An Effective Approach to the Diagnosis of Acute Viral Hepatitis in Bangladesh

A THESIS SUBMITTED TO THE UNIVERSITY OF DHAKA IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF PHILOSOPHY IN BIOCHEMISTRY

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Reply to the queries of the examiner:

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Following corrections are made according to the honorable examiners instructions for kind consideration and acceptance of the thesis paper:

Query no. 1 and 11: Spelling mistakes and other minor mistakes were corrected at the mentioned pages.

Query no. 2: Criteria for the diagnosis of acute viral hepatitis were added on page no. 36.

Query no. 3: In figure 19 (page 63) the total percentages exceed 100 as the mixed infection were not excluded during calculation of the percentage for individual virus. One sample which has co-infection were counted twice during calculation of percentages.

Query no. 4: In table 6 (page 63) sum of the multiple infection and percentage were corrected to 81 and 15.98 respectively.

Query no. 5: Table 7 has been corrected. There were some mistakes in the table 7. Previously prepared table was attached to the thesis papers. The remaining test were done later and in place of corrected table, previous table was attached. The sample no. is 507 and the positive no. of cases is 58 and the actual percentage is 11.4%.

Query no. 6: Same mistakes also happened in the cases of IgM anti-HBc and IgM anti HEV. Total no. of anti-HEV. IgM positive cases were 289 and 38 cases which positive for only anti-HEV.IgG were considered positive for HEV and the percentage for the Hepatitis E virus was calculated as 64.5% from the formula (327/507)X100. Same thing also happened in case of hepatitis B virus. HBsAg positive in 109 cases and 23 cases positive for anti-HBcIgM were considered collectively to calculate the percentage for HBV. The calculation was (132/507)X100.

Query no. 7: The total no. of cases positive for anti-HEV.IgG now mentioned in the legend of figure 19 and 20.

Query no. 8: The mixed infections are within the study population. It is explained in query no.3. Total no. of sample is 507. In figure 20, the number mentioned in the figure corresponds the no. of positive cases of that virus.

Query no. 9: References were added in all the figures.

Query no. 10: References taken from the text books are corrected and written properly.

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Declaration

This dissertation has been submitted to the University of Dhaka in partial fulfilment of the requirements for the degree of Master of Philosophy (M.Phil) in Biochemistry. This study has been carried out in the Department of immunology, Bangladesh Institute of Research and Rehabilitation in Diabetes, Endocrine and Metabolic disorders (BIRDEM). No part of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institutes of learning.

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Dedicated to
My Parents &
Sk. Saadi Rahmatullah

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ABSTRACT

Serological markers of different hepatitis viruses and liver function tests are important for diagnosis, management and follow up of patients with viral hepatitis; and helps prevention of disease transmission. This study was carried out to find out the etiological agents responsible for acute viral hepatitis in Bangladesh. It also evaluated the importance of liver function tests, clinical features and risk factors associated with the disease. Serological markers for hepatitis A, B, C, D and E as well as liver function tests (Serum bilirubin, ALT: alanine transaminase, AST: aspartate transaminase, ALP: alkaline phosphatase, PT: Prothrombin time) was assayed in 507 clinically jaundiced individuals. Hepatitis E virus (HEV) was found to be the commonest (64.5%) etiology of acute viral hepatitis followed by hepatitis B virus (HBV, 26%), hepatitis A virus (HAV, 11.4%), unknown causes (11.2%) and hepatitis C virus (HCV, 2.8%). There was none found with hepatitis D virus (HDV). Among the positive cases (88.8%), 16% had co-infections with more than one virus. Most common association was observed for HEV and HBV (10%). Youngest age group had predominantly HAV infection; adolescent and young adults had mostly HEV and HBV infection, whereas senior aged group had HEV or HBV or HCV or unknown viral hepatitis. Almost everyone of the studied population had evidence of IgG,anti-HAV. Among the risk factors and environmental factors, history of previous jaundice was important for HBV (40.0%) and HEV (20.6%) as well as unknown (22.8%) viral hepatitis ($p=0.001$); history of surgery for unknown (40.4%) and HCV (40.0%) as well as HBV (29.3%) ($p<0.001$); and blood transfusion for HCV (40.0%) and HBV (12.0%) in most instances ($p<0.001$). Of the clinical findings, fever was more associated with HAV (86.5%) and HEV (69.0%); whereas hematemesis (20.0%) and ascites (40.0%) were mostly related to HCV. Hepatospleenomegaly was more prevalent among HBV (33.3%), HEV (21.8%) and unknown (28.1%) viral hepatitis. Altered and raised ALT and AST were equally important for all types of viral hepatitis ($p=0.531$ and $p=0.205$ respectively); and magnitude of rise was higher in HAV and HEV ($p<0.001$). Younger the age, higher was the chances of HAV ($p<0.001$) which was also reflected by inverse relation of HAV with marital status ($p<0.001$). Similarly, shorter the duration of jaundice, probability of HEV ($p<0.001$) and HAV ($p<0.001$) were higher; conversely, more the duration of jaundice, higher the possibility of HBV ($p<0.001$) and unknown hepatitis ($p=0.017$). Pregnancy had strong relationship with HEV ($p=0.002$). Ascites ($p=0.034$), history of jaundice ($p<0.001$), blood transfusion ($p=0.037$), multiple sex partnership ($p=0.042$), hepatospleenomegaly ($p=0.010$) significantly correlated with HBV; and some of them for

HCV and unkonwn hepatitis. This study suggests that, young patients (age<16 years) with high ALT and/or AST level should be tested for IgM anti-HAV and hepatitis B surface antigen (HBsAg). IgM anti-HAV seems unnecessary for the age group higher than 16 years while IgM anti-HEV for the age group less than 16 years. IgM anti-HEV test can be done for the age group less than 16 years when the above mentioned tests are negative. Only HBsAg positive cases can then be further investigated for IgM anti-HBc to detect acute HBV infection. If all the above mentioned tests are negative then IgG anti-HEV test can be done. Patients negative for all the above markers should then be tested for anti-HCV. If anti-HCV is negative, autoimmune hepatitis or secondary causes should be investigated.

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List of Abbreviations

ALP	: Alkaline phosphatase
ALT	: Alanine transaminase
ANOVA	: Analysis of variables
Anti-HDV	: Antibody to hepatitis D virus
Anti-HAV total	: Antibody against hepatitis A virus
Anti-HBc IgG	: IgG antibody against hepatitis B core antigen
Anti-HCV	: Antibody against hepatitis C virus
Anti-HBc,total	: Antibody against hepatitis B core antigen
Anti-HBcIgM	: IgM antibody against hepatitis B core antigen
Anti-HBs	: Antibody against hepatitis B surface antigen
Anti-HBe	: Antibody against hepatitis B e antigen
Anti-HCV	: Antibody against hepatitis C virus
Anti-HDV	: Antibody against hepatitis D virus
Anti-HIV	: Antibody against human immunodeficiency virus
AST	: Aspartate transaminase
BCIP	: Bromochloro-indolylphosphate
BIRDEM	: Bangladesh institute of research & rehabilitation in diabetes, endocrine and metabolic disorders
HAV	: Hepatitis A virus
HBV	: Hepatitis B virus
HCV	: Hepatitis C virus
HDV	: Hepatitis D virus
HEV	: Hepatitis E virus
HBsAg	: Hepatitis B surface antigen
HBeAg	: Hepatitis B e antigen
HBcAg	: Hepatitis B core antigen
EBV	: Epstain-Bar virus
CMV	: Cytomegalovirus
HSV	: Herpes simplex virus
HDV-RNA	: Hepatitis D virus ribonucleic acid
DNA	: Deoxyribonucleic acid
RNA	: Ribonucleic acid
ORF	: Open reading frame

UTR	: Untranslated region
NANBH	: Non-A, non-B hepatitis
HVR	: Hypervariable region
HDAg	: hepatitis D virus antigen
HAVAg	: Hepatitis A virus antigen
IgM Anti-HAV	: IgM antibody against hepatitis A virus
IgG Anti-HAV	: IgG antibody against hepatitis A virus
IgM Anti-HEV	: IgM antibody against hepatitis E virus
IgG Anti-HEV	: IgG antibody against hepatitis E virus
IFN	: Interferon
HBV-DNA	: Hepatitis B virus deoxyribonucleic acid
WHO	: World Health Organization
RIA	: Radioimmuoassay
EIA	: Enzyme immunoassay
PCR	: Polymerase chain reaction
HIV	: Human immunodeficiency virus
PT	: Prothrombin time
ELISA	: Enzyme-Linked immunosorbent assay
LIA	: Line immuno assay

Chapter 1

INTRODUCTION

1. INTRODUCTION

1.1 VIRAL HEPATITIS

Hepatitis is an inflammation of the liver resulting from variety of causes e.g., viruses, bacteria, toxins, drugs or excessive alcohol (1-2). 'Viral hepatitis' is the most common liver disease worldwide caused by hepatotropic viruses endemic worldwide and which cause hepatitis as the sole or predominant clinical manifestation of infection (3). The term viral hepatitis is usually used to describe infections caused by agents whose primary tissue tropism is the liver. Acute viral hepatitis is a common and sometimes serious viral infection of the liver leading to inflammation and necrosis. The viral agents of acute hepatitis can be divided into two major groups (4-5):

1.1.1 Primary hepatitis viruses:

Five major viruses are the etiologic agents responsible for most clinical cases of viral hepatitis: hepatitis A virus (HAV), hepatitis B virus (HBV), hepatitis C virus (HCV), the delta agent (HDV), and hepatitis E virus (HEV). There are reports for other viruses such as hepatitis F virus (HFV) and hepatitis G virus (HGV) (6). Primary hepatitis viruses account for approximately 95% of the case of hepatitis (4). These viruses are classified as primary hepatitis viruses because they attack primarily the hepatocytes and have little direct affect on other organ system.

1.1.2 Secondary hepatitis viruses:

Epstein-Barr virus (EBV), cytomegalovirus (CMV), herpes simplex virus (HSV), herpes zoster, yellow fever virus, enterovirus, paramyxo virus, adeno virus, hemorrhagic virus (e.g. dengue) and rubella virus. The secondary viruses involve the liver secondarily in the course of systemic infection of another body system.

1.2 CLINICAL COURSE OF VIRAL HEPATITIS

In a clinical set up, viral hepatitis can occur in acute or chronic form. The signs and symptoms of viral hepatitis are extremely variable. It can be mild, transient, and completely asymptomatic, or can be severe, prolonged, and ultimately fatal. In case of acute viral hepatitis the six diseases are clinically similar and cannot be reliably distinguished on the basis of clinical history, serum biochemical testing, or even epidemiological features (7-11). The course of viral hepatitis can take one of four forms as described below (table1) (4).

Table 1: Forms of hepatitis (4)

Form	Characteristics
Acute hepatitis	This forms of hepatitis is the typical form with associated jaundice. It has four phases: incubation, preicteric, icteric and convalescence.
Fulminant acute hepatitis	This rare form of hepatitis is associated with hepatic failure and deterioration continues and proceeds within one week to liver failure or coma (12).
Subclinical hepatitis without jaundice	This form of hepatitis accounts for persons with demonstrable antibodies in their serum but no reported history of hepatitis.
Chronic hepatitis	This form of hepatitis is accompanied by hepatic inflammation and necrosis that last at least 6 months.

1.2.1 Prodromal phase:

The onset of disease varies from abrupt to insidious. General malaise, myalgia, easy fatigability, upper respiratory symptoms (nasal discharge, pharyngitis), and severe anorexia may precede the onset of jaundice by 1 to 2 weeks. Nausea, vomiting and anorexia are frequently associated with alteration in olfaction and taste, and diarrhea or constipation may occur. Skin rash, arthritis, or serum sickness may be seen early in acute hepatitis. A low-grade fever between 100° to 102°F is more often present in hepatitis A and E than in hepatitis B or C. Abdominal pain is usually mild and constant in the right upper quadrant or right epigastrium and is often aggravated by jarring or exertion. Dark urine and clay-colored stools may be noticed by the patient from 1 to 5 days prior to the onset of clinical jaundice.

1.2.2 Icteric phase:

Clinical jaundice occurs after 5-10 days but may appear at the same time as the initial symptomatology. Most patients never develop icterus. With the onset of jaundice, there is often an intensification of the prodromal symptoms followed by progressive clinical improvement. Icterus can be detected if the bilirubin level exceeds 2.5-3.0 mg/dl. Vital signs are normal, although bradycardia can occur when significant hyperbilirubinemia is present.

Palpation often demonstrates an enlarged and slightly tender liver. However, in acute viral hepatitis, the liver usually is slightly enlarged with smooth, regular and firm edge. Spleen tip is felt in 5-25% of the patients. Tenderness can be elicited by direct palpation of the edge or by gentle percussion of the lower ribs. Enlarged lymph nodes, especially in the cervical or epitochlear areas may occur (13-16).

1.2.3 Convalescent phase:

Constitutional symptoms disappear but usually some liver enlargement and abnormalities in biochemical liver function tests are still evident. The duration of the posticteric phase is variable, ranging from 2 to 12 weeks, and usually is more prolonged in acute hepatitis B and C. Complete clinical and biochemical recovery is to be expected after 1-2 months in all cases of hepatitis A and E, and after 3-4 months in most of the uncomplicated hepatitis B and C. In remainder, biochemical recovery may be delayed. A substantial proportion of patients with viral hepatitis never become icteric.

1.2.4 Course:

The acute illness usually subsides rapidly over a period of 2 to 3 weeks. Complete clinical and laboratory recovery occur by 9 weeks in hepatitis A and by 16 weeks in hepatitis B and hepatitis C. In 5-10% cases, the course may be more protracted, and less than 1% will have an acute fulminant course.

1.2.5 Complications:

Fulminant hepatic failure, Relapsing hepatitis (Biochemical or Clinical), Cholestatic hepatitis, Post hepatitis syndrome, Chronic hepatitis, Cirrhosis, Hepatocellular carcinoma, Hepatic encephalopathy, Ascitis, Bacterial infections, Hyperbilirubinemia, Unexplained increase in serum aminotransferase, Renal complications, Glomerulonephritis, Vasculitis, Renal failure, Henoch-scholein purpura, Hematologic (Aplastic anemia, Pancytopenia, Agranulocytosis, Thrombocytopenia), Connective tissue diseases, Arthralgia, Arthritis, Papular-acrodermatitis, Urticaria, Pancreatitis, Myocarditis, Pneumonitis, Transverse myelitis, Peripheral neuropathy.

1.3 EPIDEMIOLOGY OF HEPATITIS VIRUSES

1.3.1 Hepatitis A virus:

1.3.1.1 Epidemiological pattern:

Hepatitis A has worldwide incidence and is endemic in many developing countries (figure1). Hepatitis A is still a common infection primarily among the poor, the elderly, older children, and young adults. In high endemicity zones, such as many areas of Asia, Africa, Eastern Europe and a number of South American countries, mass exposure to HAV in early childhood confers herd immunity (table2).

Table 2. Estimated incidence of reported cases of hepatitis A worldwide in 1990 (17)

Region	Estimated rate of infection (per 100,000 a year)	Cases per year (thousand)
North America	10	28
Central/South America	20-40	162
Europe	5-60	278
Africa/Middle East	20-60	251
Asia	10-30	676
Oceania	15-30	5
Total		1.4 million

1.3.1.2 Risk group (3):

Persons in institutions for custodial care and the staff who work with them are at highest risk for HAV infection. The risk among male homosexuals and intravenous drug abusers has increased, most likely due to person-to-person contact. The risk group includes

-Patients and staff in custodial institutions

-Persons in close personal contact with an infected individual

-Travelers and military personnel in areas where the disease is widespread

-Children in day care centers

-Male homosexuals

-IV drug abusers

1.3.1.3 Infectivity (3):

Twenty five to 40% of individuals are no longer infectious or shedding the virus in their feces, when symptoms first appear. In isolated cases, however, HAV can be found in stool specimens up to two weeks after the onset of symptoms. Because the period of highest

infectivity occurs during the late incubation period, patients should be considered potentially infectious for up to two weeks after onset of symptoms.

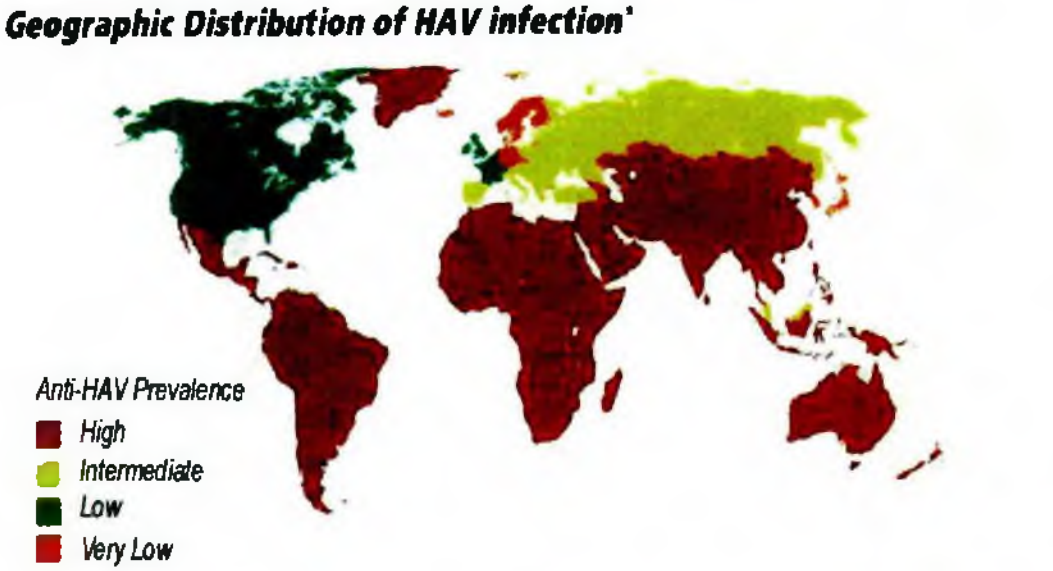


Figure1. Geographic distribution of hepatitis A virus (18)

1.3.2 Hepatitis B virus:

1.3.2.1 Epidemiological pattern:

The incidence of HBV infections and patterns of transmission vary widely in different parts of the world. It is estimated that worldwide more than 50 million new infections with HBV occur yearly, and as many as 1 million deaths annually can be attributed to the effects of this infection (19). Worldwide, it is estimated that there are 300 million HBV carriers and most new cases occur among people aged 20-39 (20).

In endemic areas such as East-Asia, sub-Saharan Africa, Eastern Europe and the Amazon basin, carrier rates range from 8% to 25%. In these areas, more than 50% of the population have had HBV infection. Most acquired the infection at birth or during childhood (21). In areas of moderate prevalence of chronic carriers (2% to 7% of the population is HBsAg-positive), the lifetime risk of becoming infected with HBV is estimated to be 20% to 60%. In these group, most exposures also occur in childhood (22). According to WHO and other studies, Bangladesh resides in this group with a prevalence rate of around 4% (23-26). In low prevalence areas such as Western Europe and North America, the prevalence of chronic carriers is less than 2%, and the lifetime risk of infection is less than 20%.

Incidence/Prevalence

Geographic Distribution of Chronic HBV Infection*

*(Note: The map of HBsAg prevalence generalizes available data, patterns may vary within countries.)

Figure2. Geographical distribution of chronic HBV infection (20)

1.3.2.2 Risk group (27):

The following groups of subjects are currently considered at risk of HBV infection:

-newborns from HBsAg carrier mothers

-household and sexual contacts of HBV chronic carriers

-health care workers

-haemodialysis patients

-clients and staff of institutions for mentally disabled persons

-inmates of correctional facilities

-users of intravenous drugs

-policemen

-sexually active heterosexual with multiple sex partners

-sexually active homosexual men

-international travellers

-military personnel employed in high ecdemic areas

1.3.2.3 Infectivity (3):

Viral spread is facilitated by the asymptomatic course of the infection and by the fact that many HBsAg carriers are unaware of their infectivity. HBeAg positive patients generally have more than 10^6 virions/ml and thus transmit HBV more frequently than HBeAg negative subjects; anti-HBe positive patients usually circulate virus in low levels so that a large volume of inoculam, such as a blood transfusion unit, is required to transmit infection. Needlestick exposures (less than 0.001ml of plasma) may not transmit HBV if HBV levels are below 10^3 virions/ml; these titres are usually found in anti-HBe patients. Only patients with HBV-DNA or HBeAg detectable by routine assays (more than 10^6 virions/ml) are usually capable of spreading HBV by minute parenteral exposures. HBsAg can be detected in other body fluids; however the level of the virus is 100-1000 fold less than in blood.

1.3.3 Hepatitis C virus:

1.3.3.1 Epidemiological pattern:

The medical importance of hepatitis C resides in its propensity to become chronic. It is generally thought that HCV infection become chronic in 50% of cases. Sporadic of community-acquired forms show a lower rate of chronicity. WHO estimates that up 3% of the world's population has been infected with HCV or 227 million individuals and there may be more than 170 million chronic carriers of HCV (28). Incidence is endemic worldwide; high incidence in Japan, Italy and Spain (figure3). The prevalence among blood donors in northern Europe and North America (<1%) is lower than that in Southern Europe and Africa (13% in Eygpt), while figures for Asia may be as high as 3%.

1.3.3.2 Risk group (3):

The following groups of subjects are currently considered at risk of HCV infection:

-intravenous drug abusers

-multiple transfusion and organ transplant recipients

-recipients of blood products

-haemodialysis patients

-health care workers

-heterosexual partners of patients with chronic hepatitis are at an increased risk

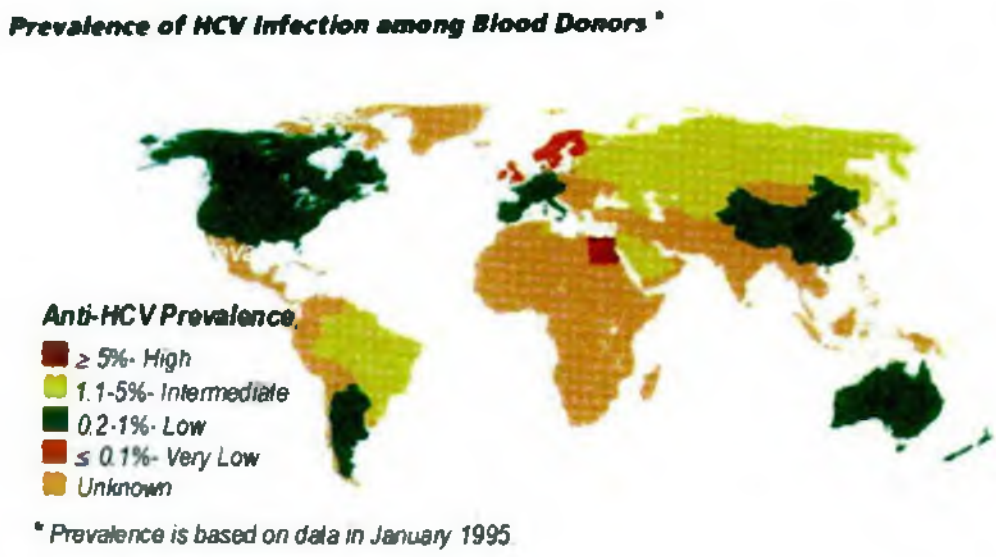


Figure3. Prevalence of HCV infection among Blood Donors (29)

1.3.3.3 Infectivity (3):

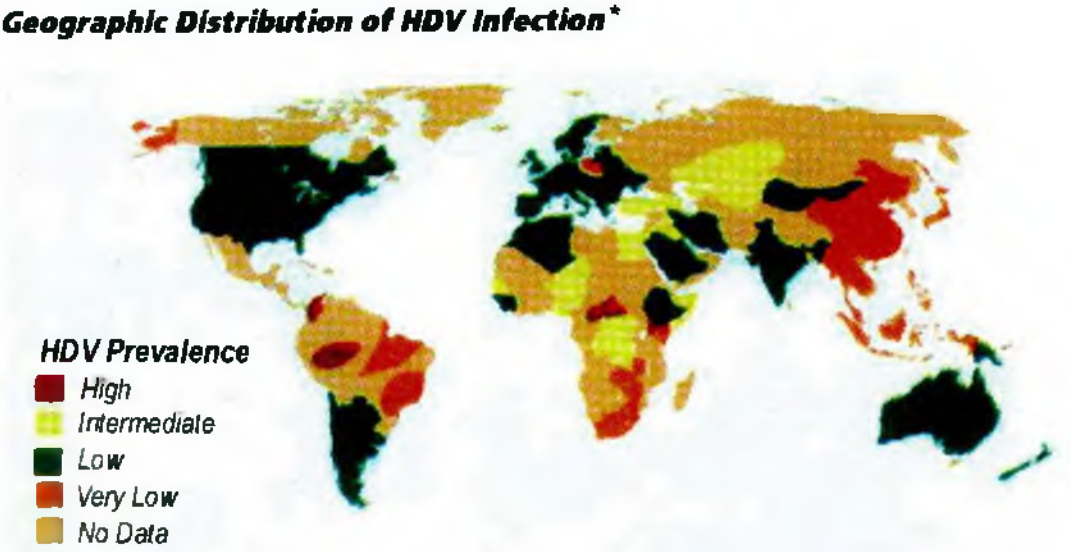
The infectivity of HCV is usually low and rarely reaches 10^6 infective doses per ml in blood. HCV has been detected in various body fluids including saliva of patients with chronic hepatitis C. Saliva was shown to be infectious when injected into a chimpanzee. Accidental needle-stick injuries with contaminated needles or sharp instruments causes infection in 3-10% of health care workers.

