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Leptospirosis: an emerging disease in cryptogenic hepatitis patients in north India

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Abstract
We assessed the role of occult HBV, leptospirosis, cytomegalovirus and Epstein-Barr virus in cryptogenic hepatitis cases.246 consecutive cases with symptoms of acute hepatitis were enrolled in the study with 30 healthy controls. ELISA for HAV, HBV, HCV and HEV were performed to rule out common viral etiology of hepatitis. Occult HBV was detected by amplification of HBV pre-core gene. IgM antibodies to leptospira, CMV and EBV were detected by ELISA.142 (57.7%) were HBV positive, 6 (2.43%) were HCV positive, 5 (2.03%) were positive for HAV and 3 (1.21%) were HEV positive. Two had occult HBV infection. 90 cases were labeled as cryptogenic. 46 (51%) of these were positive for leptospirosis, 8 (8.8%) were positive for CMV and 2 (2.2%) were positive for EBV. Mean age of these patients was 29.43years. Majority belonged to rural areas. Deranged liver functional test was observed in most of the cases. Leptospirosis emerged as a major cause of hepatitis apart from HBV in this region. It should be actively looked for during the preliminary work up of hepatitis cases so that it can be treated on time preventing significant morbidity. We recommend detection of leptospira in the preliminary work up itself in cryptogenic hepatitis if the clinical condition so merits.

Keywords: HBV; Leptospirosis; CMV; EBV.

Introduction

Hepatitis A, B, C and E viruses cause more than 80% of viral hepatitis. In the remaining 15-20% cases, the etiology of hepatitis remains uncertain and cannot be attributed to any of the known infectious and non-infectious causes of hepatitis. This category of hepatitis of unknown etiology is usually termed as cryptogenic hepatitis. Several studies have pointed to occult HBV as a leading cause of cryptogenic hepatitis. Despite the advent of modern diagnostic tools that detect occult HBV and HCV infection, several cases of hepatitis still remain unexplained. While HGV, TTV and SEN viruses are being increasingly studied, their role in hepatitis remains questionable. Bacterial etiology in causation of hepatitis remains largely unexplored during the clinical workup of patients with hepatitis. Non-hepatotropic herpes viruses are also rarely considered in the differential diagnosis of hepatitis.

Leptospirosis is a septicemic spirochaetal zoonosis with multisystemic involvement, caused by the pathogenic strains of *Leptospira interrogans*. Rural farm workers have traditionally been at high risk for leptospirosis, and it can be a significant public health problem when water and food safety are not ensured. Several epidemics of leptospirosis have occurred on Andaman and Nicobar islands and in southern and western parts of India during the past century (Sehgal, 2000). The organism has been detected in farm animals in many parts of the country (Ratnam, 1994).

The true incidence of human leptospirosis in northern India is not known either because of a lack of awareness on the part of the treating physicians or because of the lack of diagnostic techniques (Joseph and Kalra, 1966). Given the large rural population in North India, lack of safe and clean drinking water, presence of a large bovine population not to mention rodents we feel that there is a need for a pilot study to assess the prevalence of leptospira in our region.

EBV is a ubiquitous human herpes virus that is usually transmitted through close personal contact among young children and via intimate oral contact among adolescent and young adults (Hoagland, 1955).Transmission by blood transfusion also has been documented (Paloheimo and Halnonew, 1965). Hepatic involvement is exceedingly common in EBV infection and varies in severity (Horowitz and Burke, 1980).

CMV, another member of the human herpes family of viruses, transmissible through blood component transfusions is an important

cause of concern worldwide. CMV is a ubiquitous agent, and seropositivity rates in the adult population over 40 years of age worldwide are 60 to 100%, possibly due to transmission through breastfeeding, sexual contact and spread from children (Zhang *et al.*, 1995; Hecker *et al.*, 2004). Herpes viruses have been implicated in hepatitis in several studies especially CMV and EBV. All three can have a benign to severe course-effective antibacterial and antiviral drugs are available if the need arises. Thus, detection of these agents in patients where other causes have been excluded can lead to better patient care.

In this study, we assessed the role of one spirochaete i.e. leptospira and two herpes viruses - CMV and EBV - as etiological agents in cryptogenic hepatitis in Aligarh region of U.P. Such a study in patients of hepatitis has not been conducted before in this region in particular and in India in general.

