DISSERTATION ON

OCCURRENCE OF HEPATOTROPHIC VIRAL INFECTIONS AMONG PATIENTS IN TERTIARY CARE CENTER

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Certificate

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CONTENTS

INTRODUCTION

More than 40 million people worldwide are infected with HIV/AIDS, and more than 3.1 million AIDS-related deaths occurred worldwide each year. In India, there is an estimated 5.1 million people infected with HIV today[1]. Current anti-HIV drug therapy has made a tremendous difference in the lives of many people with HIV infection. Unfortunately, the life-extending benefits of anti-HIV drug treatment have opened up a new set of problems for many HIV positive people, coinfected with other viruses[2].

Hepatic abnormalities are quite common among HIV infected persons. Thousands of people with HIV are also infected, or at risk of being infected with one of the several hepatitis viruses. Some of these viruses can cause chronic infection which may result in cirrhosis, liver failure, and hepatocellular carcinoma in a proportion of patients. Apart from being more prevalent, the natural history of these hepatitis viruses may be modified in patients with HIV infection and hence their significance[3]. Also it has been claimed that certain hepatitis virus infections may exacerbate the course of HIV infection, with rapid progression to AIDS, although this observation has not yet been confirmed.

Viral hepatitis is not so much an opportunistic infection in patients with HIV infection, as it is companion infection. Thus because some forms of hepatitis are acquired through sexual spread, or parenteral contact with blood or blood products in the same way that HIV is,

hepatitis and HIV infections often coexist. The enteral forms of hepatitis (HAV and HEV) are not strongly associated with HIV infection, whereas HBV, HDV, HCV and HGV are strongly associated because of shared routes of transmission. So screening of these hepatitis viral infections becomes mandatory in HIV positive patients[3].

In case of hepatitis B, C, and D, HIV infection is associated with higher levels of replication of these hepatitis viruses, with significantly greater amounts of virus in serum than among individuals without HIV. These higher levels of replication of hepatitis viruses presumably reflect deficiencies in the ability of the immune system to control their replication[3].

Hepatitis G virus is a newly discovered virus which is strongly associated in patients with HIV infection. This virus is mostly asymptomatic, and it has been found in most of the studies that patients with HGV RNA had significantly higher CD4 counts, low HIV viral loads and slower progression to AIDS, than in patients with HIV alone[4].

Screening for these hepatitis viruses has its significance not just because they are more prevalent and alters the natural course of the disease, they do have an important role in treatment. Perhaps the most important consideration is the timing of administering treatment to coinfected patients[5].

AIMS AND OBJECTIVES

- To study the serological status of hepatotrophic viral infections like HBV and HCV among HIV positive and HIV negative patients attending Voluntary Confidential Counselling and Testing Centre (VCCTC), Government General Hospital, Chennai.

- To compare the progression of Hepatitis B virus infection among HIV positive and HIV negative study groups.

- To compare the levels of anti HBs, among HIV positive and HIV negative study groups with hepatitis B virus infection, during follow up.

- To detect the prevalence of HGV RNA using the RT-PCR among HIV positives with coexistent HBsAg and/ or anti HCV.

- To study the clinical stage and immunological status of patients with HIV infection at presentation, and their correlation with hepatotrophic viral infections.

REVIEW OF LITERATURE

HIV / AIDS

HISTORY AND INTRODUCTION

Human Immunodeficiency Virus (HIV) which produces Acquired Immunodeficiency Syndrome (AIDS) was first reported in the United States in 1981 and has since become a major worldwide epidemic.

AIDS was officially recognized for the first time in June 1981, at the Centre for Disease Control, U.S.A. in previously healthy homosexual men dying with Pneumocystis carinii pneumonia and candidiasis. In 1983 the virus causing AIDS was independently identified by a team of French scientists led by Dr.Luc Montagnier (Pasteur Institute) who called it as Lymphadenopathy Associated Virus (LAV), and American scientists team lead by Dr.Robert C.Gallo of Cancer Institute and named it as HTLV-III[7]. Jay Levy (Univ of California) isolated this virus from an AIDS patient and named it as Human Immunodeficiency Virus.

EPIDEMIOLOGY

India has had a sharp increase in the estimated number of HIV infections, from a few thousand in the early 1990s to around 5.134 million children and adults living with HIV / AIDS in 2004[8]. With a population of over one billion, the HIV epidemics in India will have a major impact on the overall spread of HIV in Asia and the Pacific and indeed worldwide.

The first case of HIV infection in India was diagnosed among commercial sex workers in Chennai, Tamil Nadu, in 1986. The greatest numbers were in Maharashtra and Gujarat in the west; Tamil Nadu and Andhra Pradesh in the south; and Manipur in the north-east[9]. The majority of the reported AIDS cases have occurred in the sexually active and economically productive 15 to 44 age group. Every 24 hours over 15,000 people are infected with HIV; half of them are between 15 to 24 years of age[10].

The epidemic continues to shift towards women and young people. It has been estimated that 38% of adults living with HIV/AIDS in India, as of the end of 2003 were women. The increasing HIV prevalence among women can consequently be seen in the increase of mother to child transmission of HIV and paediatric HIV cases.

HIV TYPES AND SUBTYPES

There are two distinct types of human AIDS viruses: HIV-1 and HIV-2[11]. These two types are distinguished on the basis of genome organization and phylogenetic (evolutionary) relationships with other primate lentiviruses. HIV-2 has only 40% genetic identity with HIV-1, and it is more closely related to simian immunodeficiency virus. A major proportion of the infection worldwide is caused by the HIV-1 virus. Based on env gene sequences, HIV-1 comprises of three distinct viral groups (M,N,0); the predominant M groups contains 9 subtypes(A-K, omitting E and I). In India and China , subtype C is the most prevalent. Similarly , six subtypes of HIV-2 (A-F) have been identified[12].

TRANSMISSION

The infection can be transmitted by

1) Blood and blood products (effective route for viral transmission)

   - Blood transfusions
   - Intra-venous drug abusers - sharing of needles
   - Health care workers: needle stick injuries - risk approximately 0.36% (depends on extent of the injury)

2) Organ transplants
3) Sexual intercourse

Both homosexual and heterosexual exposure.

4) Vertical transmission

10 to 40% of babies born to HIV infected mothers will be infected[15].

STRUCTURE

HIV is a retrovirus, a member of the Lentivirus genus. It is 120 nm in size, nucleocapsid is icosohedral, enveloped, single stranded RNA virus. HIV comprises of an outer envelope consisting of a lipid bilayer with uniformly arranged 72 spikes or knobs of Glycoprotein (gp)120 and (gp) 41. gp 120 protrudes out on the surface of the virus and gp 41 is embedded in the lipid matrix. Just below the viral envelope is a layer called the matrix, which is made from the protein p17. Inside is the

protein core (capsid) which is made from the protein p24. Inside the core are three enzymes required for HIV replication called reverse transcriptase, integrase and protease. Also held within the core is HIV's genetic material, which consists of two identical strands of RNA. Proteins p7 and p9 are bound to the RNA and are believed to be involved in regulation of gene expression[13].

GENETIC STRUCTURE

HIV has several major genes coding for structural proteins that are found in all retroviruses, and several nonstructural ("accessory") genes that are unique to HIV.

Structural Genes

gag (Group-specific Antigen): codes for p24, the viral capsid; p6 and p7, the nucleocapsid proteins; and p17, a matrix protein[13].

pol: Codes for viral enzymes, the most important of which are reverse transcriptase, integrase, and protease which cleaves the proteins derived from gag and pol into functional proteins.

env (for "envelope"): Codes for the precursor to gp120 and gp41, proteins embedded in the viral envelope which enables the virus to attach to, and fuse with target cells.

Also in common with the other retroviruses, HIV possess at either end of its genome, a segment of nucleic acid called the LTR (long terminal repeats).

Non Structural / Regulatory Genes

tat gene (transactivator of transcription) and rev gene(regulator of expression of viral proteins) are the two most significant regulatory genes.

In addition to the gag, pol, and env genes contained in all retroviruses, and the tat and rev regulatory genes, HIV-1 contains four additional genes: nef, vif, vpr and vpu, encoding the so-called accessory proteins. HIV-2 does not contain vpu, but instead harbours another gene,vpx. The accessory proteins are not absolutely required for viral replication in all in vitro systems, but represent critical virulence factors in vivo[14, 15].

IMMUNOPATHOGENESIS

Viral receptors and co-receptors

All primate lentiviruses use as a receptor the CD4 molecule, which is expressed on macrophages and T lymphocytes. A second co-receptor in addition to CD4 is necessary for HIV-1 to gain entry to cells. The second receptor is required for fusion of the virus with the cell membrane. The virus first binds to CD4 and then to the co-receptor. These interactions cause conformational change in the viral envelope, activating the gp41 fusion peptide and triggering membrane fusion. Chemokine receptors serve as HIV-1 second receptors. CCR5, the receptor for chemokines - RANTES, MIP-1 alpha, and MIP-1 beta, is the predominant co-receptor for macrophage tropic (M-tropic) strains of

HIV-1. CXCR4 (fusin), the receptor for chemokine SDF-1, is the co-receptor for lymphocyte tropic (T-tropic) strains of HIV-1. Chemokines act as potent natural inhibitors of HIV infection, as they bind and down modulate chemokine receptors that serve as critical co-receptors for HIV to gain access into cells[16].

Pathogenesis

After the HIV virus enters its human host, it immediately starts to replicate into abundance. The virus can be located inside the T4 lymphocytes (helper T cells) and in other classes of immune cells (like macrophages). Early in infection, primary HIV isolates are M-tropic. As the infection progresses, the dominant M-tropic viruses are replaced by T-tropic viruses.

Following primary infection, there is a 4 to 11 day period between mucosal infection and initial viremia, the viremia is detectable for about 8 to 12 weeks. 3-6 weeks after primary infection CD4 counts drop. Immune response to HIV occurs 1 week to 3 months after infection, plasma viremia drops, and levels of CD4 cells rebound. However, the immune response is unable to clear the infection and HIV persists in the lymph nodes. In T cells it can lie dormant indefinitely, inextricable from the cell but hidden from the victim's immune system; when the same cells are stimulated, however, it can destroy them in a burst of replication. This period of latency may last for 10 years. During this time, there is a high level of ongoing viral replication. The CD4 count

decreases steadily, when it falls to less than 200 clinical AIDS usually sets in[13,17].

Replication and Production of New Virions

After HIV enters the bloodstream, it binds its glycoprotein (gp120) antireceptor to the CD4 molecule (receptor) on the host cell's membrane (HIV co-receptor on lymphocytes is the CXCR4 chemokine receptor) and injects its core into the host cell's cytoplasm[21]. The viral core which includes two identical strands of RNA, structural proteins, and enzymes. Reverse transcriptase (the enzyme responsible for converting HIV genetic material into DNA), first makes a single strand DNA copy of the viral RNA. Then, ribonuclease (an associated enzyme) destroys the original strands of RNA. The DNA polymerase then makes a second copy of DNA using the first copy as a template. This process is called reverse transcription .The completed viral double stranded DNA is now ready to head for the cell's nucleus. Once there, a viral enzyme, called an integrase, helps to integrate the viral DNA, or provirus, into the host cell's DNA. Viral transcription is now possible and every time the cell undergoes replication, the provirus will be duplicated along with the cell's own DNA. Once the virus reaches this stage, the infection is permanent. Once the provirus is integrated into the host cell's genome, it can remain silent for years or it may become activated to produce new virions .After the above process is completed, the provirus exits the nucleus and enters the cytoplasm .In the cytoplasm viral proteins are synthesized and assembled with the viral. RNA Maturation process is

completed when the virion components exit the cell through "budding" in order to gain its envelope and glycoproteins which are HIV's final structural elements[13].

Modes of Cell Death in T cells

There are three main ways HIV incites cell death in T4 cells, namely through the "budding" process, cell-to-cell fusion (syncytium formations), and through tricking the immune system into killing healthy T cells[13,21]. The consequences of CD4 T cell dysfunction caused by HIV infection are devasting, because the CD4 T lymphocyte plays a critical role in the human immune response.

COURSE OF HIV INFECTION

Exposure to human immunodeficiency virus (HIV) does not have a single common outcome in all individuals. Even in the absence of antiviral drug treatment, several classes of clinical progression have been defined.

1. Rapid progressors, are individuals who develop the symptoms of AIDS, or end-stage HIV disease, within 2-3 years after infection[18] About 10% of individuals who become HIV-positive fit this profile.

2. Typical progressors, consists of individuals who develop end-stage disease within approximately 10 years after seroconversion.

3. Long term non-progressors consists of those who have normal CD4+T cell counts after more than a decade of HIV seropositivity without drug treatment[19]. Approximately 10% of HIV-positive individuals appear to fit this profile. Such individuals also tend to have low or undetectable levels of HIV in their blood[20].