1.3.4 Hepatitis D virus:

1.3.4.1 Epidemiological pattern:

Hepatitis D occurs worldwide but its geographical prevalence varies widely (fugure 4). The epidemiology of HDVinfection varies unpredictably in incidence and pattern from one region to another. In general, the global pattern of HDV infection corresponds to the prevalence of chronic HBV infection. In general, HDV infects on average 4% of acute hepatitis B or 2% of all acute viral hepatitis (30). In countries with a low prevalence of chronic HBV infection, HDV prevalence is generally low among both asymptomatic HBV carriers (<10%) and among patients with chronic HBV-related liver disease (<25%). In countries with moderate and high levels of chronic HBVprevalence, the prevalence of HDV infection is highly

variable. However, in most of Southeast Asia and China, where the prevalence of chronic HBV infection is very high, HDV infection is uncommon (table 3).



*(Note: The map of anti-HDV prevalence generalized available data, patterns may vary within countries.)

Figure4. Geographic distribution of HDV infection (31)

Table 3. Epidemiology of delta hepatitis: Presence of anti-HDV among chronic carriers of HBsAg around the world (32)

Continent	Carriers tested	Anti-HDV positive
South America	669	51 (7.6%)
North Americaa	940	112 (11.9%)
Asia	1651	179 (10.8%)
Africa	167	17 (10.4%)
Australia/ Ocenia	144	9 (6.3%)
Eastern Europe	1556	56 (3.6%)
Northern Europe	587	73 (12.4%)
Southern Europe	2697	484 (17.9%)

1.3.4.2 Risk group (3):

Anyone at risk for hepatitis B is at risk for hepatitis D. Those who have a history of intravenous drug use or who have chronic hepatitis B that suddenly deteriorates are more likely to have hepatitis D. Hemophiliacs and patients undergoing hemodialysis are especially

at risk because of their repeated potential exposure to hepatitis B. Also at risk are male homosexuals and individuals with multiple sexual partners.

1.3.4.3 Infectivity (3):

HDV-RNA replicates very efficiently; up to 300,000 genome copies can be found in infected hepatocytes. HDV is secreted in the blood before the onset of clinical disease and begins to decrease during the acute phase of hepatitis. HDV infectivity can be extreme during the early phase of infection.

1.3.5 Hepatitis E virus:

1.3.5.1 Epidemiological pattern:

Areas of the world can be classified as HEV-endemic based on the occurance of hepatitis E outbreaks, which have been reported throught Asia, Africa, the Middle East, and Central America, predominantly in developing countries where environmental sanitation facilities are inadequate (figure 5). The incidence of infection appears to be low in first world countries. Lack of hygienic conditions and failures to adequately treat water and sewage have been linked to massive epidemics of hepatitis E. Hepatitis E is a hazard to travellers to areas where HEV is endemic. In developed countries, hepatitis E has been reported only sporadically and usually in travellers who acquired the infection in endemic areas.

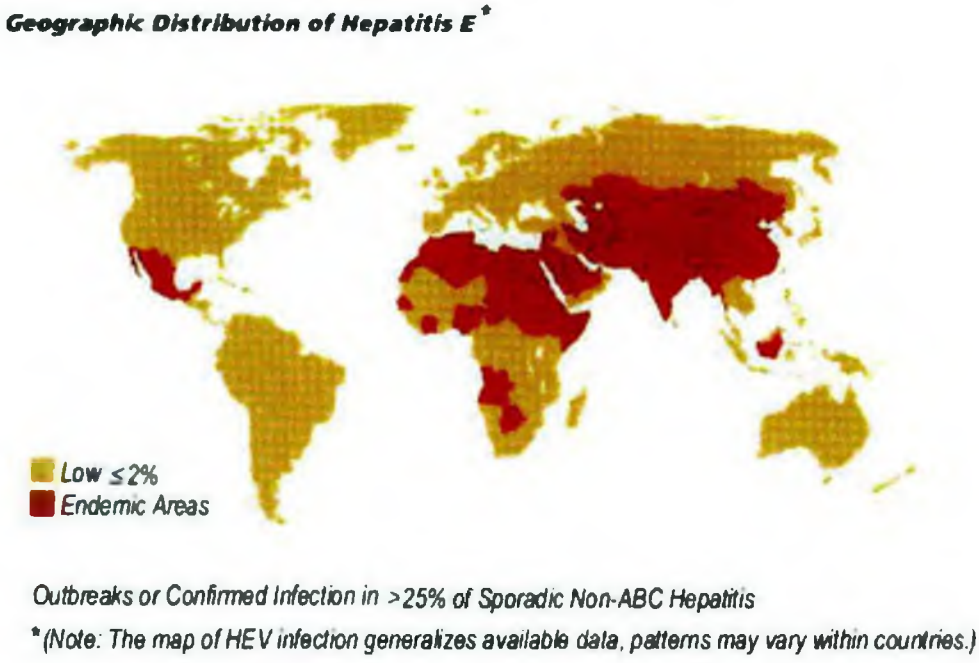


Figure 5. Geographic distribution of HEV infection (31)

1.3.5.2 Risk group (3):

The following groups of subjects are currently considered at risk of HEV infection:

-newborns from HEV positive mothers

-haemodialysis patients

-international travelers to high endemic areas

-military personnel employed in high endemic areas

1.3.5.3 Infectivity (3):

Large outbreaks are common where the source of infection is usually gross faecal contamination of drinking water supplies. Case-to-case transmission to household contacts appears to be uncommon. This suggests that a large inoculum is needed to establish infection.

1.4 GENERAL CHARACTERISTICS AND STRUCTURE OF THE HEPATITIS VIRUSES

1.4.1 Hepatitis A virus (HAV):

The hepatitis A virus (HAV) was first discovered in 1973 (33). HAV is classified within the new Heparnavirus genus of the Picornaviridae family (34). HAV is exceptionally stable among the picornaviruses in hepatotropism, stability, single antigenic structure and processing pattern of the capsid protein precursors (35-37). Hepatitis A virion is a naked, spherical particle. The particle has icosahedral symmetry with 12 pentameters and a 27 nm overall diameter (figure6) (table4) (37-39).

The virion consists of a genome of linear, single-stranded RNA of messenger sense polarity, 7480 nucleotides: it has a relatively lengthy 5′ untranslated region (5′ UTR) and a long open reading frame (ORF) encoding of a polyprotein of 2227 amino acids; this is cleaved in part by viral proteases into structural and non structural proteins (40). The former include the polypeptides of the capsid (VP1, VP2, VP3 and VP4), followed by a putative RNA helicase, an RNA-dependent RNA polymerase, and a cysteine protease (41). There are three genotypes of HAV, based on about 15% diversity in nucleotide sequences of VP1 and only one serotype is known (42).

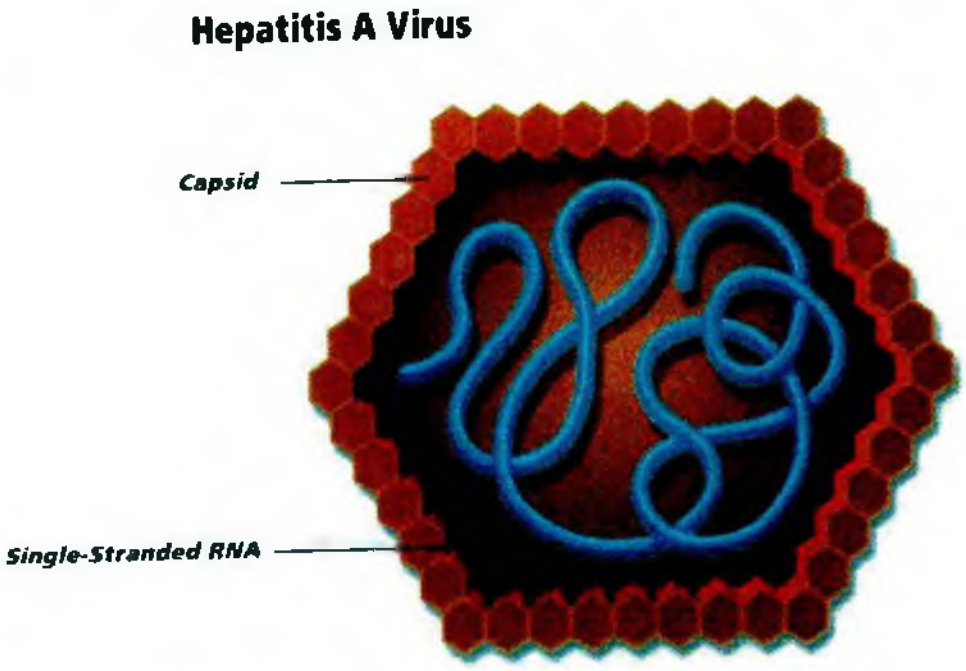


Figure 6: Schematic diagram of hepatitis A virus (104)

1.4.2 Hepatitis B virus (HBV):

In 1963, Blumberg discovered a previously unknown protein in the blood from an Australian aborigine and he termed this protein the Australian (Au) antigen (43). By 1968, other investigators notably Prince and Okochi and Murakami, had established that the Au antigen (now known as the hepatitis B surface antigen or HBsAg) was sepcifically found in the serum of type B hepatitis infected patients (44, 45). Later Dane found the virus-like particle in the seurm of type B hepatitis infected patients. This particle was considered to be the hepatitis B virus (HBV) (46).

The hepatitis B virus is a double stranded circular DNA virus with a diameter of 42 nm and a member of the hepadnaviridae family (47). The genomic size varies between various HBV subtypes, but all are approximately 3.2 kb long and 3200 nucleotides long (48). The outer protein shell is made by the hepatitis B surface (HBs) proteins. The inner protein shell is referred to as the core particle or capsid having a diameter of 34 nm (46,49). It is composed of hepatitis B core (HBc) protein and encloses the viral DNA. The core antigen (HBcAg), the e antigen (HBeAg) and the surface antigen (HBsAg) are three physical features of the virus (figure 7).

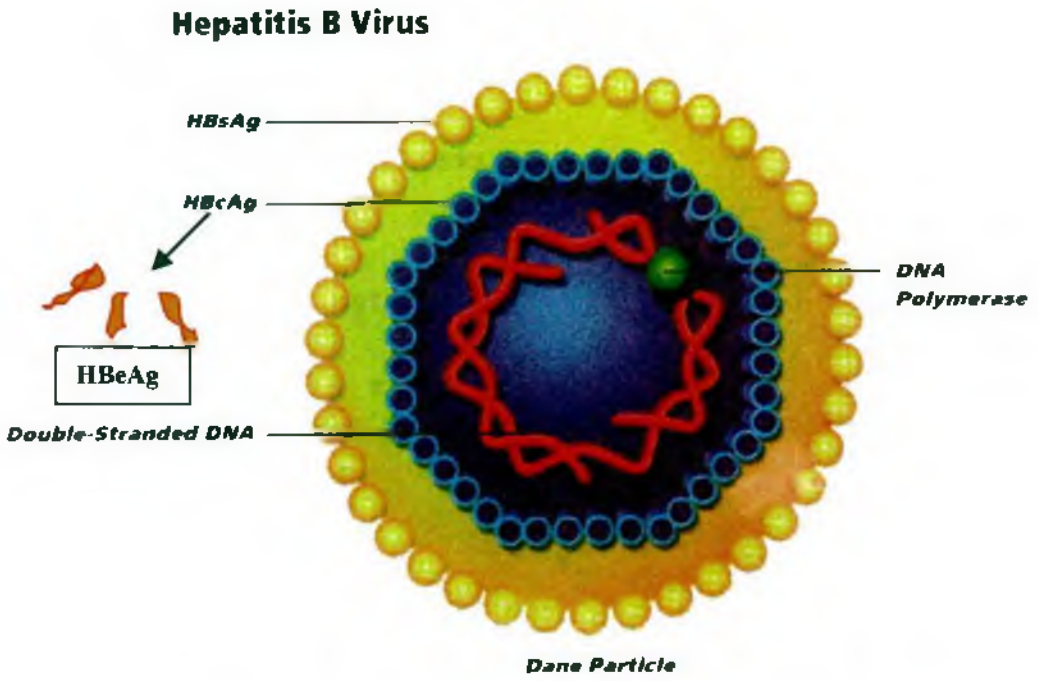


Figure 7: Schematic diagram of hepatitis B virus (104)

1.4.3 Hepatitis C virus (HCV):

Hepatitis C virus was first identified in 1989 and was shown to be the major viral cause of parenterally transmitted non-A, non-B hepatitis (NANBH) (50). It eluded identification for many years. In 1989, the genome was cloned from the serum of an infected chimpanzee (51). It is classified as putative Togavirus related to the Flavivirus and Pestiviruses of 45 to 65 nm in size (52). The possibility that HCV particles are present in the circulation as immune complex or in association with lipoproteins (53,54). The genome is made up of 9400 bases aligned in a single RNA molecule with positive polarity. It contains an open reading frame that encodes for a polyprotein precursor of 3011 amino acids, subsequently cleaved into functional and structural polypeptides. Like the other members of the flaviviridae, the HCV genome has a region in the 5′ position that codes for proteins forming the structure of the virus particle (structural proteins) and a region comprising the 3′ end that codes for enzymatic proteins (functional proteins). At the 5′ end of the genome there is a small sequence that expresses no protein (untranslated region =5′ UTR); this region maintains a consistent nucleotide homology in different viral isolates (figure 8).

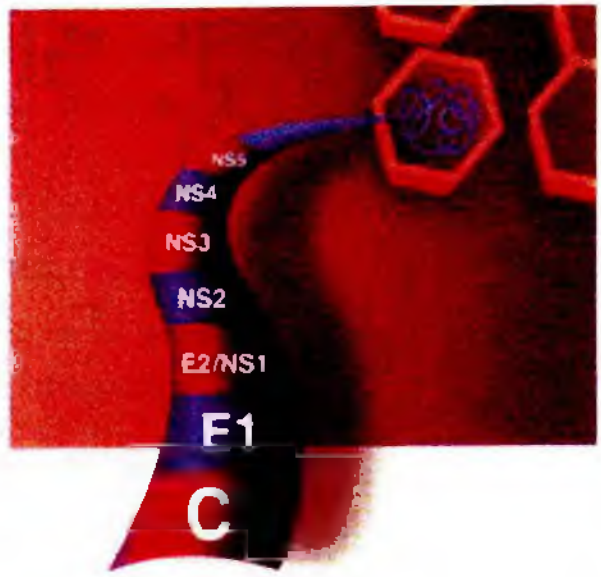


Figure 8: Structure of the HCV genome (104)

In the structural region are: gene C which codes for the nucleocapsid protein, p22 of about 22Kd, without glycosylated binding site; its binding with RNA is associated with the formation of the nucleocapsid; and E1 and E2 genes (the latter first identified as NS1), code for the envelope proteins. The E1 gene codes for a glycoprotein, gp33, which is extensively glycosylated (6 binding sites for glycosylation have been identified). The E2 gene codes for glycoprotein gp70. Analysis of the E2/NS1 regions show an aminoterminal hypervariable region (HVR1); HVR1 may represent the target of neutralizing HCV antibodies.

The non structural region of the genome contains the genes NS2, NS3, NS4 and NS5 which code for functional proteins. In particular, the protein products of NS2 (p23) and NS4 (p10 and p27) are probably associated with cell membrane functions. NS3 codes for an helicase protease (p72). NS5 produces an RNA-dependent-RNA polymerase (p70) and a protein of unknown function (p56), but probably required for the formation of a replication complex. A wide genetic variability has been demonstrated in viral isolates of HCV obtained worldwide. This classification is generally based on the analysis of the 5' UTR, the core regions, and/or the NS3 and NS5 sequences. The recent proposal for a single classification system according to phylogenetic analysis foresees the distinction of 6 types and many subtypes (55,56). Group

isolates show homologies of 88-100%, while types show homologies of 56-76%. The subtypes within each type are indicated with lower case letters of the alphabet and have an homology of 74-86%(57). Geographic distribution of HCV genotypes provides information about the origin and transmission of the virus; it has also implications for the development of vaccines and the sensitivity of the virus to antiviral therapy.

1.4.4 Hepatitis D virus (HDV):

The hepatitis D virus (HDV), formerly called the delta virus was first detected as a new nuclear antigen in the hepatocytes of patients infected with hepatitis B virus and was frequently associated with severe acute or chronic hepatitis (58).

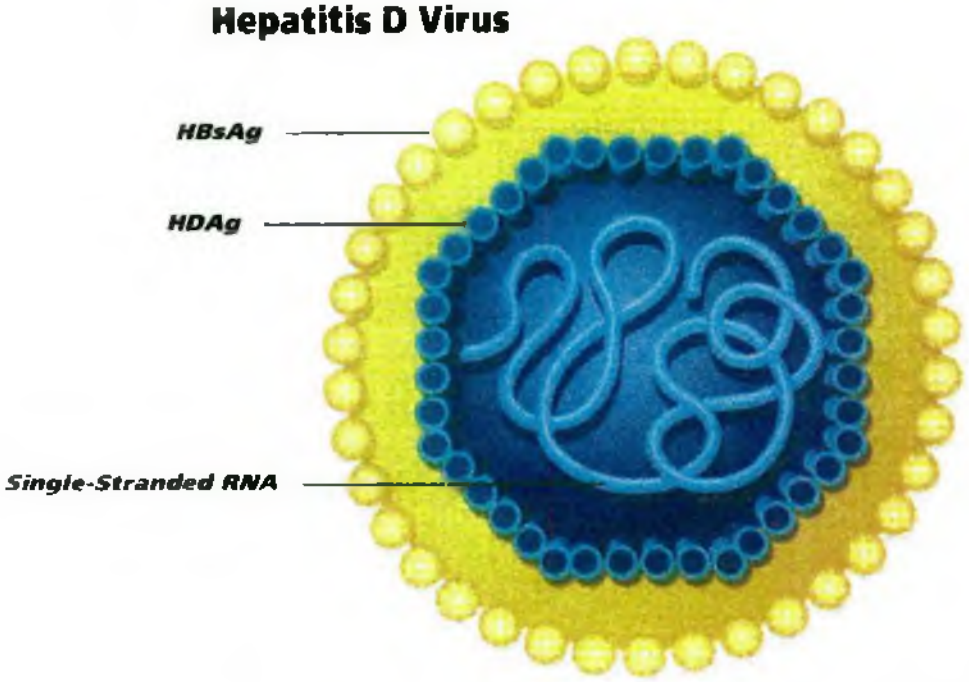


Figure 9: Structure of the HDV genome (104)

HDV is a unique small mammalian RNA virus consisting of spherical particles of about 36 nm in diameter. The RNA genome encodes for the hepatitis delta antigen (HDAg). The outer coat of HDV consists of hepatitis B surface antigen and lipid, which envelops the RNA genome and the delta antigen (figure9) (59).HDV is a defective virus, which requires HBV as a helper virus in order to replicate. Infection therefore only occurs in patients who are already infected with hepatitis B. HDV is similar to viroids and virusoids, infectious agents of plants; in analogy to virusoids it cannot form infectious particles by itself and needs the HBsAg coat of HBV to be assembled into a virion (60).The RNA genome is approximately 1700

nucleotides long, single-stranded circular, with a high G+C content and a high degree (up to 70%) of intramolecular base pairing. The molecule contains many stretches complementary to each other that, under native conditions, collapse the RNA into an unbranched rod-like structure (61). HDV RNA sequence contains a functional ORF that encodes the delta antigen and this region resides on the antigenomic strand and is expressed via translation of a specific mRNA.

The HD protein (HDAg) consists of a nuclear phosphoprotein with RNA binding activity. HDAg is usually found in patient's serum or liver in two forms of 27,000 and 24,000 kD designated as large (214 amino acids) and small (155 amino acids) HDAg, respectively. These two HDAg forms are translated from two RNA species different by a single point mutation. The large HDAg is more heavily phosphorylated than the smaller one. In virus-infected cells, HDAg is exclusively localized in the nuclei, but also found in smaller amount in cytoplasm. The small antigen accelerates the synthesis of the genome while the large antigen seems to act as an inhibitor of HDV-RNA synthesis and may be involved in packaging of the viral nucleic acid in the HBsAg envelope (62,63).

Replication of HDV RNA occurs in the cell nuclei, as both HDAg and HDV RNA are localized exclusively in the nuclei. Replication is thought to occur via a rolling circle mechanism similar to that described for the replication of viroids. Although HDV requires complementary functions of HBV for infection, it can replicate within infected cells in the absence of HBV (64). Two regions of HDV RNA, one in the genomic and the other in the antigenomic strand, demonstrate an ability to self-cleave and self-ligate in vitro in the absence of protein catalysts; they qualify, therefore, as ribozymes, i.e. a unique RNA molecule which contains in itself both genetic information and enzymatic activity (65). Three genotypes of HDV have been cloned sequenced. The most common is genotype I, reported in Italy, America, Taiwan, Nauru, France and Lebanon. Genotype II has been isolated from only one patient with chronic hepatitis in Japan and genotype III from patients with severe acute hepatitis in Peru and Columbia. There is 30% divergence in amino acid sequences among different genotypes.

1.4.5 Hepatitis E virus (HEV):

Hepatitis E virus is recently identified cause of enterically transmitted non-A, non-B (NANB) hepatitis (66). It is spherical, non enveloped, 32-34 nm in particles containing a single stranded RNA genome with positive polarity (figure10) (67-69). HEV appears to be the single

member of a novel virus genus which has provisionally been classified within the family of Caliciviridae. The host range is confined to primates. The HEV RNA is single-stranded, polyadenylated RNA of approximately 7.5 kb. The gemome encompasses three ORF(70); the larger ORF contains sequences encoding a RNA-dependent RNA polymerase, an RNA helicase, a methyltransferase and a papain-like cysteine protease (71); a second frame is thought to encode for structural proteins; a third frame probably contains a transmembrane sequence involved in transportation of the virus within the cell. There are distinct sequence divergences between isolates from different areas; HEV from Mexico exhibits only 77% sequence homology with virus isolates from Central Asia (72,73).

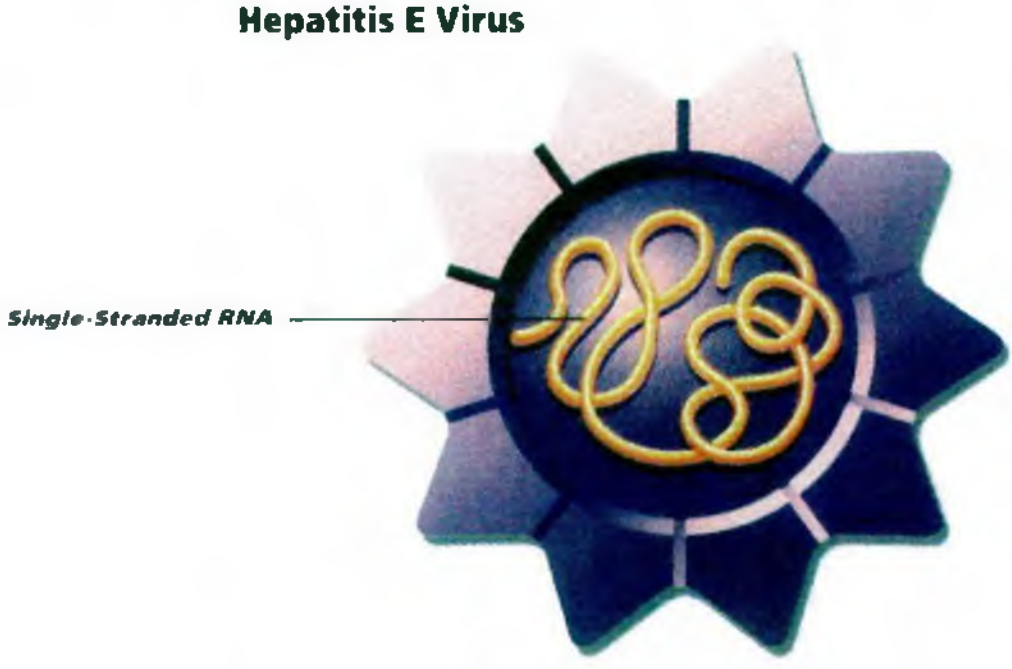


Figure 10: Structure of the HEV genome (104)

Table 4: The hepatitis viruses (74)

Types	A	B	C	D	E	G
Nucleic acid	RNA	DNA	RNA	RNA	RNA	RNA
Genomic size (kb)	7.5	3.2	9.4	1.7	7.6	9.3
Virion size (nm)	27	42	30~60	35~37	27~34	?
Transmission route (major)	p.o.	Parenteral	Parenteral	Parenteral	p.o.	?
Incubation period (month)	0.5~1.5	1~6	1~6	1~6	0.5~2	?
Onset (usually)	Acute	insidious	Insidious	Acute	acute	?
Chronicity	No	Yes	Yes	Yes	No	Yes
Chronic carrier	No	Yes	Yes	Yes	No	Yes
Results in:						
Cirrhosis	No	Yes	Yes	Yes	No	?
Liver cancer	No	Yes	Yes	No?	No	?
Immunoprophylaxis						
Passive	Yes	Yes	No*	No	No?	?
Active	Yes	Yes	No	No*	No	?
Treatment for chronic infection	-	IFN	IFN	IFN?	-	?
Natural immunity	Lifelong	lifelong	No	No	Temporary	?

p.o. =per os.; *Immunizing against HBV will prevent HDV infection; IFN= interferon

1.5 CLINICAL FEATURES OF VIRAL HEPATITIS

1.5.1 Hepatitis A hepatitis:

Hepatitis A virus (HAV) hepatitis occurs sporadically or in epidemic form and has an incubation time of 15 to 50 days (figure11). Age 5 to 15 is the group most affected, and adults are often infected by spread from children as a result of overcrowding, poor hygiene, and poor sanitation. With improved standard of living, the prevalence is decreasing worldwide. In urban areas, only about 30% of adults show IgG anti-HAV, where as in underdeveloped countries, 90% of children have the antibody by the age of 10. Young people, not previously exposed, and visiting endemic areas, are increasingly becoming affected.

