2. REVIEW OF LITERATURE

2.1 Viral Hepatitis

In the 5th century BC in Greece “epidemic- Jaundice” was the term used by Hippocrates to describe disease resembling outbreaks of viral hepatitis. Any inflammation of the liver is called hepatitis. (Regev and Schiff et al., 2000) Liver inflammation (hepatitis) can be caused by bacteria, parasites, fungal and viral infection, and chemical agents including drugs, toxins and alcohol. (Schiff et al., 2004)

Eleven viruses has been recognized as causing hepatitis. Hepatotrophic viruses are nine and two herpes viruses (Cytomegalovirus and Epstein- Barr virus) Of the nine hepatotrophic viruses, five are well characterized. Hepatitis A and hepatitis E are transmitted by faecal- oral contamination. Other major types include hepatitis B (Serum hepatitis), hepatitis C (formerly non-A, non-B hepatitis) and hepatitis D (formerly delta hepatitis). Hepatitis G and TTV (Transfusion Transmission Virus) is newly discovered virus. (Prescott L.M et al., 2005)

2.1.1 Hepatitis B Virus Morphology and Replication

Until the late 19th century, Infectious nature of the disease and the liver involvement were not recognized. The term epidemic jaundice was first described by Babylonians. In 1885, Lurmann observed post small pox vaccination led to the outbreak of jaundice. This was recognized to be caused by hepatitis B as the human lymph was used for the preparation of vaccine. Thus, there was a transmission of hepatitis B by human serum called homologous serum hepatitis. (Topley & Wilson, 10th edition)

In 1965, there was an accidentally discovery of surface protein of HBV by an anthropologist B S Blumberg. Australian antigen was identified during the discovery of polymorphic serum proteins as genetic marker in the blood of Australian aborigine (Blumberg et al. 1965) Blumberg et al. 1967 recognized the association between serum hepatitis and occurrence of Australian antigen

Classification

HBV virus was discovered in the eastern woodchuck (a marmot of the North American east coast) after the observation of liver carcinoma on them. This virus was closely related to virus affecting chimpanzees, ground squirrel hepatitis virus, gibbons, wood chunk hepatitis B virus gorillas, woolly monkeys, orang-utans, gray herons Heron hepatitis B virus (HHBV), peking ducks, (Duck hepatitis B
virus (DHBV) and other water fowls. All these hepatitis viruses are species specific. Some of them were used as experimental model for elucidating pathogenesis and replication of HBV. (Prescott L.M et al., 2005) Due to their biological and molecular similarities these viruses were defined under a common virus family hepadnaviridae. (Manson et al, 2005)

**Structure of HBV**

HBV is a DNA virus belongs to the family Hepadnaviridae, genus Orthohepadnavirus with an outer envelope (42nm) and an inner core (27nm). This envelope encloses DNA polymerase and viral genome. Three types of particles are visualized under the electron microscope. They are spherical forms, tubular or filamentous forms and complete form or Dane particles. (Jawetz et al., 2007)

Spherical forms: Spherical particle (22 nm) is more abundant forms exclusively made up of HBsAg.

Tubular or filamentous forms: Filamentous or tubular (22nm) are the second type. Spherical forms and tubular forms are antigenically identical and both are responsible for production of HBsAg in great excess.

Complete form or Dane particles: are the third types of particle. They are double- walled spherical structure, (42nm) called complete HBV- dane particle. The Dane particle is made up of outer surface envelope (Hepatitis B surface antigen) and inner nucleocapsid. Nucleocapsid (27 nm) encloses precore antigen, core antigen and partially dsDNA. (Apurba et al., 2019)

**Viral antigens**

Virulence of the organism is strongly associated with its antigens- HBsAg, HBcAg and HBeAg.