THE GENETIC STABILITY OF THE HIV VIRUS

HIV can make thousands of duplicates within a single cell, so genetic mutations are very common. Useless or inactive genetic mutations have little effect on the virus as a whole, however, if environmentally advantageous mutant genes are produced and continuously selected for survival, they are transmitted to other human hosts where they undergo further genetic mutations. These mutant genes could, in time and with enough human hosts, produce a new serotype of HIV (HIV-3, perhaps)[21]. Several mutant HIV virions can be found within a single infected individual, therefore, a vaccine developed for one mutant HIV gene may not work against the others[13].

WHO DISEASE STAGING SYSTEM FOR HIV INFECTION AND DISEASE IN ADULTS AND ADOLESCENTS

The CD4 lymphocyte count is central to the 1993 CDC classification system for HIV disease and to all of the staging systems proposed in developing countries. The Global Programme on AIDS of the World Health Organization (WHO) has proposed a simplified staging system that is clinically based and flexible enough to be used in different

parts of the world. The system is based on four groups of clinical conditions that are considered to have prognostic significance and therefore constitute stages, plus an assessment of physical activity performance expressed as a four-point score. Patients are classified according to the highest stage recorded for either clinical condition or physical activity.

CLINICAL CLASSIFICATION

Clinical Stage I:

- Asymptomatic
- Persistent generalized lymphadenopathy

Performance scale 1: asymptomatic, normal activity

Clinical Stage II:

- Weight loss <10% of body weight
- Minor mucocutaneous manifestations (seborrheic dermatitis, prurigo, fungal nail infections, recurrent oral ulcerations, angular cheilitis)
- Herpes zoster within the last five years
- Recurrent upper respiratory tract infections (i.e. bacterial sinusitis)

And/or performance scale 2: symptomatic, normal activity

Clinical Stage III:

- Weight loss >10% of body weight
- Unexplained chronic diarrhoea, >1 month
- Unexplained prolonged fever (intermittent or constant), >1 month
- Oral candidiasis (thrush)
- Oral hairy leucoplakia
- Pulmonary tuberculosis
- Severe bacterial infections (i.e. pneumonia, pyomyositis)

And/or performance scale 3: bedridden <50% of the day during last month

Clinical Stage IV:

- HIV wasting syndrome (weight loss of >10% of body weight, plus either unexplained chronic diarrohea (>1 month) or chronic weakness and unexplained prolonged fever (>1 month).
- Pneumocystic carinii pneumonia
- Toxoplasmosis of the brain
- Cryptosporidiosis with diarrhoea >1 month
- Cryptococcosis, extrapulmonary

- Cytomegalovirus disease of an organ other than liver, spleen or lymph node (e.g. retinitis).
- Herpes simplex virus infection, mucocutaneous (>1 month) or visceral
- Progressive multifocal leucoencephalopathy
- Any disseminated endemic mycosis
- Candidiasis of esophagus, trachea, bronchi
- Atypical mycobacteriosis, disseminated or pulmonary
- Non-typhoid Salmonella septicemia
- Extrapulmonary tuberculosis
- Lymphoma
- Kaposi's sarcoma
- HIV encephalopathy (clinical findings of disabling cognitive and/or motor dysfunction interfering with activities of daily living, progressing over weeks to months, in the absence of a concurrent illness or condition, other than HIV infection, which could explain the findings).

And/or performance scale 4: bedridden >50% of the day during last month.

THE NEW CDC STAGING SYSTEM

CDC has revised the classification system for HIV infection to emphasize the clinical importance of the CD4+ T lymphocyte count in the categorization of HIV-related clinical conditions

The previous CDC staging system divided the spectrum of HIV illness into four (I - IV). This has been replaced with a three-tier system-catogories A, B and C[23].

LABORATORY CLASSIFICATION

Lymphocytes	*CD 4+/mm^3*	*Clinical Stage*			
		I Asymptomatic	*II Early*	*III Intermediate*	*IV Late*
> 2000	> 500	1A	2A	3A	4A
1000- 2000	200 - 500	1B	2B	3B	4B
< 1000	< 200	1C	2C	3C	4C

Criteria for HIV infection for persons ages greater than 13 years:

a. repeatedly reactive screening tests for HIV antibody (e.g., enzyme immunoassay) with specific antibody identified by the use of supplemental tests (e.g., Western blot, immunofluorescence assay);

b. direct identification of virus in host tissues by virus isolation

c. HIV antigen detection; or

d. a positive result on any other highly specific licensed test for HIV.

DIAGNOSIS OF HIV INFECTION

The methods for diagnosis can be broadly classified into direct and indirect methods

Direct methods:

1. Virus culture
2. P24 antigen detection
3. Detection of Viral RNA / Pro-viral DNA.

Indirect methods:

Tests for screening

1. Rapid / simple tests
2. ELISA

Tests for confirmation

The Western Blot / Immunoblot

HIV AND HEPATOROPHIC VIRAL INFECTIONS

In HIV infected individuals, a wide range of in vivo antigenic stimuli seem to serve as cellular activators. Active infection by any virus substantially increases HIV viremia. So concomitant viral infections with hepatitis viruses may serve as cofactors of AIDS[11].

Since the beginning of the AIDS pandemic, hepatic problems have been among the most common features of the disease. Despite the introduction of highly active antiretroviral therapy (HAART) in 1995 and 1996, most HIV-infected patients continue to have liver disorders.

COMMON HEPATOTROPHIC VIRAL INFECTIONS IN AIDS PATIENTS

Hepatitis B

Hepatitis C

Hepatitis D (coinfection with HBV)

Hepatitis G

Viral hepatitis is an important public health problem worldwide. Hepatitis may be caused by several viruses that have in common hepatotropism and tendency to cause liver injury. They can be broadly grouped into those that are blood borne(Hepatitis B,C,D,G) and those that are enterically spread(Hepatitis A and E).

Initially, hepatitis aquired by blood transfusion, fresh frozen plasma infusion and administration of vaccines was called as “serum hepatitis” or “homologous serum jaundice”. Later it was renamed as “Hepatitis B” [24]. Later hepatitis C virus was identified as an agent causing post transfusion non-A non-B hepatitis[25]. Later it became evident that 15 to 20% of the PT-NANB cases could not be attributed to any of the hepatitis viruses that was discovered so far at that time, and thus the search for newer hepatitis agent led to the discovery of flavi-like

virus, called GB virus[26] or Hepatitis G virus. It is readily transmitted by blood transfusion, intravenous drug use and may spread sexually. HGV has been reported in adults and children throughout the world. HGV infection has been reported in 10 to 20% of adults with chronic HBV or HCV infection, indicating coinfection is a common occurrence. The overall prevalence of HGV infection among HIV positives was 52.7%, with seroprevalence of 32.1% and viremia prevalence of 20.6%[27].

Infection with hepatitis viruses transmitted through parenteral route and sexual contact (B,C,G and D)are more common with HIV infection when compared to enteric hepatitis viruses (A,D,E). Because these viruses are transmitted in similar fashions, it is not surprising that most HIV seropositive persons are also infected with HBV, HCV or HGV. Although all these forms of hepatitis occur commonly in patients not infected with HIV, they are somewhat more prevalent and their natural history may be modified in patients with HIV infection, hence their significance. Prevalence rate of these hepatitis viruses with HIV varies in different geographical situations. It depends mostly on the high risk status of the patient.

In addition to these hepatitis viruses, there are the universally prevalent viruses such as cytomegalovirus, adenovirus, herpes simplex virus etc., which can cause hepatitis as part of the clinical presentation, though their primary sites of replication may be in other regions of the body.

HEPATITIS B

Hepatitis B virus was discovered by Blumberg et al in 1967 in the blood of an Australian aborigine and thus its antigen is also called Australia antigen. Using electron microscopic methods, D S Dane eventually discovered virus like particles that carried this antigen on their surface, in the serum of hepatitis B patients[28].

EPIDEMIOLOGY

India has been placed into the intermediate zone of prevalence of hepatitis B (2-7% prevalence rates) by the WHO[29]. Nearly 40 million people, out of the global HBV infection pool of 350 million, are from India. Approximately 5 to 10% of the patients with HBV infection do not clear HBsAg but become HBsAg carriers. As many as 0.2% to 1.0% of adults in the United States are chronic HBsAg carriers. The prevalence of the carrier state is even higher in high risk populations such as homosexuals (6%) and Intravenous drug abusers (7%)[30].The average estimated carrier rate of hepatitis B virus (HBV) in India is 4%, with a total pool of approximately 36 million carriers. Wide variations in social, economic, and health factors in different regions may explain variations in carrier rates from one part of the country to another[31].

TRANSMISSION

The virus is highly infectious and minute amounts of blood / body fluids as little as 0.01 microlitre can transmit the infection. Transmission of HBV is mainly by the parenteral route which involves direct contact with body fluids. The most common routes are

- Direct or indirect contact with infected blood (needle sticks,cuts etc)
- IVDU (sharing needles)
- Unprotected sexual contact
- Transfusion of infected blood and blood products
- Vertical transmission (perinatal transmission).

STRUCTURE

Hepatitis B virus belongs to hepadnavirus family with a compact DNA genome 3.2 kb in length. The virus particle (virion) is a complex double shelled structure having an external diameter of 42 nm with a nucleocapsid core (27nm) and lipoprotein coat. This particle is called Dane particle. It represents the complete HBV. It replicates in the nuclei of infected hepatocytes and posses a distinct antigen called Hepatitis B core antigen (HBcAg). The virion core antigen contains DNA polymerase and double stranded DNA molecule. The major structural protein is lipoprotein viral coat and is called Hepatitis B surface antigen (HBsAg).

TYPES AND SUBTYPES

HBV has several antigenic subdeterminants, one of which is known as “a”, found in all individuals with HBsAg, and others termed d, y, w and r, which combine with “a” to produce four main HBV subtypes (adw, ayw, adr and ayr)[30].

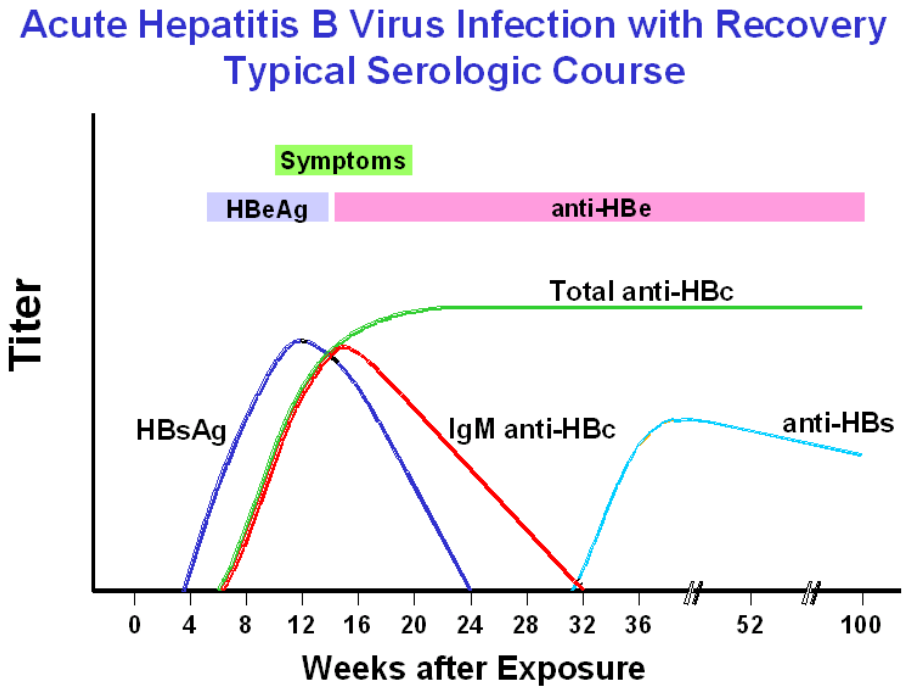
PATHOGENESIS

The incubation period of HBV infection is about 30-180 days, during which the patient has no signs and symptoms but the virus may be detected in the blood stream. Hepatitis B surface antigen is synthesized in the cytoplasm of infected hepatocytes in excess quantities than that needed for viral assembly. The excess material in the form of small particles, either 22nm spherical particles or small tubular particles of varying lengths are released in the blood stream. Since these smaller particles contain only the major viral protein HBsAg they are considered non-infectious but act as markers of infection. Occasionally complete virus occurs in blood stream. The virion has an other marker i.e. Hepatitis B “e” antigen (HBeAg) which is closely related to core antigen correlates with active viral replication, and therefore associated with high viral load and infectivity[32].

The first detectable viral marker is HBsAg followed by hepatitis B e antigen (HBeAg) and HBV DNA. Titers may be high during the incubation period, but HBV DNA and HBeAg levels begin to fall at the onset of illness and may be undetectable at the time of peak clinical illness. Core antigen does not appear in blood, but antibody to this antigen (anti-HBc) is detectable with the onset of clinical symptoms. IgM - anti HBc is used in an important diagnostic assay for acute hepatitis B infection. Before current molecular assays were available, it was the only marker detectable at the time between when HBsAg disappears and anti-HBs appears, the "window period". Patients who clear the virus lose

HBsAg and develop anti-HBsAb. Anti-HBsAb is a long-lasting antibody and is associated with immunity. The presence of anti-HBsAb and anti-HBcAb (IgG) indicates recovery and immunity in a previously infected individual, whereas a successful vaccination response produces antibody only to HBsAg[30].