Clinically, the hepatitis is usually mild, particularly in children, in whom it is frequently subclinical or passed off as gastroenteritis. The disease is more serious and prolonged in adults. The nephrotic syndrome has been reported with immune complex-mesangial, proliferative glomerulonephritis (75).

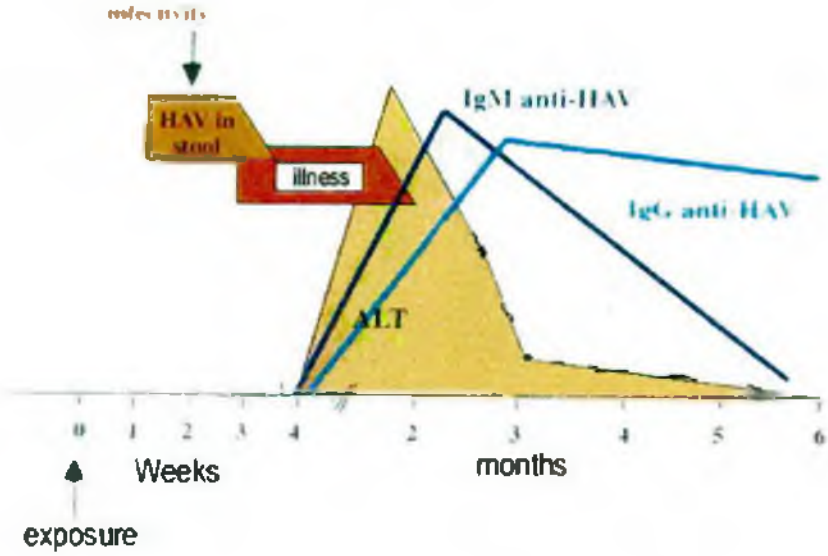


Figure 11: The course of acute hepatitis A (3)

ALT, alanine aminotransferase; HAV, hepatitis A virus.

1.5.1.1 Cholestatic Hepatitis A:

This form of hepatitis A affects adults (76). The jaundice lasts 1 to 4 months and itching is severe. Serum IgM anti-HAV is positive. The prognosis is excellent.

1.5.1.2 Relapsing Hepatitis A:

Occasionally after 30 to 90 days, the patient suffers a relapse. The serum transaminase levels have never returned to normal. The disease resembles the original attack clinically and biochemically, and virus A is found in the stool (77). The relapse may last several months, but recovery eventually ensues. Relapsing HAV is associated with continuing viremia and shedding virus in stools during the relapse phase (78). The pathogenesis probably involves mechanisms responding to the continuing antigenic stimulation. Rarely, the relapse can be associated with arthritis, vasculitis, and cryoglobulinemia (79).

1.5.2 Hepatitis B hepatitis:

1.5.2.1 Acute HBV infection:

The incubation period of hepatitis B virus (HBV) is about 6 weeks. The clinical course of acute HBV infection may be anicteric. The high carriage rate of serum markers in those who give no history of acute HBV infection suggests that subclinical episodes must be extremely frequent (figure12). The nonicteric case is more liable to become chronic than the icteric one.

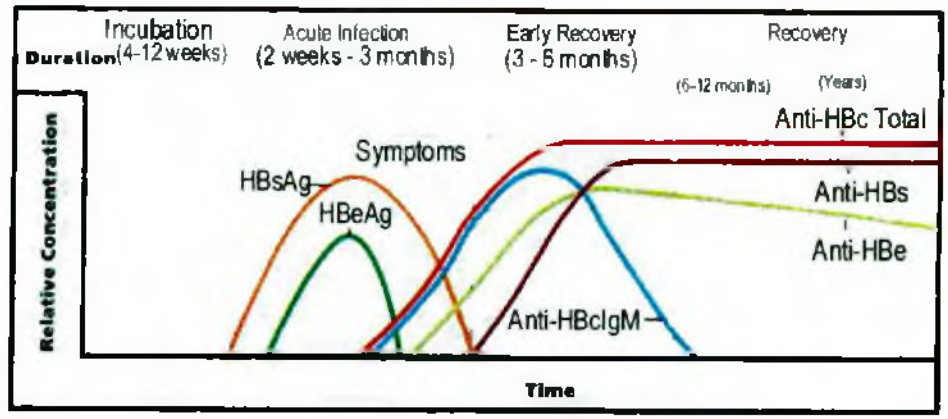


Figure 12: The course of acute type B hepatitis. HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B e antigen; anti-HBe, antibody to hepatitis B e antigen; anti-HBs, antibody against hepatitis B surface antigen; anti-HBclgM, IgM type antibody to hepatitis B core antigen; anti-HBc Total, total antibody to hepatitis B core antigen. (3)

The clinical attack diagnosed in the adult tends to be more severe than for hepatitis A or C, however; the overall picture is similar. The self-limited, benign icteric disease usually lasts less than 4 months; jaundice rarely exceeds 4 weeks. Occasionally, a prolonged benign course is marked by increased serum transaminase values for more than 100 days. Relapses are rare. Cholestatic hepatitis, with prolonged deep jaundice, is unusual.

There may be features suggesting immune complex disease. This is shown in the prodormal period by a serum sickness-like syndrome. This develops about 1 week before jaundice. It can be associated with an icteric or an anicteric attack. The syndrome has also been described with chronic HBV. Fever is usual. The skin lesion is urticarial and, rarely children show a papular acrodermatitis. The arthropathy is symmetrical and nonmigratory and affects small joints.Serum rheumatoid factor is negative.It is usually transitory, but can persist.A fulminant course of hepatitis B in the first 4 weeks is related to an enhanced immune response

with more rapid clearing of virus. Antibodies to surface and e antigen increase and multiplication of virus ceases (80). In fulminant hepatitis B, the surface antigen may be in low titer or undetectable. The diagnosis may be made only by finding serum IgM anti-HBc. Another viral hepatitis, superimposed on a symptomless hepatitis B carrier, may precipitate a fulminant course. The new agent may be virus A, delta virus, or hepatitis C virus.

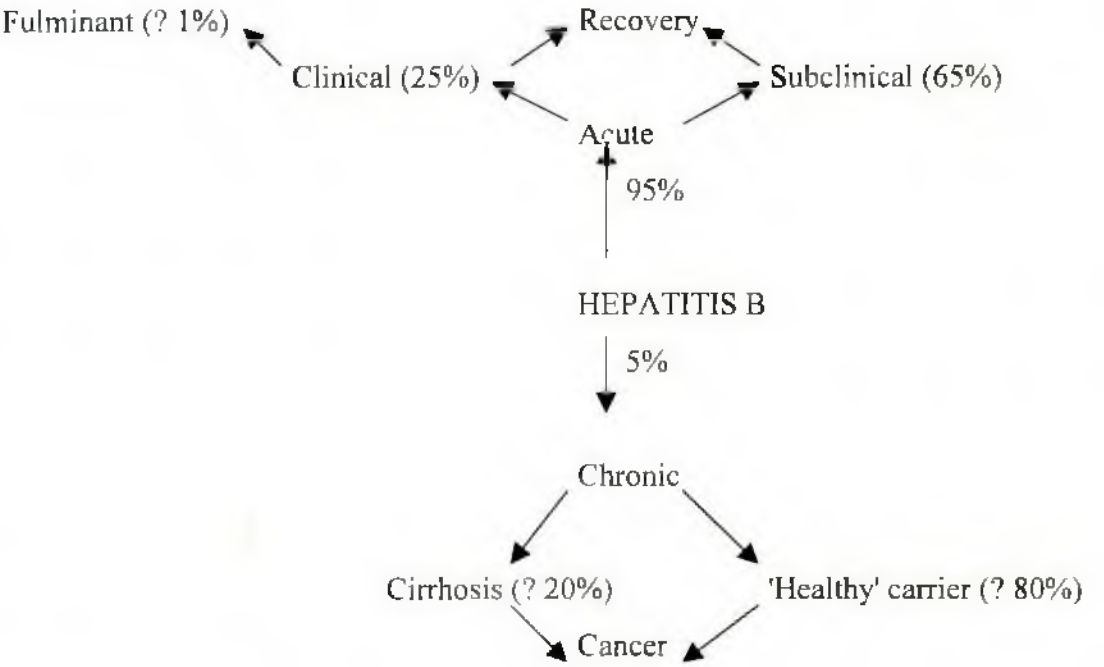


Figure 13: The effect of exposure to HBV. (121)

Reactivation of hepatitis B viral replication is well recognized after cancer chemotherapy, methotrexate, for rheumatoid arthritis (81), organ transplantation, or the administration of corticosteroids to HBeAg-positive patients. The result is icteric hepatitis, nonfatal hepatic failure, or death (82). Spontaneous exacerbations may also occur as a result of reactivation of HBV replication (figure13) (83).

1.5.2.2 Chronic HBV infection:

An individual is considered chronically infected if HBsAg is present for more than six months (figure14). The very young and the very old are at particular risk for chronic HBV infection. Chronic HBV hepatitis is found predominantly in males. The condition may follow an unresolved acute HBV. The acute attack is usually mild and of 'grumbling' type.

The patient having an explosive onset and deep jaundice usually recovers completely. After an attack, the serum transaminase levels fluctuate with intermittent jaundice. The patient may be virtually symptom-free with only complain of fatigue and being generally unwell.

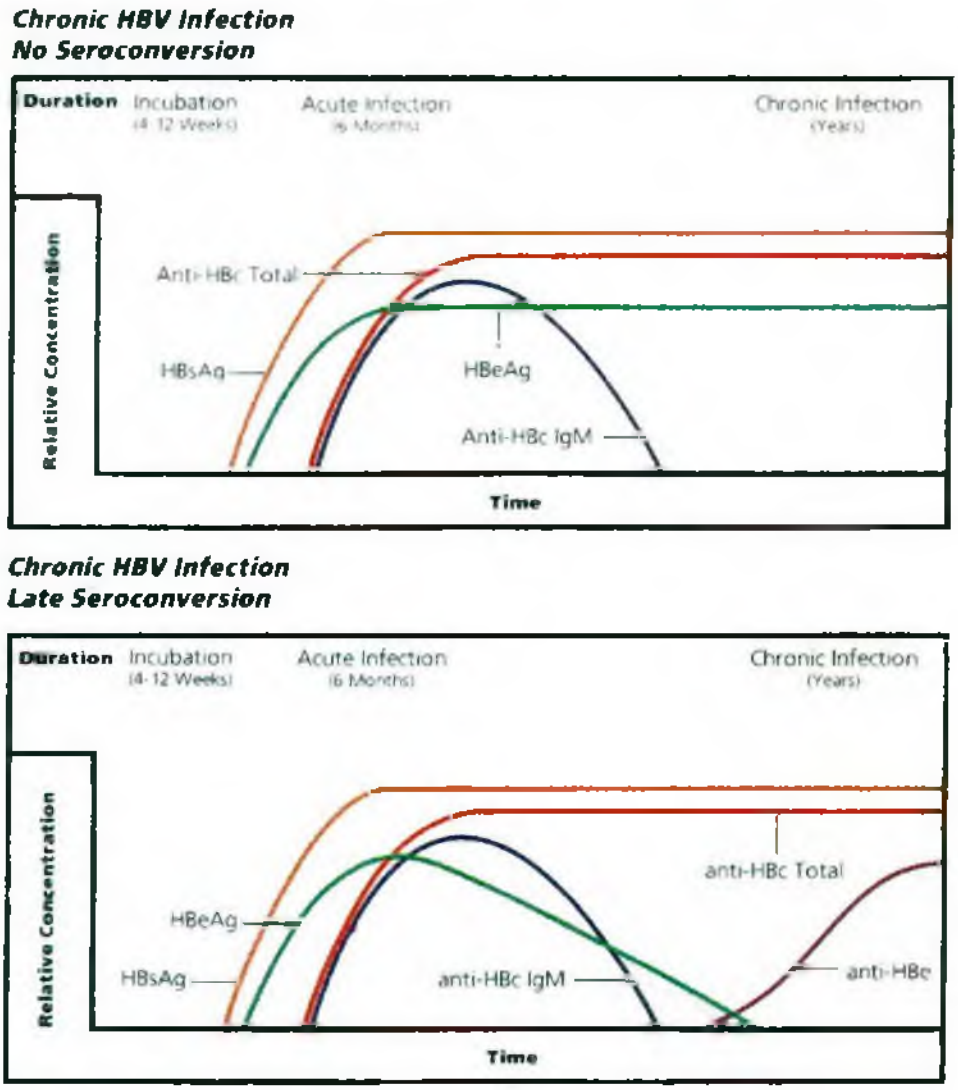


Figure 14: The course of chronic type B hepatitis. (৪)

Chronic hepatitis is often a silent disease. Symptoms do not correlate with the severity of liver damage. In about one-half of cases, presentation is as established chronic liver disease with jaundice, ascites, portal hypertension. Encephalopathy is unusual at presentation.

Many patients, usually with established stable cirrhosis evolving slowly over many years, present with hepatocellular carcinoma. An apparently stable patient with chronic HBV disease may have a clinical relapse. This is marked by increasing fatigue and usually rises in

serum transaminase values. Relapse may be related to seroconversion from an HBeAg-positive state to an HBeAg-and HBV-DNA-negative one. Liver biopsy shows an acute lobuler-type hepatitis, which usually subsides, and the serum transaminase values fall. Seroconversion may be spontaneous in 10-15% of patients per annum (84). HBV-DNA can remain positive even when anti-HBe has developed. In some HBeAg-positive patients, flare-ups of viral replication and transaminase elevation are found without eventual clearing of HBeAg (85). Spontaneous reactivation from HBeAg negative to HBeAg and HBV-DNA positive has also been described. The clinical picture ranges from absence of manifestations to fulminant liver failure (85). Reactivation is particularly severe in those who are human immunodeficiency virus (HIV) positive.

1.5.3 Hepatitis C hepatitis:

1.5.3.1 Acute HCV Hepatitis:

The incubation period is probably 5 to 12 weeks. Only 25% of sufferers are jaundiced and the patient may be completely asymptomatic. The anti-HIV-positive patient may have a rapidly progressive course (86). A fulminant course, however, is rare, and co-infection with another virus must be considered. Post transfusion hepatitis C is liable to be overlooked unless serum transaminases are being monitored frequently. This is especially in the postoperative setting, where malaise and fatigue are expected in the first few weeks after surgery. Serum transaminase levels are only moderately elevated–about 15 times the upper limit of normal at 7 to 8 weeks after infection. Multiple peaks are usual for 3 months after the onset. These multiple peaks are seen in 70%, 21% have two peaks, and only 0.5% a single peak. Serum hepatitis C-RNA can be detected 1 to 2 weeks after infection (87).

In those that recover completely, serum HCV-RNA is lost, but HCV antibody persists for months. At 1 year, the majority of sufferers from post-transfusion hepatitis will still have raised serum transaminase levels. Most of these patients will develop chronic hepatitis and 20% will develop cirrhosis (Fig 15.). The disease is insidious and marked by fluctuant serum transaminases.

1.5.3.2 Chronic HCV Hepatitis:

The hepatitis C infection may have been acquired as long as 25 years previously. The frequency of chronicity is the same in the community-acquired sporadic disease as in the parenterally transmitted one. The patient may present insidiously with fatigue as the major symptom. The patient feels below par, the degree of which is variable. Chronic hepatitis C is

often asymptomatic. The course is slow, marked by fluctuating "yo-yo" transaminases over many years (figure 15). Every elevation probably represents an episode of hepatitis C viremia perhaps because of a quasi species. At presentation, the serum transaminase level rarely exceed six times the upper limit of normal, and the mean is about three times normal (figure16).

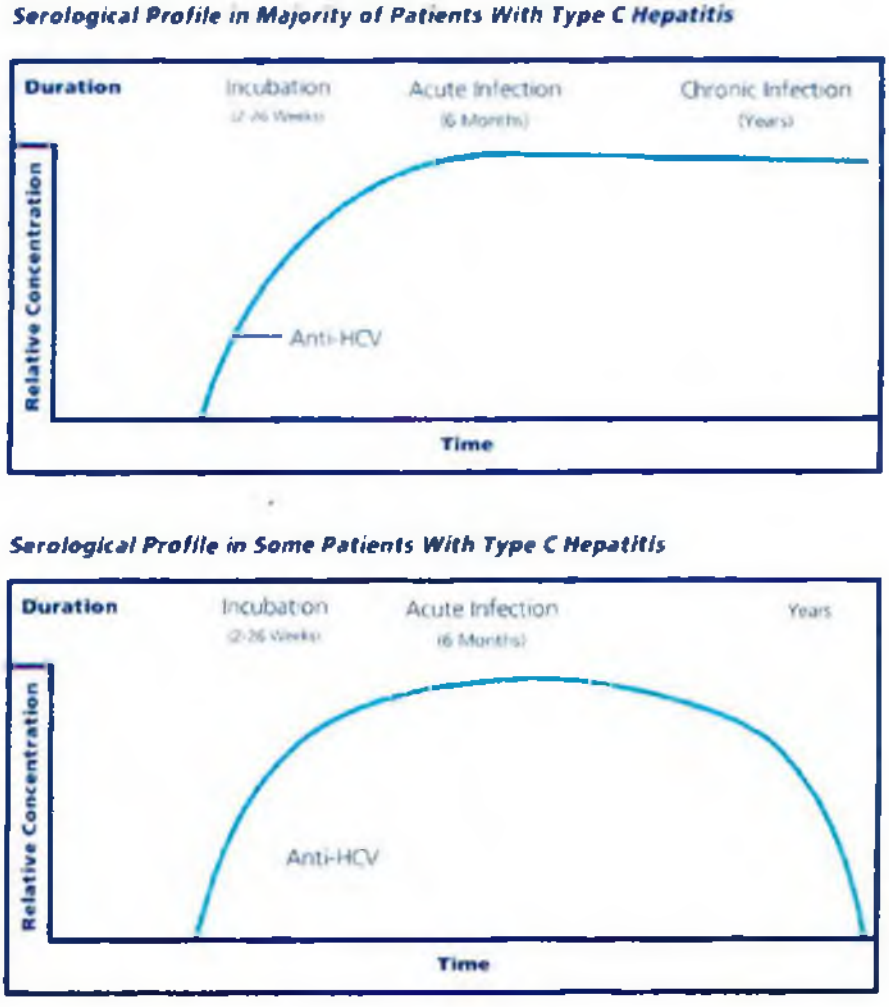


Figure 15: The serological course of post-transfusion chronic HCV infection. Serum HCV RNA appears very early. Anti-HCV positivity is delayed. Serum alanine aminotransferase (ALT) shows characteristics fluctuations. (5)

Fluctuating transaminases in the absence of alcoholism are virtually diagnostic of HCV infection. During the peaks, symptoms, particularly fatugue, increase. Normal transaminase

levels can be reported for weeks or months, even though the HCV-RNA remains positive (88).

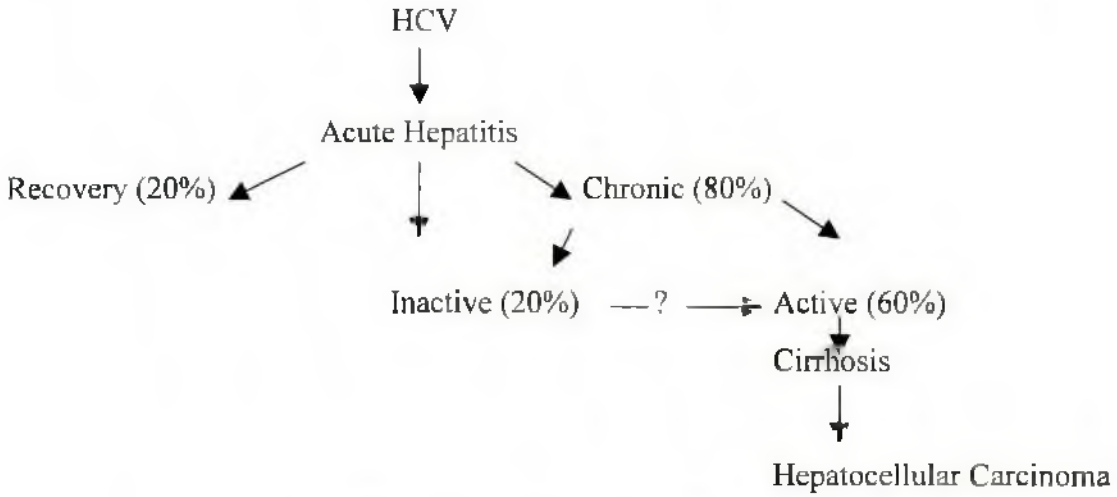


Figure 16: The effect of exposure to the hepatitis C virus. (121)

1.5.4 Hepatitis D hepatitis:

Hepatitis delta agent is capable of infection only when activated by the presence of HBV. It resembles satellite viruses of plants (viroids), which cannot replicate without another specific virus. The interaction between the two viruses is very complex. Synthesis of delta virus may depress the appearance of hepatitis B viral markers in infected cells and even lead to elimination of active viral replication. Hepatitis B and delta infection may be simultaneous (coinfection) or delta may infect a chronic HBsAg carrier (superinfection).

With co-infection, the acute delta hepatitis is usually self-limited, as the delta cannot outlive the transient HBV antigenemia. The clinical picture is usually indistinguishable from hepatitis resulting from HBV alone. However, a biphasic rise in aspartate transaminase may be noted, the second rise being due to the acute effects of delta (89). With superinfection, the acute attack may be severe and even fulminant, or may be marked only by a rise in serum transaminase levels. Delta infection should always be considered in any HBV carrier, usually clinically stable, which has a relapse. Co-infection is more likely to be followed by recovery, with the patient becoming immune to delta. Superinfection is followed by a complete recovery in only 10% of patients and there is a high chronicity rate. Both modes of infection can have a fulminant course.

The HBV-infected patient who acquires delta virus has more active necroinflammation and develops cirrhosis more rapidly than for chronic hepatitis or other viral etiologies. However,

cirrhosis remains in a stable condition for long periods and there is prolonged survival. In certain circumstances, delta virus can be present without HBsAg positivity (90). This is seen in HIV-infected patients and also after hepatic transplantation for HBV and HDV end-stage disease. Hepatocellular carcinoma in a complication of HDV infection but less common in HBsAg carriers with HDV than HBV alone.

1.5.5 Hepatitis E hepatitis:

Infection with hepatitis E virus (HEV) accounts for sporadic and major epidemics of viral hepatitis in developing countries. In general, hepatitis E resembles hepatitis A. It affects young adults and is rare in children (91). This occurrence in young adults is in contrast to HAV. It is possible that the sufferer may have been infected previously in childhood but has lost detectable antibody. Volunteer studies have given an incubation period of 22 to 46 days for blood and 34 to 36 days feces (92). Onset is abrupt; 100% of patients are jaundiced and there are no extrahepatic features. HEV accounts for acute liver failure in endemic region (93). It has also been associated with fulminant disease (94). The course may be cholestatic. Chronicity does not develop. The mortality rate is very high (about 25%) in woman in the last trimester of pregnancy. The severe picture in pregnancy is more common in epidemics than in sporadic cases.

1.6 TRANSMISSION OF THE HEPATITIS VIRUSES

1.6.1 Transmission of HAV:

The principle route of HAV infection is the fecal oral route. The two major ways of HAV transmission are epidemics from a common source, such as contaminated food or water, and direct person to person contact. Parenteral transmission is extremely rare, but can follow transfusion of blood from a donor during the incubation stage of the disease (95).

Direct person to person spread by the fecal oral route is the most important means of transmissions of hepatitis A virus. High titers of virus are present in the feces from late in the incubation period, when the patient is maximally infectious, until the first week of clinical illness. Transmission of HAV via fecally contaminated food or water is well described and may be associated with outbreaks of disease. Such outbreaks are dramatic because of the explosive nature of spread (96). Food-borne and waterborne transmissions continue to be important routes of transmission in developing countries and are particularly relevant to the

susceptible traveler. Food may be contaminated with HAV during preparation by an infected food handler or before handling, as is typically the case with shellfish. A great many outbreaks of hepatitis A have been reported in association with consumption of raw or partially cooked bivalve mollusks, such as mussels, oysters, and clams. Transient viremia has been documented after HAV infection but percutaneous transfer of blood or serum is an infrequent route of HAV transmission. HAV is not transmitted by the sexual route or by vertical transmission with the exception of sexual oral-anal contact.

1.6.2 Transmission of HBV:

Transmission of HBV occurs by the parenteral or inapparent parenteral route. Percutaneous exposure to blood, sexual transmission, and perinatal transmission, which probably results from mucous membrane exposures to maternal blood or amniotic fluid, account for virtually all HBV infections in humans. The source of most HBV infections is probably exposure to body fluids of chronic carriers. Contact with infected blood through improper use of unsterilized needles, scarification and tattooing, sharing of razors and toothbrushes, and transfusion with "uncontrolled" blood account for some transmission in all endemic areas. In intermediate and high endemicity areas, transmission may occur at the time of birth (perinatal transmission) and by close contacts between HBsAg carrier parents and their children or close contact among children (horizontal transmission).

Kissing is not thought to be a significant means of transmission, but biting may be. Infection by feces, urine, tears, breast milk, bile, or pancreatic juice has never been demonstrated even though HBsAg or HBV particles have been detected in such fluid (97).

1.6.3 Transmission of HCV:

HCV infection is acquired in various ways. It could be from an infected blood transfusion, the indication for which may be trauma or obstetric complication or all varieties of surgical operations. The amount of blood transfused can be as little as one unit. The condition is more common in woman, perhaps because they have more operations. Parenteral drug abusers and recipients of blood products such as hemophiliacs are at risk. In many instances, the mode of infection is unknown. Patients with community-acquired non-A, non-B hepatitis are 50% anti-HCV positive (98,99). These patients have no risk factor such as transfusion or drug abuse, and their mode of infection is uncertain.