Materials and Methods

246 consecutive cases [159 male (64.64%), 87 female (35.36%); mean age was 31.99 yrs; ± 14.02] with symptoms of acute liver disease attending the Medicine outpatient department or admitted in the Medicine wards of J.N. Medical College, were recruited in the study over a ten month period from June 2009 to March 2010. This study was conducted in the Department of Microbiology, J.N. Medical College, A.M.U., Aligarh. A written informed consent was obtained from each patient. The study was cleared by the Institutional Ethical Committee of J.N. Medical College. All patients underwent complete physical examination and detailed clinical history was elicited from them. Patients with autoimmune hepatitis, alcoholic hepatitis and drug-induced hepatitis were excluded from the study.

Healthy control individuals

The control group consisted of 30 healthy people [22 (73.34%) men and 8 (26.66%) women; mean age 37 yrs] which were randomly selected from the blood bank.

Collection and transport of sample

Seven ml blood was collected aseptically from all patients. Serum was separated by centrifugation, aliquoted and stored at -20°C till further tests were performed. A battery of biochemical and hematological investigations were conducted.

Routine investigations

Liver function tests, prothrombin time and total bilirubin were performed. Specified investigations like ultrasonographic examination of liver, upper GI endoscopy and liver biopsy was performed wherever feasible.

Serological tests for hepatitis viruses

The sera were first tested for hepatitis A, B, C, and E virus markers using commercially available ELISA kits according to the manufacturer's instructions: HAV IgM and HEV IgM antibodies were detected by ELISA kits (DRG International, Inc., USA). Sera of all patients were subjected to detailed screening for HBV serological markers i.e. HBsAg (J. Mitra & Co. Pvt. Ltd., India), Anti-HBc Plus (Monolisa^{TM}, Bio-Rad, France), IgM anti HBc (DRG International, Inc., USA), HBeAg (DRG International, Inc., USA), anti HBe (Radim, Italy) and HCV Antigens for Core, NS3, NS4 & NS5 by 3rd generation immunoassay test (J. Mitra & Co. Pvt. Ltd., India).

Amplification of HBV DNA

Finally, we performed the PCR for HBV core gene in all HBV serologically positive patients to reconfirm HBV infection as well as in all serologically negative patients to detect the occult HBV infection. DNA was extracted from 100 *µ*l of serum by phenol chloroform extraction and ethanol precipitation, and the pellet was dissolved in 30 *µ*l of DNAase and RNAase free sterile water and directly subjected to PCR-based amplification. PCR was carried out for the amplification of a 321 base pair sequence of the core gene of HBV using the primer sequences synthesized by Operon, Germany (Genetix). HBV DNA was amplified in a PCR thermal cycler (Labnics, USA) for 40 cycles using the following primer sequences: forward primer, 5-TA(C/T) TAG GAG GCT GTA GGC AT-3; reverse primer, 5-AGA ATA GCT TGC CTG AGT GC-3. A 2-*µ*l DNA sample was mixed with the reaction mixture and a 25-*µ*l amplification reaction was performed. The reaction mixture contained 1x PCR buffer, $MgCl_2$ (1.5 mM), 200 Mm dNTP's, a 20 pmol concentration of each primer; and 2.5 units of Dream Taq DNA polymerase (MBI Fermentas, USA).

Detection of leptospira, CMV and EBV

Patients negative for all the hepatitis viruses and 30 healthy controls were tested for evidence of recent leptospiral, EBV, CMV infections by

specific leptospira IgM antibody using the commercially available ELISA kit (DRG International, Inc., USA), CMV IgM and EBV-VCA IgM antibody (Calbiotech, Inc, USA). The test procedure was performed according to the protocol provided along with the kit. The results for leptospira IgM ELISA were interpreted according to manufacturer's instructions, i.e. values, 0.0-0.3 OD units (DRG ELISA) were considered negative, 0.5-<1.0 OD units were equivocal and >1.0 OD units were positive. For samples showing equivocal results, another blood sample was drawn after a period of 10 or more days, and the test was repeated. Negative and positive controls were kept with each test run. Results for CMV IgM and EBV VCA IgM were interpreted according to manufacturer instructions, i.e. Ab index < 0.9 were negative, 0.9-1.1 were equivocal and >1.1 would be considered positive for both infections.

Results

Distribution of A, B, C, and E hepatitis viruses

Of the 246 consecutive patients with hepatitis, 115 (46.74%) were HBsAg positive, 6 (2.43%) were positive for anti-HCV, 5 (2.03%) were positive for anti-HAV IgM while anti-HEV IgM were positive in 3 (1.21%) patients. On analyzing the HBV serological markers patients a total of57.7 % were found to be HBV positive as shown in (table 1). 142 cases were positive for anti-HBc total among which anti-HBc IgM was present in 60 persons. Of the remaining 82 positive for anti-HBc total, 30 were anti-HBs positive while 52 were chronic carriers. Two (1.52%) cases were identified as occult HBV infection in which all HBV serological markers were negative except anti-HBc total in one case and none was positive in the other.

Table 1: Distribution of HBV serological markers in positive cases.

Patients	HBsAg	anti-HBc Total	anti-HBc IgM	HBeAg	anti-HBe	anti-HBS
Positive	115	142	60	45	65	30
Negative	131	104	186	201	181	216

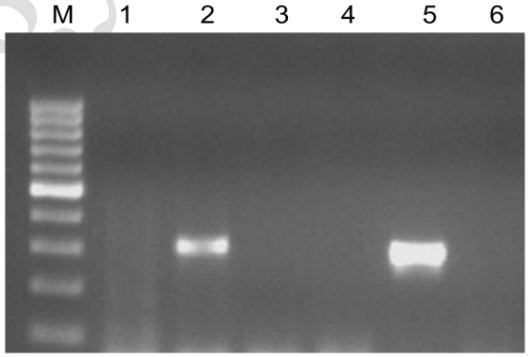

Figure 1: Shows *M*, 100 bp ladder; *lane2 & 5* show the 321 bp product obtained with F &R primers of core gene (HBV) while *lane* 1, 3 &4 show negative samples and lane 6 is negative control.

Prevalence of leptospiral, CMV, and EBV infection

Ninety (36.5%) patients were negative for the HBV, HCV, HAV and HEV infection which were then investigated for acute leptospiral, CMV, and EBV infection. Among these cases, surprisingly, leptospirosis was detected in 46 (51%) [p <0.017; CI 95%: -.2987 to .0305] out of the 90 patients while 8 (8.8%) and 2 (2.2%) patients were positive for acute CMV and EBV infection respectively (as shown in table 2). Among the healthy controls, one patient each was positive for leptospira and CMV both and none was positive for EBV. The O.D. in the leptospira patients was extremely high and ranged from 1.08 to 2.733 while in the control group, O.D. was much below the cutoff. Repeat samples were requested in 8 cases. In one case, a fourfold rise in O.D. was observed. Similarly, the Ab index of CMV and EBV in cases of cryptogenic hepatitis were well above the cutoff ranging from 1.1 to 2.482 and 1.04 to 1.12 respectively.

Table 2: Infectious etiology in 90 cases with cryptogenic hepatitis.

	Leptospira IgM	CMV IgM	EBV IgM
No. of positive cases in cryptogenic hepatitis	46/90 (51%) P<0.017	8/90 (8.8%)	2/90(2.2%)
No. of positive cases in control group	1/30 (3.33%)	1/30 (3.33%)	0/30(0%)

Clinico-epidemiologic profile of leptospira, CMV, and EBV infected patients

The leptospira positive patients, hailed from various parts of U.P., Delhi and Haryana, however, majority of patients belonged to Aligarh region of Uttar Pradesh. Mean age of cryptogenic hepatitis patients was 28.7 yrs ± 13.73 ranging from 6.5 yrs to 65 yrs. old. Male patients were 46 while there were 44 female patients. Most of the patients were from rural (65.35%) areas surrounding Aligarh. Major epidemiological risk factors were their occupation followed by direct contact with contaminated water and alcoholism. Most of the patients were farmers 31 (34.4%), laborers 12 (13.3%), 39 (43.3%) had animal contact, 43 (47.7%) gave history of using unclean ponds and dirty surroundings. Patients with primary CMV and EBV infection had no particular epidemiological profile and there was no significant urban- rural divide.

The clinical profile is given in Table 3. In leptospirosis, fever (97.8%) predominated followed by jaundice (76%), anorexia (58.6%), headache (45.6%) nausea/vomiting (36.9%). One patient infected with leptospira expired. Common symptoms of CMV and EBV were similar with sudden onset of fever, headache, chills, severe myalgia, and hepatosplenomegaly.

Table 3: Different clinical features of leptospirosis, CMV and EBV infected patients.

Clinical features	No. of Leptospirosis infected patients (%)	No. of CMV infected patients (%)	No. of EBV infected patients (%)
Jaundice	35(76)	1(12.5%)	-
Fever	45(97.8)	6(75%)	2(100%)
Headache	21(45.6)	1(12.5%)	-
Anorexia	27(58.6)	7(87.5%)	-
Vomiting/Nausea	17(36.9)	-	1(50%)
Myalgia	16 (34.7)	3(37.5%)	2(100%)
Hepatomegaly	17(36.9%)	2(25%)	2(100%)
Splenomegaly	5(10.86%)	2(25%)	1(50%)
Conjuctival suffusion	6(13)	(28%)	-
Diarrhea	4(8.6)	-	-
Abdominal pain	31(67.3)	-	-
Respiratory symptoms	9(19.5)	3(37.5%)	1(50%)
Lymph adenitis	-	2(25%	2(100%)

In cases of leptospirosis, the mean ALT and AST, ALP was raised in a majority of patients 15 (32.6%). Some had only raised bilirubin, ALP, AST or ALT alone. In CMV and EBV, the transaminases were moderately elevated (as shown in table 4).

Table 4: Biochemical profiles of Leptospirosis, EBV and CMV infected patients.

Parameters	Leptospira	EBV	CMV	Reference Range
SGOT	31.3IU/L	23IU/L	39.42IU/L	2-20 IU/L
SGPT	32.61IU/L	24.2IU/L	39.142IU/L	2-15 IU/L
ALP	16.46KAU/100ml	14.3KAU/100ml	18.85KAU/100ml	3-13 KAU/100ml
Serum Bilirubin	2.84mg/100ml	1.4mg/100ml	2.72mg/100ml	0.2-1.0 mg/100ml

*****All values shown in table 3 are mean values.

Discussion

In our study, 156 (63.4%) patients were infected with hepatitis A-E viruses including 2 (0.81%) cases of occult HBV infection. This is the first report of occult HBV infection from this region. Contrary to other reports, prevalence of occult HBV infection in this study was quite low (Vaishali *et al.,* 2004). Majority of patients were infected with HBV (56.91%) followed by HCV (2.43%), HAV (2.03%) and HEV (1.21%). In the 90 cryptogenic cases, leptospira was detected in 51% cases, 8.8% had cytomegalovirus infection and 2.2% were Epstein-Barr virus infected cases. By detecting these additional bacterial and viral causes of hepatitis, the 90 (36.5%) cases labeled as cryptogenic shrank to 34 (13.82%). An unprecedentedly high prevalence of leptospirosis was found in cryptogenic hepatitis patients in our region. CMV positivity at 8.8% was also high. Three of these patients were immunocompromised and remaining five were healthy individuals. EBV accounted for only 2.2 % cases. The unexpectedly high prevalence of 51% cases of leptospira is a cause for concern. The most common presenting complaints in these patients were fever, jaundice and anorexia. The liver enzymes were moderately raised in our study. Serum bilirubin levels elevate as part of the obstructive disease due to capillaritis in the liver. Levels of hepatocellular transaminases are elevated less often and less significantly (usually <200 U/L). Jaundice and bilirubinemia disproportional to hepatocellular damage is common in leptospirosis. There have been reports of emergence of leptospirosis from Chandigarh (6.3%), Himachal Pradesh (22%), Punjab (29.8%), Haryana (44%), Western U.P. (9.5%), Eastern U.P. (3.1%) and Bihar (3.1%) (Sunil *et al.,* 2010).

Several studies have focused on herpes viruses as etiologic agents of hepatitis in immunocompetent adults (Cisneros-Herreros and Herrero-Romero, 2006; Vujacich *et al.,* 2006; Just-Nübling *et al.,* 2003). Primary cytomegalovirus (CMV) infection may give rise to more or less severe clinical illness in such patients. The predominant clinical complaint in these patients was fever with hepatitis. Similar presentations were observed in other studies (Vujacich *et al.,* 2006; Just-Nübling *et al.,* 2003). The liver enzymes were moderately raised. The differential diagnosis of CMV infection with hepatic involvement against acute hepatitis A, B, C and E can be based on the absence of jaundice, fever, intense asthenia and comparatively lower elevation of transaminases in such cases.

In the acute phase of infectious mononucleosis, elevated transaminases are found in 80% of patients, while jaundice is noted in only 5.0-6.6% (Markin, 1994). Hepatitis owing to primary EBV infection is usually mild and self-limited, although the mechanism is unclear. Rarely, it results in hepatic failure with severe jaundice in fatal infectious mononucleosis (Markin *et al.,* 1987). Hepatic involvement with infectious mononucleosis varies in severity and its frequency varies with age, which is estimated to be 10% in young adults and 30% in the elderly (Lawee, 2007). EBV infections are often associated with mild hepatocellular hepatitis, can go undetected, and resolves spontaneously. The elevated aminotransferases are usually less than five-fold normal levels, and bilirubin may be elevated in up to 5%, which may be due to intrahepatic cholestasis or hemolytic anemia

(Adams *et al.,* 2006). In our two EBV positive cases, both were immunocompetent adult patients and had mild hepatitis with moderate elevation of aminotransferases.