Co-detection of HBsAg and anti HBs was seen in occasional patients recovering from acute hepatitis B, and it has been found in upto 30% of patients with chronic hepatitis[33]. This co-occurrence may be due to simultaneous infection with different subtypes of HBV[34]. This is also an indication that immune response to viral envelope is being activated in these patients, and this has been proposed as an evidence of greater degree of inflammatory activity in such patients and poor prognosis[35].



The risk of developing chronic infection (or the carrier state), defined as the persistence of HBsAg in the blood for more than 6 months, is dependent on the age and immune function of the patient at the time of initial infection. Patients with chronic infection will spontaneously clear surface antigen at a rate of 0.5% a year[32],[36].

DIAGNOSIS

In the appropriate clinical context, acute hepatitis B is diagnosed by detecting HBsAg and IgM core antibody, or core antibody alone, in the window period. IgM core antibodies are lost within 6 to 12 months of the onset of illness. Biochemically, serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels increase to between 500 to 5000U/L and fall after the acute phase of infection. Serum bilirubin level seldom increases above 10 mg/dL, alkaline phosphatase and prothrombin time are usually normal or mildly elevated. Loss of HBsAg and the development of HBsAb denotes recovery from the acute infection and the development of immunity.

Chronic hepatitis B is defined as the persistence of HBsAg in the serum of a patient for at least six months. Patients with chronic infection may be divided into those with evidence of active replication, typically associated with abnormal transaminases and higher viral loads, and those in the non-replicative state, which is associated with decreased markers of liver inflammation and damage, and lower viral loads. Transaminases may be normal, or increased anywhere from 1 to 10 times the upper limit of normal in chronic infection. The clinical outcome

of infection is correlated with e Ag status, conversion to e antigen negative, e Ab-positive in patients with chronic hepatitis B typically leads to decreased inflammation, with normalizing transaminases and decreased levels of HBV DNA in serum "the inactive carrier" state.

Chronic HBV infection is associated with a 10-fold increase in the risk of developing hepatocellular carcinoma (HCC). This risk is further magnified in the setting of ongoing inflammation: in patients with both HBsAg and HBeAg, the risk increases to 60-fold compared with the general population. It is therefore recommended that HBV carriers, particularly those at highest risk (men over age 45, patients with cirrhosis, and those with a family history of liver cancer) should be screened with ultrasound and alpha-fetoprotein for HCC, probably every 6 months[36].

The most useful detection methods are ELISA for HBV antigens and antibodies and PCR for viral DNA.

HIV AND HBV CO-INFECTION

Course of hepatitis B with concurrent HIV infection

The frequency and type of changes seen in the course of HBV infection among HIV-seropositive persons appear to vary with the order and age at which HBV and HIV are acquired. In HIV-infected patients, chronic hepatitis B has an unfavorable course compared with monoinfected patients, and the risk of liver-associated mortality is significantly increased.

Upto 95% of all HIV-infected patients have been infected with hepatitis B, and approximately 10-15% have chronic hepatitis B. Because primary HBV infection leads to chronic hepatitis in 2-5% of immunocompetent adults, HIV-infected patients experience chronicity about three to five times more often. A possible reason for this is the HIV-associated T-cell defect. A polarization to a Th2-type response could result in the inhibition of specific cellular defense mechanisms[37,38].

Data from the Multicenter AIDS Cohort Study (MACS) have demonstrated the unfavorable influence of HIV infection on hepatitis B[39]. HIV-positive patients possibly experience more frequent reactivation episodes of chronic hepatitis B than HIV-negative patients. Despite the worsening described, initially the clinical course is usually more benign in HIV-positive patients, although viral replication is increased. This seems contradictory at first, but can be explained by the impairment of cellular immunity, which may lead to an increase in viral replication, but at the same time also reduces hepatocyte damage. Therefore, transaminases in HBV / HIV-coinfected patients are frequently only mildly increased.

Influence of HBV on HIV infection

HBV infection neither leads to a more rapid decline of CD4+ cells nor to an increased frequency of AIDS-defining events. However, the reduction in HIV-associated mortality has led to an increase in mortality resulting from liver-related complications. In addition, HAART-related

hepatotoxicity develops about three times more frequently in patients with chronic hepatitis B. Whether or not the prognosis of HBV/HIV-infected patients is changed by HAART and HBV effective therapies, remains to be seen[37].

HEPATITIS C VIRUS

HCV was discovered in 1989 and is the most common cause of post transfusion non-A non-B hepatitis.

STRUCTURE

HCV is an enveloped, single stranded RNA virus, 50-60 nm in diameter with a genome of 10,000 nucleotides[25]. HCV is a member of and shares relationship with Flaviviruses. The genome encodes for three structural proteins; capsid protein(C-20KDa) and two envelope glycoproteins (EI-gp35 and E2-gp70) and several nonstructural proteins (NS)i.e. NS2, NS3, NS4A, NS4B, NS5A, NS5B have been identified.

EPIDEMIOLOGY AND TRANSMISSON

The prevalence of HCV antibodies in blood in developed countries ranges from 0.4-2%. The prevalence of HCV in Indian blood donors ranges from 0.6 to 2.4%[40]. 50% of HCV infection is reported to progress to chronic infection of which 20% may progress to cirrhosis and hepatocellular carcinoma.

Coinfection with HIV and HCV occurs frequently, due to the fact that both are transmitted via the same pathways (parenteral, sexual,

vertical). 240,000 people (30% of HIV-infected individuals) are estimated to be infected with both viruses in the USA. As HCV is ten times more infectious than HIV on blood-to-blood contact, intravenous drug users and recipients of blood products are particularly susceptible to coinfection.

In contrast, sexual transmission of HCV occurs significantly less frequently than HBV or HIV. As a result, HCV is rare in homosexual men and coinfection is more seldom in this group. The risk of transmission probably depends on the number of sexual partners and the performance of sexual practices that are prone to injuries. In total, about 4-8% of all HIV-infected homosexuals are also infected with HCV.

Perinatal transmission of hepatitis C is rare in immunocompetent individuals(<1%). The transmission rate rises with increasing immunosuppression in HIV-positive mothers, and is estimated to be as high as 20%. On the other hand, HIV-positive mothers treated effectively with HAART do not appear to have an increased risk for materno-fetal transmission of the hepatitis C virus.

Coinfection of HCV with HBV is associated with a worse outcome.

TYPES AND SUBTYPES

Hepatitis C virus can be grouped into atleast six genotypes .In the USA and Western Europe, genotypes 1a and 1 b are most common, followed by types 2 and 3. Genotype 4a is found at high frequency in the Middle East, particularly in Egypt. In Africa, infection with the genotypes

2, 4 and 5 is most predominant, whereas genotypes 1, 3 and 6 prevail in South East Asia[41].

CLINICAL COURSE AND PATHOGENESIS

Course in HIV/HCV co-infected patients

The clinical course of hepatitis C and HIV coinfection is determined by the HIV-associated immunosuppression. Progression of immunosuppression accelerates the course of hepatitis C. In the American Multicenter Hemophilia Cohort Study (MHCS) it was proved that rapid progression of liver disease was found particularly in patients with CD4+ T-cell counts below 100/m l. In the group of HIV-negative but HCV-positive patients, there was not a single case of liver failure during the same period of observation. In this group, the latent period until liver failure or hepatocellular carcinoma developed is estimated to be 30-40 years[42].

Patients co-infected with HIV and HCV seem to be at significantly greater risk of progression to cirrhosis than patients infected with HCV alone. Fibrosis is more severe, cirrhosis is more common, and mortality due to end-stage liver disease is greater in co-infected patients than in patients with HCV alone. HCV viral loads are generally higher with concomitant HIV infection, which may account for the more rapid clinical progression of HCV in co-infected patients.

Diagnosis

Anti HCV antibodies can be detected in 50 to 70% of the patients at the onset of symptoms, whereas in others appearance is delayed 3-6 weeks. Antibodies are directed against core, envelope, and NS3 and NS4 proteins and tend to be replatively low in titer. Nucleic acid based assays (RT-PCR) detect the presence of circulating HCV RNA and are useful for monitoring patients on antiviral therapy. Nucleic acid assays are used to genotype HCV isolates[11].

HEPATITIS G VIRUS

Hepatitis G virus is an enveloped RNA virus which belongs to the Flaviviridae family.

HISTORY

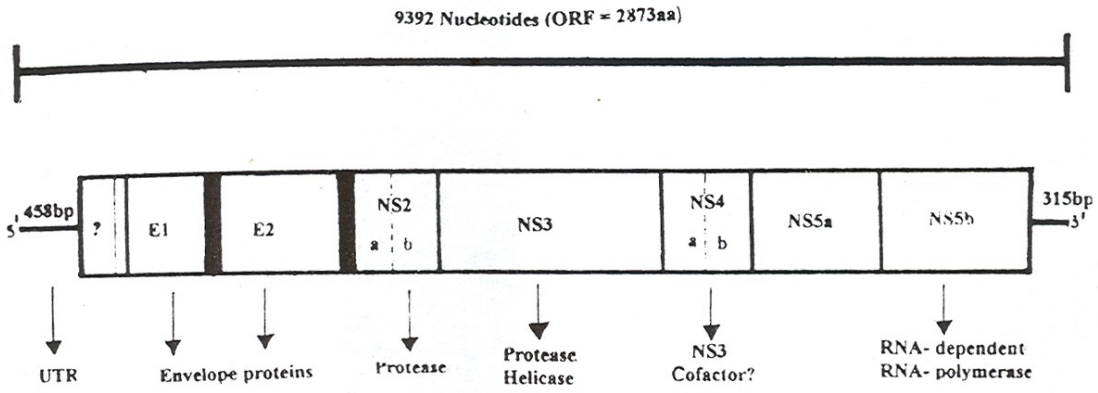
The discovery of GBV-C dates back to 1967, when a team of German investigators induced self-limiting hepatitis in tamarin monkeys that had been inoculated with serum from a surgeon with the initials G.B, who had developed acute hepatitis in 1964[43]. In 1995, a team of researchers at Abbott Laboratories isolated three viruses from tamarin that had been inoculated with the GB agent, which went on to be called GBV-A, GBV-B and GBV-C. GBV-A and GBV-B were only detectable in nonhuman primates, and that GBV-C was the GB virus responsible for hepatitis in humans.

At about the same time, researchers at Genelabs Technologies made their own discovery in chimpanzees infected with sera from a

patient infected with HCV[44]. A second virus was isolated in this patient, which the Genelabs team called the hepatitis G virus (HGV).

It was determined that the genomic organization of these viruses is similar to that of HCV and all are considered to be members of the Flaviviridae family. Comparison of the polyprotein sequences of GBV-C and HGV revealed a 95% identity with each other, and has led workers to believe that they are closely related, but different isolates of the same virus[45]. For purpose of convenience, these two agents will henceforth be collectively referred to as HGV.

STRUCTURE



Genetic organization of HGV.

HGV is a positive sense, single-stranded RNA virus with sequence identity with various flaviviruses. The genome has approximately 9,400 nucleotides and contains a large open reading frame that encodes a polyprotein of more than 2,800 amino acids. HGV genome is preceded by 5'untranslated region (5'UTR), followed by a long open reading frame, terminating with the 3'UTR. The presumed polyprotein contains

structural envelope 1 (E1) and 2 (E2) glycoproteins at the amino terminal end, followed by non structural (NS) proteins (NS2, NS3, NS4a, NS4b, NS5a, NS5b) at the carboxy terminal end. The HGV polyprotein does not appear to encode a nucleocapsid protein, and the 5'UTR contains an internal ribosome entry site[46].Untranslated regions at the 5' and 3' ends by (5'UTR / 5'NCR and 3'UTR / 3'NCR respectively), flank the open reading frame of the genome.

HGV bears close similarity to GBV-A, GBV-B and HCV with sequence homologies of 43.8%, 28.4% and 26.8% to each of them respectively. Unlike HCV however, in the E1 and E2 regions of HGV genome, there appear to be no hypervariable regions. Additionally, most if not the entire core region, that is seen in the HCV genome, is missing for HGV[47].

This virus encodes for genes, for a serine protease, a helicase and an RNA-dependent RNA polymerase, consistent with other pesti- and flaviviruses.

EPIDEMIOLOGY AND TRANSMISSION OF HGV

HGV is mainly a blood borne virus infection with world wide distribution. Its prevalence varies in different geographical locations. HGV is predominantly spread through parenteral routes, principally intravenous drug abuse and blood transfusion. HGV prevalence among general population and blood donors have also found to be high. The prevalence of HGV RNA in general population is around 1 to 2% and in voluntary blood donors it ranges from 0.5 to 4%[48] and in India it is about 4%[49]. High rates of HGV of more than 30% were seen in injection drug users[50].

HGV infection has been sought in populations at high risk for sexually transmitted diseases. It is found in higher rates among homosexuals and commercial sex workers. Prevalence of HGV RNA in homosexual and bisexual men was found to be 11%[51] and in commercial sex workers 20%[52]. Maternal-fetal transmission rates was found to be more than 30%[53].