Other potential routes of transmission such as inapparent parenteral or permucosal exposures in the transmission of the virus (e.g., medical intervention, tattooing, acupuncture and vertical, sexual, accidental needlestick, and household transmission) are also discussed.

1.6.4 Transmission of HDV:

Like HBV, HDV is a blood borne virus and may thus be transmitted by parenteral contact with blood or blood products. Parenteral transmission of HDV occurs through the direct injection of contaminated blood and blood derivatives or through interpersonal contacts (100).

Common methods of transmission of HDV infection include intravenous drug abuse, transfusion, sexual contact, and nosocomial infection. In addition, inapparent parenteral spread is probably responsible for spread of HDV infection within families. The most frequent victims of HDV are drug-addicts; specially among the HBsAg carrier addicts. The transfusion risk of hepatitis D from blood screened for the HBV is very low. Nosocomial transmission of HDV is of limited concern. Sexual promiscuity is a major mode of inapparent parenteral transmission of HDV. Vertical transmission represents an inconspicuous mode for the transmission of HDV, although it is important in maintaining the endemicity of HBV in developing countries. Intrafamilial spread has been noted from southern Italy (101).

1.6.5 Transmission of HEV:

The disease is enterically transmitted, usually by sewage-contaminated water. The viral genome has been found in the stools of sufferers (102). The principle route of HEV transmission is the oral-fecal route. The main vehicle of transmission is water contaminated by feces. Food-borne transmission has been postulated to be the cause of some outbreaks (103). Unlike HAV, person-to-person transmission of HEV appears to be uncommon. Sexual contact has not been identified as a risk factor for transmission. Transmission of HEV by administration of blood products is unlikely to occur. Person-to-person infection is uncommon and vertical transmission rare.

1.7 SEROLOGY AND DIFFERENTIAL DIAGNOSIS OF VIRAL HEPATITIS

1.7.1 Clinical evidence of hepatitis (104):

A patient presenting with flu-like, general, nonspecific symptoms may have the flu or any of a variety of other conditions, including viral hepatitis. A significant number of patients with hepatitis may be asymptomatic. To arrive at a diagnosis, one must include:

-The patient's medical history (previous illness and therapies)

-The patient's risk-behavior history (lifestyle, sexual preference, drug use, recent travel, medical history of close personal contacts, etc.)

-Physical examination

-Blood test results (ALT, AST, ALP, bilirubin, and prothrombin time)

-Serological testing for the hepatitis viruses.

1.7.2 Liver function tests (104):

When the initial work up indicates possible liver disease- risk factors, swollen or painful liver- the physician is likely to order blood tests for liver enzymes and bilirubin to evaluate liver function. Liver enzymes are typically elevated in patients with hepatitis. Patients with symptoms suggestive of liver disease who have elevated liver enzymes are considered candidates for follow-up testing to determine whether specific viral hepatitis agents are causing the liver inflammation.

1.7.3 Serological assessment and diagnosis of viral hepatitis:

Immunoassays are available for many of the hepatitis markers. Proper selection and interpretation of these tests requires some knowledge of the serological events that accompany acute and chronic viral hepatitis. Each test is unique for a specific antigen or antibody. Test results can provide physicians with the specific information needed to determine the type and, in some cases, the status of an infection or to identify patients who are immune to certain hepatitis infections.

1.7.3.1 Serology and diagnosis of HAV (104):

Hepatitis A virus is rarely detectable in serum. By the onset of symptoms, however, antibody to hepatitis A virus (anti-HAV) is almost always detectable. IgM Antibody to the Hepatitis A Virus (IgM anti-HAV) is the immunoglobulin M (IgM) class of antibody to HAV. The initial response to an HAV infection consists almost entirely of IgM antibody. The antibody initially rises rapidly in titer (figure17).

Three to six months after an HAV infection, IgM anti-HAV falls to undetectable levels. Therefore, acute hepatitis A is diagnosed by detecting IgM anti-HAV in serum. The IgM anti-HAV assay is specific for diagnosing or confirming an acute hepatitis A infection, usually within the past three to six months.

Total antibody to the hepatitis A virus (total anti-HAV) assay detects the presence of immunoglobulins G and M (IgG and IgM) and indicates past infection and immunity to hepatitis A. IgG antibody levels rise quickly after the virus is cleared and decline slowly over many years. Total anti-HAV cannot confirm a recent infection, as can IgM anti-HAV, and is primarily useful in epidemiologic studies and immunity panels.

Hepatitis A Diagnostic Profile

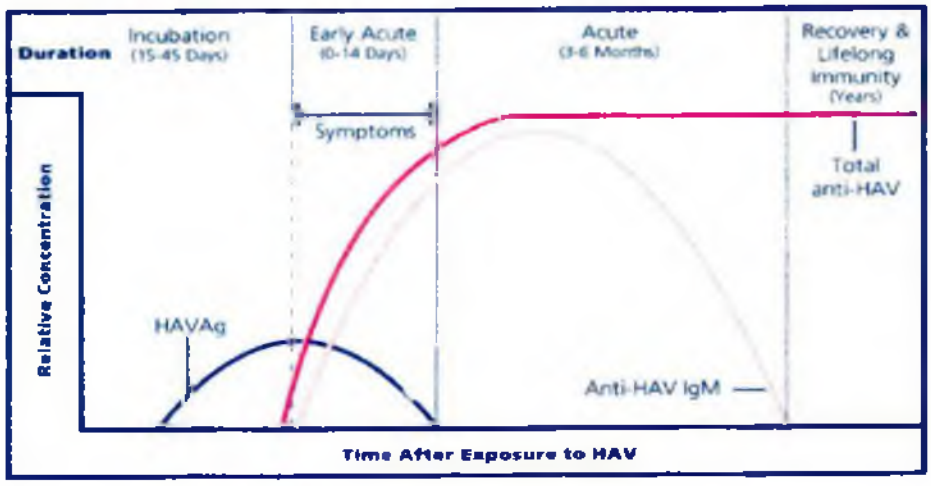


Table 17: Diagnostic profile of hepatitis A virus. HAVAg, hepatitis A virus antigen; Anti-HAV IgM, IgM type antibody to hepatitis A virus; Total anti-HAV, total antibodies against hepatitis A virus. (৩)

1.7.3.2 Serology and diagnosis of HBV:

Three antigen system and their corresponding antibodies are associated with HBV infections. Hepatitis B surface antigen (HBsAg) and its antibody- anti-HBs, Hepatitis B core antigen and its antibodies- IgM anti-HBc and IgG anti-HBc, Hepatitis B e antigen (HBeAg) and its antibody- anti-HBe. HBsAg is the first serologic marker to appear following HBV infection.

Detectable in serum 30-60 days after exposure to HBV, HBsAg generally peaks at, or shortly after, the onset of elevated liver enzymes during the acute phase of the disease. HBsAg is one of the markers in the acute hepatitis panel of tests. With clinical improvement, HBsAg falls and subsequently disappears. In general, liver enzymes return to normal levels when surface antigen disappears. Thus, the presence of HBsAg in serum indicates the presence of HBV and the potential infectious state of the person. If HBsAg continues to be present for longer than six months, the patient is likely to be an HBV chronic carrier who can transmit the disease. HBeAg appears at about the same time that surface antigen is first detected in serum. HBeAg continues to be detected in persons with acute or chronic HBV infection. The presence of HBeAg indicate high infectivity.

The serologic profile in chronic carriers usually includes the HBsAg, HBeAg, and total antibody to the core antigen. Antibody to the hepatitis B core antigen (anti-HBc) can be detected in all patients infected with HBV at one to four weeks after the appearance of surface antigen. Because it is detectable for the remainder of the patient's life, anti-HBc is an indicator of current or previous HBV infection and is helpful in screening programs. This marker is sometimes used with anti-HBs to determine which individuals have been previously exposed to, or are potential chronic carriers of, HBV and, therefore do not need vaccine. The marker does not appear to be associated with recovery from or immunity to hepatitis B. IgM anti-HBc appears when the anti-HBc is first detected. It is usually present for up to six months after the onset of symptoms, and then falls to undetectable levels. IgM anti-HBc is a marker of recent acute HBV infection and is part of the acute hepatitis testing panel. The simultaneous presence of IgM anti-HBc and HBsAg indicates an ongoing hepatitis B infection. The disappearance of e antigen is followed shortly by the appearance of its antibody: anti-HBe. Seroconversion is usually occurs at or around the peak of clinical symptoms. This conversion suggests that the infection is being resolved. Anti-HBe, therefore, indicates the patient's reduced infectious state.

Anti-HBs appears during convalescence, usually after HBsAg has disappeared and recovery is complete. Anti-HBs is the major protective antibody against HBV. When it develops following infection, anti-HBs persists for life and is, therefore, a marker of recovery and immunity. Anti-HBs also develops after inoculation with HBV vaccine, although levels of antibody conferred by vaccination wane over time.

In diagnosing viral hepatitis, the physician can test for the presence of four critical markers using the acute hepatitis panel: IgM anti-HEV, IgM anti-HAV, HBsAg, IgM anti-HBc, and

anti-HCV. Results from this panel of tests help differentiate hepatitis A, B, C and E. Monitoring of an ongoing HBV infection can be done using a panel of tests. This monitoring panel consists of HBsAg, HBeAg, and anti-HBe. If HBsAg persists for six months or longer, the patient most likely to has a chronic HBV infection. If HBsAg continues to be present in follow-up specimens, the physician should assess the extent and progress of liver disease with a detailed history, physical examination, and repeated liver function tests.

HBeAg is a serological marker for infectivity. Healthy or inactive HBV carriers will be positive for HBsAg but not be positive for HBeAg and will have normal levels of ALT and AST. Persistent HBsAg and HBeAg in patients with elevated ALT levels indicate the likelihood of chronic active hepatitis B. These patients should be evaluated as potential candidates for IFN therapy. Hepatitis B viral DNA is found in the core of the virus. Because the DNA present is proportional to the number of infectious viral particles, HBV-DNA is accurately indicates active viral replication. HBV-DNA is used to monitor the efficacy of chronic HBV therapy.

(figure12)

1.7.3.3 Serology and diagnosis of HCV:

Anti-c22 and anti-c33c are often the first antibodies to be produced after infection. The conformational epitopes of c33 are particularly immunogenic. Antibody against NS5 can also be present in the early phase of infection. The pattern of anti-HCV differs according to the phase of infection and to differences in biological responses of different individuals. In the acute phase of disease seroconversion to anti-HCV is generally late; the antibody usually increases 3 to 6 months after the onset of hepatitis C and exposure to the virus. The appearance of antibody may coincide with or follow the transaminase peak. Anti-HCV generally persists in patients in the infection progresses to chronicity. In resolving cases

the antibody disappears within 6 to 12 months; however anti-HCV may be detectable for up to 4 years. The appearance of anti-c100-3 is delayed and often associated with progression to chronicity. In patients who resolve chronic hepatitis C by antiviral treatment, anti-c-100-3 diminishes and disappears in 24 months while anti-c22 and c33 persist indefinitely. The presence of anti-c-100-3 in asymptomatic patients is associated with viremia.

As yet, no antigen test is available for HCV. Anti-HCV testing is helpful in diagnosing individuals with signs, symptoms, and biochemical evidence of hepatitis and those who have a history of NANB hepatitis. Patient who test positive for anti-HCV should be considered

infected and infectious until proven otherwise. Anti-HCV testing should be repeated at regular intervals for one year in cases of negative but the patient's clinical signs and symptoms suggest HCV infection. As false positive results can occur; supplemental assays have been developed to define the specificity of a positive anti-HCV assay. Such tests are based on immunoblot technology.

1.7.3.4 Serology and diagnosis of HDV:

Two markers distinguish hepatitis D. The circulating antigen, hepatitis D antigen (HDAg), indicate an acute infection. It is, however, typically present transiently in very low levels and has little usefulness as a diagnostic marker. Total antibody to the HD antigen, anti-HD, is an indicator of past or current delta infection. High titers of the antibody indicate a chronic or active infection.

HDV infection may be diagnosed by detecting HDAg in serum during early infection and by the appearance of anti-HDV during or after infection. The test for HDAg and total anti-HDV is commonly available.

1.7.3.5 Serology and diagnosis of HEV:

Diagnosis is made during the acute phase of illness by the demonstration of HEV-RNA and the IgM antibody to HEV (IgM anti-HEV); this antibody is present in high titers at the onset of disease and persists only for a few months. Viremia lasts at least 2 weeks in nearly all infected patients.

During the convalescence, IgG anti-HEV becomes the predominant antibody, reaching high titers in a few months; it is then detectable life-long. As in the case of hepatitis A the finding of IgG class antibodies is not in itself diagnostic of hepatitis E; the diagnosis is made on the concomitant presence of IgM anti-HEV. IgG anti-HEV increases in prevalence as a function of age and lower socio-economic status.

The diagnosis requires specific serologic testing for antibody markers of exposure to the HEV. Several EIAs using recombinant HEV proteins have been developed to detect IgM and IgG to HEV. In addition, the polymerase chain reaction (PCR) has been used to detect fecal shedding and viremia.

1.8 THE RATIONALE OF THE PRESENT STUDY

Clinical jaundice due to viral hepatitis is a common health problem in Bangladesh. At least five distinct primary hepatotrophic viruses named alphabetically A to E are responsible for the disease. Irrespective of the etiology, clinical presentation of different viral infections are similar. The exact etiology can not be established on the basis of clinical diagnosis only. But the etiological diagnosis is necessary for immediate management and follow up of the patients as well as for prevention of the disease transmission.

Several studies have been carried out in acutely jaundiced patients in Bangladesh. In 1984, Islam et al reported that HBV infection accounts for 25% cases of acute viral hepatitis in Bangladesh. However, they tested only HBsAg for acute HBV infection (105). Hlady et al reported that in 1990 that enterically transmitted non-A, non-B hepatitis is an important cause of acute hepatitis in Bangladesh. In that study, only 19 samples were included and only one sample was tested for anti-HEV.IgM (106). In 1996, M Khan and N Ahmed reported that in acute viral hepatitis, hepatitis A, B, C, and E, accounts for 12.5%, 35%, 3.5% and 19.5% cases respectively. But the paper failed to provide detailed methodology, patient recruitment, and other relevant information. Moreover, tests for hepatitis delta virus was not included in that study (107). So, the studies undertaken previously not give any guideline for the diagnosis of acute viral hepatitis in Bangladesh in a precise and cost-effective way.

In recent years, enzyme immunoassays (EIA) are commercially available for serological diagnosis of the etiology of different types of viral hepatitis in addition to biochemical tests. All the biochemical and serological tests can be performed for various types of viral hepatitis. But doing all the tests may be financially a burden for the patient in most cases. Therefore, the present study was carried out to find out the exact etiologic agents and to precise the tests in light of specific type of viral hepatitis that can help diagnosis of acute hepatitis in cost-effective way. Moreover, the present study might help clinicians selecting minimum tests of acute viral hepatitis for investigation in a cost-effective way.

1.9 THE AIMS OF THE PRESENT STUDY

This study was carried out:

- To find out the etiologic agents, demographics, risk factors of acute viral hepatitis in Bangladesh.
- To evaluate the role of biochemical tests in the diagnosis of clinically jaundiced patients.
- To select a minimum number of serological tests for the diagnosis of acute viral hepatitis.
- To compare the clinical presentation, biochemical and serological profiles and risk factors associated with different types of acute viral hepatitis.

Chapter 2

MATERIALS AND METHODS

2. MATERIALS AND METHODS

2.1 STUDY SUBJECTS

The present cross sectional study comprised of 507 clinically jaundiced patients with suspected acute viral infection [age: 33±16 years; sex (m/f): 373/134]. The study was carried out in the department of Immunology of Bangladesh Institute of Research and Rehabilitation in Diabetes, Endocrine and Metabolic Disorders (BIRDEM). Study subjects were referred to the Department of Immunology at BIRDEM hospital by physicians to detect virus(s) causing jaundice. The purpose of the study was clearly explained to each of the subjects and they were recruited only after they had given full consent to participate in the study. Approval of the Ethical Review Committee of BIRDEM was duly taken before carrying of the study.

2.1.1 Criteria for the diagnosis of acute viral hepatitis

Patients with jaundice of duration no more than six weeks referred to the BIRDEM Hospital by their physicians with flu-like symptoms-such as fatigue, joint and/or muscle pain, loss of appetite, nausea, vomiting, diarrhea, fever etc.- were recruited in the present study. Patients with history of taking drugs and/or alcohol were excluded initially from the study.

Recruited patients, after collection of blood, tested immediately for prothrombin time and biochemical markers (bilirubin, ALT, AST and ALP). Patients with high bilirubin and ALT and/or AST level were finally included in the study. Patients with high bilirubin and ALP level but normal ALT and AST level were then excluded from the study.

Finally recruited patients were tested for all the acute markers of viral hepatitis. The samples were tested for hepatitis A (Anti-HAV,IgM and Anti-HAV,total), hepatitis B (HBsAg, Anti-HBc,IgM and Anti-HBc,total), hepatitis C (Anti-HCV and Anti-HCV confirmatory by LIA), hepatitis D (HDAg and Anti-HDV only in the cases of HBV positive) and hepatitis E (Anti-HEV,IgM and Anti-HEV,IgG) virus infections. Serological markers for hepatitis viruses and liver function tests were rechecked in all the cases.

2.2 SAMPLE COLLECTION AND PRESERVATION

Five ml of venous blood was collected from each of the subjects and 1.8ml blood was taken into test tube containing anti-coagulant (3.8% Na-citrate) for prothombin time. Prothrombin time test was done within half an hour of collection. Rest of the blood was poured into autoclaved microcentrifuge tubes. Serum was separated immediately and tested for biochemical markers. Rest of the serum samples were preserved immediately at -70^{0}C for assay of serological markers.

2.3 ANALYTICAL METHODS

2.3.1 Liver function tests

Alanine transaminase (ALT), aspartate transaminase (AST) and alkaline phosphatase (ALP), were assayed by chemistry analyzer RA-50 (Bayer, UK) using commercial reagents (Randox Laboratories Ltd., UK and Bayer, UK). Prothrombin time was estimated by automated

analyzer Coag-a-mate II (Organon Teknika, Netherland) using commercial thromboplastin reagent (Pacific hemostasis, USA).

2.3.1.1 Prothrombin Time

Principle of the determination of prothrombin time

The one-stage prothrombin time (PT) measures the clotting time of plasma after adding a source of tissue factor (thromboplastin) and calcium. The recalcification of plasma in the presence of tissue factor generates activated Factor Xa (F.Xa). F.Xa in turn activates Prothrombin to thrombin, which converts fibrinogen to an insolouble fibrin clot.

Reagents for the determination of prothrombin time

Thromboplastin-D, a lyophilized extract of acetone dehydrated rabbit brain; $CaCl_2$ with buffers and stabilizers.

Normal and Abnormal plasmas: Coagulation control Level 1, 2 and 3.

Level 1 is a lyophilized normal plasma. Level 2 and 3 are adjusted to mimic moderately and severely deficient plasma, respectively.

Procedures for the determination of prothrombin time

Thromboplastin-D reagent vial was reconstituted with distilled water. The reagent was warmed to 37^0C before the assay. An aliquot of 100 µl of the test plasma was added to cuvette and warmed to 37^0C. Then 200 µl of warmed Thromboplastin-D was forcibly added to the test plasma and the time of clot formation was observed in the automated analyzer, Coag-a-mate II. Normal and abnormal controls were also checked with each group of assays.

2.3.1.2 ALT assay

Principle of ALT assay

$$\alpha\text{-ketoglutarate} + \text{L-alanine} \xrightleftharpoons{\text{ALT}} \text{L-glutamate} + \text{pyruvate}$$

$$\text{pyruvate} + \text{NADH} + \text{H}^+ \xrightleftharpoons{\text{LD}} \text{L-lactate} + \text{NAD}^+$$

ALT catalyzes the reversible transfer of an amino group from alanine to α-ketoglutarate. Pyruvate formed by this reaction is then converted to lactate. The activity of ALT is determined from the oxidation of NADH. Decrease in absorbance due to oxidation of NADH to NAD^+ is measured at 340 nm.

Reagents for ALT assay

a) Buffer/Substrate: Tris buffer; L-alanine

b) Enzyme/Coenzyme/α-oxoglutarate: α-ketoglutarate; LD; NADH

Procedure for ALT assay

Reagent (a) was ready to use. One vial of reagent (b) was reconstituted with the appropriate volume of reagent (a) before the assay. 100 µl of sample was added to 1000 µl of reagent (b) and mixed. Initial absorbance was read after 1 minute and then after 1, 2, and 3 minutes. This assay was carried at 37^0C, absorbance was mesured at 340 nm using 1 cm light path cuvette. ALT activity is expressed as U/L and calculated using the following formula:

U/L= 1746 X Δ A 340 nm/min

2.3.1.3 AST assay

Principle of AST assay

α-ketoglutarate + L-aspartate $\xrightleftharpoons{\text{AST}}$ L-glutamate + oxaloacetate

oxaloacetate + NADH + H^+ $\xrightleftharpoons{\text{MDH}}$ L-malate + NAD^+

AST catalyzes the reversible transfer of an amino group from aspartate to α-ketoglutarate. Oxaloacetate formed by this reaction is then converted to malate. The activity of AST is determined from the oxidation of NADH. Decrease in absorbance due to oxidation of NADH to NAD^+ is measured at 340 nm.

Reagents for AST assay

a) Buffer/Substrate: Tris buffer; L-aspartate

b) Enzyme/Coenzyme/α-ketoglutarate: α-ketoglutarate; MDH; LD; NADH

Procedure for AST assay

Reagent (a) was ready to use. One vial of reagent (b) was reconstituted with the appropriate volume of reagent (a) before the assay. 100 µl of sample was added to 1000 µl of reagent (b) and mixed. Initial absorbance was read after 1 minute and then after 1, 2, and 3 minutes. This assay was carried at 37^0C, absorbance was mesured at 340 nm using 1 cm light path cuvette. ALT activity is expressed as U/L and calculated using the following formula:

U/L= 1746X Δ A 340 nm/min

2.3.1.4 ALP assay

Principle of ALP assay

p-nitrophenylphosphate + H_2O $\xrightarrow{ALP}$ phosphate + p-nitrophenol

p-nitrophenylphosphate ia hydrolysed to p-nitrophenol and phosphate. Absorbance increased in a given time is proportional to the activity of the enzyme and is determined at 405 nm.

Reagents for ALP assay

a) Buffer: Diethanolamine buffer

b) Substrate: p-nitrophenylphosphate

Procedure for ALP assay

Reagent (a) was ready to use. One vial of reagent (b) was reconstituted with the appropriate volume of reagent (a) before the assay. Ten µl of sample was added to 500 µl of reagent (b) and mixed. Initial absorbance was read and then after 1, 2, and 3 minutes. This assay was carried at 37^0C, absorbance was mesured at 405 nm using 1 cm light path cuvette. ALP activity is expressed as U/L and calculated using the following formula:

U/L= 3300 X Δ A 405 nm/min.

2.3.2 Viral markers

Assay of the serological markers for hepatitis A (Anti-HAV,IgM and Anti-HAV,total), hepatitis B (HBsAg, Anti-HBc,IgM, Anti-HBc,total), hepatitis C (Anti-HCV, Anti-HCV LIA), hepatitis D (HDAg and Anti-HDV) [Diasorin, Italy] and Hepatitis E (Anti-HEV,IgM and Anti-HEV,IgG) (Gene Lab, Singapore) were performed by commercial enzyme immunoassay kits following the manufacturer's instructions.

2.3.2.1 IgM anti-HAV assay

Principle of IgM anti-HAV assay

Qualitative IgM anti-HAV determination is an immunometric assay, based on the antibody capture ELISA (Enzyme-linked Immunosorbent Assay) technique. The presence of IgM anti-HAV allows the HAV and the enzyme tracer (anti-HAV antibody (mouse monoclonal) conjugated to HRP (horseradish peroxidase)) to bind to the solid phase. Therefore, the enzyme activity is proportional to the IgM anti-HAV concentration present in samples or controls. Measurement of enzyme activity is performed by adding a colourless

chromogen/substrate solution. The enzyme action produces a colour which can be measured by ELISA reader.

Reagents for IgM anti-HAV assay

a) Coated strips: Wells are coated with antibody to human IgM (mouse monoclonal).

b) Enzyme tracer (50X): Anti-HAV antibody (mouse monoclonal) conjugated to HRP in tris buffer, proteins, stabilizers and preservatives.

c) Negative control: Human serum non-reactive for IgG and IgM anti-HAV, diluted 1:100 with phosphate (PBS) buffer. The solution contains proteins, stabilizers, an inert dye and preservatives.

d) Positive control: Human serum reactive for IgM anti-HAV and non-reactive for IgG anti-HAV, diluted with phosphate buffer. The solution contains proteins, stabilizers and preservatives.

e) HAV: Formaldehyde-inactivated human HAV. The solution contains human serum non-reactive for IgG and IgM anti-HAV, PBS buffer, proteins and preservatives.

f) Sample diluent: PBS buffer, BSA (Bovine serum albumin), human IgG, an inert dye and preservatives.

g) Wash buffer (25X): PBS buffer, Tween 20 and preservatives.

h) Chromogen: Tetramethylbenzidine (TMB) derivative in buffer solution.

i) Substrate: Buffer solution containing H_2O_2.

j) Blocking reagent: 1N sulphuric acid solution.

Preparation and stability of reagents for IgM anti-HAV assay

Coated strips: Ready to use, the strips should be kept at 2-8^{0}C. The bag should be opened at room temperature. Unused strips are preserved after sealing the bag at 2-8^{0}C.

Enzyme tracer: The solution should be diluted at 1:50 with HAV. After dilution, it can be stored for one week at 2-8^{0}C.

Negative control, positive control, blocking reagent: Ready to use, should be stored at 2-8^{0}C.

