.7%)	24 (11.9%)	
Mean age in years	40.0±6.03 years	42.6±8.87 years	0.50

Table 4:- Comparison of Lft (Mean) (Seropositive OBI and Sseronegative OBI.

	OBI (seropositive)	**OBI (seronegative)**	**P value**
AST(MEAN)	52IU/l	48IU/l	0.46
ALT	52IU/l	51IU/l	0.45
ALP	88IU/l	90IU/l	0.66
S.BILURUBIN	2.2mg/dl	1.mg/dl	0.56
S.ALBUMIN	3.4mg/l	3.2mg/dl	0.67

Table 5:- Risk factor analysis among OBI cases with OBI negative cases.

	OBI +	**OBI-**	**P Value**
H/O alcohol intake	3 (50.0%)	32 (15.9%)	0.06
H/O past CLD	0	17 (8.5%)	1.0
H/O previous treatment	1 (16.7%)	8 (4.0%)	0.23
H/O Malignancy	0 (0%)	0 (0%)	-
H/O family history of CLD	1 (16.7%)	5 (2.5%)	0.16
H/O previous HBV exposure	0 (0%)	1 (0.5%)	1.0
H/O blood transfusion	0 (0%)	1 (0.5%)	1.0

Table 6:- Correlation of Total anti HBc by ELISA with RTPCR for OBI diagnosis.

	TOTAL ANTI HBc ELISA POSITIVE	**TOTAL ANTI HBc ELISA NEGATIVE**
RTPCR HBV DNA+ (N=6)	4 (66.66%)	2 (33.33%)
RTPCR HBV DNA- (N=201)	11 (5.47%)	190 (94.52%)

Kappa value = 0.354; p value <0.001

Discussion:-

OBI is a clinical condition which is difficult to diagnose and it mainly has two important characteristics: Absence of HBsAg, and low viral replication. Researchers have suggested that the lack of HBsAg in OBI may be due to rearrangements in the HBV genome that interfere with gene expression or lead to the production of an antigenically modified S protein.[7] This study was done with the aim to assess the prevalence of OBI in a tertiary care hospital and the role of Total antiHBc as surrogate marker of OBI and diagnosis of OBI by PCR.

In India, majority of research on OBI has been done on blood donors. A total of 207 individuals were enrolled in this OBI screening study, mean age being 42.53±8.80 years (table 1). Majority of the patients were male 182(87.9%) and 25 were female (12.1%). The prevalence of OBI in this study(table 2,3) was found to be 7.24% using total antibody against the core antigen(Anti HBc) as surrogate marker of OBI which is lower than other studies in India (10.2–58.8%).[8,9] . However, HBV DNA PCR is recommended for accurate diagnosis of OBI. Overall OBI prevalence using HBV DNA PCR was 2.96 percent in this study, with 1.93 percent having seropositive OBI and 0.96 percent having seronegative OBI. Rizvi et al in their study [10] reported a higher OBI prevalence of 25.6% using antiHBc as surrogate marker and 7.6% OBI prevalence using HBV PCR . The prevalence of seropositive OBI was higher than that of seronegative OBI in the present study . OBI was found to range from 3.9 percent in Kolkata (eastern India) and 0.78 percent in New Delhi (northern India) to 0.05 percent in Chandigarh (north-western India) and 0.15 percent in Vellore in studies conducted across India (southern India) using the HBV DNA PCR.[8,11,12] According to past studies which were conducted,based on the endemicity of HBV infection, countries were divided into three groups: high, intermediate, and low endemic areas. Hepatitis B virus (HBV) has been discovered in approximately 200 percent of the world's population, with 350 million individuals living with the infection long-term. With a disease burden of around 50 million people (prevalence of 2–7%, with an average of 4%), India is in the intermediate endemicity zone. In tribal regions, pockets of increased endemicity can be identified, where the high burden is sustained by intracaste marriages, tribal customs, illiteracy, and a lack of access

to health-care resources. [13] OBI is found in a wide range of people. It is estimated that 1% to 95% of people worldwide are affected, depending on several factors that influence prevalence rates, including: (1) geographical variations (endemicity), (2) patient characteristics, such as the presence of concomitant disorders like diabetes, and (3) the various diagnostic procedures used, each with a different sensitivity.[14,15]

In a study published in 2019,Raimando G et al discovered that serum was negative for all HBV infection serological indicators in roughly 20% of OBI, 50% were positive for hepatitis B core antibody (anti-HBs), and 35% were positive for hepatitis B surface antibody (anti-HBc). Based on these HBV antibody profiles, OBI can be categorised as seropositive or seronegative, with seronegative patients testing negative for both anti-HBc and anti-HBs people have lowest HBV levels .[16]