Few data exist on the prevalence of HGV in HIV infected patients. In one study[54] HGV RNA was detected in 22.3% of HIV-infected patients.

In another study conducted in Belgium the HGV RNA prevalence was found to be 34%[55]. Curiously, two studies of HIV patients showed slower rate of progression of HIV infection in HGV co-infected patients, although it is possible that this is due to a confounding variable and not the biologic effect of HGV[50].

Studies on HGV infection in acute liver disease showed that HGV RNA was detected in 9% of the patients with acute non A-E hepatitis, and coinfections with acute HBV 32%, HCV 20%. The prevalence of HGV in patients with hepatitis B was significantly higher than those with hepatitis C or hepatitis non-A-E[56].

Prevalence of HGV RNA in HIV infected HBsAg positive patients was 22%,HIV infected anti HCV positive patients was found to be 16% and HIV infected without markers for HBV and HCV was found to be 3%[70]. HGV infection may exacerbate HCV and HBV infection[57].

HGV REPLICATION

HGV replicates like other positive stranded RNA viruses, with a replicative negative strand intermediary. In a recent study it was found that both genomic and antigenomic strands of HGV were identified in liver, which implied that HGV replicates in human hepatocytes[58]. Some studies concluded that it is highly unlikely that HGV replicates in or infects hepatocytes and the site of HGV replication may be in mononuclear cells in bone marrow or spleen, but not in peripheral blood mononuclear cells [59]. In another study on HGV, it was found that the spleen, bone marrow, kidney and liver biopsies were found to be uniformly positive for both negative and positive strands of HGV RNA[60].

PATHOGENESIS

Early studies identified HGV in patients with fulminant hepatitis, suggesting that HGV might play a role in the etiology of the disease.

Recent studies have shown that HGV may be associated with mild acute hepatitis but, even though it commonly results in chronic infection, does not seen to be associated with significant chronic hepatitis or liver disease[61,63].

HGV clearance is common in immunocompetent subjects, occurring in approximately 60 to 75% of HGV infected persons, along with the development of antibodies against the envelope glycoprotein E2[62].

Currently, HGV is only associated with a mild rise in transaminases during acute infection not unusual for an acute viral infection. Persistent infection is probably not associated with significant liver disease, although some groups disagree. The high prevalence of HGV infection in the general population makes demonstration of disease association particularly difficult.

Although the evidence suggests that HGV is probably not hepatotropic, the virus is replicating somewhere in the infected individual. Many researchers have argued that HGV may be a latent, well adapted virus of low virulence.

IMMUNE RESPONSE TO HGV

HGV RNA appears shortly after infection with HGV, becoming detectable as soon as 2 to 3 weeks after exposure. In 50% to 75% of cases, the infected person successfully clears HGV infection. In these cases, HGV RNA disappears as anti-E2 becomes detectable over an interval of several months. Seroconversion to E2 is evidence of past HGV infection and confers immunity against further infection with HGV. In a minority of cases, HGV infection becomes persistent for many years[50]. Several studies have suggested that younger age at time of infection and concurrent or ongoing immunosuppression may favour persistent infection. Infection with the hepatitis G virus can lead to persistent infection in 15 – 30% of adults. Possible mechanisms of HGV persistence, as found in some patients, remain to be elucidated[65].

Longitudinal study revealed that anti-E2 developed either during viraemia or some years after seronegativity for HGV RNA. Hence the antibody response itself seemed not to play a major role in the clearance of HGV though anti-E2 was associated with the clearance of HGV RNA. In contrast to HCV, spontaneous recovery is frequently observed in HGV infections[64].

Immunity to HGV seems to be long-lasting, because circulating antibody to E2 could still be detected 14 years after seroconversion. Reactivation of HGV after anti-E2 immune response has never before

been observed. In some cases of impaired immunocompetence, the loss of these antibodies may induce the reactivation of HGV.

CLINICAL FEATURES

Four different stages of the clinical course of the infection with HGV have been noted so far 1. Asymptomatic 2. Light Acute hepatitis (When the laboratory values and histological changes are quickly back in normal status) 3. Persistent Infection (When the laboratory values and histological changes persist) 4. Chronic Infection (When histological finds are of that of steatosis with persistent or chronic active hepatitis with fibrosis or Cirrhosis[66]. Most of the patients show minor elevation in aminotransferase levels that last until the clearance of HGV RNA[67].

METHODS OF DETECTION FOR HGV

The laboratory diagnosis of HGV infection is currently based on the measurement of antibodies against HGV envelope antigen (E2 antibodies) and on molecular biological assays to detect HGV RNA[68]. However, anti-E2 antibodies are not detectable during HGV active replication ,but they rather seem to be associated with a past infection after the clearance of HGV viremia[69]. Therefore, the direct detection of HGV RNA in biological specimens appears to be the most reliable method for the diagnosis of ongoing HGV infection[70].

CURRENT INFECTION

The reverse transcriptase polymerase chain reaction(RT-PCR) has been the most widely used diagnostic tool for the detection of HGV

RNA. RT-PCR ELISA was more sensitive than RT-nested PCR for detection of HGV RNA. HGV was originally identified utilizing degenerate oligonucleotide primers derived from the putative NS3 region of the genome and serum-derived DNA as the template[71]. Use of degenerate primers to the NS3 region have also shown high sensitivity in HGV RNA detection[72].

The second molecular assay utilized oligonucleotide primer pairs from the 5' untranslated region of the virus. Initially primers specific for the NS3 region were used[73]. Later primers derived from the 5'NCR was found to be more efficient than those derived from the NS3 and NS5 regions[74].

Automated probe-based assays have been established for the detection of HGV(Abbott Lcx probe system).

Hepatitis G virus infection has also been detected by a modified PCR technology which incorporates digoxigenin-labelled nucleotides into the amplicon. Primers from the non-coding region and the NS5 region of HGV are utilized for a single round amplification. Using a streptavidin surface and a biotin-labelled capture probe, the labelled nucleic acid is bound through the capture probe to the surface, and the amplified nucleic acid is detected using antibody to digoxigenin[75]. The amplified products can also be detected by a commercial assay(GEN-ETI-K-DEIA,sorin Biomedica).

HGV quantitation was performed using a research-based branched DNA (bDNA) assay with a set of probes directed at the 5' untranslated region[76].

A RT PCR using mutant HGV cDNA competitor has been used in recent studies[59].

PAST INFECTION

An ELISA was used for the qualitative determination of IgG antibodies to the HGV E2 antigen. Five purified prokaryotically expressed recombinant proteins were coated to microtitre plate and used for ELISA. Eight well-expressed recombinant proteins were separated by SDS-PAGE and transferred electrophoretically to nitrocellulose for immumoblotting[77].

Anti-HGV(E2) was detected by using an enzyme-linked immunosorbent assay recently commercialized by Boehringer Mannheim-ROCHE to the HGV E2 antigen located putative on the envelope of the virus (Anti-Hgenv Enzymun-test). The E_2 transmembrane protein was bound onto streptavidin-coated microtiter plates, which were incubated with the diluted specimen, and the antibodies directed against E_2 protein were detected using an anti-human IgG-peroxidase conjugate and 2,2' azino di-[3 etylbenzothiazoline sulfonate] (ABST) as peroxidase substrate. Extinction was measured at 405 nm (Anti-HGenv, Boehringer Mannheim, Mannheim, Germany)[78].

HGV COINFECTION WITH HIV

Hepatitis G appears to be a harmless hepatitis virus which seems to slow HIV disease progression dramatically. The prevalence of HGV RNA in HIV patients was found to be 15%. Survival was significantly longer for those who tested positive for HGV[79].

In another study, the effects of HGV were found to be independent of age, HIV-1 load, HCV load, CD4 and CD8 T cell counts, and CC chemokine receptor 5 (CCR5) genotype[80].

HGV virus did not prevent the entry of HIV in the cell or the depletion of CD4 cells, but it did have an inhibitory effect in replication and HIV growth in cell culture. This effect did not appear to be a result of cellular toxicity, considering that HGV replication in peripheral-blood mononuclear cells appeared to be noncytopathic and did not inhibit the synthesis of cellular proteins[81].

In addition, some studies suggest that HIV-1 replication is directly reduced by HGV. Various stages of the HIV life cycle may be affected by HGV, including retroviral binding and fusion to target cells through the high-affinity receptor and several chemokine coreceptors, internalization and reverse transcription, integration into the host-cell genome to create the HIV provirus, viral transcription, translation and viral morphogenesis; another possibility is a series of interactions that involve CCR5. Hence, the internalization of chemokine receptors is an effective mechanism to block cellular entry of HIV into the cells [83].

The interaction between HIV and HGV is unusual and curious, and it is beneficial to patients who are dually infected. It is, therefore, of great interest to understand the mechanisms involved in this interaction, because it could result in progress in our understanding of viral pathogenesis and in a contribution towards the development of novel HIV-1 treatment strategies.

HGV AND HBV

HGV coinfection does not lead to the activation of hepatic pathological changes and does not speed up the progression of fibrosis in patients with chronic hepatitis B infection. HGV has at most a mild hepatic pathogenicity[84].

Reciprocal influence between HBV, HCV and HIV appears rather complex, HBsAg carriage seeming to exert per se a negative effect on HCV replication, particularly in HIV-negative patients, suggesting that interactions between hepatitis viruses should always be analysed in the light of HIV status[85].

HGV infection may not affect HBV replication. Liver is the site of HGV replication, but HGV probably also replicates in extrahepatic tissues. HGV hepatic pathogenicity is probably mild and further studies are still needed[84].

HGV AND HCV

A high frequency of HGV coinfection existed in the hepatitis C patients. A reverse relationship was found to exist between HCV RNA concentration and HGV infection frequency.

In coinfections of HGV with HCV, this virus did not affect the clinical course of HCV. Peak serum ALT level, peak bilirubin level, histologic severity of viremia, frequency of chronic hepatitis, and response to interferon are virtually identical in patients with combined HGV and HCV infection and in those with HCV infection alone. Likewise HGV also has no apparent influence on the clinical course of acute disease among patients with hepatitis A, B or C[56].

HGV AND HCV IN HIV INFECTED PATIENTS

Coinfection with HCV and HGV was present in 17% of the HIV infected individuals and 8.6% only had HGV among HIV positives. Patients positive for HGV-RNA showed clinical and analytical characteristics similar to those found in HGV-RNA negative patients. Among the HCV- HGV coinfected and those presenting HGV as the only virus, it was observed that the coinfected group presented alterations in transaminases and predominance of parenteral transmission as a risk factor for HIV, whereas the HGV group presented normal transaminases and predominance of sexual transmission. No differences were perceived in mean CD4 and HIV-RNA values in both groups. Being positive for HGV in HIV-positive patients does not influence the presence of hepatic disease that in these patients is frequently accompanied by coinfection with other hepatotropic viruses. Moreover, it does not seem to influence the viremia of the HIV, or the CD4 cell counts[86].

MATERIALS AND METHODS

DETAILS OF THE STUDY

Setting:

This study was conducted in serologically confirmed HIV positive and negative patients attending VCCTC, Government General Hospital, Chennai.

Period of study:

March 2004 to February 2006.

Venue of study:

Study was performed in the Institute of Microbiology, Madras Medical College, Chennai and The Department of Virology, King Institute of Preventive Medicine, Chennai.

Methodology:

Pre and post test counselling were given to the patients attending VCCTC, and informed consent was got from them for testing. Strict confidentiality regarding the results were maintained.

For ELISA, blood samples were collected from the patients. Serum was separated, and stored at -20°C in cold storage, until use.

For all the ELISAs performed, the manufacturer's testing protocol instructions were strictly followed and results were interpreted only when the validity criteria were satisfied.

HIV SEROLOGY

Antibodies to HIV-1 and HIV-2 were detected using Enzyme Linked Immunosorbent Assay. Serum samples reactive for 1st ELISA were subjected to 2nd ELISA which utilizes a different principle from the first one. The sample is considered reactive, only if the second ELISA confirms the report of the first.

ELISA for detecting antibodies to HIV-1 and HIV-2 in patients serum was performed using Microlisa (J Mitra& Co Ltd, India) / InTec (InTec Products, Inc; China).

Microlisa HIV test is a second generation enzyme immunoassay based on indirect ELISA. It detects anti HIV antibodies to HIV-1 and /or HIV-2 with equal reactivity. The antigen used is a complex of recombinant proteins, representing immunodominant epitopes, gp 41, C terminus of
gp 120 of HIV -1 (including Subgroups O & C) and gp 36 of HIV-2.

InTec is an ELISA based, double antigens “sandwich” immunoassay, which employs a variety of recombinant HIV antigens- gp120/ gp41/ gp36/ p24 immobilized at the bottom of microtiter wells, and recombinant antigens- gp120/ gp41/ gp36/ p24 coupled with horseradish peroxidase (HRP) as conjugate solution.

HEPATITIS B SEROLOGY

HBsAg:

ELISA for detecting hepatitis B surface antigen in the patients serum was performed using Hepalisa (J Mitra& Co Ltd,India)/ Microscreen (Span Diagnostics Ltd, India).