HAV: Ready to use, should be stored at 2-8^{0}C. This solution is used to dilute the enzyme tracer. Throughly mix before withdrawing an aliquot to dilute the enzyme tracer.

Sample diluent: Ready to use, should be stored at 2-8^{0}C. The solution is used to dilute samples.

Wash buffer: The contents of the vial is diluted 1:25 with distilled water, which may be stored for one week at 2-8^{0}C. The reagent is used to rinse wells.

Chromogen: The solution is mixed 1:1 with substrate. After dilution, chromogen/substrate can be stored for 8 hours at room temperature, away from the light.

Substrate: The solution is mixed 1:1 with chromogen.

Assay procedure for IgM anti-HAV

Aliquots of 100 µl negative control, positive control and diluted samples (1:100 with sample diluent) were dispensed into their respective wells and covered with sealer to prevent evaporation. Wells were incubated at 37^0C for 1 hours ± 5 minutes. The diluted enzyme tracer solution was prepared at the end of first incubation and contents of wells were discarded and strips were rinsed five times with wash buffer to remove unbound materials. Then 100 µl diluted enzyme tracer was dispensed into all wells, covered with sealers and incubated again at 37^0C for 1 hour ± 5 minutes. Chromogen/substrate was prepared at the end of the second incubation and strips were rinsed five times with wash buffer. Then 100 µl chromogen/substrste was dispensed into all wells and incubated for 30 ± 2 minutes at room temperature in a dark place followed by adding 100 µl blocking reagent into all wells. Absorbance was measured at 450/630 nm using ELISA reader.

Calculation and interpretation of results for IgM anti-IIAV

The cut-off value is determined by adding 0.100 to the mean absorbance of the negative control values (NCx).

Cut-off value = NCx + 0.100

The presence or absence of IgM anti-HAV is determined by comparing the absorbance value of the unknown samples to that of the cut-off value. The unknown samples with absorbance values greater than or equal to the cut-off value should be considered reactive for IgM anti-HAV. The unknown samples with absorbance values less than the cut-off value should be considered non-reactive.

2.3.2.2 Total anti-HAV assay

Principle of Total anti-HAV assay

The method for qualitative or quantitative anti-HAV determination is a competitive immunometric assay, based on the ELISA (Enzyme-linked Immunosorbent Assay) technique. Anti-HAV present in the test sample and anti-HAV antibody (mouse monoclonal) to the solid phase compete for a fixed and limited quantity of HAV present in the neutralizing solution. The amount of HAV coupled to the solid phase will thus proportionally decreases as the anti-HAV concentration in the test samples increases. After removing the unbound reagents, the enzyme tracer is incubated in the well. The quantity of tracer bound to the solid phase through HAV and consequently the enzyme activity are inversely proportional to the

concentration of anti-HAV present in samples or controls. Measurement of enzyme activity is performed by adding a colourless chromogen/substrate solution. The enzyme action produces a colour which can be measured by ELISA reader.

Reagents for Total anti-HAV assay

a) Coated strips: Wells are coated with antibody to human IgM (mouse monoclonal).

b) Enzyme tracer (50X): Anti-HAV antibody (mouse monoclonal) conjugated to HRP in tris buffer, proteins, stabilizers and preservatives.

c) Negative control: Human serum non-reactive for IgG and IgM anti-HAV, diluted 1:100 with phosphate (PBS) buffer. The solution contains proteins, stabilizers, an inert dye and preservatives.

d) Positive control: Human serum reactive for IgM anti-HAV and non-reactive for IgG anti-HAV, diluted with phosphate buffer. The solution contains proteins, stabilizers and preservatives.

e) HAV: Formaldehyde-inactivated human HAV. The solution contains human serum non-reactive for IgG and IgM anti-HAV, PBS buffer, proteins and preservatives.

g) Sample diluent: PBS buffer, BSA (Bovine serum albumin), human IgG, an inert dye and preservatives.

g) Wash buffer (25X): PBS buffer, Tween 20 and preservatives.

h) Chromogen: Tetramethylbenzidine (TMB) derivative in buffer solution.

i) Substrate: Buffer solution containing H_2O_2.

j) Blocking reagent: 1N sulphuric acid solution.

Preparation and stability of reagents for Total anti-HAV assay

Coated strips: Ready to use, the strips should be kept at 2-8^{0}C. The bag should be opened at room temperature. Unused strips are preserved after sealing the bag at 2-8^{0}C.

Enzyme tracer: The solution should be diluted at 1:50 with HAV. After dilution, it can be stored for one week at 2-8^{0}C.

Negative control, positive control, blocking reagent: Ready to use, should be stored at 2-8^{0}C.

HAV: Ready to use, should be stored at 2-8^{0}C. This solution is used to dilute the enzyme tracer. Throughly mix before withdrawing an aliquot to dilute the enzyme tracer.

Sample diluent: Ready to use, should be stored at 2-8^{0}C. The solution is used to dilute samples.

Wash buffer: The contents of the vial is diluted 1:25 with distilled water, which may be stored for one week at 2-8^{0}C. The reagent is used to rinse wells.

Chromogen: The solution is mixed 1:1 with substrate. After dilution, chromogen/substrate can be stored for 8 hours at room temperature, away from the light.

Substrate: The solution is mixed 1:1 with chromogen.

Assay procedure for Total anti-HAV

Aliquots of 100 µl negative control, positive control and diluted samples (1:100 with sample diluent) were dispensed into their respective wells and covered with sealer to prevent evaporation. Wells were incubated at 37^0C for 1 hours ± 5 minutes. The diluted enzyme tracer solution was prepared at the end of first incubation and contents of wells were discarded and strips were rinsed five times with wash buffer to remove unbound materials. Then 100 µl diluted enzyme tracer was dispensed into all wells, covered with sealers and incubated again at 37^0C for 1 hour ± 5 minutes. Chromogen/substrate was prepared at the end of the second incubation and strips were rinsed five times with wash buffer. Then 100 µl chromogen/substrste was dispensed into all wells and incubated for 30 ± 2 minutes at room temperature in a dark place followed by adding 100 µl blocking reagent into all wells. Absorbance was measured at 450/630 nm using ELISA reader.

Calculation and interpretation of results for Total anti-HAV

The cut-off value is determined by adding 0.100 to the mean absorbance of the negative control values (NCx).

Cut-off value = NCx + 0.100

The presence or absence of IgM anti-HAV is determined by comparing the absorbance value of the unknown samples to that of the cut-off value. The unknown samples with absorbance values greater than or equal to the cut-off value should be considered reactive for IgM anti-HAV. The unknown samples with absorbance values less than the cut-off value should be considered non-reactive.

2.3.2.3 HBsAg assay

Principle of HBsAg assay

Qualitative HBsAg determination is a direct, non-competitive sandwich assay, based on the Enzyme-Linked Immunosorbent Assay (ELISA). Incubation of serum samples containing HBsAg at 37^0C in the wells coated with anti-HBs mouse monoclonal antibody allows binding of HBsAg with anti-HBs. After washing, a second incubation with Enzyme tracer [sheep anti-HBs antibody conjugated to horseradish peroxidase (HRP)], allows binding to the bound HBsAg. The enzyme activity is therefore proportional to the HBsAg concentration present in

samples or controls. Measurement of bound enzyme activity is performed by adding a chromogen/substrate solution. The enzyme action produces a colour which can be detected with ELISA reader.

Reagents for HBsAg assay

a) Coated strips: Wells are coated with anti-HBs mouse monoclonal antibody, having the same reactivity for ad and ay subtypes.

b) Enzyme tracer (50X): anti-HBs antibody (sheep) conjugated to HRP, Tris buffer, stabilizers and preservatives.

c) Negative control: Human plasma non-reactive for HBsAg and preservatives.

d) Positive control: Heat-inactivated recalcified human plasma at a concentration of 0.5-1.5 PEI U/ml HBsAg (subtype ad and ay) and preservatives. HBsAg standard preparation from Paul-Ehrlich-Institute (PEI, Germany).

e) Tracer diluent: Buffer solution, proteins and preservatives.

f) Wash buffer (25X): PBS, Tween 20 and preservatives.

g) Chromogen: Tetramethylbenzidine (TMB) derivative in buffer solution.

h) Substrate: Buffer solution containing H_2O_2.

i) Blocking reagent: 1N sulphuric acid solution.

Preparation and stability of reagents for HBsAg assay

Coated strips: Ready to use, the strips should be kept at 2-8^{0}C. The bag should be opened at room temperature. Unused strips are preserved after sealing the bag at 2-8^{0}C.

Enzyme tracer: The solution should be diluted at 1:50 with tracer diluent. After dilution, it can be stored for one week at 2-8^{0}C.

Negative control, positive control, blocking reagent: Ready to use, should be stored at 2-8^{0}C.

Tracer diluent: Ready to use, should be stored at 2-8^{0}C. This solution is used to dilute the enzyme tracer.

Wash buffer: The contents of the vial is diluted at 1:25 with distilled water, which may be stored for one week at 2-8^{0}C. The reagent is used to rinse wells.

Chromogen: The solution is mixed 1:1 with substrate.

Substrate: The solution is mixed 1:1 with chromogen.

Assay procedure for HBsAg

Aliquotes of 100 µl negative control, positive control and samples were dispensed into their respective wells and covered with sealer to prevent evaporation. Wells were incubated at 37^{0}C for 30 ± 2 minutes with shaking at 1430 ± 20 rpm/G value or 1 hour ± 5 minutes at

37^0C without shaking. The diluted enzyme tracer solution was prepared at the end of first incubation. After first incubation, contents of wells were discarded and strips were rinsed five times with wash buffer to remove unbound materials. Then 100 µl diluted enzyme tracer was dispensed into all wells, covered with sealers and incubated again at 37^0C for 30 ± 2 minutes with shaking at 1430 ± 20 rpm or 1 hour ± 5 minutes at 37^0C without shaking. Chromogen/substrate was prepared at the end of the second incubation and strips were rinsed five times with wash buffer. Then 100 µl chromogen/substrste was dispensed into all wells and incubated for 30 minutes at room temperature in a dark place followed by adding 100 µl blocking reagent into all wells. Absorbance was measured at 450/630 nm using ELISA reader.

Calculation and interpretation of results for HBsAg

The cut-off value is determined by adding 0.050 to the mean absorbance of the negative control values (NCx).

Cut-off value = NCx + 0.050

The presence or absence of HBsAg is determined by comparing the absorbance value of unknown samples to that of the cut-off value. The unknown samples with absorbance values greater than or equal to the cut-off value should be considered reactive for HBsAg. The unknown samples with absorbance values less than the cut-off value should be considered non-reactive.

2.3.2.4 IgM anti-HBc assay

Principle of IgM anti-HBc assay

Qualitative IgM anti-HBc determination is an immunometric assay, based on ELISA method. The presence of IgM anti-HBc allows the HBcAg and the enzyme tracer (human anti-HBc antibodies conjugated to HRP) to bind to the solid phase. Therefore, the enzyme activity is proportional to the IgM anti-HBc concentration present in samples or controls. Measurement of enzyme activity is performed by adding a chromogen/substrate solution. The enzyme action produces a colour which can be measured by ELISA reader.

Reagents for IgM anti-HBc assay

a) Coated strips: Wells are coated with antibody to human IgM (mouse monoclonal).

b) Enzyme tracer (50X): Human anti-HBc antibodies conjugated to HRP in tris buffer, proteins, stabilizers and preservatives.

c) Negative control: Human serum non-reactive for all HBV markers, diluted at 1:200 with phosphate buffer. The solution contains proteins, stabilizers and preservatives.

d) Positive control: Human serum reactive for IgM anti-HBc and HBsAg, diluted at 1:200 with phosphate buffer. The solution contains proteins, stabilizers and preservatives.

e) HBcAg: Recombinant, proteins and preservatives

f) Sample diluent: PBS, BSA, human IgG, murine IgG and preservatives.

g) Wash buffer (25X): PBS, Tween 20 and preservatives.

h) Chromogen: TMB derivative in buffer solution.

i) Substrate: Buffer solution containing H_2O_2.

j) Blocking reagent: 1N sulphuric acid solution.

Preparation and stability of reagents for IgM anti-HBc assay

Coated strips: Ready to use, the strips should be kept at 2-8^{0}C. The bag should be opened at room temperature. Unused strips are preserved after sealing the bag at 2-8^{0}C.

Enzyme tracer: The solution should be diluted at 1:50 with HBcAg. After dilution, it can be stored for one week at 2-8^{0}C.

Negative control, positive control: Should be diluted at 1:20 with sample diluent before use to a final 1:4000 dilution. The diluted solutions are stable for one week at 2-8^{0}C.

HBcAg: Ready to use, should be stored at 2-8^{0}C. This solution is used to dilute the enzyme tracer.

Sample diluent: Ready to use, should be stored at 2-8^{0}C. The solution is used to dilute samples and controls.

Wash buffer: The contents of the vial is diluted 1:25 with distilled water, which may be stored for one week at 2-8^{0}C. The reagent is used to rinse wells.

Chromogen: The solution is mixed 1:1 with substrate.

Substrate: The solution is mixed 1:1 with chromogen.

Assay procedure for IgM anti-HBc

Aliquots of 100 µl diluted negative control, positive control and samples were dispensed into their respective wells and covered with sealer to prevent evaporation. Wells were incubated at 37^{0}C for 1 hours ± 5 minutes. The diluted enzyme tracer solution was prepared at the end of first incubation and contents of wells were discarded and strips were rinsed five times with wash buffer to remove unbound materials. Then 100 µl diluted enzyme tracer was dispensed into all wells, covered with sealers and incubated again at 37^{0}C for 1 hour ± 5 minutes. Chromogen/substrate was prepared at the end of the second incubation and strips were rinsed

five times with wash buffer. Then 100 µl chromogen/substrste was dispensed into all wells and incubated for 30 ± 2 minutes at room temperature in a dark place followed by adding 100 µl blocking reagent into all wells. Absorbance was measured at 450/630 nm using ELISA reader.

Calculation and interpretation of results for IgM anti-HBc

The cut-off value is determined by adding 0.100 to the mean absorbance of the negative control values (NCx).

Cut-off value = NCx + 0.100

The presence or absence of IgM anti-HBc is determined by comparing the absorbance value of unknown samples to that of the cut-off value. The unknown samples with absorbance values greater than or equal to the cut-off value should be considered reactive for IgM anti-HBc. The unknown samples with absorbance values less than the cut-off value should be considered non-reactive.

2.3.2.5 Total anti-HBc assay

Principle of Total anti-HBc assay

Qualitative total anti-HBc determination is a competitive assay, based on ELISA technique. Anti-HBc present in the sample and labelled anti-HBc antibodies compete for a fixed quantity of HBcAg. The quantity of enzyme tracer (human anti-HBc antibodies conjugated to HRP), bound to the solid phase through HBcAg and consequently the enzyme activity is inversely proportional to the anti-HBc concentration present in samples or controls. Measurement of enzyme activity is performed by adding a chromogen/substrate solution. The enzyme action produces a colour which can be measured by ELISA reader.

Reagents for Total anti-HBc assay

a) Coated strips: Wells are coated with recombinant HBcAg.

b) Enzyme tracer (50X): Anti-HBc antibodies conjugated to HRP in tris buffer, proteins, stabilizers and preservatives.

c) Negative control: Human serum non-reactive for all HBV markers and preservatives.

d) Positive control: Human plasma reactive for anti-HBc and preservatives.

e) Tracer diluent: Human serum non-reactive for all HBV markers in PBS and preservatives.

f) Wash buffer (25X): PBS, Tween 20 and preservatives.

g) Chromogen: TMB derivative in buffer solution.

h) Substrate: Buffer solution containing H_2O_2.

i) Blocking reagent: 1N sulphuric acid solution.

Preparation and stability of reagents for Total anti-HBc assay

Coated strips: Ready to use, the strips should be kept at 2-8^{0}C. The bag should be opened at room temperature. Unused strips are preserved after sealing the bag at 2-8^{0}C.

Enzyme tracer: The solution should be diluted at 1:50 with tracer diluent. After dilution, it can be stored for one week at 2-8^{0}C.

Negative control, positive control, blocking reagent: Ready to use, should be stored at 2-8^{0}C.

Tracer diluent: Ready to use, should be stored at 2-8^{0}C. This solution is used to dilute the enzyme tracer.

Wash buffer: The contents of the vial is diluted at 1:25 with distilled water, which may be stored for one week at 2-8^{0}C. The reagent is used to rinse wells.

Chromogen: The solution is mixed 1:1 with substrate.

Substrate: The solution is mixed 1:1 with chromogen.

Assay procedure for Total anti-HBc assay

Aliquotes of 50 µl negative control, positive control and samples were dispensed into their respective wells. Then 100 µl diluted enzyme tracer was immediately dispensed into all wells and covered with sealer to prevent evaporation. Wells were incubated at 37^{0}C for 2 hours ± 10 minutes. Chromogen/substrate was prepared at the end of the incubation and strips were rinsed five times with wash buffer followed by adding 100 µl chromogen/substrste into all wells and incubated for 30 ± 2 minutes at room temperature in a dark place. Then 100 µl blocking reagent was dispensed into all wells. Absorbance was measured at 450/630 nm using ELISA reader.

Calculation and interpretation of results for Total anti-HBc assay

The cut-off value is determined by adding the mean absorbance for the negative control values (NCx) multiplied by 0.2 to the mean absorbance for the positive control values (PCx) multiplied by 0.8.

Cut-off value = 0.2 NCx + 0.8 PCx

The presence or absence of anti-HBc is determined by comparing the absorbance value of the unknown samples to that of the cut-off value. The unknown samples with absorbance values less than or equal to the cut-off value should be considered reactive for anti-HBc. The unknown samples with absorbance greater than the cut-off value should be considered non-reactive.

2.3.2.6 Anti-HCV assay

Principle of anti-HCV assay

Qualitative determination of antibody to HCV is an immunometric assay, based on ELISA method. Incubation of serum samples containing antibody to HCV at 37^0C in the wells coated with polypeptides of the structural and non-structural regions of HCV allows binding of anti-HCV. After washing, a second incubation with enzyme tracer (goat IgG to human IgG conjugated to HRP) allows binding to the bound anti-HCV. The enzyme activity is therefore proportional to the concentration of antibody to HCV present in samples or controls. Measurement of bound enzyme activity is performed by adding a chromogen/substrate solution. The enzyme action produces a colour which can be detected with ELISA reader.

Reagents for anti-HCV assay

a) Coated strips: Wells are coated with the following polypeptides of the structural and non-structural regions of HCV: C22 and C33 (recombinant), NS4 and NS5 (synthetic). The strips are ready to use and should be stored at 2-8^0C.

b) Enzyme tracer (100X): IgG raised in goats to human IgG conjugated to HRP, stabilizers and preservatives. The solution should be diluted at 1:100 with tracer diluent just before use. The working enzyme tracer cannot be stored and should be used immediately after preparation.

c) Negative control: Human serum non-reactive for antibodies to HCV and preservatives. The solution should be diluted 1:21 with sample diluent.

d) Positive control: Heat-inactivated human plasma reactive for antibodies to HCV and preservatives. The solution should be diluted 1:21 with sample diluent.

e) Tracer diluent: Protein stabilizer solution and preservatives. The reagent is ready to use and should be stored at 2-8^0C. The solution is used to dilute the enzyme tracer.

f) Sample diluent: Protein solution, detergents and preservatives. The reagent is ready to use and should be stored at 2-8^0C. The solution is used to dilute controls and samples.

g) Wash buffer (25X): PBS buffer, Tween 20 and preservatives. The solution is diluted at 1:25 with distilled water, can be stored for one week at 2-8^0C and is used to rinse wells.

h) Chromogen: TMB derivative in buffer solution. The solution should be mixed 1:1 with substrate.

i) Substrate: Buffer solution containing H_2O_2. The solution should be mixed 1:1 with chromogen.

j) Blocking reagent: 1N sulphuric acid solution.

Assay procedure for anti-HCV

Aliquots of 200 µl diluted negative controls, positive controls and samples (1:21) were dispensed into their respective wells and covered with sealer to prevent evaporation. Wells were incubated at 37^0C for 30 ± 2 minutes with shaking at 1430 ± 20 rpm/G or 1 hour ± 5 minutes at 37^0C without shaking. The diluted enzyme tracer solution was prepared at the end of first incubation. After first incubation, contents of wells were discarded and strips were rinsed five times with wash buffer to remove unbound materials. Then 100 µl diluted enzyme tracer was dispensed into all wells, covered with sealers and incubated again at 37^0C for 30 ± 2 minutes with shaking at 1430 ± 20 rpm or 1 hour ± 5 minutes at 37^0C without shaking. Chromogen/substrate was prepared at the end of the second incubation and strips were rinsed five times with wash buffer. Then 100 µl chromogen/substrate was dispensed into all wells and incubated for 30 minutes at room temperature in a dark place followed by adding 100 µl blocking reagent into all wells. Absorbance was measured at 450/630 nm using ELISA reader.

Calculation and interpretation of results for anti-HCV assay

The cut-off value is determined by adding 0.200 to the mean absorbance of the negative control values (NCx).

Cut-off value = NCx + 0.200

The presence or absence of antibody to HCV is determined by comparing the absorbance value of unknown samples to that of the cut-off value. The unknown samples with absorbance values greater than or equal to the cut-off value should be considered reactive for antibody to HCV. The unknown samples with absorbance values less than the cut-off value should be considered non-reactive.

2.3.2.7 Anti-HCV Line Immunoassay (LIA)

Principle of anti-HCV LIA assay

This qualitative determination of antibody to HCV is a modified western blot assay. The test sample is incubated in a test trough together with the multiple antigen-coated test strip. Specific HCV-antibodies, if present in the sample, will bind to the individual HCV antigen lines on the strip. Subsequently, an affinity-purified goat anti-human IgG (H+L) labeled with alkaline phosphatase is added. Upon a positive reaction, this labeled anti-human antibody becomes bound to any HCV antigen/antibody complex previously formed. Incubation with enzyme substrate produces a dark brown color in proportion to the amount of specific

antibody present in the sample. Color development is stopped with sulphuric acid. If the sample contains no HCV-specific antibodies, the labelled anti-human antibody cannot be bound and no background color develops. Positive or negative results are determined by visually comparing the intensity of an antigen line with the control lines.

Reagents for anti-HCV LIA

a) Coated strips: Antigens representing core, NS1, NS3, NS4 and NS5 proteins of HCV have been coated as discrete lines on a nylon strips with plastic backing. Four control lines are coated on each strips: one anti-streptavidine control line, one strong positive control line (anti-human IgG), one moderate positive control line (all human IgG) and one weak positive control line (all human IgG). A total of six HCV lines are also coated: line 1 and 2 consist of several core epitopes, line 3 contains several NS1 epitopes, line 4 contains several NS3 epitopes, line 5 contains several NS4 epitopes and line 6 contains several NS5 epitopes. The strips are ready to use and should be stored at 2-8^{0}C.

b) Conjugate (100x): Goat anti-human IgG (H+L chain) labelled with alkaline phosphatase in TRIS buffer containing protein stabilizers and 0.1% sodium azide as a preservative. The solution should be diluted at 1:100 with conjugate diluent just before use. Diluted conjugate is stable for 8 hours at room temperature if kept in the dark.

c) Negative control: Human serum containing antibodies to HCV in PBS, sodium chloride, Triton, protein stabilizers, prediluted 1:100 in sample diluent. The reagent is ready to use and contains 0.1% sodium azide as a preservative.

d) Positive control: Human serum in PBS containing sodium chloride, Triton, protein stabilizers, prediluted 1:100 in sample diluent and non-reactive for antibodies to HCV. The reagent is ready to use and contains 0.1% sodium azide as a preservative.

e) Conjugate diluent: PBS, sodium chloride, Triton, protein stabilizer and 0.1% sodium azide as a preservative. The reagent is ready to use and should be stored at 2-8^{0}C. The solution is used to dilute conjugate.

f) Sample diluent: PBS, sodium chloride, Triton, protein stabilizer and 0.1% sodium azide as a preservative. The reagent is ready to use and should be stored at 2-8^{0}C. The solution is used to dilute samples.

g) Wash buffer (5x): PBS buffer, sodium chloride, Triton, and 0.5% sodium azide as a preservative. The solution is to be diluted 1:5 with distilled water, can be stored for one week at 2-8^{0}C and is used to rinse strips.

h) Substrate (100x): Bromochloro-indolylphosphate (BCIP) in dimethylformamide (R 20/21, R 36, R 61, S 45, S 53). The solution should be diluted 1:100 with substrate buffer.

i) Substrate buffer: Contains TRIS buffer sodium chloride and 0.1% sodium azide as a preservative. The reagent is ready to use and is used to dilute substrate.

j) Blocking reagent: Ready to use 0.2N sulphuric acid solution.

Assay procedure for anti-HCV LIA

Identified each test trough for controls and specimens and placed it into the tray. Required number of LIA test strips were taken from their container, identified the strips, and added one strips to each of the test troughs. Strips were placed coated membrane side up into the trough using a pair of forceps. To each test trough, 1 ml sample diluent was dispensed. Aliquots of 10 µl sample or 1 ml negative control and positive control were dispensed into appropriately labelled trough and covered with sealer to prevent evaporation. The tray was incubated by placing the tray horizontally on an orbital mixer (at 160 rpm) and agitate at room temperature (15-30^{0}C) for overnight (16±2 hours). The diluted enzyme tracer solution was prepared at the end of the incubation. After the incubation, aspirate the liquid and wash each trough three times with wash buffer. Then 1 ml prepared conjugate was dispensed into all troughs, covered with sealers and incubated again on an orbital mixer (at 160 rpm) and agitate at room temperature (15-30^{0}C) for 30 minutes. Substrate solution was prepared at the end of the second incubation and troughs were washed two times with wash buffer and one time with substrate buffer. Then 1 ml of the prepared substrate solution was dispensed into all troughs, covered with sealers and incubated again on an orbital mixer (at 160 rpm) and agitate at room temperature (15-30^{0}C) for 30 minutes. At the end of the incubation, after aspiration of the liquid, 1 ml blocking reagent added to each trough and covered with sealers and incubated again on an orbital mixer (at 160 rpm) and agitate at room temperature (15-30^{0}C) for 10 to 30 minutes. After the incubation, test strips were removed from the trough and placed them on absorbent paper. Developed strips retain their color if stored in the dark.