In this study, the prevalence of OBI among anti-HBc seropositives was found to be 1.96 percent. The variability in OBI prevalence is most likely attributable to the use of HBsAg assays of varied sensitivity, the prevalence of HBV, and the type of population studied. [17]

Regarding the demographic profile of OBI cases (table 4), 83.3 percent were male with age group 36-45 years while 1(16.7 percent) patient was female in the age group 26-35 years. There was no statistically significant difference regarding age and gender among OBI cases and patients with no OBI . There is very less data on age and gender of individuals with OBI available in previous studies. Most of the studies show generally higher prevalence of HBV infection in men. In the current study, out of 207 total number of patients 182 were male (87.9%)and 25 were female(12.1%) because of which the OBI prevalence might be more in males in this study as the male participants were already more in number. One study on OBI showed 100% males in Western Europe (on a small number of cases), 62% in South East Asia (genotype B/C) and 88% in Italy, Spain, Poland (genotype D). The authors recommended that the difference might be related to the generally more efficient anti-viral activity of the female immune system but this hypothesis would require further studies to be supported by firm evidence. [10,11,12,18] .There was a study conducted by Diarra B et al in 2018 and it was found that majority of OBI carriers mainly constituted of men (9/16) in the age group 31–50.[91] In a study condusted in areas of dominance of genotype A2 (Europe), B/C (South East Asia) and D (Mediterranean basin), median age, irrespective of gender,OBI transmission ranges between 45 and 55 years [10,11,12].

Out of total 207 patients , in 115 (55.6%) patients LFT was normal and deranged in 92(44.4%) patients. All cases of OBI had deranged LFT , with mean AST,ALT , bilirubin levels on the higher side in OBI cases when compared to Non OBI cases.. And among OBI cases (table 5), it was found that LFT mean values of OBI seropositive cases were comparatively more as compared to OBI seronegative cases. AST range in OBI positive cases was 48-58U/l, ALT range 40-62U/l, ALP range 24-146U/l, total bilirubin range was 1.3-4mg/dl, Total albumin mean was in a range of 2.4-4g/dl. This is similar to the findings of Rizvi et al [10] where total bilirubin levels were significantly deranged in OBI patients. Although occult hepatitis B infection has been documented in a variety of clinical circumstances, it is unknown if occult HBV causes liver damage. Persistent HBV infections might frequently experience flare-ups in viral replication and liver damage with elevated liver enzymes. A study done previously showed that during the enzyme flares, HBV DNA was detected in 7/7 study patients versus 3/8 controls, $p = 0.026$ suggesting a relationship between occult hepatitis B and liver damage. It was found that HBV infection can cause flare-ups in viral replication, which were linked to elevated liver enzymes.[19] Anti-HBc reactive samples were tested for LFTs in a study conducted by Dhawan HK etal. The serum bilirubin levels were all normal, and two (18%) of the samples had elevated liver enzymes. Increased liver enzyme levels in blood donors indicate underlying hepatitis or liver injury, which can only be ruled out by additional tests like hepatic ultrasonography, liver biopsy, and genetic testing.[20]

Risk factor analysis (table 6) in present study showed that history of alcohol intake was the most common risk factor seen in 50% (3/6) patients out of total 6 OBI cases however it was found to be statistically not significant (P value was >0.05) . In fact there was no statisitical significance between any risk factors among OBI positive and negative cases indicating that none of the risk factors were associated with OBI. In India, alcohol is currently the most common cause of CLD, and this representation has arisen over the previous few years. This is attributed to growing alcohol consumption rates rather than a true drop in HBV infection prevalence (which has remained steady over time) or the influence of vaccination (as the vaccine programme in India on mass basis started only in 2011).[13] Very few studies have analyzed risk factor among OBI cases. One study conducted by Azarkar et al [19] showed that

OBI was significantly associated with some risk factors like diabetes, tattooing , tooth surgery, imprisonment, and multisexual behaviors were associated although these have not been seen or reported anywhere else in the literature.

The most reliable OBI biomarker is low-level serum HBV DNA detected using a sensitive, quantitative HBV PCR assay . In the absence of HBsAg and antiHBs antibody, when HBV DNA test is unavailable or intermittent viremia is suspected, detection of anti-HBc antibody, a surrogate marker of OBI, is beneficial.[13] When OBI is characterised only by the presence of anti-HBc antibody, erroneous anti-HBc positivity and negativity in OBI detection should be taken into account. Not everyone who tests positive for anti-HBc also tests positive for HBV DNA. Furthermore, the lack of antiHBc antibody does not rule out seronegative OBI.[6] Although anti-HBc is not an ideal OBI biomarker still it indicates the risk .