Hepalisa detects Hepatitis B surface antigen based on direct sandwich principle, and Microscreen is third generation Elisa for screening of HBsAg.

HBeAg:

Hepatitis B envelope antigen was detected in all HBsAg positive patients using HBeAg EIA (Diazorin, ETI-EBK plus, Ref No:140). It is an indirect, non-competetive assay based on the use of polystyrene microwells coated with mouse monoclonal antibodies to hepatitis B envelope antigen. An enzyme tracer containing horseradish peroxidase labeled mouse monoclonal antibody to HBeAg detects any captured HBeAg.

Anti HBs:

Antibodies to hepatitis B surface antigen were detected in all HBsAg positive patients, during their follow up after 6 months. Qualitative/ Quantitative determination of antibodies to hepatitis B surface antigen using anti-HBs EIA (Diazorin, ETI-AB-AUK-3, Ref

p001603). It is quantitative\ qualitative, direct, non competitive, sandwich assay.

HEPATITIS C SEROLOGY

Anti HCV antibody (anti HCV) was tested in the Anti HCV EIA (Zhongshan bio-tech Co Ltd, China). This is a third generation enzyme immunoassay for the qualitative determination of antibody to hepatitis C virus in human serum/ plasma.

HEPATITIS G VIRUS

RT- PCR FOR DETECTION OF HGV RNA

Collection and processing of clinical samples:

Blood samples were collected in nuclease free EDTA coated tubes. Samples once collected were clearly labeled and stored in refrigerator till centrifugation. After centrifugation, plasma samples were stored at 2 to 8ºC for upto 6 hours. For long term storage aliquoted plasma samples were stored at -60º C, until use.

For RT-PCR testing, the recommendations of Kwok and Higuchi were followed to prevent sample contamination[6]. The assay was standardized with positive controls (RNA extracts/ cDNA from known positives) got from Department of Virology, CMC vellore. Subsequently all the assays were performed with one positive control and distilled water controls after every third patient's sample.

HGV RNA EXTRACTION FROM PLASMA/SERUM

Materials required

- QIA amp Min Elute Virus Spin Kit(Cat No:57704, Qiagen, Germany).
- Sterile 1.5ml/2ml microcentrifuge tubes.
- 50, 200, 500µl adjustable pipettes and tips.
- Microcentrifuge adjustable to 13K
- Vortexer
- Heating block

HGV RNA was extracted using QIA amp Min Elute Virus Spin Kit, according to manufacturers' instructions, from 200µl of plasma.

Principle and procedure of QIA amp Min Elute Virus Spin Kit

As the first step, viral lysis was done with proteases and lysis buffer added to 200µl sample and incubated at 56ºC for 15 min. Lysis buffer is Buffer AL (containing 28 µg/ml of carrier RNA). Carrier RNA enhances binding of viral nucleic acids to the QIAamp membrane, especially if there are very few target molecules in the sample and also reduces the chance of viral RNA degradation. The denaturing conditions of the lysis buffer also serves to inactivate RNases.

To this alcohol was added to adjust buffer conditions and to provide ideal binding conditions. The lysate was transferred to the QIA amp Min Elute column containing silica based membrane. The viral nucleic acids were adsorbed onto silica based membrane and the impurities were removed, as the lysate is drawn through by centrifugation. This was followed by two wash steps with the provided wash buffer (AW2 Buffer) and ethanol which efficiently removed all the inhibitors of the RT PCR reaction. The columns were spin dry and incubated at 56ºC for 3 min to dry membrane completely as ethanol carryover may cause problems in downstream applications. The RNA was eluted finally in 100 µl of elution buffer provided with the kit.

The extracted RNA was used immediately for c DNA synthesis.

REVERSE TRANSCRIPTION

Materials required

- QIA amp one step RT PCR Kit (Cat No:210210, Qiagen, Germany)
- Sterile 0.5 ml microcentrifuge tube
- 10, 20, 100 µl adjustable pipettes and tips
- vortexer
- Gene Amp PCR System 2400 thermal cycler (Perkin –Elmer, USA)
- Viral RNA

- Forward and Reverse primers specific for 5'NCR region of the HGV genome (Cat No: Primers_ (2 OD), Qiagen, Germany).

PCR PRIMERS USED FOR AMPLIFICATION AND DETECTION OF HGV RNA

HGV Primer	No of bases	Nucleotide sequence (5' – 3')
5'NCR forward primer	20	CGG CCA AAA GGT GGT GGA TG
5'NCR reverse primer	19	CGA CGA GCC TGA CGT CGG G

Procedure

All the reactions were carried out on ice.

Preparation of Master Mix

Master Mix was prepared with its final concentration containing 1x RT Buffer having 12.5 mM MgCl 2, 400 µM of each dNTPs , 1x concentration of Q solution, 0.6 µM of Primer A, 0.6 µM of Primer B and 2µl of Qiagen one step RT PCR enzyme mix per reaction.

To 40µl of Master Mix, 10µl of template RNA (extracted from patients plasma) was added to make it to a total volume of 50µl per reaction.

Known positive and negative controls were included in every reaction. The negative control lacks template RNA, in order to detect possible contamination of the reaction components.

HGV PCR

Thermal cycling conditions included initial temperature of 50°C for 30 min for reverse transcription, followed by 45 cycles of 94°C for 1 minute and 30 seconds, 55°C for 1 minute and 30 seconds, and 72°C for 1 minute and 30 seconds followed by a final extension step at 72°C for 10 minutes in the thermal cycler.

Pre and post PCR reactions were carried out in different rooms. Aerosol barrier tips were used at all micropipetting steps. Change of disposable plastic gloves after handling of each sample was followed.

Detection of amplified products by Gel detection method

The amplification product was analysed in 2% agarose gel with 0.5µl of ethidium bromide and viewed under UV illumination (Alpha Innotech, USA). Phi 174x Hae digest (Boehringer Mannheim- Roche, Gmbh, Germany) was used as the molecular weight standard for each assay.

The specific amplified product from the 5'NCR region of the HGV genome was 186 bp in size.

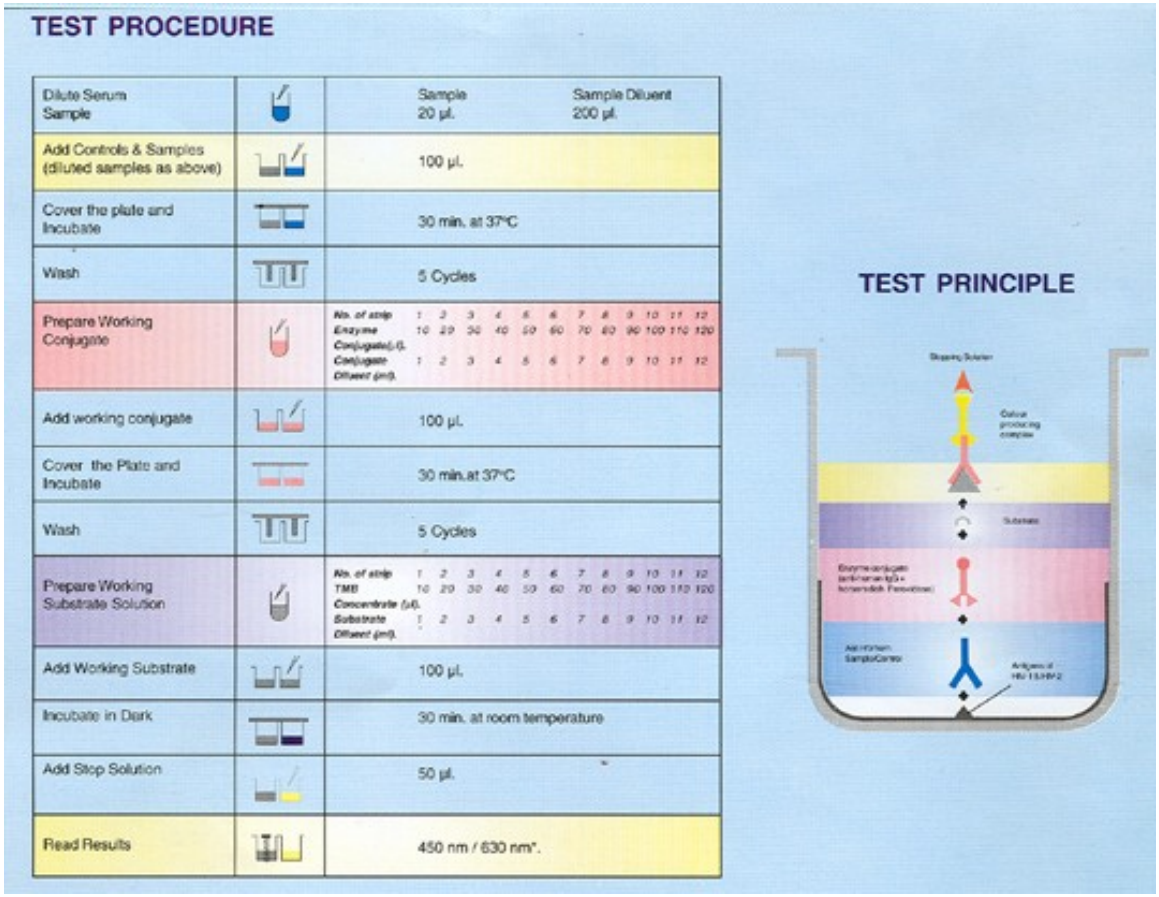
Determination of CD4\CD8 counts

CD4 and CD8 counts were determined with the help of FACS count system (Becton Dickinson Immunocytometry systems).

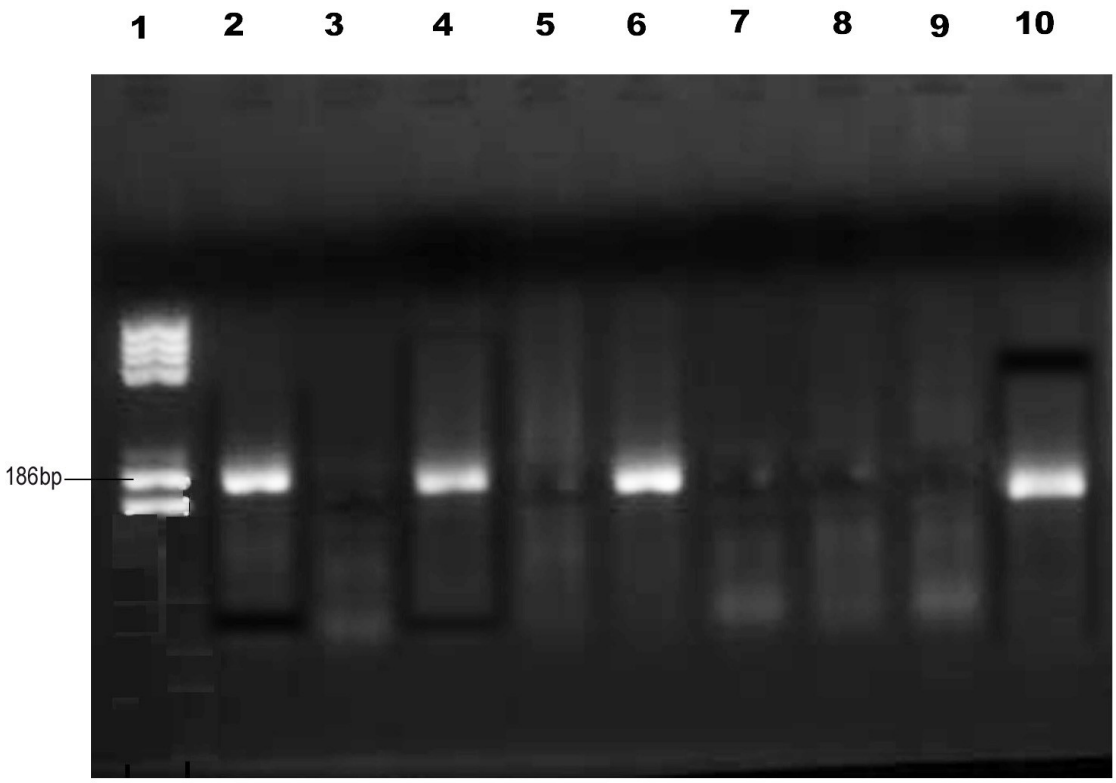
Control runs were set up each time when the instrument is run, or when a new lot of reagents were used.

About 3 ml of blood samples were collected in K3 EDTA (liquid) VACUTAINER tubes and tested on the same day of collection.

ELISA - HIV

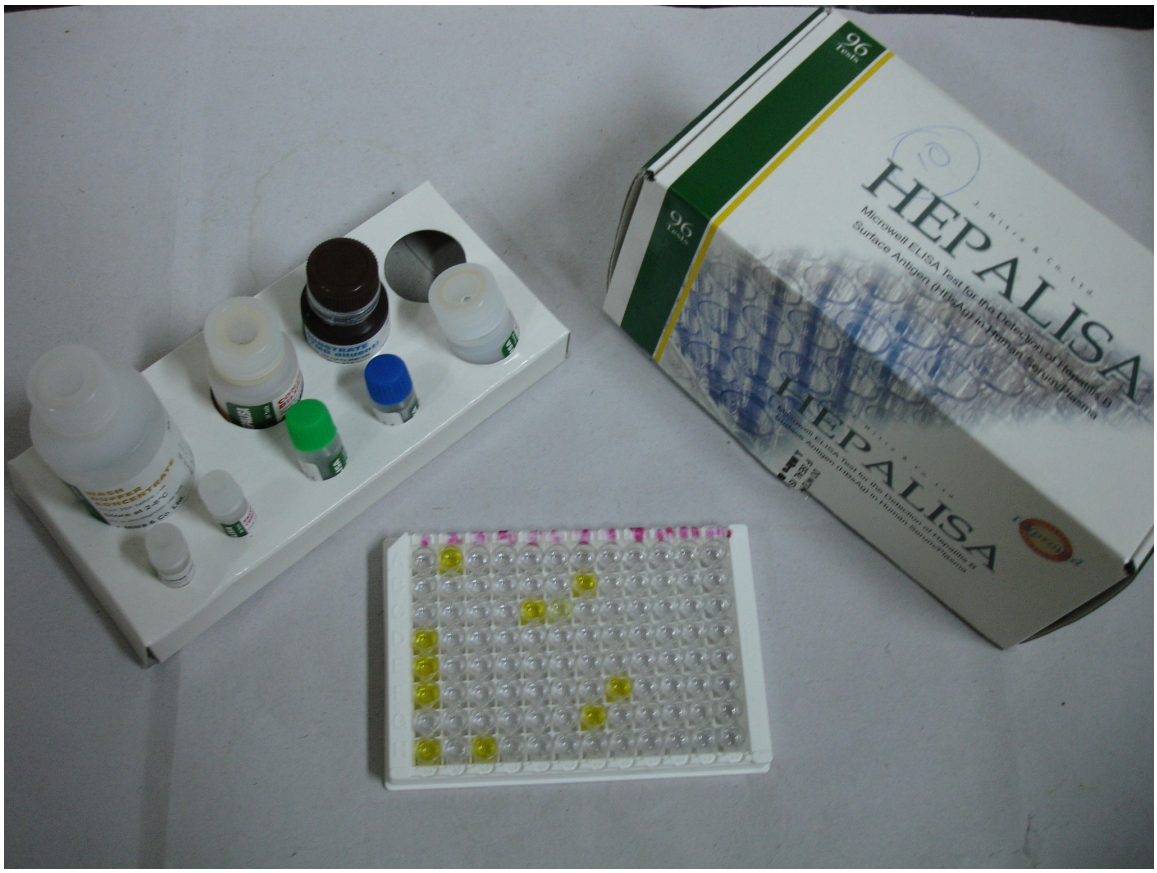


ETHIDIUM BROMIDE STAINED AGAROSE GEL PRODUCTS AFTER RT-PCR FOR THE 5' NCR REGION OF HGV GENOME



Lane 1	-	Hae III digest of ϕX174
Lane 2	-	Positive Control
Lane 3	-	Negative Control
Lane 4,6,10	-	Positive Samples
Lane 5,7,8,9	-	Negative Samples

ELISA - HBsAg



RESULTS

TABLE I - AGE AND SEX DISTRIBUTION

Age in years	*HIV positives (n = 500)*		*Total*	*HIV negatives (n = 300)*		*Total*
	Male	*Female*		*Male*	*Female*	
15 - 20	3	1	4 (0.8%)	1	0	1 (0.3%)
20 - 29	72	69	141 (28.2%)	25	12	41 (13.7%)
30 - 39	175	85	260 (52%)	130	60	190 (63.3%)
40 - 49	68	11	79 (15.8%)	45	12	57 (19%)
> 50	15	1	16 (3.2%)	10	1	11 (3.7%)
Total	333	167	500	211	89	300
%	66.6	33.4		70.3	29.7	

- Maximum number of HIV positives were between 30 to 39 years (median age-34 years)

- The male: female ratio was 1.9: 1

TABLE II
PREVALENCE OF HBSAG AND ANTI HCV AMONG HIV POSITIVES AND NEGATIVES

HIV Status	*HBsAg*	*Percentage*	*Anti HCV*	*Percentage*
HIV positives (n = 500)	24	4.8	20	4
HIV negatives (n = 300)	26	8.6	10	3.3

Prevalence of Hepatitis B surface antigen was more both among HIV positives and negatives, when compared to anti HCV antibodies.

TABLE III
MARKERS OF HBV

HIV status	*HBsAg positives in 1st screening*	*%*	*HBsAg positives in 2nd screening (after 6 months)*	*%*	*HBe Ag*
HIV positives (n = 500)	24	4.8	5	20.8	4
HIV negatives (n = 300)	26	8.6	2	7.6	2

- The persistence of Hepatitis B surface antigen after 6 months was three times more among the HIV positives, when compared to the HIV negative study group.

- The presence of HBeAg which indicates active replication was also more among HIV positives when compared to negative study group.

TABLE IV
ANTI HBS LEVELS AMONG HBSAG POSITIVES
AFTER 6 MONTHS FOLLOW UP

Study Group	*HBsAg*	*Anti HBs levels*	
		1- 10 IU/L	*> 10 IU/L*
HIV positives (n = 500)	24	7 (29.1%)	1 (4.1%)
HIV negatives (n = 300)	26	13 (50%)	4 (15.4%)

- HIV negative patients had higher antibody levels, when compared to HIV positive study group.

- Among the 5 patients who had chronic HBV infection (Table III), 2 had coexistent anti-HBs in their serum.

TABLE V
PROBABLE ROUTES OF TRANSMISSION OF HIV

Transmission Route	*HIV positives (n = 500)*	*%*
Heterosexual	420	84
Homosexual	6	1.2
Bisexual	9	1.8
IVDUs	16	3.2
Unknown	49	9.8

- Heterosexual route was the major mode of transmission of HIV .
- The heterosexuals also included the spouse of infected men.

TABLE VI
PROBABLE ROUTES OF TRANSMISSION OF HBV AND HCV

Routes of transmission	*HBsAg Positives (n = 24)*	*%*	*Anti HCV positives (n =20)*	*%*
Heterosexual	16	66.7	3	15
Homosexual	2	8.3	1	5
IVDUs	3	12.5	14	70
Unknown	3	12.5	2	10

- Heterosexual route was the major mode of transmission of HBV.
- HCV was more prevalent among IVDUs
- Among the total 16 IVDUs (Table II), 2 (12.5%) were positive for both HBsAg and Anti HCV

TABLE VII
WHO STAGING FOR HIV POSITIVES WITH AND WITHOUT HEPATOTROPHIC VIRUS INFECTIONS

Study population		*Clinical Stage I*			*Clinical Stage II*			*Clinical Stage III*			*Clinical Stage IV*		
		A	***B***	***C***	***A***	***B***	***C***	***A***	***B***	***C***	***A***	***B***	***C***
HIV positives with hepatotrophic virus infection (n = 42)	HIV+ HBV+ (n=24)	1	2	0	1	6	10	0	1	3	0	0	0
	HIV+ CV+ (n =20)	1	4	2	0	4	7	0	1	1	0	0	0
Total (n = 42)		2	6	2	1	10	15	0	2	4	0	0	0
HIV positives without hepatotrophic virus infection (n = 458)		60	79	3	29	105	42	2	28	60	0	3	47
Total HIV positives (n = 500)		62	85	5	30	115	57	2	30	64	0	3	47

Category A: CD4 counts > 500

Category B: CD4 counts 200- 500

Category C: CD4 counts <200

- Majority of the HIV positives without hepatotrophic viral infections were in Clinical Stage II Category B of HIV infection.

- Majority of the patients who were positive for hepatitis viral markers were in Clinical Stage II Category C of HIV infection

TABLE VIII
COMPARISON OF CD4 COUNTS AMONG HIV POSITIVES WITH AND WITHOUT HEPATITIS MARKERS

Study Group	*CD4 + / mm^3*			*Mean± SD*
	A> 500	*B 200-500*	*C < 200*	
HIV with hepatitis markers (HBsAg & anti HCV) (n = 42)	3 (7.1%)	16 (38.1%)	23 (54.3%)	210.11± 120.9
HIV without hepatitis markers (HBsAg & anti HCV) (n = 458)	91 (19.9%)	217 (47.3%)	150 (32.8%)	316.43±177.9

HIV positives with hepatotrophic virus infections had less mean CD4 counts, when compared to HIV positives without hepatotrophic virus infections.

TABLE IX
ALT LEVELS AMONG HIV POSITIVES WITH COEXISTENT HEPATITIS VIRUS INFECTIONS

Study Group	*Hepatitis Virus Markers*	*ALT Values*			*Mean ±SD*
		Normal	*< 3 times ULN**	*> 3 times ULN**	
HIV Positives (n= 500)	HBsAg positives (n=24)	18 (75%)	2 (8.3%)	4(16.7%)	49.5±43.2
	Anti HCV positives(n=20)	6 (30%)	7(35%)	7(35%)	71.5±42.5
HIV negatives (n=300)	HBsAg positives (n= 26)	14(53.8%)	10(38.5%)	2(7.7%)	37.5±25.5
	Anti HCV positives (n = 10)	3(30%)	5 (50%)	2 (20%)	53.8±35.3

*ULN- Upper Limit of Normal

Mean serum ALT levels were more among the HIV positive patients who were co-infected with HBV and / or HCV when compared to HIV negative study group.

TABLE X
PREVALENCE OF HGV RNA AMONG HIV POSITIVES

Study group	*Hepatitis virus marker status*	*HGV RNA*	*%*
HIV Positives (n = 500)	HBsAg positives (n = 24)	6	25
	Anti HCV positives (n = 20)	7	35

The prevalence of HGV RNA was more in anti HCV positive patients when compared to HBsAg positive patients, among the HIV positive study group.

TABLE XI
PROBABLE ROUTES OF TRANSMISSION OF HGV AMONG HBV AND HCV POSITIVE INDIVIDUALS WITH HIV INFECTION

Routes of transmission	*HGV positives (n = 13)*	
	HIV+ HBV+ (n = 6)	*HIV+ HCV+ (n = 7)*
Heterosexual	3 (50%)	1 (14.3%)
Homosexual	1 (16.7%)	0
IVDUs	0	6 (85.7%)
Unknown	2 (33.3%)	0

Sexual route was the predominant mode of transmission of HGV among HBV positives with HIV, whereas parenteral route was the major mode of transmission of HGV among HCV positives with HIV infection.

TABLE XII
COMPARISON OF CD4 COUNTS BETWEEN HGV RNA POSITIVE AND NEGATIVE INDIVIDUALS

Study group	*HGV Status*	*CD4 +/ mm³*			*Mean ±SD*
		> 500	*200- 500*	*< 200*	
HIV+/ HBV+ (n= 24)	HGV + (n= 6)	2(33.33%)	2(33.33%)	2(33.33%)	308.3±204.3
	HGV – (n=18)	0	7(38.9%)	11(61.1%)	181±78.5
HIV+/ HCV+ (n= 20)	HGV + (n=7)	0	5 (71.4%)	2(28.6%)	198.1±55.5
	HGV – (n=13)	1(7.7%)	4(30.8%)	8(61.5%)	212.4±135.3

- HGV positive individuals had higher mean CD_4 counts when compared to HGV negative individuals, among the HIV positive and HBsAg positive study group.
- There was no significant difference observed between the HGV positive and negative patients, among the HIV positive & anti HCV positive study group.

TABLE XIII
COMPARISON OF ALT LEVELS BETWEEN HGV RNA POSITIVES AND NEGATIVE INDIVIDUALS

Study Group	*HGV status*	*ALT Values*			*Mean±SD*
		Normal	*< 3 times ULN**	*> 3 times ULN**	
HIV+ / HBV+ (n= 24)	HGV + (n=6)	1 (16.7%)	3 (50%)	2 (33.3%)	65.7±42.3
	HGV - (n=18)	13 (72.2%)	3 (16.7%)	2 (11.1%)	44.0±43.2
HIV+/ HCV+ (n=20)	HGV + (n=7)	1 (14.3%)	3 (42.9%)	3 (42.9%)	79.0±36.6
	HGV - (n=13)	5 (38.5%)	4 (30.8%)	4 (30.8%)	67.5±50.6

Mean serum ALT levels were more among HGV positive individuals, when compared to HGV negative individuals.

DISCUSSION

Hepatotrophic virus infections occur more commonly among HIV positive individuals. Their prevalence depends on the geographical area & high risk behaviour; and their immunopathogenesis differs among HIV infected and non infected individuals. Institution of Highly Active Anti Retroviral Therapy (HAART) also requires a knowledge about these viruses. The present study is focused on the prevalence of the hepatotrophic viruses and their clinical course which would help the physician in appropriate management and treatment.

In the present study, patients between 30 to 39 years of age were predominantly affected with HIV. The average age is 34.5 years (Table I). This correlated well with the previous datas available, where the predominant age group was given under a broader range of 30-49 years[9]. HIV mainly affects people in sexually active age group, which indirectly implies sexual route may be the major mode of transmission of HIV.

There was a male predominance, with male: female ratio being 1.9:1 in our study (Table I). In the previous studies conducted in 1998 the male: female ratio was about 3:1[87]. Latest study by Cacala SR et al in 2006 has shown male: female ratio of 1.4:1[88], which correlates well with the present study. The increase in the number of females may be due to the effective awareness created among them, which has made

them come to voluntary confidential counseling and testing center for HIV testing.

The prevalence of HBsAg was 4.8% and anti HCV was about 4%, among the HIV positives in our study. Among the HIV negatives, 8.6% were HBsAg positive and 3.3% were anti HCV positive (Table II). In a study conducted by S M Ahsan et al in Mumbai during 2002 showed a seroprevalence of about 3.5% for HBsAg and 8% for anti HCV, among HIV positives[89]. In a study conducted by chandrasekaran S et al in Madurai showed a prevalence rate of 18.2% for HBsAg and 3.4 % for anti HCV, among suspected hepatitis cases who were HIV negatives[90]. The differences in the prevalence observed are due to the variations in the prevalence rates among different geographical situations, and depends mostly on the high risk status of the patient.

In our study, among the 24 HIV positives who had HBsAg in their serum during the initial screening, when they were screened after 6 months follow up, 5 (20.8%) had persistent HBsAg in their serum which indicated their chronicity. In HIV negative study group, among the 26 who had HBsAg in their serum during the initial screening, only 2 (7.6%) had persistent HBsAg in their serum and had chronic hepatitis B infection (Table III). Chronicity was about 3 times more among the HIV positives when compared to the HIV negatives in our study. Mandells principles and practice of infectious diseases textbook states that approximately 5 to 10% of the patients with HBV infection do not clear HBsAg, but become chronic HBsAg carriers; and among the HIV

positives infected with HBsAg about 10-15% have chronic hepatitis B infection[30]. HIV infected patients experience chronicity about three to five times more often than HIV negatives, and this correlates well with our study.

Around 4 (16.6%) of the HIV positives and 2(7.6%) of the HIV negatives who had HBsAg in their serum during initial screening, had HBeAg in their serum, and were in active replicative state (Table III). HIV positives with hepatitis B infection were more infectious and had a poor clinical outcome when compared to HIV negatives in our study, and this correlates well with the previous studies[30].

In the present study, anti HBs levels were accessed after 6 months among the patients who were HBsAg positive during the initial screening. This was done to access the clearance of HBsAg and development of protective anti HBs levels. Among the 24 HIV positives, who were HBsAg positive initially (before 6 months), only one had anti HBs level in protective levels (more than 10 IU/L), and in 7 anti HBs was just detected but below the protective levels. Among the 26 HIV negative & HBsAg positive study group, after 6 months four had anti HBs levels in protective levels (more than 10 IU/L), and in 13 anti HBs was just detected but below the protective levels (Table IV).

Among the 5 HIV positive patients who had chronic hepatitis B infection, 2 had coexistent anti HBs in their serum, in our study (Table IV). As per shiels et al(1987) co-occurrence of HBsAg and anti HBs has

been observed in occasional patients recovering from acute hepatitis B, and it has been found in upto 30% of patients with chronic hepatitis which correlates well with our study[33]. This co–occurrence is associated with a greater degree of inflammatory activity and poor prognosis.

Heterosexual route was the major mode of transmission of HIV which accounted for about 84% of the total, in the remaining about 3.2% were IVDUs, 1.2% were homosexuals, 1.8% were bisexuals and in about 9.8% the mode of transmission was not known (Table V). The heterosexual population also included the spouse of infected men. Most of the males gave history of contact with multiple partners and unsafe sex. A recent study conducted by NACO in 2005 showed that about 85% of the transmission of HIV was through sexual route and about 2.4% were IVDUs[9], which correlates well with the present study. Heterosexual route is the major route of transmission of HIV in India .

The major mode of transmission of hepatitis B virus was heterosexual route which was about 66.7%. Among the rest, about 8.3% were homosexuals, 12.5% were IVDUs and in 12.5% the mode of transmission was not known. Among the HIV positive homosexuals who were seropositive for HBsAg[2], one developed carrier state (Table VI). As per Mandells principles and practice of infectious diseases, risk for transmission of HBV in heterosexuals is about 41%, homosexuals 9% and IVDUs 15%[30]. Transmission of HBV by heterosexual route was more in the present study, since the transmisson of the hepatotrophic viruses depends on the geographical area.

Majority of the HIV positives with anti HCV antibodies were IVDUs (70%). Among the remaining, about 15% were heterosexuals , 5% were homosexuals and in about 10% the route of transmission was not known(Table VI). As per Miriam J. Alter et al(2006), 72-95% of injection drug users, 1-12% of homosexuals and 9-27% of heterosexuals had HCV infection, among the HIV infected patients[91], and this correlates well with the present study.

In the present study, majority of the HIV positives without hepatotrophic virus infections (22.9%) were in Clinical Stage II Category B of infection. Most of the patients (35.7%) who were seropositive for hepatitis viral markers were in Clinical Stage II Category C of HIV infection (Table VII). In a study conducted by SV Jalgoankar et al, 2003, there was a significant difference between the symptomatic- CDC stage IV(71.4%) and asymptomatic- CDC stage II (28.6%) of HIV infected patients who had coexistent HBsAg or anti HCV in their serum[92]. This study correlates well with the present study which also showed the difference. The incidence of co-infection rises with the disease progression, hence the difference.

Majority of the HIV positives with hepatotrophic virus infections (HBV and or HCV), had CD4 counts below 200 (54.8%) in our study. All the patients with chronic hepatitis B infection were in this category. Whereas in HIV positives with no detectable hepatitis viral markers in the serum, only 32.8% had CD4 counts below 200 (Table VIII). In previous studies among HIV positive patients with HBV/ HCV coinfection

have shown that they are associated with lower CD4 counts[3] which correlates well with the present study. This carriers its significance because in a previous study (American Multicenter Hemophilia Cohort Study) it was proved that rapid progression of liver disease was found particularly in patients with CD4+ counts below 100/m l.

In the present study, among the HIV positives, four hepatitis B carriers had ALT levels more than 3 times the upper limit of normal and were active carriers of hepatitis B infection. One patient (HIV and HBsAg positive) who was a carrier, neither had HBeAg nor elevated ALT levels and was a healthy carrier of HBV infection. Among the HIV negative and HBsAg positive study group 2 (7.7%) had ALT levels more than 3 times the upper limit of normal , whereas 4 (16.7)% of the HIV positive and HBsAg positive study group had ALT levels more than 3 times the upper limit of normal. This implies that the rise in ALT levels is more among HIV positives when compared to HIV negatives (Table IX). In a previous study by Gregory J Dore et al, in National Centre in HIV Epidemiology and Clinical Research, Australia, among the 23 HIV positives who were positive for HBsAg , 8 had abnormal ALT levels (more than upper limit of normal)[93] which was about 6 in the present study.

Among the HIV positives who had coexistent anti HCV, 7(35%) had ALT levels more than 3 times the upper limit of normal. Among the HIV negatives who had coexistent anti HCV 2(20%) had ALT levels more 3 times than upper limit of normal (Table IX). In a previous study by Margaret J Koziel et al (2002), among the HIV positives with HCV

coinfection, 31% had normal ALT levels, 42% had ALT levels less than 2 times ULN & ALT levels were more than 2 and 3 times the ULN in 15% and 12%, respectively[94]. This correlates well with the present study. As per Conry-Cantilena C et al (1996), around 30% of patients with chronic HCV infection had elevated ALT levels, 40% had ALT levels close to the upper limit of normal (<2 times the upper limit of normal) and 30% had normal ALT[95], which correlates well with the present study.

In the present study, prevalence of HGV RNA in HIV infected HBsAg positive patients was about 25 %, and HIV infected anti HCV positive patients was found to be 35%. Two patients who had chronic hepatitis B infection had coexistent HGV RNA (Table X). Prevalence of HGV RNA among HIV infected, anti HCV positives was more when compared to HIV infected HBsAg positives in our study. As per Bonacini M et al(1998), prevalence of HGV RNA in HIV infected HBsAg positive patients was 22 %, HIV infected anti HCV positive patients was found to be 16%[54]. The prevalence rate varies in our study, since it depends on the geographical area and high risk status of the patient.

In the present study, among the HGV positives majority of them were IVDUs(46%), about 31% were heterosexuals, 15.38% were homosexuals and in about 8% the route of transmission was not known. Sexual transmission was the predominant mode of transmission of HGV among HBV positives with HIV (50%), whereas parenteral route was the major mode of transmission of HGV among HCV positives with HIV (85.7%)(Table XI). As per Kleinman et al (2001), high rates of HGV of

more than 30% were seen in injection drug users[50], which correlates well with the present study. As per Stark K et al(1996), prevalence of HGV RNA in homosexual and bisexual men was found to be 11%[51], which correlates well with our study.

In the present study, around 2 (33.33%) of the HIV positives who had coexistent HBV and HGV infections had CD4 counts below 200, whereas about[11] 61.1% of the HIV positive, HBsAg positive and HGV negative study group had CD4 counts below 200. Among the HIV positive, HCV positive and HGV positive study group, only 2 (28.6%) had CD4 counts below 200, whereas about 8 (61.5%) had CD4 counts below 200 among the HIV positive, HCV positive, HGV negative study group (Table XII). Majority of the patients who were positive for HGV RNA along with HBV, had higher CD4 counts when compared to HGV RNA negatives with HBV among HIV positive patients. As per T.Tsertsvadze et al(2001), CD4 count was higher in patients with HIV, HBV and HGV combined infections in comparison with HIV positive, HBV positive and HGV negative patients[96], which correlates well with the present study.

About 2 (33.3%) out of total 6 HIV positive, HBV positive and HGV positive individuals had ALT levels more than 3 times the upper limit of normal, whereas among the HIV positive, HBV positive and HGV negative study group about 2(11.1%) of the total 18 had raised ALT levels more than 3 times the upper limit of normal (Table XIII). As per

T.Tsertsvadze et al (2001), Significantly higher ALT levels were

observed in the subgroup of patients with HIV, HBV and HGV combined infections in comparison with HIV positive, HBV positive and HGV negative individuals[96], which correlates well with the study. In the present study , 3 (42.9%) of the HIV positive, HCV positive and HGV positive study group had raised ALT levels (more than 3 times ULN) when compared to 4(30.8%) of the HIV positive, HCV positive and HGV negative study group. A previous study by Lopez Calvo et al (2003)[97] also confirmed the same.

SUMMARY AND CONCLUSION

- In the present study 500 HIV positive and 300 HIV negative patients attending VCCTC, Government General Hospital, Chennai were screened for the coexistence of hepatotrophic virus infections. Majority of the HIV positives were in the sexually active age group of 30 – 39 years (52%), with males being more than females in the ratio of 1.9: 1.

- Heterosexual route was the major mode of transmission of HIV (84%).

- Hepatitis B surface antigen was the more prevalent viral marker among HIV positives (4.8%) and HIV negatives (8.6%), when compared to anti HCV antibodies.

- Transmission of HBV was mainly through sexual route (75%) and transmission of HCV was mainly through parenteral route(70%), among the HIV positives.

- Among the 5 HIV positives who had chronic HBV infection, 4 were ‘chronic active carriers’ with CD4 counts below 200 & ALT levels more than 3 times ULN and one was a ‘Healthy carrier’ who neither had HBeAg, nor raised ALT levels..

- The chronicity of HBV was about 3 times more common among the HIV positives(20.8%), when compared to the HIV

negatives(7.6%), which implies that HIV positives with HBV infection were more infectious and had a poor prognosis when compared to HIV negative study group.

- HIV negative study group had higher mean anti-HBs levels, when compared to HIV positive study group, during their follow up after 6 months. This implies that HIV associated depressed cellular defence mechanism may be the cause for diminished anti-HBs levels. Among the 5 HIV positives who had chronic hepatitis B infection, 2 had co-existent anti HBs in their serum.

- Majority of the HIV positives with hepatitis B or C infections had lower mean CD4 counts, when compared to HIV positives without hepatotrophic virus infections. This shows that hepatotrophic viruses affect the course of HIV infection.

- Among the HBsAg and anti HCV positives, ALT levels were raised more among the HIV positive study group when compared to HIV negatives signifying that the course of hepatitis B and C is accelerated in HIV coinfection.

- HGV infection occurs with high frequency in patients with HIV and HCV combined infections (35%), signifying the common modes of transmission of HGV and HCV.

- Among the HIV positive study group, HBsAg positives with HGV co-infection had higher mean CD4 counts, when

compared to HBsAg positives without HGV co-infection. There was no significant difference observed between the HCV positives with or without HGV co-infection. This implies that the presence of HGV RNA is associated with milder HIV status in individuals with HIV and HBV combined infection.

- Among the HIV positive study group, HGV positive patients with hepatitis B and / or hepatitis C co-infections had higher mean ALT levels, when compared to HGV negative individuals. This implies that, presence of HGV RNA was associated with severe hepatitis in patients with HIV and HBV or HCV combined infections.