Interpretation of results for anti-HCV LIA

The identity and location of the antigen and control lines coated on the strip are as follows:

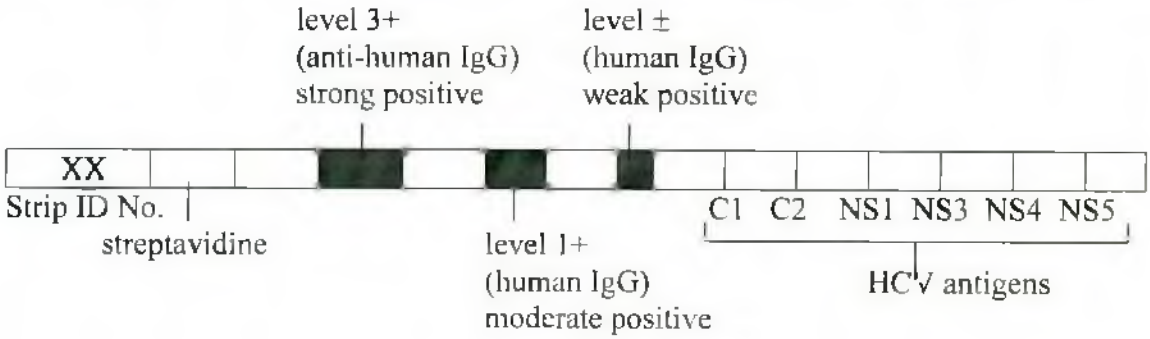


Figure 18: HCV LIA strip

The intensity of the reaction on the control lines on each strip is used to assign the reactivity rating for each antigen line on that strip:

Intensity of antigen line reaction	R	Rating
Lower than level ±	R < ±	-
Higher than or equal to level ± but lower than level 1+	± ≤ R < 1+	±
Equivalent to level 1+	R = 1+	1+
Higher than level 1+ but lower than level 3+	1+ ≤ R < 3+	2+
Equivalent to level 3+	R = 3+	3+
Higher than level 3+	R > 3+	4+

A sample is considered non-reactive for HCV antibody if all HCV antigen lines have a negativereactivity rating. A sample is considered reactive to HCV antibody if one HCV antigen line has a reactivity rating of 2+ or higher or if at least two HCV antigen lines have a reactivity rating of minimum 1+ or higher. A sample is considered intermediate if reactivity rating of 1+ or ± is seen on one antigen line with or without other antigen lines presenting a reactivity rating of ± and a follow-up of the same patient should be requested.

2.3.2.8 HDAg assay

Principle of HDAg assay

Qualitative HDAg determination is a direct, non-competitive sandwich assay, based on the ELISA technique. The presence of HDAg allows the tracer to bind to the solid phase. Therefore, the enzyme activity is proportional to the HDAg concentration present in samples or controls. Measurement of enzyme activity is performed by adding a chromogen/substrate colorless solution. The enzyme action on chromogen/substrate produces a colour which can be detected with ELISA reader.

Reagents for HDAg assay

a) Coated strips: Wells are coated with human IgG anti-HD. The wells are ready to use. The strips should be kept at 2-8^{0}C. The bag should be opened at room temperature. Unused strips were preserved after sealing the bag at 2-8^{0}C.
b) Enzyme tracer (50X): Human IgG anti-HD conjugated to HRP, stabilizers and preservatives. The solution should be diluted at 1:50 with tracer diluent. After dilution, it can be stored for one week at 2-8^{0}C.
c) Negative control: Human serum negative for HDV markers and preservatives. Ready to use, should be stored at 2-8^{0}C.

d) Positive control: Recombinant HDAg, phosphate buffer, proteins and preservatives. Ready to use, should be stored at 2-8^{0}C.

e) Nonidet P-40: 2% solution of non-ionic detergent Nonidet P-40, PBS buffer at pH 7.4 and preservatives. The solution is ready to use and should be stored at 2-8^{0}C.

f) Tracer diluent: Human serum negative for HDV markers, PBS buffer, proteins and preservatives. The reagent is used to dilute the enzyme tracer. The solution is ready to use and should be stored at 2-8^{0}C.

g) Wash buffer (25X): PBS buffer, Tween 20 and preservatives. Ready to use, may be stored for one week at 2-8^{0}C. The contents of the vial is diluted at 1:25 with distilled water. This solution is used to rinse wells.

h) Chromogen: Tetramethylbenzidine (TMB) derivative in buffer solution. Just before use, dilute the 1:50 with substrate. After dilution, chromogen/substrate cannot be stored. Chromogen solution should be stored at 2-8^{0}C

i) Substrate: Buffer solution containing H_2O_2 (0.005%), citrate buffer and preservatives. The reagent is ready to use and used to dilute the chromogen. Substrate solution should be stored at 2-8^{0}C

j) Blocking reagent: 1N sulphuric acid solution. Ready to use, should be stored at 2-8^{0}C.

Assay procedure for HDAg

Aliquotes of 25 µl Nonidet P-40 plus 75 µl negative control or positive control or samples were dispensed into their respective wells and covered with sealer to prevent evaporation. Wells were incubated overnight (18-22 hours) at room temperature. The diluted enzyme tracer solution was prepared at the end of first incubation. After first incubation, contents of wells were discarded and strips were rinsed five times with wash buffer to remove unbound materials. Then 100 µl diluted enzyme tracer was dispensed into all wells, covered with sealers and incubated at 37^{0}C for 2 hours ± 10 minutes at 37^{0}C. Chromogen/substrate was prepared at the end of the second incubation and strips were rinsed five times with wash buffer. Then 100 µl chromogen/substrste was dispensed into all wells and incubated for 30 minutes at room temperature in a dark place followed by adding 100 µl blocking reagent into all wells. Absorbance was measured at 450/630 nm using ELISA reader.

Calculation and interpretation of results for HDAg

The cut-off value is determined by adding 0.150 to the mean absorbance of the negative control values (NCx).

Cut-off value = NCx + 0.150

The presence or absence of HDAg is determined by comparing the absorbance value of unknown samples to that of the cut-off value. The unknown samples with absorbance values greater than or equal to the cut-off value should be considered reactive for HDAg. The unknown samples with absorbance values less than the cut-off value should be considered non-reactive.

2.3.2.9 Anti-HDV assay

Principle of anti-HDV assay

Qualitative anti-HD determination is a simultaneous competitive assay, based on the ELISA technique. Anti-HD present in the sample and labeled anti-HD antibodies compete for a fixed quantitiy of HDAg bound to the solid phase. The quantity of enzyme tracer bound to the solid phase and consequently the enzyme activity is inversely proportional to the anti-HD concentration present in samples or controls. Measurement of bound enzyme activity is performed by adding a chromogen/substrate solution. The enzyme action produces a colour which can be detected with ELISA reader.

Reagents for anti-HDV assay

a) Coated strips: Wells are coated with recombinant HDAg. The strips are ready to use and should be kept at 2-8^{0}C.

b) Enzyme tracer (50X): Human Anti-HD antibodies conjugated to HRP, tris buffer, stabilizers and preservatives. The solution should be diluted at 1:50 with tracer diluent and enzyme tracer can be stored at 2-8^{0}C.

c) Negative control: Human serum non-reactive for HDV markers and preservatives. The solution is ready to use and should be stored at 2-8^{0}C.

d) Positive control: Human anti-HD antibodies, buffer and preservatives. The solution is ready to use and should be stored at 2-8^{0}C.

e) Tracer diluent: Human serum non-reactive for HDV markers in PBS, proteins and preservatives. The reagent is ready to use and should be stored at 2-8^{0}C. The solution is used to dilute the enzyme tracer.

f) Wash buffer (25X): PBS, Tween 20 and preservatives. The solution is diluted at 1:25 with distilled water, can be stored for one week at 2-8^{0}C and is used to rinse wells.

g) Chromogen: TMB derivative in buffer solution. The solution should be mixed 1:1 with substrate.

h) Substrate: Buffer solution containing H_2O_2. The solution should be mixed 1:1 with chromogen.

i) Blocking reagent: 1N sulfuric acid solution.

Assay procedure for anti-HDV

Aliquots of 50 µl negative control, positive control and samples were dispensed into their respective wells and 100 µl diluted enzyme tracer was dispensed into all wells and covered with sealer to prevent evaporation. Wells were incubated at 37^0C for 3 hours ± 15 minutes. Chromogen/substrate was prepared at the end of the incubation and contents of wells were discarded and strips were rinsed five times with wash buffer to remove unbound materials. Then 100 µl chromogen/substrate was dispensed into all well and incubated for 30 ± 2 minutes at room temperature in a dark place followed by adding 100 µl blocking reagent into all wells. Absorbance was measured at 450/630 nm using ELISA reader.

Calculation and interpretation of results for anti-HDV

The cut-off value is determined by adding to the mean absorbance for the negative control values (NCx) multiplied by 0.5 to the mean absorbance for the positive control values (PCx) multiplied by 0.5.

Cut-off value = 0.5 NCx + 0.5 PCx

The presence or absence of anti-HD is determined by comparing the absorbance of unknown samples to that of the cut-off value. The unknown samples with absorbance values less than or equal to the cut-off value should be considered reactive for anti-HD. The unknown samples with absorbance values greater than the cut-off value should be considered non-reactive.

2.3.2.10 IgM anti-HEV assay

Principle of IgM anti-HEV assay

Qualitative IgM anti-HEV determination is an immunometric assay, based on ELISA method. Human serum of plasma, diluted in diluent buffer, are incubated in the HEV antigen coated wells. IgM anti-HEV, if present, will bind to the solid phase HEV antigens. The wells are thoroughly washed to remove unbound materials and a mouse monoclonal anti-human IgM labeled with HRP is added to the wells. This labeled antibody will bind to any antigen-antibody complexes previously formed and excess unbound labeled antibodies are removed by washing. Therefore, the enzyme activity is proportional to the IgM anti-HEV concentration present in samples or controls. Measurement of enzyme activity is performed by adding a substrate solution containing hydrogen peroxide and o-Phenylenediamine Dihydrochoride (OPD.2HCl). The enzyme action produces a yellow-orange colour. Reaction

is terminated by addition of sulfuric acid. The intensity of color can be measured by ELISA reader.

Reagents for IgM anti-HEV assay

a) Coated strips: Wells are coated with three recombinant HEV antigens which correspond to the structural regions of the hepatitis E virus. Should be store at 2-8°C.

b) Conjugate (50X): Mouse monoclonal anti-human IgM labeled with HRP. Contains 0.02% thimerasol as preservative. Should be store at 2-8°C.

c) Non-reactive control: Normal human serum non-reactive for HCV, HEV, HBsAg, and HIV-1. This solution contains thimerasol and sodium azide as preservatives. Should be store at 2-8°C.

d) Reactive control: Inactivated human serum containing a high titer of antibodies specific for HEV. This solution contains thimerasol and sodium azide as preservatives. Should be store at 2-8°C.

e) Diluent: TRIS based saline solution containing heat treated normal goat serum, BSA, and stabilizers. Contains Bronidox as preservative. Should be store at 2-8°C.

f) Plate wash concentrate (20X): PBS, Tween 20 and contains chloroacetamide as preservative. Should be store at 2-8°C.

g) Substrate buffer: Citrate buffer solution containing H_2O_2. Store in the dark at 2-8°C.

h) Substrate tablet: o-Phenylenediamine.2HCl. Should be store at 2-8°C.

i) Stop solution: 2M sulfuric acid solution. Should be store in the dark at 2-8°C.

Preparation and stability of reagents for IgM anti-HEV assay

Coated strips: Ready to use, the strips should be kept at 2-8°C. The bag should be opened at room temperature. Unused strips are preserved after sealing the bag at 2-8°C.

Working conjugate: The solution should be diluted at 1:50 with diluent. After dilution, it can be stored for one week at 2-8°C.

Negative control, positive control and samples: Should be diluted at 1:21 with diluent before use.

Diluent: Ready to use, should be stored at 2-8°C. This solution is used to dilute the conjugate, controls and samples.

Wash buffer: The contents of the vial is diluted 1:20 with distilled water, which may be stored for one week at room temperature. The reagent is used to rinse wells.

Substrate solution: Dissolve one OPD tablet into 5 ml substrate buffer. Substrate solution must be prepared fresh prior to use and shield it from light until ready to use.

Assay procedure for IgM anti-HEV

Aliquots of 200 µl of diluent was dispensed into all wells and then 10 µl of negative control, positive control and samples were dispensed into their respective wells. After complete mixing wells were covered with sealer to prevent evaporation. Wells were incubated at 37^0C for 30 minutes. The working conjugate solution was prepared at the end of first incubation and contents of wells were discarded and strips were rinsed six times with wash buffer to remove unbound materials. Then 100 µl working conjugate solution was dispensed into all wells, covered with sealers and incubated again at 37^0C for 30 minutes. Working substrate solution was prepared before 15 minutes of the second incubation and strips were rinsed six times with wash buffer. Then 100 µl working substrate solution was dispensed into all wells and incubated for 15 minutes at room temperature in a dark place followed by adding 50 µl stop solution into all wells. Absorbance was measured at 490/620 nm using ELISA reader.

Calculation and interpretation of results for IgM anti-HEV

The cut-off value is determined by adding 0.400 to the mean absorbance of the non-reactive control (NRCx).

Cut-off value = NRCx + 0.400

The presence or absence of IgM anti-HEV is determined by comparing the absorbance value of unknown samples to that of the cut-off value. The unknown samples with absorbance values greater than or equal to the cut-off value should be considered reactive for IgM anti-HEV. The unknown samples with absorbance values less than the cut-off value should be considered non-reactive.

2.3.2.11 IgG anti-HEV assay

Principle of IgG anti-HEV assay

Qualitative IgG anti-HEV determination is an immunometric assay, based on ELISA method. Human serum of plasma, diluted in diluent buffer, are incubated in the HEV antigen coated wells. IgM anti-HEV, if present, will bind to the solid phase HEV antigens. The wells are thoroughly washed to remove unbound materials and an affinity purified anti-human IgG labeled with HRP is added to the wells. This labeled antibody will bind to any antigen-antibody complexes previously formed and excess unbound labeled antibodies are removed by washing. Therefore, the enzyme activity is proportional to the IgG anti-HEV concentration present in samples or controls. Measurement of enzyme activity is performed by adding a substrate solution containing hydrogen peroxide and o-Phenylenediamine Dihydrochoride

(OPD.2HCl). The enzyme action produces a yellow-orange colour. Reaction is terminated by addition of sulf uric acid. The intensity of color can be measured by ELISA reader.

Reagents for IgG anti-HEV assay

a) Coated strips: Wells are coated with two recombinant HEV antigens which correspond to the structural regions of the hepatitis E virus. Should be store at 2-8°C.

b) Conjugate (1000X): Goat anti-human IgG labeled with HRP. Contains 0.02% thimerasol as preservative. Should be store at 2-8°C.

c) Non-reactive control: Normal human serum non-reactive for HEV, HBsAg, and HIV-1. This solution contains thimerasol and sodium azide as preservatives. Should be store at 2-8°C.

d) Reactive control: Inactivated human serum containing a high titer of antibodies specific for HEV. This solution contains thimerasol and sodium azide as preservatives. Should be store at 2-8°C.

e) Diluent: TRIS based saline solution containing heat treated normal goat serum, BSA, and stabilizers. Contains Bronidox as preservative. Should be store at 2-8°C.

f) Plate wash concentrate (20X): PBS, Tween 20 and contains chloroacetamide as preservative. Should be store at 2-8°C.

g) Substrate buffer: Citrate buffer solution containing H_2O_2. Store in the dark at 2-8°C.

h) Substrate tablet: o-Phenylenediamine.2HCl. Should be store at 2-8°C.

i) Stop solution: 2M sulf uric acid solution. Should be store in the dark at 2-8°C.

Preparation and stability of reagents for IgG anti-HEV assay

Coated strips: Ready to use, the strips should be kept at 2-8°C. The bag should be opened at room temperature. Unused strips are preserved after sealing the bag at 2-8°C.

Working conjugate: The solution should be diluted at 1:1000 with diluent. After dilution, it can be stored for one week at 2-8°C.

Negative control, positive control and samples: Should be diluted at 1:21 with diluent before use.

Diluent: Ready to use, should be stored at 2-8°C. This solution is used to dilute the conjugate, controls and samples.

Wash buffer: The contents of the vial is diluted 1:20 with distilled water, which may be stored for one week at room temperature. The reagent is used to rinse wells.

Substrate solution: Dissolve one OPD tablet into 5 ml substrate buffer. Substrate solution must be prepared fresh prior to use and shield it from light until ready to use.

Assay procedure for IgG anti-HEV

Aliquots of 200 µl of diluent was dispensed into all wells and then 10 µl of negative control, positive control and samples were dispensed into their respective wells. After complete mixing wells were covered with sealer to prevent evaporation. Wells were incubated at 37^0C for 30 minutes. The working conjugate solution was prepared at the end of first incubation and contents of wells were discarded and strips were rinsed six times with wash buffer to remove unbound materials. Then 100 µl working conjugate solution was dispensed into all wells, covered with sealers and incubated again at 37^0C for 30 minutes. Working substrate solution was prepared before 15 minutes of the second incubation and strips were rinsed six times with wash buffer. Then 100 µl working substrate solution was dispensed into all wells and incubated for 15 minutes at room temperature in a dark place followed by adding 50 µl stop solution into all wells. Absorbance was measured at 490/620 nm using ELISA reader.

Calculation and interpretation of results for IgG anti-HEV

The cut-off value is determined by adding 0.500 to the mean absorbance of the non-reactive control (NRCx).

Cut-off value = NRCx + 0.500

The presence or absence of IgG anti-HEV is determined by comparing the absorbance value of unknown samples to that of the cut-off value. The unknown samples with absorbance values greater than or equal to the cut-off value should be considered reactive for IgG anti-HEV. The unknown samples with absorbance values less than the cut-off value should be considered non-reactive.

2.4 STATISTICAL ANALYSIS

All data are expressed in frequencies, percentages and mean±SE unless mentioned otherwise. Comparison between two groups was done by Chi-square or Student's t-test as applicable whereas for multiple groups, one-way ANOVA was utilized. Correlation analysis was done by Spearman or Pearson's linear model as applicable. Predictive capability of various biochemical and related parameters over different viruses were checked by multiple regression analysis. P values ≤ 0.05 were considered as significant.

Chapter 3

RESULTS

3. RESULTS:

This study investigated 507 clinically jaundiced patients recruited on the basis of referral by their respective physician.

3.1 DEMOGRAPHIC PROFILE:

Mean (±SD) age of the subjects was 33 (±16) years with sex distribution of 373:134 (male:female). Of them, 60.4% were married, 93.1% were Muslim, 6.3% Hindu, and only 0.6% were Christian; whereas 31.8% were service holder, 27% students, 14.4% unemployed, 11.4% business holder and 15.4% were doing no particular job. Of 134 women, 17 (3.4%) were pregnant.

Mean (±SE) duration of jaundice was 19 (±1) days. Fulminant hepatitis was suspected in 16 (3.2%). Family history of jaundice was recorded in 109 (21.5%), history of blood transfusion in 28 (5.5%), history of surgery in 101 (19.9%). Of the other risk factors, 57 (11.2%) were taking medication, 7 (1.4%) were alcoholics, 10 (2.0%) were addicts and 103 (20.3%) were smokers. About 2% subjects gave history of multiple sex partnership.

Among the environmental factors, area of living, type of house used, sharing of beds, intake of food, drinking water, recent travel were investigated. 417 (82.4%) were living in town, 87 (17.2%) in village and only 2 (0.4%) in the slum area. About housing, 186 (53.8%) were living in their own house, 126 (36.4%) in rented house and 34 (9.8%) in mess while 80 (23.2%) were living alone and 265 (76.8%) were sharing beds. 81 (23.3%) were taking foods in their own house and 266 (76.7%) at hotel or restaurent while 51 (14.7) were taking boiled water, 19 (5.5%) tap water, 50 (14.5%) tube well water and 266 (65.3%) had no particular habit of water intake. Of the total patients 138 (51.9%) gave history of recent travel (table 5).

Table 5: Demographic profile of the studied subjects

Character	Frequency	Character	Frequency
Total number of subjects	507	Addict	10 (2.0)
Age (Years, mean ± SD)	33±16	Smoker	103 (20.3)
Sex (male: female)	373(73.6): 134(26.4)	Multiple sex partner	09 (1.8)
Marital status (married/unmarried)	306 (60.4):201(39.6)	Living area (n =506)	
Religion		Town	417 (82.4)
Muslim	472 (93.1)	Village	87 (17.2)
Hindu	32 (6.3)	Slum	02 (0.4)
Christian	3 (0.6)	House (n=346)	
Budhist	0 (0.0)	Own	186 (53.8)
Occupation (n=493)		Rented	126 (36.4)
Service	157 (31.8)	Mess	34 (9.8)
Student	133 (27.0)	Beds (n=345)	
Unemployed	71 (14.4)	Alone	80 (23.2)
Business	56 (11.4)	Sharing	265 (76.8)
Others	76 (15.4)	Recent food intake (n=347)	
Pregnant women (n=134)	17 (3.4)	Home	81 (23.3)
Duration jaundice (day, mean ± SE)	19± 1.06	Hotel/restaurant	266 (76.7)
Fulminant hepatitis	16 (3.2)	Drinking water (n=346)	
Family history of recent jaundice	109 (21.5)	Boiled	51 (14.7)
History of blood transfusion	28 (5.5)	Tap	19 (5.5)
History of surgery	101 (19.9)	Tube well	50 (14.5)
Drug (other than used for addiction)	57 (11.2)	Others	226 (65.3)
Alcohol	07 (1.4)	Recent travel (n=266)	138 (51.9)

Within parentheses are percentages

3.2 FREQUENCY OF VARIOUS HEPATITIS, AGE GROUP DISTRIBUTION AND SEROMARKERS:

As shown in figure 19, the highest frequencies observed with hepatitis E virus infection (64.5%), followed by hepatitis B virus infection (26.0%), hepatitis A virus (11.4%), unknown (11.2%) and hepatitis C virus (2.8%). Of the hepatitis B virus infected patients tested for hepatitis D virus, none were revealed to have evidences of hepatitis D virus.

Depicted in table 6 are the frequencies of patients having more than one hepatitis viral co-infection among the total patients. It was found that 51 (10.1%) had co-infection with hepatitis B and E, 20 (3.9%) had hepatitis A and E, 5 (1.0%) had hepatitis B and C, 4 (0.8%) had hepatitis C and E and only 1 (0.2%) had hepatitis A and B infection.

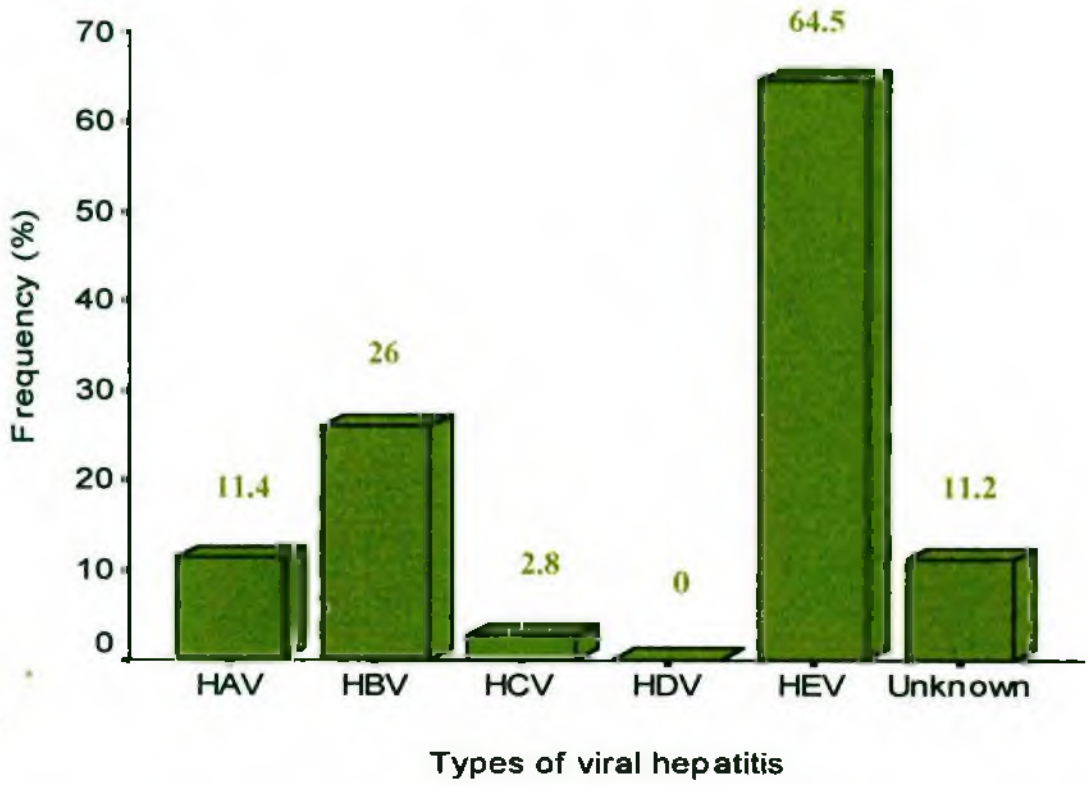


Figure 19: Percent distribution of various hepatitis infection (n=507)

Percentages were calculated individually for the viruses from 507 samples. Samples positive for more than one viruses were not excluded during calculation for different viruses. Samples positive for anti-HBc,IgM (n=23) were considered positive for HBV; HCV positive cases were confirmed by LIA; Only HBsAg positive cases were tested for HDV; Cases negative for all viral markers but positive for IgG, Anti-HEV (n=38) were considered positive for HEV; Groups are not mutually exclusive; HAV: hepatitis A virus; HBV: hepatitis B virus; HCV: hepatitis C virus; HDV: hepatitis D virus; HEV: hepatitis E virus.