OBI can occur after infection, with antibodies to the hepatitis B surface antigen (anti-HBs) and persistent low-level viraemia, escape mutants undetected by HBsAg testing, or healthy carriage with antibodies to the hepatitis B e antigen (anti-HBe) and the hepatitis B core antigen (anti-HBc) (anti-HBc). Anti-HBe and, later, anti-HBc may become undetectable in the latter circumstance over time.

In the present study, it was found that there is a fair level of agreement (table 7) between total antibody against core antigen by ELISA and RTPCR (Kappa value = 0.354) .This corelation between total antibody against core antigen ELISA and RTPCR was found to be statistically significant as p value <0.001 is less than 0.05.

Some researchers believe that the HBsAg immunoassay s inferior sensitivity for detecting HBV compared to polymerase chain reaction (PCR) is to blame for the emergence of OBI. However, the reduced replication rate of HBV seen in OBI cannot be explained by this variation in assay sensitivity. The precise underlying mechanisms of OBI, which could be complex, remain unknown. Both host and viral variables appear to play a role in viral replication inhibition and infection control.[21]

In earlier studies, evolution of mutations in gene of HBV surface antigen resulted in virus escaping which is undetectable in HBsAg-negative patients by common serological tests hence making it difficult to diagnose Occult HBV infection (OBI) as stated earlier too. It can be diagnosed by highly sensitive and specific molecular biology techniques like the real time PCR.[22] Sensitive detection and quantification of HBV-DNA is essential for assessment of occult HBV infection and therefore Real time PCR assays with remarkable analytical and clinical sensitivity, calibrated against the WHO 1st International standard (HBV-DNA values are reported in the international format (IU/mL)) are needed .[23]

The gold standard for OBI diagnosis is the detection of HBV DNA from the liver, as cccDNA persists in the hepatocytes and HBV DNA is many a times detected in the liver when HBV DNA is absent in serum.[6] As obtaining liver tissue is an invasive procedure ,hence obtaining hepatic HBV DNA is difficult in clinical practice. In addition, real-time PCR based assays for serum (or plasma) HBV DNA detection have been used with better sensitivity to detect OBI in majority of cases. To improve the sensitivity of the test, at least 1 mL of serum should be collected when a blood sample is used. Using oligonucleotide primers specific for distinct HBV genomic areas and complementary to highly conserved nucleotide sequences, DNA extracts should be amplified by highly sensitive nested PCR or a real-time PCR approach that can detect fewer than 10 copies of HBV DNA. Each PCR experiment should contain appropriate negative and positive controls. In addition, periodic testing for HBV DNA will improve diagnosis of OBI especially in high-risk patients, as intermittent viremia can occur in occult HBV infection.[24] When highly sensitive HBV DNA testing cannot be performed, anti-HBc could be used as a possible surrogate marker for identifying potential seropositive OBI cases. [24]

In this study, among the 6 OBI cases confirmed by quantitative real time PCR for HBV DNA , highest HBV DNA level was 141550IU/ml(LOG 5.15IU/ml) and lowest was 912IU/ml (LOG2.9IU/ml). A study conducted by Bajpai et al in New delhi [24]observed that HBV-DNA levels in OBI cases ranged from log 2.19 to 4.93 IU/ml with median log value of 3.16 IU/ml .It was observed in the present study that on correlating LFT with viral load , mean AST , ALT ,S BIL,S ALB was found to be comparatively lower in OBI cases with HBV DNA <2000IU/ml as compared to those with HBV DNA >2000IU/ml although it was not statistically significant. A study conducted by Dhawan et al, suggested a relationship between occult hepatitis B and liver damage. HBV infection was seen to be showing flare-ups in viral replication, which were linked to elevated liver enzymes.[5,20]

Conclusion:-

OBI has high prevalence among patient of CLD. All HBsAg negative with total anti HBc positive individuals are probable OBI and it is recommended that all such cases should be screened with PCR for HBV DNA.

For confirmed OBI, HBsAg negative patients should be tested for Total anti HBc by ELISA and HBV DNA by PCR. It is recommended that real time HBV DNA PCR should be performed in all HBsAg negative patients with chronic hepatic inflammation (deranged LFT).

Total Anti HBc by ELISA may be used as a surrogate marker for diagnosis of OBI if molecular facility is not available.

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