In our study, leptospira prevalence was second only to HBV infection. We suggest that it should be investigated during the preliminary workup itself. It diagnosed and treated on time, leptospirosis is easily treatable and significant morbidity and mortality can be prevented. Leptospirosis, a septicemic zoonosis with multisystemic involvement, caused by the pathogenic strains of *Leptospira interrogans* appears to be an emerging public health problem in North India and especially so in the Aligarh region. Rural farm workers are at high risk of contracting leptospirosis, and it can be a significant public health problem when water and food safety are not ensured. As was seen in our study a predominantly rural poor population was infected. In 1998, researchers warned that, unless adequate public health measures were initiated, large leptospirosis epidemics were possible in areas where the disease had not been previously reported (Singhal and Sood, 1998). In addition, they recommended improving clinical diagnosis and conducting systematic epidemiologic studies for control of the disease (Singhal and Sood, 1998). Leptospirosis occurs as two recognizable clinical syndromes. Anicteric leptospirosis is a self-limited disease similar to a mild flulike illness. icteric leptospirosis, also known as Weil disease, is a severe illness characterized by multi-organ involvement or even failure. Two distinct phases of illness are observed in the mild form—the septicemic (acute) phase and the immune (delayed) phase. In icteric leptospirosis, the 2 phases of illness are often continuous and indistinguishable. At disease onset, clinically predicting the severity of disease is not possible. Subsequent sequelae depend on the serovar involved and the health, nutritional status, and age of the patient, as well as the rapidity of definitive and supportive treatment.

Leptospira has been recognized in India since 1931 (Sambasiva *et al.,* 2003). Early diagnosis is essential. If untreated the illness can progress rapidly and mortality rates are high in severe cases. It is therefore important to actively investigate leptospirosis in cases of cryptogenic hepatitis (Cumberland *et al.,* 1999). Reported prevalence of leptospirosis studies from different regions shows a wide disparity. Interestingly, the present study found that 51% of cases had serological evidence of leptospirosis, which is definitely higher than the previous studies in north India (Sunil *et al.,* 2010; Manocha *et al.,* 2004).

Detection of IgM antibodies to leptospira by ELISA is now widely used in diagnosis of leptospirosis, which is more sensitive than MAT and most laboratories prefer IgM ELISA formats for the diagnosis of leptospirosis. Further, this test is reactive even in early cases of leptospirosis when MAT may be negative. MAT does not have any diagnostic significance in 1st week and antibodies peak around 3rd week. Moreover, it is a very highly complex test to perform and interpret, and therefore can be done only in reference laboratory at Andaman in India. Earlier studies showed IgM antibodies could be detected by ELISA from as early as the fifth day to the 60th day after acute infection with leptospirosis (Sumathi *et al.,* 1995).

The prevalence of leptospirosis is higher in rural as compared to the urban population, mainly due to greater exposure to livestock, nearby dirty surroundings and unclean ponds. Other reason for high prevalence of leptospirosis in this area may also be due to the overuse of fertilizers commonly used in agriculture, which makes the pH of the water and soil alkaline, thereby allowing leptospira to survive for a longer time and thus facilitating its transmission as reported before (Bharadwaj *et al.,* 2002). The frequency of clinical features among leptospira positive patients showed a wide variation. Similar variations in symptoms of leptospirosis have been reported earlier (Sumathi *et al.,* 1995; Park *et al.,* 1989).

Conclusion

The result of our study point out that leptospiral infection is more prevalent than CMV and EBV and not uncommon in this region. Due to its protean clinical presentation, diagnosis on clinical grounds alone is difficult. We recommend that a high index of suspicion for leptospirosis should be maintained in patients of cryptogenic hepatitis especially so in the presence of history of rural background, animal contact, and contact with water contaminated with urine of rodents. Early diagnosis of this potentially dangerous disease is imperative in preventing fatalities caused by leptospirosis. In this study, one mortality occurred due to leptospiral infection. Thus, in this region, leptospirosis infection should be actively looked for followed by CMV and EBV if leptospiral infection is negative.

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