Table 6. Frequency of infection by multiple viruses among jaundiced patients (n=507)

Name of the virus	Number of positive cases	Percentage (%)
HAV+HBV	01	0.2
HAV+HEV	20	3.9
HBV+HCV	05	1.0
HBV+HEV	51	10.1
HCV+HEV	04	0.8
Total	**81**	**15.98**

HAV: hepatitis A virus; HBV: hepatitis B virus; HCV: hepatitis C virus, HEV: hepatitis E virus

Figure 20 displays the age distribution of the patients infected by different viruses. Almost cent percent of the patients falling under 5 years of age group had HAV infection. Most of the

patients falling under 6 years to 35 years had HEV infection but a moderate number had also HBV infection and a very few had unknown virus infection while almost none had any HAV, HDV or HCV infection. Patients falling under the group of 46 to >60 age group, had again moderately higher frequencies for HEV infection but a good number had HBV or unknown viral hepatitis and less than 15% had HCV infection while none had HDV or HAV infection in this age group.

IgM,anti-HAV seromarker was positive in 11.4% (58/507) and IgG,anti-HAV in 98.4% of 305 adult patients tested for the marker. HBsAg was positive in 21.5% (109/507), anti-HBc,IgM in 14.8% (75/507) and of the tested 44 patients having low level of anti-HBcIgM, 68.2% (30/44) were positive for anti-HBc,total. Anti-HCV was positive in 2.8% (14/507) and anti-HEV,IgM in 57.0% (289/507), anti-HEV,IgG in 68.4% (305/446) while none of 132 patients with hepatitis B virus infection were positive for HDAg or anti-HDV (table 7).

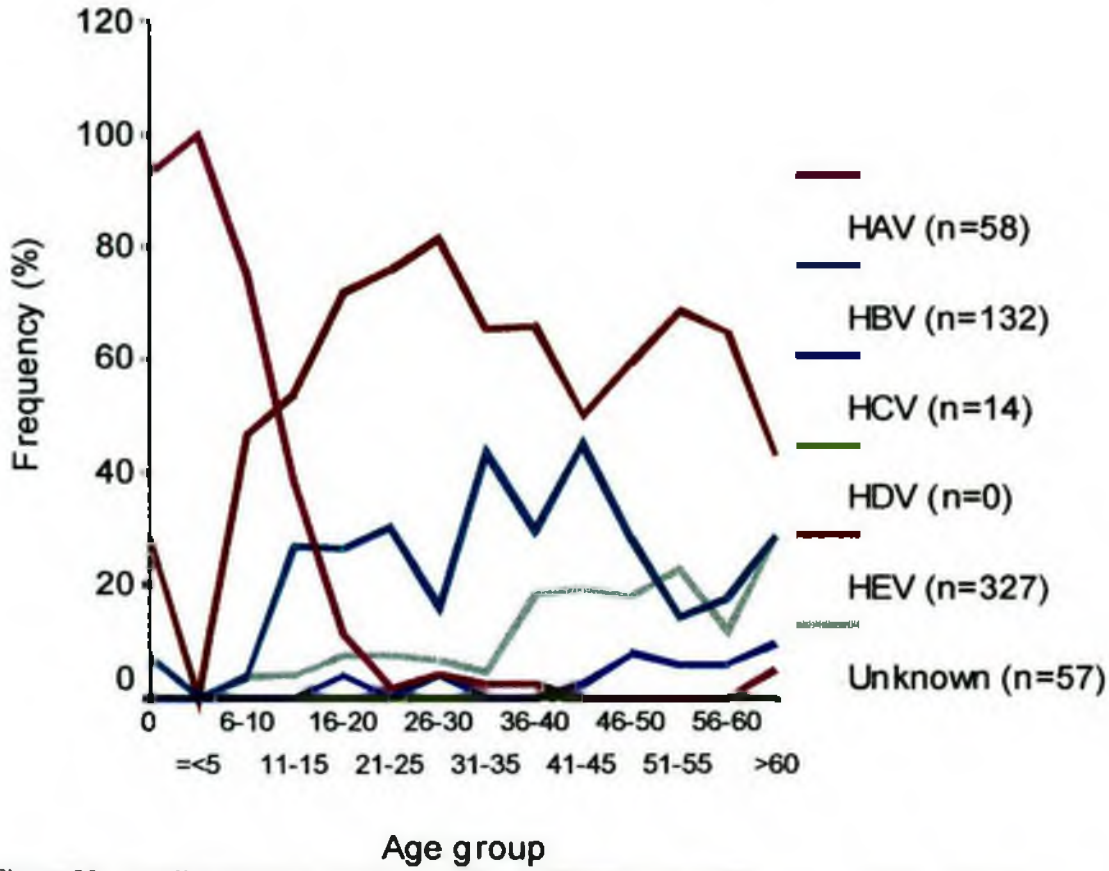


Figure 20: Age distribution of various acute viral hepatitis (n=507)
Percentages were calculated individually for the viruses from 507 samples. Samples positive for more than one viruses were not excluded during calculation for different viruses. Samples positive for anti-HBc,IgM (n=23) were considered positive for HBV; Cases negative for all viral markers but positive for IgG, Anti-HEV (n=38) were considered positive for HEV; Groups are not mutually exclusive; HAV: hepatitis A virus; HBV: hepatitis B virus; HCV: hepatitis C virus; HDV: hepatitis D virus; HEV: hepatitis E virus.

Table 7: Status of seromarkers in patients infected by various hepatitis viruses

Virus type	Seromarker	Positive (n, %)	Negative (n, %)
HAV			
	IgM, Anti-HAV (n=507)	58 (11.4)	449 (88.6)
	IgG, Anti-HAV* (n=305)	300 (98.4)	5 (1.6)
HBV			
	HBsAg (n=507)	109 (21.5)	398 (78.5)
	IgM, Anti-HBc (n=507)	75 (14.8)	432 (85.2)
	Total, Anti-HBc† (n=44)	30 (68.2)	14 (31.8)
HCV			
	Anti-HCV (n=507)	14 (2.8)	493 (97.2)
	HCV LIA‡ (n=14)	14 (100.0)	0 (0.0)
HDV§			
	HDAg (n=132)	00 (0.0)	132 (100)
	Anti-HDV (n=132)	00 (0.0)	132 (100)
HEV			
	IgM, Anti-HEV (n=507)	289 (57.0)	218 (43.0)
	IgG, Anti-HEV (n=446)	305 (68.4)	141 (31.6)

*To see the immune status among adults

†Total anti-HBc test was done only to confirm the positivity of IgM, Anti-HBc

‡HCV LIA done only on anti-HCV positive cases

§Only HBsAg positive cases were tested for HDV

HAV: hepatitis A virus; HBV: hepatitis B virus; HCV: hepatitis C virus; HDV: hepatitis D virus; HEV: hepatitis E virus

3.3 RISK FACTORS AND ENVIRONMENTAL FACTORS OF VIRAL HEPATITIS:

Frequency of previous jaundice was highest in hepatitis B virus (40%), followed by unknown variety (22.8%), hepatitis E virus (20.6%), and hepatitis A virus (8.1%), but none in hepatitis C virus (p=0.001). History of recent jaundice in the family members was statistically same in all types of hepatitis (p=0.464) and ranged from 14% to 29.7%. Highest frequency for history of surgery was found in unknown (40.4%) and Hepatitis C virus (40%) followed by hepatitis B virus (29.3%), hepatitis E virus (16.7%), and hepatitis A virus (10.8%) (p=0.000) whereas blood transfusion was highest in hepatitis C virus (40%) followed by hepatitis B virus (12%), unknown (10.5%), and hepatitis E virus (3.2%), but none in hepatitis A virus (p=0.000). History of smoking was highest in hepatitis B virus (26.7%) followed by unknown (22.8%), hepatitis E virus (21.4%), hepatitis C virus (20%), and hepatitis A virus (2.7%) (p=0.059). Very few were alcoholics (range 0-3.5%, p=0.604) or addicted (0-5.3%, p=0.395) (table 8).

Assessment of standard of living revealed that most of the patients in all groups were town dwellers (hepatitis A virus: 83.8%; hepatitis B virus: 73.3%; hepatitis C virus: 80.0%; hepatitis E virus: 87.3%; unknown: 77.2%). However, most of the remaining patients were living in villages (10.8%, 26.7%, 20.0%, 12.7% and 22.8% respectively) and 5.4% of the hepatitis A virus patients but none of the other virally infected patients were living in the slum area (p=0.050). About housing, there was no statistical difference among the different virus infected groups (p=0.289). However, most of them were living in own houses (hepatitis A virus: 63.3%; hepatitis B virus: 64.4%; hepatitis C virus: 60.0%; hepatitis E virus: 52.0%; unknown: 57.4%) whereas a moderate number (hepatitis A virus: 36.4%; hepatitis B virus: 33.9%; hepatitis C virus: 20.0%; hepatitis E virus: 35.6%; unknown: 34.0%) were living in the rented houses. Sharing of beds in the household was not statistically different among various hepatitis virus infected groups but cent percent of hepatitis C virus were sharing beds, which were 86.4% for hepatitis B virus, 78.7% for unknown, 72.7% for hepatitis A virus and 71.2% for hepatitis E virus (p=0.109). Cent percent of hepatitis C virus patients were taking food outside home followed by 90.9% in hepatitis A virus, 79.7% in hepatitis B virus, 77.1% in hepatitis E and 67.3% in unkonwn viruses (p=0.242). Type of drinking water as catagorized by boiled, tap, tubewell and other sources was not statistically different for the various virally infected groups (p=0.427). About recent travelling, 59.4% of the hepatitis E virus patients had such history followed by 55.0% in unknown, 50.0% in HCV, 38.1% in HBV and 30.0% in HAV (p=0.085) (table 9).

Table 8: Various risk factors of viral hepatitis

Risk factors*	Viruses [No. observed (%)]					χ2	p
	HAV (n=37)	HBV (n=75)	HCV (n=5)	HEV (n=252)	Unknown (n=57)		
History of previous jaundice	3 (8.1)	30 (40.0)	0 (0.0)	52 (20.6)	13 (22.8)	19.799	0.001
Recent jaundice of family member	11 (29.7)	18 (24.0)	1 (20.0)	58 (23.0)	8 (14.0)	3.592	0.464
History of surgery	4 (10.8)	22 (29.3)	2 (40.0)	42 (16.7)	23 (40.4)	21.418	<0.001
Blood transfusion	0 (0.0)	9 (12.0)	2 (40.0)	8 (3.2)	6 (10.5)	23.449	<0.001
Multiple sex partner	0 (0.0)	4 (5.3)	0 (0.0)	4 (1.6)	0 (0.0)	6.854	0.144
Smoking	1 (2.7)	20 (26.7)	1 (20.0)	54 (21.4)	13 (22.8)	9.072	0.059
Drugs (used for medication)	1 (2.7)	12 (16.0)	1 (20.0)	25 (9.9)	13 (22.8)	11.587	0.021
Alcohol	0 (0.0)	2 (2.7)	0 (0.0)	3 (1.2)	2 (3.5)	2.728	0.604
Addiction	0 (0.0)	2 (2.7)	0 (0.0)	4 (1.6)	3 (5.3)	4.080	0.395

*Patients having infection with more than one virus were excluded from this analysis; Kruskal one-way ANOVA
HAV: hepatitis A virus; HBV: hepatitis B virus; HCV: hepatitis C virus; HEV: hepatitis E virus

Table 9: Environmental factors for viral hepatitis

Environmental factors *	Viruses [no. observed/no. of virus positive (%)]					χ^2	p
	HAV	HBV	HCV	HEV	Unknown		
Standard of living							
Town	31/37 (83.8)	55/75 (73.3)	4/5 (80.0)	219/251 (87.3)	44/57 (77.2)		
Village	4/37 (10.8)	20/75 (26.7)	1/5 (20.0)	32/251 (12.7)	13/57 (22.8)	9.481	0.050
Slum	2/37 (5.4)	0/75 (0.0)	0/5 (0.0)	0/251 (0.0)	0/57 (0.0)		
House							
Own	7/11 (63.6)	38/59 (64.4)	3/5 (60.0)	92/177 (52.0)	27/47 (57.4)		
Rented	4/11 (36.4)	20/59 (33.9)	1/5 (20.0)	63/177 (35.6)	16/47 (34.0)	4.983	0.289
Mess	0/11 (0.0)	1/59 (1.7)	1/5 (20.0)	22/177 (12.4)	4/47 (8.5)		
Beds							
Alone	3/11 (27.3)	8/59 (13.6)	0/5 (0.0)	51/177 (28.8)	10/47 (21.3)	7.565	0.109
Sharing beds	8/11 (72.7)	51/59 (86.4)	5/5	126/177 (71.2)	37/47 (78.7)		
Recent food habit							
Home	1/11 (9.1)	12/59 (20.3)	0/5 (0.0)	41/179 (22.9)	16/49 (32.7)	5.473	0.242
Outside home (hotel/restaurant)	10/11 (90.9)	47/59 (79.7)	5/5	138/179 (77.1)	33/49 (67.3)		
Drinking water							
Boiled	1/11 (9.1)	6/58 (10.3)	0/5 (0.0)	29/179 (16.2)	8/49 (16.3)		
Tap	0/11 (0.0)	4/58 (6.9)	0/5 (0.0)	9/179 (5.0)	3/49 (6.1)	3.849	0.427
Tube well	3/11 (27.3)	6/58 (10.3)	0/5 (0.0)	25/179 (14.0)	7/49 (14.3)		
Others	7/11 (63.6)	42/58 (72.4)	5/5	116/179 (64.8)	31/49 (63.3)		
Recent travel	3/10 (30.0)	16/42 (38.1)	2/4 (50.0)	82/138 (59.4)	22/40 (55.0)	8.189	0.085

*Patients having infection with more than one virus were excluded from this analysis; Kruskal one-way ANOVA
HAV: hepatitis A virus; HBV: hepatitis B virus; HCV: hepatitis C virus; HEV: hepatitis E virus

3.4 CLINICAL FINDINGS:

Among the clinical symptoms, frequency of fever was highest among HAV (86.5%), followed by 69.0% in HEV, 64.9% in unknown, 56.0% in HBV and 40.0% in HCV (p=0.015). There was also statistical difference for vomiting (p<0.001; frequencies ranging from 16.0% to 56.8%), ascites (p<0.001; frequencies ranging from 0.4% to 40.0%), nausea (p=0.070; frequencies ranging from 30.7% to 51.4%) and hematemesis (p=0.070; frequencies ranging from 0.0% to 20.0%). However, there was no statistical differences among the various viral infection for the symptoms of itching (p=0.326), anorexia (p=0.328), diarrhoea (p=0.397), melena (p=0.235), abdominal pain (p=0.457), weight loss (p=0.338), bleeding (p=0.264) and yellow urine (p=0.191) though a good number of patients in every group had itching, anorexia, abdominal pain, yellow urine (table 10).

In analyzing the clinical signs, cent percent of the HAV and HCV whereas near cent percent of HBV (98.6%), HEV (97.6%) and unknown (91.2%) groups had clinical jaundice (p=0.976); but most had mild or moderate jaundice and a few had jaundice of severe category (range: 8.1% to 20.0%). About 24.0% of HAV were anaemic followed by 20.0% in HCV, 17.6% in unknown and 14.7% in both HBV and HEV (p=0.649), but most were of mild category. Liver was enlarged in 33.3% of HBV, 28.1% in unknown, 21.8% in HEV, 20.0% in HCV and 18.9% in HAV (p=0.267) but very few had spleenomegaly (p=0.014; frequencies ranging from 0 to 6.7%) (table 11).

Table 10: Clinical symptoms observed among various hepatitis virus infection

Clinical symptoms	Viruses (no. observed, %)					χ^2	p
	HAV (n=37)	HBV (n=75)	HCV (n=5)	HEV (n=252)	Unknown (n=57)		
Fever	32 (86.5)	42 (56.0)	2 (40.0)	174 (69.0)	37 (64.9)	12.274	0.015
Itching	15 (40.5)	35 (46.7)	0 (0.0)	108 (42.9)	22 (38.6)	4.641	0.326
Anorexia	29 (78.4)	51 (68.0)	4 (80.0)	194 (77.0)	38 (66.7)	4.628	0.328
Diarrhoea	2 (5.4)	2 (2.7)	1 (20.0)	19 (7.5)	5 (8.8)	4.064	0.397
Nausea	19 (51.4)	23 (30.7)	2 (40.0)	118 (46.8)	20 (35.1)	8.667	0.070
Vomiting	21 (56.8)	12 (16.0)	2 (40.0)	91 (36.1)	22 (38.6)	20.125	<0.001
Hematemesis	0 (0.0)	2 (2.7)	1 (20.0)	5 (2.0)	1 (1.8)	8.681	0.070
Melena	0 (0.0)	4 (5.3)	0 (0.0)	6 (2.4)	4 (7.0)	5.549	0.235
Ascitis	4 (10.8)	8 (10.7)	2 (40.0)	1 (0.4)	4 (7.0)	35.658	<0.001
Abdominal pain and mass	20 (54.1)	31 (41.3)	3 (60.0)	100 (39.7)	22 (38.6)	3.639	0.457
Weight loss	16 (43.2)	18 (24.0)	2 (40.0)	78 (31.0)	17 (29.8)	4.535	0.338
Bleeding	0 (0.0)	2 (2.7)	0 (0.0)	3 (1.2)	3 (5.3)	5.236	0.264
Yellow urine	32 (86.5)	50 (66.7)	3 (60.0)	176 (69.8)	37 (64.9)	6.109	0.191

Patients having infection with more than one virus were excluded from this analysis; Kruskal one-way ANOVA
HAV: hepatitis A virus; HBV: hepatitis B virus; HCV: hepatitis C virus; HEV: hepatitis E virus

Table 11: Clinical signs observed among various hepatitis virus infection

Clinical signs	Viruses (no. observed, %)					$\chi 2$	p
	HAV (n=37)	HBV (n=75)	HCV (n=5)	HEV (n=252)	Unknown (n=57)		
Jaundice							
Mild	17 (45.9)	34 (45.3)	3 (60.0)	103 (40.9)	23 (40.4)		
Moderate	17 (45.9)	31 (41.3)	1 (20.0)	115 (45.6)	19 (33.3)		
Severe	3 (8.1)	9 (12.0)	1 (20.0)	28 (11.1)	10 (17.5)		
Total	**37 (100)**	**74 (98.6)**	**5 (100)**	**246 (97.6)**	**52 (91.2)**	**0.470**	**0.976**
Anemia							
Mild	9 (24.3)	11 (14.7)	1 (20.0)	36 (14.3)	9 (15.8)		
Moderate	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.4)	1 (1.8)		
Severe	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)		
Total	**9 (24.3)**	**11 (14.7)**	**1 (20.0)**	**37 (14.7)**	**10 (17.6)**	**2.475**	**0.649**
Liver							
Enlarged	7 (18.9)	25 (33.3)	1 (20)	55 (21.8)	16 (28.1)	5.207	0.267
Spleen							
Enlarged	1 (2.7)	5 (6.7)	0 (0.0)	1 (0.4)	3 (5.3)	12.512	0.014

Patients having infection with more than one virus were excluded from this analysis; Kruskal one-way ANOVA
HAV: hepatitis A virus; HBV: hepatitis B virus; HCV: hepatitis C virus; HEV: hepatitis E virus

3.5 BIOCHEMICAL MARKERS IN HEPATITIS:

Serum bilirubin, ALT and AST were raised in greater than 80.0% patients in all the hepatitis groups but there was statistical difference for serum bilirubin (p=0.012) only among the groups. Though not statistically different among the groups, moderately higher number of patients had raised ALP and Prothro mbin Time (p=0.109 for ALP and p=0.337 for Prothrombin Time) (table 12).

Comparison of quantitative values (mean±SE) for bilirubin revealed no statistical difference among the groups (p=0.436). On the other hand, ALT was highest in HAV (1263±161), followed by HEV (1130±70), HCV (735±371), HBV (729±91) and unknown (440±88) which were statistically different (p<0.001). Similarly, AST was also elevated in HEV (1022±102), followed by HAV (1004±217), HCV (676±612), HBV (658±122) and unknown (344±85) having statistical difference among the groups (p=0.007). ALP was raised moderately in all the groups with statistical difference among the groups (p=0.010) but highest in unkonwn (361±87) and lowest in HCV (126±21) and other three in between. However, Prothrombin Time was statistically same (p=0.877) among the groups though in all groups it was mild to moderately high (table 13).

Table 12: Status of biochemical markers in various viral hepatitis

Biochemical markers	Viruses [no. raised/no. tested, (% raised)]					$\chi 2$	p
	HAV	HBV	HCV	HEV	Unknown		
Total bilirubin status	35/37 (94.6)	73/75 (97.3)	4/5 (80)	243/245 (99.2)	53/56 (94.6)	12.784	0.012
ALT status	37/37 (100)	66/69 (95.7)	5/5 (100)	229/235 (97.4)	50/53 (94.3)	3.164	0.531
AST status	21/21 (100)	32/34 (94.1)	2/2 (100)	95/97 (97.9)	24/27 (88.9)	5.925	0.205
ALP status	23/29 (79.3)	28/52 (53.8)	1/4 (25)	98/170 (57.6)	25/44 (56.8)	7.564	0.109
Raised prothrombin time	11/23 (47.8)	33/45 (73.3)	2/3 (66.7)	91/144 (63.2)	23/34 (67.6)	4.548	0.337

Patients having infection with more than one virus were excluded from this analysis; Kruskal one-way ANOVA
HAV: hepatitis A virus; HBV: hepatitis B virus; HCV: hepatitis C virus; HEV: hepatitis E virus

Table 13: Mean (±SE) values of biochemical markers observed in various viral hepatitis

Biochemical markers	Viruses (no. of viruses tested (Mean±SE))					F	p
	HAV	HBV	HCV	HEV	Unknown		
Serum total bilirubin	37 (6.82±0.98)	75 (8.92±0.94)	5 (6.34±2.03)	245 (8.63±0.42)	56 (7.65±0.91)	0.949	0.436
ALT	37 (1263±161)	69 (729±91)	5 (735±371)	235 (1130±70)	53 (440±88)	7.579	<0.001
AST	21 (1004±217)	34 (658±122)	2 (676±612)	97 (1022±102)	27 (344±85)	3.673	0.007
ALP	29 (325±34)	52 (247±25)	4 (126±21)	170 (217±11)	44 (361±87)	3.403	0.010
Prothrombin time	23 (16±2)	45 (14±0.5)	3 (14±2)	144 (15±0.7)	34 (15±0.8)	0.301	0.877

Patients having infection with more than one virus were excluded from this analysis;One-way ANOVA
HAV: hepatitis A virus; HBV: hepatitis B virus; HCV: hepatitis C virus; HEV: hepatitis E virus

3.6 CORRELATIONS:

Table 14 shows correlations of various factors with various types of hepatitis. HAV correlated statistically with IgM,anti-HAV ($p<0.001$), fever ($p=0.001$), vomiting ($p<0.001$), SGPT ($p=0.009$) and alkaline phosphatase ($p<0.001$) whereas inversely with HBsAg ($p<0.001$), anti-HEV,IgM ($p<0.001$), anti-HEV,IgG ($p<0.001$), age ($p<0.001$), marital status ($p<0.001$), history of previous jaundice ($p=0.002$), history of surgery ($p=0.052$), history of blood transfusion ($p=0.051$), duration of clinical jaundice ($p<0.001$) and smoking ($p=0.001$).

HBV correlated statistically with HBsAg ($p<0.001$), age ($p=0.063$), marital status ($p=0.011$), ascites ($p=0.034$), history of previous jaundice ($p<0.001$), history of blood transfusion ($p=0.037$), multiple sex partnership ($p=0.042$), enlarged liver ($p=0.080$), enlarged spleen ($p=0.010$), duration of clinical jaundice ($p<0.001$), and anti-HBcIgM ($p<0.001$); while inversely with anti-HAV,IgM ($p=<0.001$), anti-HEV,IgM ($p<0.001$), anti-HEV,IgG ($p=0.017$), fever ($p=0.005$), vomiting ($p=0.001$), SGPT ($p=0.084$) and alkaline phosphatase ($p=0.073$).

HCV correlated statistically with anti-HCV ($p<0.001$), age ($p=0.014$), ascites ($p=0.001$), history of blood transfusion ($p=0.008$); whereas inversely with anti-HEV,IgM ($p=0.028$), itching ($p=0.007$), SGPT ($p=0.012$) and alkaline phosphatase ($p=0.004$).

HEV correlated statistically with anti-HEV,IgM ($p<0.001$), anti-HEV,IgG ($p<0.001$), pregnancy ($p=0.002$), SGPT ($p<0.001$) and SGOT ($p=0.002$); while inversely with anti-HAV,IgM ($p<0.001$), HBsAg ($p<0.001$), anti-HCV ($p=0.003$), ascites ($p<0.001$), history of previous jaundice ($p=0.058$), surgery ($p<0.001$), history of blood transfusion ($p=0.001$), spleenomegaly ($p=0.004$), duration of clinical jaundice ($p<0.001$) and anti-HBcIgM ($p<0.001$).