- The present study throws light upon the co existence of HIV with Hepatotrophic viruses in a tertiary care center like ours.

- Only very few studies have been performed in India analyzing the coexistence of HGV with HIV. Our study can be considered as pioneer study in this context. The results aid the central HIV programme implementing agencies, especially in the field of ART to give importance to hepatitis G virus, along with hepatitis B and hepatitis C viral infections.

ABBREVIATIONS

HIV	– Human Immuno Deficiency Virus
AIDS	– Acquired Immuno Deficiency Syndrome
VCCTC	- Voluntary Confidential Counselling and Testing Centre
HBV	- Hepatitis B virus
HCV	- Hepatitis C virus
HGV	– Hepatitis G virus
ELISA	- Enzyme Linked Immuno Sorbent Assay
RT PCR	– Reverse Transcriptase- Polymerase Chain Reaction
ALT	– Alanine Amino Transferase
HAART	– Highly Active Anti Retroviral Therapy

APPENDIX

PROFORMA

Name: Address:

PID No:

Age: Occupation:

Sex – Male/Female

Income: <500, 1000-3000, 3000-5000, More than 5000

Marital Status: Married/Unmarried/Widow/Widower/ Separated

Heterosexual/ Homosexual

Sexual Habits – Pre marital / Extra Marital

Contact with Women in Prostitution – Yes/No

Last Contact:

Use of Condom – Yes/No

HISTORY

H/o. STD – Yes/No

Genital Ulcer – Yes/No

Genital Discharge – Yes/No

H/o Lymphadenopathy – Yes/No

Treatment for STD/HIV – Yes/No

Fever – Yes/No Duration – Days/weeks/Months

Loss of Appetite – Yes/No

Weight Loss - <10%, 10-25%, 25-50%, > 50%

Dysphagia – Yes / No Duration – Days/weeks/Months

Fatigue – Yes /No Duration – Days/weeks/Months

Diarrhea – Acute/Chronic

Pain Abdomen – Yes/No

Jaundice – Yes/No

Pulmonary Symptoms – Yes/No

Headache – Yes/No

Seizure – Yes / No

Neurologic deficit – Yes/No

Personal habits – Smoking/Alcohol/Drugs

EXAMINATION

Height Weight

Anemia – Yes/No Lymph Node – Yes/No

Oral Candidiasis –

Cardiovascular System – Normal/Abnormal

Respiratory System – Normal/Abnormal

Abdomen – Normal /Abnormal

Central Nervous System – Normal / Abnormal

INVESTIGATIONS

TC – DC - Hb–

Platelet Count – ESR –

Liver Function Tests: (Patients on admission)

S. Bilirubin – Total: Direct –

SAP –

SGOT – SGPT –

S.T. Proteins – S. Albumin –

Ultrasonogram –

ELISA

HIV 1 and 2 –

HBs Ag –

Anti Hbs Ag –

HBe Ag (If HBs Ag +ve) –

Anti HCV Ab –

PCR

HGV RNA –

FLOW CYTOMETRY

CD4/ CD8 –

ELISA

All the ELISAs were performed by strictly following the manufacturer's testing protocol instructions and results were interpreted only when the controls were satisfactory.

HIV ELISA

I.MICROLISA

MICROLISA HIV test is an enzyme immunoassay based on Indirect ELISA. Recombinant proteins gp 41, C terminus of gp120 for HIV-1, and gp 36 for HIV-1 and HIV-2 representing immunodominant epitopes are coated onto microtiter wells .

Test procedure

- 100 µl of the sample diluent was added to A1 well as blank
- 100 µl of Negative Control was added to wells B1 and C1 and 100µL of Positive Control was to the wells D1, E1 and F1 respectively.
- 100 µl of sample diluent was added in all the wells starting from G1 followed by the addition of 10 µl of sample from G1.
- Cover seal was applied and incubated for 37°C for 30 min.
- Wells were washed well with working wash solution according to the wash procedure.
- 100 µl of working conjugate solution was added to all the wells including A1.
- Cover seal was applied and incubated for 37°C for 30 min.
- Wells were washed well with working wash solution according to the wash procedure.

- 100 µl of working substrate solution was added to all the wells including A1.
- The microtitre plate was incubated at room temperature (20-30°C) for 30 min in dark.
- 50 µl of stop solution was added and the absorbance was read at 450 nm within 30 minutes in ELISA reader after blanking A1.

Validity Criteria

Blank must be < 0.100

Negative control must be ≤ 0.150

Positive control must be ≥ 0.50

Calculation of results

Cut off value = $\frac{\text{Mean of Negative control + Mean of positive control}}{6}$

II. InTec – HIV ELISA(3rd generation)

InTec is an ELISA based, double antigens "sandwich" immunoassay, which employs a variety of recombinant HIV antigens: some (gp 120/gp41/gp36/p24) immobilized at the bottom of microtiter wells, and others (gp120/gp41/gp36/p24) coupled with horseradish peroxidase (HRP) as conjugate solution.

Test Procedure

- 50 µl of Positive Control was added to wells B1, C1 and D1. 50µL of Negative Control was to the wells E1, F1 and G1 respectively. Well A1 was treated as blank.
- 50 µl of sample was added starting from well H1 .
- 100 µl of enzyme conjugate working solution was added into all the wells except for blank.
- Cover seal was applied and incubated for 37°C for 60 min.
- Wells were washed well with working wash solution according to the wash procedure.
- 50 µl of color A and 50 µl of color B was added to each well.
- Cover seal was applied and incubated for 37°C for 20 min
- 50 µl of 2 M sulfuric acid was added to all the wells and the absorbance was read at 450 nm or 450 and 630 nm as reference within 30 minutes in ELISA reader.

Validity Criteria

Blank must be $\leq$ 0.100

Negative control must be $\leq$ 0.100

Positive control must be $\geq$ 0.500

Calculation of results

Cut off value = Mean of positive control x 0.1 + 0.050

HBV ELISA

I.HBsAg ELISA

Hepalisa

HEPALISA is a solid phase enzyme linked immunosorbent assay based on the "Direct Sandwich" principle. The microwells are coated with Monoclonal antibodies with high reactivity for HBsAg.

Test Procedure

- 100 µl of Negative Control was added to wells B1 and C1. 100µL of Positive Control was to the wells D1, E1 and F1 respectively. Well A1 was treated as blank.
- 100 µl of sample was added in each well starting from G1
- Cover seal was applied and incubated for 37°C for 30 min.
- Wells were washed well with working wash solution according to the wash procedure.
- 100 µl of working enzyme conjugate was added to all the wells except A1.
- Cover seal was applied and incubated for 37°C for 30 min.
- Wells were washed well with working wash solution according to the wash procedure.
- 100 µl of working substrate solution was added to all the wells including A1
- The microtitre plate was incubated at room temperature (20-30°C) for 30 min in dark.
- 50 µl of stop solution was added and the absorbance was read at 450 nm within 30 minutes in ELISA reader after blanking A1(use of a reference filter of 630nm is preferred).

Validity Criteria

Blank must be < 0.100

Negative control must be 0.005 < NC< 0.150

Positive control must be ≥ 0.5

Calculation of results

Cut off value = Mean of positive control x 0.1 + 0.050

II.HBeAg ELISA

The assay is direct, non competitive test based on the use of polystyrene microwells coated with mouse monoclonal antibodies to Hepatitis B e antigen.

Test Procedure

- 50 µl of incubation buffer was added to all the wells, except for the blank well. Dispense reagents into the strip wells according to the following scheme, leaving an empty well for the blank.

Reagents	No of Replicates	Volume
Calibrator	3	100 µl
Negative control	1	100 µl
Positive control	1	100 µl
Samples	1	100 µl

- The plate was incubated for 2 hours at 37° C
- Wells were washed well with working wash solution according to the wash procedure.

- 100 µl of working enzyme tracer was added to all the wells, except A1
- The plate was incubated for 1 hour at 37° C
- Wells were washed well with working wash solution according to the wash procedure.
- 100 µl of working substrate solution was added to all the wells including A1
- The microtitre plate was incubated at room temperature (20-30°C) for 30 min in dark.
- 100 µl of stop solution was added and the absorbance was read at 450 nm within 30 minutes in ELISA (use of a reference filter of 630nm is preferred).

Validity Criteria

Blank must be $0.000 \leq BLK \leq 0.150$

Calibrator must be $-0.020 < \text{Cal mean} < 0.120$

Negative control must be $-0.020 < NC < 0.120$

Positive control must be $0.500 < PC < 2.500$

$PC - NC > 0.450$

Calculation of results

Cut- off value = Cal mean + 0.060

III. ANTI HBS ELISA

It is quantitative\ qualitative ,direct, non competitive, sandwich assay. Wells are coated with human HBsAg to detect anti HBs from sample or calibrator. Enzyme tracer is human HBsAg conjugated to horseradish peroxidase (HRP).The presence of anti HBs allows the enzyme tracer to bind to the solid phase. The enzyme activity is therefore proportional to the concentration of anti HBs present in the samples or calibrators.

Test procedure

- 100 µl of incubation buffer was added into all wells except for blank A1.
- 100 µl of Negative control was added to the well B1 and 100 µl of Calibrators 1, 2, 3, 4 were added to wells C1, D1, E1 and F1.
- 100 µl of sample was added starting from G1.
- Cover seal was applied and incubated for 37°C for 2 hrs ± 10 min.
- Wells were washed well with working wash solution according to the wash procedure
- 100 µl of working enzyme tracer was added to all the wells, except A1.
- Cover seal was applied and incubated for 37°C for 1 hr ± 5 min.
- Wells were washed well with working wash solution according to the wash procedure

- 100 µl of working substrate solution was added to all the wells including A1
- The microtitre plate was incubated at room temperature (20-30°C) for 30 ± 2 min in dark.
- 200 µl of blocking reagent was added to all the wells and the absorbance was read at 405/ 630 nm within 30 minutes in ELISA (use of a reference filter of 630nm is preferred).

Validity Criteria

Negative control must be NC < 0.100

Calibrator 1 must range between $0.035 \leq$ Cal 1 ≤ 0.300

The ratio of Calibrator 1 to negative control must be Cal 1 / NC ≥ 2.0

The ratio of Calibrator 4 to Calibrator 3 must be Cal 4 / Cal 3 ≥ 1.2

Calculation of results

The absorbance value at 405 / 630 nm for each calibrator were ploted in log- log

co-ordinates on the ordinate (Y axis) as a function of the concentration of anti- HBs antibody expressed as IU/L on the abscissa (X axis).

A calibration curve is obtained. Directly from the calibration curve, read the anti- HBs concentration of each sample expressed as IU/L.

HCV ELISA

Zhongshan HCV

It is a third generation enzyme immunoassay for the qualitative determination of antibody to hepatitis C virus in human serum/plasma.

Test procedure

- 100 µl of the sample diluent was added to wells A1 and B1 as blank control.
- 100 µl of Negative Control was added to wells C1 and D1 . 100µL of Positive Control was to the wells E1 and F1 respectively.
- 100 µl of sample diluent was added in all the wells starting from G1 followed by the addition of 10 µl of sample from G1.
- Cover seal was applied and incubated for 37°C for 30 min.
- Wells were washed well with working wash solution according to the wash procedure
- 50 µl of conjugate was added to all the wells except for blank control.
- Cover seal was applied and incubated for 37°C for 20 min.
- Wells were washed well with working wash solution according to the wash procedure.
- 50 µl of substrate solution A and 50 µl of substrate solution B is added to each well, including blank.
- Cover seal was applied and incubated for 37°C for 10 min in dark.

- 50 μl of stop solution is added to all the wells including blank control.
- The absorbance was determined for each well at 450 nm with ELISA reader.

Validity Criteria

Blank must be < 0.100

Negative control must be ≤ 0.100

Positive control must be ≥ 0.600

Difference between the mean of positive and negative control must be ≥ 0.500

Calculation of results

Cut off value = 0.15 + mean absorbance of negative control.

DETERMINATION OF CD4 / CD8 COUNT BY FLOW CYTOMETRY

CD4 and CD8 counts were determined with the help FACS count system (Becton Dickinson Immunocytometry systems).

Control runs were set up each time when the instrument is run, or when a new lot of reagents were used.

One reagent tube pair was used for each patient.

TEST PROCEDURE

- One reagent tube pair is labeled with patient accession number or number that identifies the tube of blood.
- The pair was vortexed upside down for 5 secs and upright for 5 secs.
- The reagent tube pair was opened with the coring station.
- The patients whole blood sample was mixed well by inverting the tube 5 times.
- 50 µl of the whole blood was pipetted into each of the two reagent tubes. The pipettes were changed between tubes.
- The tubes were capped and vortexed upright for 5 secs.
- The tubes were incubated for 60 to 120 minutes at room temperature (20 to 25 ° C).
- The tubes were then uncapped, and 50 µl of fixative solution was added into each of the two reagent tubes. The pipettes were changed between tubes.
- The reagent tubes were recapped with new caps and vortexed upright for 5 secs.
- The tubes were run on the FACS count instrument and CD4 / CD8 results were obtained.

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