HBsAg: Hepatitis B surface Antigen is antigenically complex. It contains two components- (i) reactive antigen ‘a’ epitope- common group and (ii) type specific antigens d/y and w/r. Four subtypes of HBsAg have been observed: adw, ayw, adr and ayr; Only one member of each pair being present at a time. (Apurba et al., 2019)

Hepatitis B core Antigen (HBcAg): This forms the intracellular protein enclosed with in the nucleocapsid. (Ananthanarayan & Paniker, 9th edition; Apurba et al., 2019)
Hepatitis B precore antigen (HBeAg): It is a soluble non-particulate nucleocapsid protein. (Ananthanarayan & Paniker, 9th edition)

Typing of HBV

a) Serotypes

HBV shows a distinct geographical distribution. Dominant ‘a’ antigen being shared by all have less importance in immunity. Subtype ayw is common in Northern India; West Asia through the Middle East. In Europe, Australia and the America adw is common. In Far East, South and East India adr is common. ayr is very rare. (Ananthanarayan & Paniker, 9th edition)

b) Genotypes

Well characterized eight major genotypes (A-H) of HBV are accepted in circulation, worldwide including two additional genotypes. (I and J) (Huy et al, 2008; Tatematsu et al, 2009) HBV genotypes A and D are common in India. (Vivekanandan et al. 2004; Gandhe et al. 2003) The genome has a compact structure with four overlapping genes. They are S gene, C gene, P gene and X gene.

Hepatitis B Virus Mutants

Emergence of mutant strains of HBV has been documented. Mutations in various genes of HBV are Pre-core mutants, Escape mutants and YMDD mutation.

Stability of HBV

HBV is a highly susceptible virus. Infectivity can be lost by dry heat at 160°C for 1 hour and autoclaving at 121°C for 20 minutes. (Robinson et al., 1995) HBV can be inactivated by 70% isopropyl alcohol, 80% ethyl alcohol for 2 minutes, a combined β-propiolacton and UV irradiation, 2% aqueous glutaraldehyde for 5 minutes. Antigenicity is lost after exposure to 0.25% sodium hypochlorite for 3 minutes. The virus retains infectivity when stored at 30°C - 32°C. In dried blood, HBV can withstand for a week and it can be stored for 15 years at -15°C. (Robinson et al., 1995)

Replication of HBV

The genomic replication of hepatitis B among the hepadnavirus is quite different. The replication of the DNA present as circular dsDNA genomes takes place with the help of reverse transcriptase
enzyme. The DNA gets released in the nucleus once it infects the cell. The pregenome of large kilobase of RNA are produced in the nucleus by transcription using the host RNA polymerase. The viral proteins-core protein are produced in the cytoplasm as the RNA moves into them. The RNAs are translated to produce polymerase have 3 activities (DNA polymerase, Reverse transcriptase, RNase H). Immature core proteins are produced by association of RNA pregenome with core protein and DNA polymerase. RNA with the help of protein primer transcribes itself in the presence of reverse transcriptase into a DNA copy of the pregenome RNA. The RNAaseH degrades the pregenome RNA. The fragments of RNA serve as primer for DNA polymerase. This fragments forms the minus DNA and dsDNA genomes and thus the nucleocapsid is completed. (Prescott LM et al., 2005)

2.1.2 Epidemiology of HBV

Two billions of global population suffer from HBV infection and nearly 450 million people were chronically infected by 2002. (WHO, 2002) nearly 1 million deaths occur every year due to HBV complications globally. More documented in Asia, Africa and West Pacific regions. (Liberek et al, 2007)

High Endemicity

Hepatitis B is highly endemic in South East Asia, China, Sub-Saharan Africa and Amazon Basin regions. Here 8% of them are CHB carrier. 70-95% of the population have the evidence of acute or chronic HBV infection. (Hou et al, 2005)

Intermediate Endemicity

The Middle East, Japan, part of South American regions and Eastern and South Europe document 10-60% of infection indicating the moderate endemicity of HBV infection. Among them 2-7% are chronic carrier. (Hou et al, 2005)

Low Endemicity

Low endemic of HBV infection was seen among 5-7% of the population in North America, North and Western Europe, and Australia. Upto 0.5-2% among them were chronic carriers. (Mc Quillan et al, 1989)
Transmission of HBV

Transmission occurs perinatal, sexual, parenteral (blood transfusion), percutaneous (drug users and unsterile needles) and body secretions like semen, breast milk, urine, faeces, sweat and saliva. (Prescott L.M et al., 2005) No transmission of virus is possible through food, water, insects, vectors and aersols. (Hou et al, 2005)

Perinatal transmission

Vertical transmission is possible by three routes postnatal transmission through breast milk, transplacental transmission of HBV in utero and natal transmission during delivery. (Hou et al, 2005)

Sexual transmission

Globally sexual transmission is a major source of HBV infection. Documentation of sexual transmission is higher in low endemic areas. Higher risks of infections are among the homosexual men than the heterosexual transmission. (Alter et al., 2003) The risk of infections depends upon the number of sex partners and duration of sexual activity. (Alter et al., 1994)

Parenteral Transmission

The parenteral transmission includes household contact, transfusion, dialysis, acupuncture, injection drug use, health care setting, tattooing and ear piercing. (Schiff et al., 2004)

2.1.3 Risk factors and clinical features

High risk of infections is seen among patients on haemodialysis and frequent transfusions, intravenous drug users, those who come in contact with infected blood products and sexual contact with an infected person. (Hou et al., 2005) In hospital settings, physicians, nurses and healthcare workers are at high risk. Reuse of disposable needles, improper sterilization of needles may be the possible modes of transmission. (Alter et al., 2003)

Clinical signs of HBV

Hepatitis B infections may be asymptomatic or symptomatic following one to three months of incubation period. History of fever, abdominal discomfort, fatigue, nausea, loss of appetite other symptoms appears. The virus infects hepatic cells and cause tissue degeneration with release of
associated enzymes like liver transaminases into blood streams. The liver damage causes accumulation of bilirubin in skin and tissues and results in jaundice. Chronic HBV may develop hepatocellular carcinoma. (Prescott L.M et al., 2005)

**Pathogenesis of HBV**

HBV carriers have minimal liver injury and are asymptomatic. These carriers have extensive and intrahepatic replication of viruses which are not directly cytotoxic to cells. Hence they are asymptomatic. Pathogenesis of HBV and its immune response are incompletely understood. Major histocompatibility complex (MHC) complex class II restricted with CD4+ helper T cells and class I restricted with CD8+ cytotoxic T lymphocytes involve in immune response. In acute infection antiviral cytotoxic T-lymphocyte are directed against multiple epitopes with in the polymerase, HBV core, and envelope proteins. In case of carriers of HBV infection, there is an increased in viral specific T cell response and can be assayed from peripheral blood. In both the condition the antibody (anti HBs) responses are vigorous, but the antibodies are not detectable in carriers due to the excess of circulating HBsAg. Thus the cytotoxic T lymphocytes in the immune response of HBV infection play a vital and central role in viral clearance. (Chisari FV et al., 1995; Chisari FV et al., 1996)

**Primary infection**

After an incubation period of 4 to 10 weeks HBsAg becomes detectable in the blood in case of primary infection. This is followed by anti- HBc antibodies against the HBV core antigen. The IgM isotype indicates the early infection. (Hoofnagle JH, 1995) HBsAg is detected as the viremia is established in acute infection. A very high titer of viruses- $10^9$ to $10^{10}$ virions per millilitre are detected during acute infection. (Ribeiro RM, 2002) HBeAg may be detected in primary infection. (Kajino K, 1994)

HBV induced hepatocellular damage does not increase alanine aminotransferase level until it is a well-established infection. It is a T cell mediated immune response that causes hepatocellular damage. Virus titre in the blood and hepatocytes begins to drop during the above changes. Due to the non cytolytic clearance mechanism, infections can be cleared from all the hepatocytes without hepatic destructions. Due to the clearance of infection HBsAg and HBeAg disappears and free anti HBs becomes detectable from the circulation. HBV is a self-limited infection which is defined by disappearance of the viral
antigens and appearance of antibody to HBs. However, low levels of HBV DNA may persist for many years in the blood. (Prince AM, 2001).

**Persistent infection**

Acute infection is usually a self-limiting infection unlike persistent HBV infection where the continuous virus production and HBsAg remains in the blood stream lifelong. The levels of viremia are generally low in case of chronic infections. Presence of HBeAg is usually presented with high titer of HBV (10^7 to 10^9 virions per millilitre) in the blood, indicating active viral replication and highly infectious status of the patients. In persistently infected people 5 to 10% per year have a tendency for seroconversion and positive for antiHBe antibodies. In them HBeAg starts to disappear from the blood. The immune mediated destruction of the infected hepatocytes leads to transient rise in the levels of alanine aminotransferase along with the disappearance of HBeAg. The ongoing immune attack is the natural process of HBV persistence in the infected liver cells. An inadequate immune response eradicates the infection which reduces the number of infected cells and lowers the circulating viral load. This is the reason for low levels of virimea in chronic infections in compared with the acute infection or primary infection. (Prescott)

**2.1.4 Diagnosis and detection of HBV**

The diagnosis of HBV infection is based on the detection of serological and biochemical markers. The biochemical markers for assessment of liver functions are aspartate aminotransferase (AST), alanine aminotransferase (ALT) and total and direct bilirubin (TSB). (Keeffe et al., 2006).

**2.1.5 Seromarkers**

Serological methods for detecting HBV infection are done by ELISA method. HBV antigens and antibody detection helps us to classify the patient as acute or chronically infected patients. (Jawetz et al., 2007)

**Detection of HBV Antigens**

Detection of HBsAg

The first serological marker to appear in the blood after infection is Hepatitis B surface Antigen. If they persist more than 6 months and then it indicates CHB infection. (Keeffe et al., 2006) The
appearance of HBsAg in the blood takes place at an average of 6-8 weeks, after the exposure to HBV. ALT becomes abnormal. Jaundice sets in within 3-5 weeks of infection. (Alter, 2003)

Detection of HBeAg

Active viral replication is detected by the presence of HBeAg in the serum. Absence of HBeAg does not equate to absence of replication. In few cases HBeAg are undetectable, in such cases infection may be with HBV negative for HBeAg. Most of the HBV infection HBsAg and HBeAg appears simultaneously or few days later. (Keeffe et al., 2006)

Detection of HBcAg

The highly intracellular antigen is Hepatitis B core antigen. They are expressed only in infected hepatocytes hence not detectable in serum (Lin and Kirchner, 2004).

Detection of HBV Antibodies

Anti-HBs Antibodies

HBsAg is replaced by antiHBs as acute infection is resolving. In 80% of the patients antiHBs persist, indicating life time immunity. (Hollinger and Liang et al., 2001) Occasionally, in chronically infected patients, both HBsAg and antiHBs are detectable but the significance of this finding is not known. (Keeffe et al, 2006)

Anti- HBc Antibodies

The Presence of anti- HBc in serum, the first antibody to appear indicates current or past HBV infection. In acute infection, high titers Anti- HBc IgM are present and they disappear within 6 months. Even in some cases of chronic infection, IgM antiHBc persist. (Hollinger and Liang, 2001) At the onset of illness high level of IgM specific antiHBc may be detected. The appearance of this antibody indicates viral replication as well as antibody against internal core component 27nm of hepatitis B virus. antiHBc IgG persist lifelong in patients recovered from acute HB infection and detected after 6 months of infection. To screen the population at risk HBsAg, antiHBs and antiHBc Total were screened. (Jawetz et al., 2001; Schiff et al., 2004)
Anti-HBe Antibodies

Anti HBe is associated with low risk for disease progression. However it is not protective against latter development of HCC. Anti HBe indicates chronic HBV infection and the onset of non-replicative phase. The seroconversion of HBeAg to AntiHBe usually considers as the end point for HBV therapy patients. (Keeffe et al., 2006)

2.1.6 Prophylaxis

Preventive and control measures against HBV infection are as follows: i) avoiding contact with infected blood and secretions. ii) minimizing needle stick injuries. iii) active prophylaxis with two recombinant vaccines. iv) passive prophylaxis- Hepatitis B immunoglobulin (within 7 days)

HBV carriers should be instructed properly to keep the blood loss under the control. If contaminated they have to disinfect along with regular washing. They should avoid injuries as far as possible. Transmission during the surgeries from highly viremic carriers is also documented. HBsAg screening during the blood and plasma donation is a must as minimal requirements among the donors. Vaccination of children worldwide is recommended by WHO during the first year of life. If there is a possibility of perinatal transmission, vaccination soon after the birth is mandatory

Treatment

Immune modulators

Seroconversion from HBeAg to antiHBe is in many cases induced by interferon which are used for the treatment of chronic hepatitis B patients. Both the interferon α and β used in the treatment as they induce sustained cessation of viral replication, patient respond with good clinical prognosis. Careful usage of interferon is a must as they are harmful in patients with cirrhosis and hence not recommended in late phase of chronic liver diseases.

Immune therapy

Vaccines with preS1-, preS2-, and S, are capable of inducing a Th2 response against the HBV antigens. They donot reduce viremia. A protective Th1 and antiHBs response is effectively produced by the DNA vaccine among the healthy humans.
Antiviral substances

Toxic nucleoside analogues were available in early 1980s and 1990s. To improve HBV-associated liver diseases and effective in reduction in viremia are possible by oral lamivudine. The development of resistance is the disadvantage and the limiting factor for use of lamivudine as a permanent therapy (Bonino et al. 2003) Adefovir is a nucleotide analogue used in chronic hepatitis B infection both in active HBeAg sero positive and seronegative conditions. (Marcellin et al. 2003) (Hadziyannis et al. 2003) Lamivudine- resistant HBV infections are effectively treated with Entecavir but possibilities of development of resistance is always anticipated. (Tenney and Levine et al. 2004)

2.2 Human leukocyte Antigen System (HLA)

HLA is the major histocompatibility complex (MHC) in humans. The antigen was first identified and categorized using alloantibodies against leukocytes. (Terasaki et al., 1990) In the human genome it has most significant region with respect to autoimmunity, inflammation and infection. (Kaufman et al., 1999; Tamori et al., 2013). HLA antigens are found on the surface of the cells. They are the proteins defense against the infectious agents. HLA is a protein which can present self and non self peptides to the receptors on T cells. This helps them to sustain as well as adapt the immunity and self-tolerance. These HLA proteins are located on the short arm of chromosome 6. It measures 3.6MB in length. And more than 200 genes both functional and non-functional genes located in this region. (Complete et al., 1999; Shinna et al., 1999)

The HLA Antigens

The complete HLA region has 3 subgroups designated as class I, II, and III. The HLA class I region have 12 non coding genes, 3 classical and 3 non classical genes, together 19 HLA class I gene. Class II region consist of alpha and beta chains of HLA classical class II DR, DQ and DP. Both HLA class I and II molecules presents antigenic peptides to T cells. But each protein binds to a different range of peptides. The IPD-IMGT/HLA database release 3.31.0 in January 2018 released a total of 17,695 HLA alleles (4,802 alleles in class II and 12,893 alleles in class I) Among 4,802 class II alleles, 2,146, 1,178, and 965 alleles were recorded in HLA-DRB1, -DQB1, and -DPB1 genes. Among the 12,893 class I alleles, 4,181, 4,950, and 3,685 alleles were recorded in HLA-A, -B, an-C genes, respectively. HLA Class III region is responsible for intracellular peptide processing, hormones, complement pathway, and other evolving characteristics. (Jiang et al., 2013; Li et al., 2012)
2.2.1 Structure of HLA Class II molecule

Class II region comprises a series of sub regions, all enclosing A and B genes that encodes α and β chains (Marsh et al., 2005) which both span the plasma membrane. Both the chain forms two domains (α1 and β1). The peptide binding groove is formed in the middle of the two domains. The DR gene family contains nine DRB genes (DRB1 to DRB9) and a single DRA gene. The HLA-DR antigen specificities (i.e., DR1 to DR18) are determined by the polymorphic DRβ1 chains which are encoded by DRB1 alleles. Each DQ and DP families have an unexpressed pseudogenes and one expressed gene for α and β chains. DQ molecules are formed by the DQB1 and DQA1 gene products. The DPA1 and DPB1 gene products associate to form DP molecules. (Beck et al., 2000) The dissemination of HLA Class II antigens is restricted to the “immune competent” cells, including macrophages, endothelial cells, activated T- lymphocytes and B- lymphocytes. Helper (CD4) T cells is linked with HLA Class II molecules.

It is located centromeric to the class I region and is 800kb long. The encoding genes of α- and β-chain have a similar structures, each one with six exons corresponding to the two extracellular domains, the signal sequence, the cytoplasmic tail, the transmembrane region, and the 3’ untranslated region. The DRA1 and DRB1 loci, which encode α- and β- chain of the DR molecule is the most telomeric. The gene for the DQA1 and DQB1, which encode α- and β- chain of the DQ molecule are situated centromeric (50 to 100kb) to the DR- gene. The DPA1 and DPB1 loci, which encode α- and β- chain of the DP molecule, are positioned 500 kb centromeric to the DRB genes. Other genes situated on the class II region are LMP, HLA- DM, TAP1 and TAP2 which encodes proteins that are involved in peptide loading and presentation. (Brown et al., 1993; Engelhard et al., 1994)

HLA-DQB1, DPB belongs to the HLA class II beta chain paralogs. This molecule is a heterodimer. DQB consists of an alpha (DQA) and a beta chain (DQB). DPB consists of an alpha (DPA) and a beta chain (DPB). It contains six exons and the beta chain is approximately 26-28 kDa. Exon 1 encodes the leader peptide, exons 2 and 3 encode the two extracellular domains, exon 4 encodes the transmembrane domain and exon 5 encodes the cytoplasmic tail. Both the alpha chain and the beta chain of DQ molecule contain the polymorphisms specifying the peptide binding specificities, resulting in up to four different molecules.
Presence of multiple allelic variants of a gene that allows genetic variants to exist in a population is called Polymorphism. A polymorphic gene or locus has a high frequency of genetic variants. HLA genes are the most polymorphic genes in humans. Over centuries, evolutionary pressure resulted in generation of this polymorphism. (Bjorkman, et al., 1987; Klein et al., 2000) Limited polymorphism makes an entire population becomes susceptible to infectious agent for whom all individuals would be unable to respond, while widespread polymorphism allows least proportion of the population to bind and present antigens derived from invading pathogens. (Sita Naik et al., 2003)

**HLA class II genes association with HBV across the world**

<table>
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<th>OR</th>
<th>p value</th>
<th>Country</th>
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HLA DRB1*07:01, -DQB1*03:01 and -DPB1*09:01 associations in various diseases across the world

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<td>Allergic fungal sinusitis</td>
<td>8.22</td>
<td>0.01</td>
<td>USA</td>
<td>Mark S et al, 2004</td>
</tr>
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</table>
2.2.2 Molecular Genetic Techniques

Polymerase Chain Reaction

The Polymerase Chain Reaction (PCR) is a recently developed and revolutionary new system for investigating the DNA nucleotide sequence of a particular region of interest in any individual. (Michael et al., 1995) The first step in this technique is to obtain DNA from the nuclei of an individual. The double stranded DNA is then denatured by heat into single stranded DNA. Oligonucleotide primer sequences are then closed to flank a region of interest. The oligonucleotide primer is a short segment of complementary DNA to act as a starting point for reconstruction of double stranded DNA at that site. From one copy of DNA it is thus possible to make two. These two copies can then, in turn, are denatured, reassociate with primers and produce four copies. This cycle can then be repeated until there is a sufficient copy of the selected portion of DNA to isolate on a gel. (Shankarakumar et al., 2004)

Sequence Specific Priming (SSP)

The oligonucleotide primers were used to start the PCR have sequences complementary to known sequences which are characteristic to certain HLA specificities. For example, the primers, which are specific to HLA DR15, will not be able to instigate the PCR for HLA-DR17. (Shankarakumar et al., 2004)