Unknown hepatitis correlated statistically with age ($p<0.001$), marital status ($p=0.002$), history of surgery ($p<0.001$), history of blood transfusion ($p=0.079$), addiction ($p=0.058$) and duration of clinical jaundice ($p=0.017$); while inversely with anti-HAV,IgM ($p=0.004$), HBsAg ($p<0.001$), anti-HEV,IgM ($p<0.001$), anti-HEV,IgG ($p<0.001$), SGPT ($p<0.001$), SGOT ($p<0.001$) and anti-HBc.IgM ($p=0.001$).

Table 14: Correlations of various factors with various types of hepatitis

Determinants of 'r'	HAV		HBV		HCV		HEV		Unknown	
	r	p	r	p	r	p	r	p	r	p
Age	-0.464	**<0.001**	0.083	0.063	0.109	**0.014**	-0.016	0.721	0.201	**<0.001**
Marital status	-0.405	**<0.001**	0.113	**0.011**	0.063	0.158	0.014	0.756	0.135	**0.002**
Duration of clinical jaundice	-0.183	**<0.001**	0.182	**<0.001**	0.058	0.191	-0.173	**<0.001**	0.107	**0.017**
Pregnancy	-0.067	0.132	-0.061	0.172	0.035	0.427	0.139	**0.002**	-0.066	0.136
Fever	0.142	**0.001**	-0.124	**0.005**	-0.064	0.149	0.042	0.352	-0.013	0.770
Itching	-0.005	0.918	0.023	0.603	-0.119	**0.007**	0.030	0.497	-0.025	0.580
Vomiting	0.157	**<0.001**	-0.144	**0.001**	0.075	0.093	0.031	0.485	0.020	0.653
Ascitis	0.045	0.311	0.094	**0.034**	0.141	**0.001**	-0.206	**<0.001**	0.047	0.293
History of previous jaundice	-0.138	**0.002**	0.191	**<0.001**	0.021	0.632	-0.084	0.058	-0.003	0.939
History of surgery	-0.068	0.052	0.008	0.859	0.037	0.412	-0.167	**<0.001**	0.182	**<0.001**
History of blood transfusion	-0.087	0.051	0.093	**0.037**	0.117	**0.008**	-0.145	**0.001**	0.078	0.079
Multi sex partnership	-0.048	0.278	0.090	**0.042**	-0.023	0.611	-0.025	0.573	-0.048	0.282
Smoking	-0.151	**0.001**	0.058	0.193	-0.025	0.571	0.016	0.718	0.022	0.621
Addiction	-0.051	0.252	0.013	0.773	-0.024	0.591	-0.043	0.334	0.084	0.058
Liver	-0.033	0.458	0.078	0.080	0.015	0.731	-0.063	0.155	0.028	0.526
Spleen	-0.015	0.733	0.115	**0.010**	-0.026	0.556	-0.129	**0.004**	0.068	0.127
SGPT	0.120	**0.009**	-0.079	0.084	-0.115	**0.012**	0.176	**<0.001**	-0.227	**<0.001**
SGOT	0.066	0.331	-0.068	0.316	-0.072	0.288	0.206	**0.002**	-0.245	**<0.001**
Alkaline phosphatase	0.260	**<0.001**	-0.095	0.073	-0.152	**0.004**	-0.069	0.195	0.018	0.737
Anti-HAV,IgM	1.000	**<0.001**	-0.199	**<0.001**	-0.063	0.163	-0.236	**<0.001**	-0.131	**0.004**
HBsAg	-0.190	**<0.001**	0.871	**<0.001**	0.028	0.526	-0.355	**<0.001**	-0.171	**<0.001**
Anti-HBc, IgM	-	-	0.719	**<0.001**	-0.005	0.915	-0.283	**<0.001**	-0.150	**0.001**
Anti-HCV	-0.063	0.167	0.042	0.356	1.000	**<0.001**	-0.133	**0.003**	-0.061	0.180
Anti-HEV,IgM	-0.160	**<0.001**	-0.228	**<0.001**	-0.098	**0.028**	0.845	**<0.001**	-0.409	**<0.001**
Anti-HEV,IgG	-0.401	**<0.001**	-0.113	**0.017**	0.080	0.093	0.536	**<0.001**	-0.371	**<0.001**

r: correlation coefficient; HAV: hepatitis A virus; HBV: hepatitis B virus; HCV: hepatitis C virus; HEV: hepatitis E virus

3.7 MULTIPLE REGRESSION ANALYSIS:

Findings for HAV and HCV were not statistically fit for multiple regression analysis. For HBV, HBsAg ($p<0.001$), anti-HEV,IgM ($p<0.001$), marital status ($p=0.010$), blood transfusion ($p=0.032$), enlarged spleen ($p=0.024$) and anti-HBc,IgM ($p<0.001$) were independent predictors. For HEV, absence of anti-HAV,IgM ($p<0.001$), absence of HBsAg ($p<0.001$), absence of anti-HCV ($p<0.024$), anti-HEV,IgM ($p<0.001$), anti-HEV,IgG ($p<0.001$), absence of surgery ($p=0.003$), low SGPT ($p=0.065$), and absence of anti-HBcIgM ($p=0.007$) were independent predictors. On the other hand, absence of anti-HAV,IgM ($p<0.001$), absence of HBsAg ($p<0.001$), absence of anti-HEV,IgM ($p<0.001$), absence of anti-HEV,IgG ($p<0.001$), age ($p=0.003$), lack of hematemesis ($p=0.056$), surgery ($p=0.022$), low total bilirubin ($p<0.001$), absence of anti-HBcIgM ($p<0.001$) and no history of recent jaundice in the family ($p=0.044$) were independent predictors for unknown hepatitis viruses (table 15).

Table 15: Multiple regression analysis for prediction over the type of viral hepatitis

Independent variables	HBV		HEV		Unknown	
	β	p	β	p	β	p
Age	-0.040	0.158	-0.020	0.596	0.185	**0.003**
Marital status	0.069	**0.010**	0.002	0.960	-0.015	0.795
Religion	-0.002	0.909	0.005	0.824	0.040	0.297
Pregnancy	-0.039	**0.042**	-0.015	0.554	0.021	0.612
Duration of clinical jaundice	-0.025	0.224	-0.009	0.728	-0.027	0.539
Fever	-0.021	0.242	-0.003	0.890	-0.055	0.165
Itching	-0.015	0.420	-0.00	0.867	-0.041	0.294
Anorexia	-0.009	0.629	0.006	0.790	0.007	0.869
Nausia	-0.021	0.267	0.008	0.732	-0.056	0.165
Vomiting	0.029	0.131	-0.010	0.695	0.046	0.262
Hematemesis	0.010	0.589	0.020	0.448	-0.081	0.056
Melena	-0.003	0.890	0.040	0.120	0.027	0.512
Ascitis	-0.002	0.934	-0.041	0.153	-0.047	0.314
Urine color	0.009	0.648	-0.014	0.584	0.032	0.427
History of previous jaundice	-0.021	0.280	-0.004	0.867	-0.003	0.945
Family member recent jaundice	0.015	0.411	0.025	0.291	-0.080	**0.044**
History of Surgery	-0.013	0.486	-0.074	**0.003**	0.093	**0.022**
History of Blood transfusion	0.041	**0.032**	-0.017	0.512	-0.010	0.803
Multiple sex partnership	-0.025	0.164	-0.011	0.639	-0.005	0.898
Smoking	0.019	0.318	0.020	0.421	-0.041	0.324
Addiction	0.026	0.151	-0.029	0.224	0.024	0.539
Living condition	0.017	0.353	-0.016	0.520	-0.013	0.755
Jaundice	0.001	0.942	0.000	0.994	0.000	0.998
Liver	-0.009	0.645	0.023	0.357	-0.002	0.954
Spleen	0.046	**0.024**	-0.016	0.560	0.063	0.161
Total Bilirubin	0.019	0.310	0.035	0.150	-0.143	**<0.001**
SGPT	0.002	0.918	-0.046	0.065	-0.011	0.783
Anti-HAV,IgM	0.027	0.267	-0.147	**<0.001**	-0.374	**<0.001**
HBsAg	0.688	**<0.001**	-0.148	**<0.001**	-0.290	**<0.001**
Anti-HBc, IgM	0.402	**<0.001**	-0.078	**0.007**	-0.174	**<0.001**
Anti-HCV	0.018	0.340	-0.057	**0.024**	-0.067	0.102
Anti-HEV,IgM	0.079	**<0.001**	0.660	**<0.001**	-0.386	**<0.001**
Anti-HEV,IgG	0.029	0.167	0.170	**<0.001**	-0.432	**<0.001**

β : regression coefficient

HBV: hepatitis B virus; HEV: hepatitis E virus

Chapter 4

DISCUSSION

DISCUSSION

Viral hepatitis is an important cause of morbidity and mortality worldwide. However, the incidence varies from region to region (108). It is now established that viral hepatitis is an important public health problem in South Asian countries (109-113). This study analyses the etiology of acute viral hepatitis in Bangladesh for the first time in which all five types of hepatotrophic viruses has been analyzed in a group of patients referred to the laboratory of the Department of Immunology of BIRDEM for serological tests of various hepatitis by their respective clinicians.

It was explored that, among the 507 studied cases, irrespective of age most common hepatitis in acute jaundice was HEV (64.5%); HBV (26%) being second common one whereas about 11% were HAV, very few HCV and the rest were of unknown cause. However, when age is considered, individuals of less than 16 years showed predominantly HAV followed by HEV. There was none with evidence of HDV. It was found that 16% had co-infections with more than one hepatitis viruses, of which most common association was observed for HEV and HBV (about 10%). Youngest age group had almost exclusively HAV infection; adolescent and young adults had mostly HEV and HBVinfection, whereas the aged group had HEV or HBV or HCV or unknown viral hepatitis and none of the studied population had HDV infection. It was interesting to observe that almost everyone of the adult population (>16 years) studied, had evidence of IgG-anti-HAV indicating exposure to HAV in their early life. Among the risk factors and environmental factors, history of previous jaundice was important for HBV and HEV as well as unknown viral hepatitis; history of surgery for unknown and HCV as well as HBV; and blood transfusion for HCV and HBV in most instances. Among the clinical findings, fever was frequently associated with HAV and HEV, nausea was equally related to all groups; whereas hematemesis and ascites were significantly related to HCV. Hepatospleenomegaly was more prevalent among HBV, HEV and unknown viral hepatitis. Altered and raised ALT and AST were equally important for all types of viral hepatitis; but magnitude of rise was higher in HAV and HEV. Younger the age, higher was the chances of HAV which was also reflected by inverse relation of HAV with marital status. Similarly, shorter the duration of jaundice, probability of HEV and HAV were higher; while longer the duration of jaundice, higher the possibility of HBV and unknown hepatitis. Pregnancy had strong relationship with HEV. Ascites, history of jaundice, blood transfusion, multiple sex

partnership, hepatospleenomegaly were important for prediction of HBV; and some of them for HCV and unknown hepatitis.

Based on the episode of hepatitis E outbreaks in Asia, Africa, the Middle East, and Central America; HEV endemic occurs predominantly in developing countries where water and sanitation facilities are inadequate andunhygienic. The incidence of HEV infection appears to be low in first world countries. Lack of hygienic conditions and failure to adequately treat water and sewage have been linked to massive epidemics of hepatitis E. Hepatitis E is a potential health hazard to travelers to areas where HEV is endemic. Enterically transmitted non-A non-B hepatitis associated with an outbreak of Jaundice in Dhaka was reported in 1990. However, the causes were not further investigated to specify the type of virus which could be possibly hepatitis E (106). A recent study in Haiti among Bangladeshi soldiers with acute hepatitis showed acute Hepatitis E (114). Moreover, hepatitis E has been reported among the travelers to Bangladesh (115). There are some other studies which also suggests that hepatitis E is the leading cause of acute viral hepatitis in the Indian subcontinent (116-117). Their findings strengthen earlier suspicions that enterically transmitted non-A Non-B hepatitis may be the most important cause of acute viral hepatitis in Bangladesh. Supporting the above speculation, in the present study, hepatitis E was found to be the most frequent and common type of clinical acute viral hepatitis among adults in Bangladesh accounting for more than half (64.5%) of the cases. Most of the patients falling under 6 to 35 years of age had HEV infection and highest frequency was observed among the age group 20 to 35 years. Patients falling under the group of 46 to >60 years, had again moderately higher frequencies for HEV infection which resemble with other Indian subcontinental studies (118).

The incidence and prevalence of HBV infection varies widely in different parts of the world. It is estimated that worldwide more than 50 million new infections with HBV occur yearly, and as many as 1 million deaths annually can be attributable to the effects of this infection (19). Worldwide, it is estimated that there are 300 million HBV carriers and most new cases occur among people aged 20-39 (20). In areas of moderate prevalence of chronic carriers (2% to 7% of the population is HBsAg-positive), the lifetime risk of becoming infected with HBV is estimated to be 20% to 60%. In these group, most exposures also occur in childhood (22). According to WHO and other studies, Bangladesh resides in this group with a prevalence rate of around 4% (23-26). A previous study in Bangladesh has reported 25.9% as the incidence for HBV among jaundiced patients (109). In the current study, the HBV (26%) was found as

the second most prevalent etiology of acute viral hepatitis. HBV infection was observed in the age of 11 to 20 years and most risk group is 31 to 45 years.

Among the hepatitis viruses, hepatitis A virus has worldwide incidence in many developing countries and still a common infection primarily among the poor, the elderly, older children, and young adults. Whereas, in high endemicity zones, such as many areas of Asia, Africa, Eastern Europe and a number of South American countries, mass exposure to HAV in early childhood confers herd immunity (17). In this study, HAV was the third highest etiology of acute viral hepatitis which accounts 11.4% of the total subjects. It was observed that almost all of the patients falling under 5 years of age as well as most of the patients falling within the age of 16 years had HAV infection; and a few cases which were found positive for HAV over the age of 16 had co-infection. Verifying the cause of negativity of HAV among the adults, anti-HAV,IgG test was done on 305 adult patients, which revealed that 98.4% were positive for the marker with sufficient amount of antibody titre. Therefore, it can be assumed that most of the patients became exposed to HAV in their childhood. Therefore, acute marker for the HAV may be unnecessary for the adult patients (age >16 years) as exposure to the virus once, renders life-long immunity against HAV (120).

The medical importance of hepatitis C resides in its propensity to become chronic. It is generally thought that HCV infection become chronic in 50% of cases. Sporadic community-acquired forms show a lower rate of chronicity. WHO estimates that up to 3% of the world's population has been infected with HCV or 227 million individuals and there may be more than 170 million chronic carriers of HCV (28). Incidence is endemic worldwide; high incidence in Japan, Italy and Spain. The prevalence among blood donors in northern Europe and North America (<1%) is lower than that in Southern Europe and Africa (13% in Eygpt), while figures for Asia may be as high as 3%. It was found that hepatitis C virus was an uncommon cause of acute viral hepatitis. Only 14 (2.8%) cases were found positive for anti-HCV. Among these patients 5 were found together with HBV and 4 with HEV i.e., only 5 patients had infection with only HCV.

Hepatitis D occurs worldwide but its geographical prevalence varies widely. The epidemiology of HDV infection varies unpredictably in incidence and pattern from one region to another. In general, the global pattern of HDV infection corresponds to the prevalence of chronic HBV infection. In countries with a low prevalence of chronic HBV

infection, HDV prevalence is generally low among both asymptomatic HBV carriers (<10%) and among patients with chronic HBV-related liver disease (<25%). In countries with moderate and high levels of chronic HBVprevalence, the prevalence of HDV infection is highly variable. However, paradoxically, in most of Southeast Asia and China, where the prevalence of chronic HBV infection is very high, HDV infection is uncommon. Present findings also correlate with the above reports. Patients positive for HBV, were tested for HDV, but none were found positive for hepatitis D virus. Although no HDV was found in the present study with HBV, other dual infections were not uncommon. Among the positive cases, several types of co-infection were observed. The most common co-infection was HBV and HEV (10.1%), other co-infections are HAV and HEV (3.9%), HBV and HCV (1.0%), HCV and HEV (0.8%) and only one case found positive for HAV and HBV (108).

Cases of acute viral hepatitis that cannot be attributed to the known five viruses collectively referred to as unknown or putative sixth agent in this study. Usually it is the most difficult of the forms of hepatitis to establish with certainty (119). Diagnoses that must be excluded include syphilis, mononucleosis, cholangitis, Wilson's disease, autoimmune chronic active hepatitis, Budd-Chiari syndrome and drug-related liver injury (119). In particular, drug hepatotoxicity must always be considered before a person is said to have hepatitis of unkonwn origin. In this study, patients were included after excluding the above criteria but we did not test the sample for secondary hepatitis viruses. So the putative sixth agent which account 11.2% of the total subjects and almost equal to the HAV in this study may be the secondary hepatitis viruses or other agents.

The data from this study confirmed the well-known observation that clinical findings are not too useful in identifying the etiology of acute viral hepatitis. Since jaundice and dark urine were components of the case definition, almost all patients with acute viral hepatitis had these signs. Other frequent findings were anorexia, fever, nausea, vomiting, ascites, hematemesis, abdominal pain, malaise and easy fatigability etc. and the frequency of these signs and symptoms and of abnormal concentrations of bilirubin, ALT and AST did not differ significantly among the six etiologic type of acute viral hepatitis. However, hepatospleenomegaly was important for HBV, HCV and unknown groups.

Among the risk factors, previous jaundice was found as risk factors for HBV, history of surgery was found as the most important risk factors for unknown cases followed by HCV

and HBV, history of blood transfusion was the main risk factors for HCV (29) followed by HBV while travelling was an important risk for HEV infection though not too infrequent for the other hepatitis viruses (115).

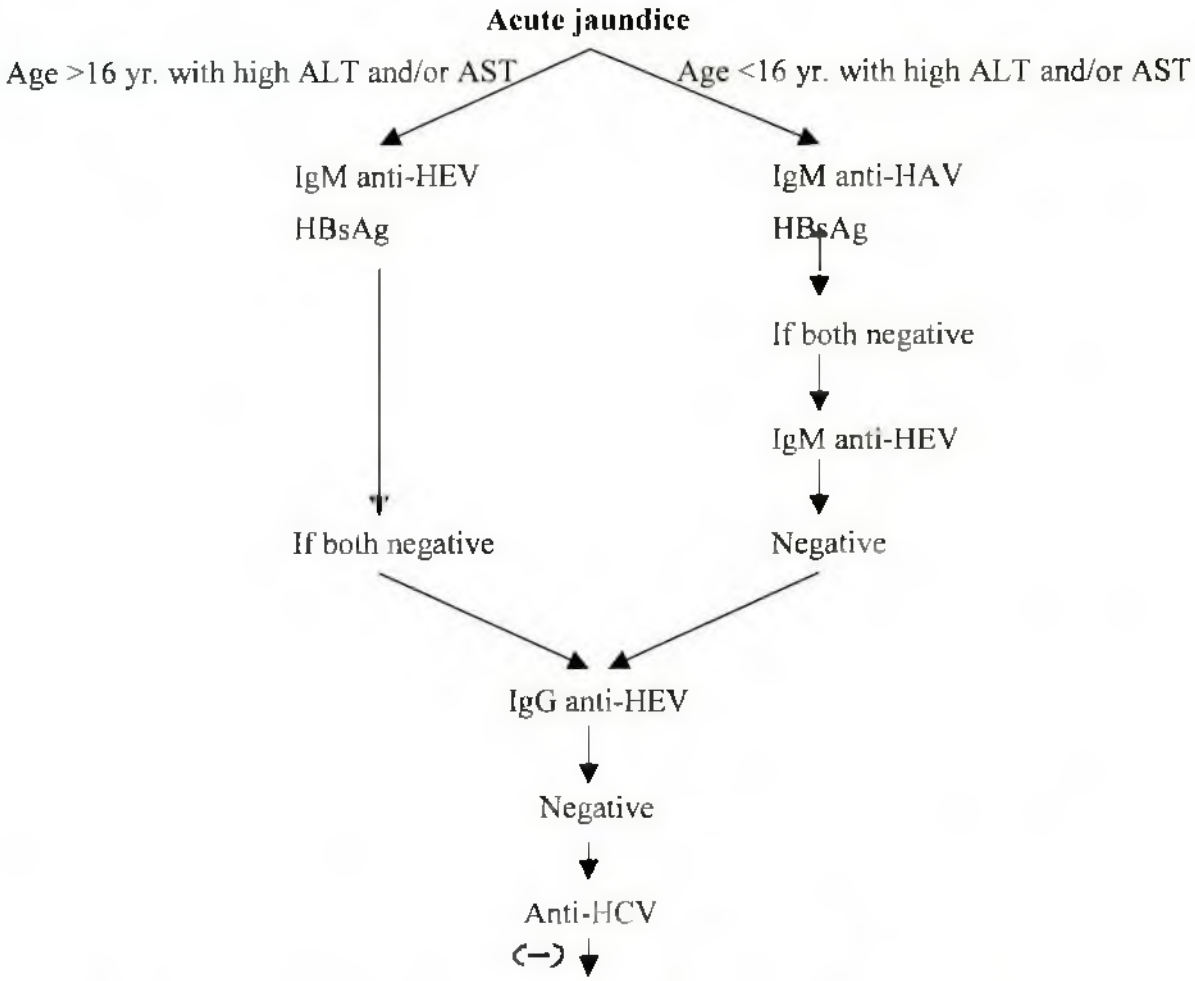
In the present study, pregnancy had a close relationship with HEV as is reported by other investigator (117). Longer the duration of jaundice, greater the possibility of HBV or unknown; whereas shorter duration of jaundice was more suggestive of HAV and HEV. Ascites, history of previous jaundice, history of surgery and multiple sex partnership were important predicting factors for HBV and to some extent for HCV and unknown acute hepatitis. Similar findings have also been reported by other investigators (108).

Therefore, the present study, in conclusion, indicates:

1. Four of all five recognized hepatitis viruses (HAV, HBV, HCV and HEV) are generally appeared to be responsible for acute viral hepatitis in Bangladesh.
2. Unknown agent(s) is accounts for a noticeable figure of acute icteric hepatitis.
3. HDV is rarest (none reported so far) cause for acute hepatitis.
4. HEV is the commonest etiology of acute viral hepatitis among clinically jaundiced patients in Bangladesh.
5. HAV is only found among children under 16 and very rarely above this age. Therefore, anyone above that age with acute viral hepatitis may not need to be investigated for anti-HAV, IgM to identify the etiology of jaundice. It may need only to exclude when no other viral etiology is found.
6. All above 20 years is probably immuned to hepatitis A due to naturally occurring infection and probably subclinical or clinical infection of hepatitis A has resulted the immunity (120). However, age-clustered children study is required to assess whether vaccination against HAV is necessary in Bangladesh where hepatitis A exposure is highly endemic.
7. Anti-HAV, IgG can be considered as a marker of immune competence for humoral immunity against HAV infection in general population.

8. Among clinically jaundiced patients suspected for acute viral hepatitis but negative for IgM anti-HAV, IgM anti-HEV should be investigated for anti-HEV,IgG or other hepatitis as: HBV, HCV and unknown.
9. Hepatitis C is a less common cause of acute viral hepatitis and markers for HCV should be suggested when other causes of viral hepatitis is excluded.
10. HBV infection is clinically more important than others. Therefore, HBsAg should also be advised whenever anti-HEV,IgM or anti-HAV,IgM are tested.

Therefore, in light of the findings in the present study, the following algorithm-based approach for analysis of acute viral hepatitis could be suggested for cost-effectiveness:



Adv: test for EBV, CMV, LKM antibodies (autoimmune hepatitis) etc.

Chapter 5

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Appendix: Data collection sheet

Lab/Case ID: Pt Stay: Date:
Name: Age: Yrs Sex: M/ F
Occupation: Marital status: M / S Pregnancy:
Address: Telephone:

Clinical Presentation

1. Onset and Duration of Jaundice: Date: Day:
2. Associated Symptoms
   (Fever/ Itching/Anorexia/ DNV/ H&M/Ascities/RU AbdominalPain&Mass/ Wt.loss / Bleeding/ Urine/ Stool)
3. Other Complaints -

Related History

1. History of previous Jaundice Yes / No
   If Yes Number of Attacks Duration
   Cause (if known)
2. History of Recent or Recurrent Jaundice with family member (living in the same house) Yes/ No
   If Yes Relation with the family member
3. History of Surgery (including Dental procedures/Needle Prick/Anaesthesia) Yes/ No
   If Yes Brief Description
4. History of Blood Transfusion Yes/ No
   If Yes Brief Description
5. Other Important Relevant Information (Sexual/Drug/Alcohol/Smoking)
6. History of hospitalization Yes/ No
   If Yes Brief Description

Personal and Social Information

Standard of living: Slum/Village/Town Own house/Rented/Mess Alone/Sharing beds
Smoking habits
Addiction
Food intake (Recent): Home/Outside home(Restaurant)
Drinking water: Boiled/Tap/Tube well/Others
History of recent visits: If yes Sanitation of the visited area Good/Bad

Physical Examination

Jaundice: Mild/ Moderate/ Severe Anaemia: Mild/ Moderate/ Severe
Liver: Enlarged / None Spleen: Enlarged/ None

Biochemical Tests

Bilirubin	mg/dl on	Bil day.....
ALT/SGPT	IU/L on	SGPT day.....
Alk phosphatase	IU/L on	AlkP day.....
AST/SGOT	IU/L on	SGOT day.....
Prothrombin Time:	Patient: ..... Sec	Control: ..... Sec

Viral Markers

Anti-HAV, IgM:	HBsAg:	Anti-HCV:	HDAg:	Anti-HEV, IgM:
Anti-HAV, IgG:	Anti-HBc, IgM:	Anti-HCV LIA:	Anti-HDV:	Anti-HEV, IgG:
	Anti-HBc, total:			
	HBeAg:			
	Anti-HBe:			
	Anti-HBs:			

Comments: