Chapter 1

Introduction

Hepatitis B Virus (HBV) is transmitted by blood and is considered a major challenge for blood safety because of associated morbidity and mortality. Transfusion-associated HBV continues to be a major problem in India, and more so in patients receiving repeated transfusions. Hepatitis B surface antigen (HBsAg) is mandatory HBV screening in India (Chaudhuri et al., 2003). It has been proved that some HBsAg non reactive but anti-HB core antibody (Total; Anti-HBcAb) reactive individuals continue to replicate HBV (Yotsuyanagi et al., 2001). Therefore, the non reactive result of HBsAg in the blood can not reflect that the person is completely free from HBV. Blood containing Anti-HBcAb with or without detectable presence of HBsAg might be infectious.

Blumberg et al. (1965) First revealed serum protein like the lipoprotein antigen. But soon the evidence accumulated that it might have something to do with hepatitis. Parallel to that, (Prince, 1967) was specifically looking for a “serum hepatitis (SH) antigen” in the blood of hepatitis B patients and reported it in 1968, but soon he realized that it was identical to australia antigen. Subsequently, various groups confirmed that austrelia antigen or serum hepatitis antigen (Au/SH-Ag) was truly a marker for acute or chronic hepatitis B and that there were apparently healthy Austrelia antigen or serum hepatitis antigen (Au/SHAg) carriers.

In 1992, Anti-HBcAb was introduced in the screening process in USA. It is now required by USFDA as an additional screen for HBV (AABB 17th Ed). However, in India Anti-HBcAb is an optional investigation but not mandatory. Window period has been a challenging situation that has proposed threat to blood safety. Therefore, screening methods should focus on reducing window period and enhance blood safety. However, this increases the requirement of resources. Recently, Nucleic Acid Test (NAT) is one such methodology which is gaining wide acceptance in India. This test has been approved for screening of blood donors but is not yet mandatory in India.

The permutation combination of use of HBsAg (mandatory blood screening investigation), Anti-HBcAb and NAT (optional blood screening investigation) for HBV screening may offer a new opportunity to enhance blood safety and blood screening programmes. However, the feasibility of implementing as policy should be considered based on pathogenicity, morbidity, mortality, endemicity, ethics and available resources since the requirements for infrastructure, financing, staffing levels, training and quality systems and the overall costs of implementation may far outweigh any potential benefit in terms of increased blood safety. The present study was carried out to study the role of anti- HBc antibody and HBV NAT in blood screening.

Screening of HBsAg and HBV DNA by NAT would appear preeminent for countries with a normal to high prevalence of HbsAg and on the other hand, HBV DNA by NAT and anti-HBc testing may be superior to HBV DNA by NAT and HBsAg testing for low prevalence countries. Early detection of pathogens probably could reduce transmission of transfusion transmitted infections like hepatitis B virus for the safety of blood products in the future (Klein, 2005; Snyder, and Dodd, 2001).

In India, screening for antibody to HIV 1 and 2, HCV and HBsAg is mandatory by law. However, the screening for Anti HBV core antibodies IgM and IgG is employed by various blood banks to decrease the transmission of HBV in case of HBsAg negative blood.

Nowadays the use of nucleic acid testing (NAT) has been implemented to detect the manifestation of occult hepatitis B virus in blood donors which is measured a potential risk for transfusion of hepatitis B virus (Chaudhuri et al. 2003). However, the use of NAT to avoid transfusion of hepatitis B virus may use a new opportunity to blood screening method, the viability for applying ID- NAT, should be considered for early detection of HBV for reduced window period. ID-NAT sreening implementation may far compensate any potential benefit in terms of increased blood safety. The present study has been carried out to find the correlation with HBcAb.

1.1 Relation to the Post Transfusion Hepatitis by HBV through Blood Transfusion

Relation to the post transfusion hepatitis by hepatitis B virus was first describd by Blumberg. He believed that Australia antigen was indeed a polymorphic serum protein like the lipoprotein antigen which he had discovered before. But soon evidence accumulated that it might have something to do with hepatitis, which Blumberg first revealed in a publication in 1967 (Blumberg et al. 1965). Similar to that, Alfred Prince (New York Blood Center) was specifically looking for a "serum hepatitis (SH) antigen" in the blood of hepatitis B patients and reported on it in 1968, but soon realized that it was identical to Australia antigen AuAg (Prince AM, 1968). Subsequently, various groups confirmed that Au/SH-Ag was actually a marker for acute or chronic hepatitis B and that there were apparently healthy Au/SHAg carriers. Post Transfusion Hepatitis (PTH) is a major concern of blood safety. Although, incidence of PTH has declined significantly, but the management of PTH still remains an expensive affair.

The risk of hepatitis virus transmission from transfusions has reduced significantly from that of the 1940s when PTH first came to light. PTH was first reported in the US by Beeson in 1943. Beginning of hepatitis B surface antigen screening and change to volunteer donors for whole-blood donations in the late 1960s and early 1970s led to an extensive reduction in PTH cases. However, up to 10% of the recipients continued to develop PTH, most cases of which were found as unknown non-A, non-B viral agent. The cost of each life- year gained was $9010, that is nearly 20.5 times that of each Quality Adjusted Life Years (QALY) gained ($5769), which is 13.1 times the annual per capita Gross National Product (GNP) of the Indian population (Aggarwal et al., 2002).

In developing countries such as India, marginal cost effectiveness and cost utility of therapy were compared adversely with annual per capita income. Therefore, HBV screening remains an essential investigation to enhance blood safety across the globe. Contributory reason for this can be an underlying fact that NAT has been developed in countries where more focus lies on HIV prevention, and have lower hepatitis B prevalence (France et al., 2012).

Although NAT screening for HBV is available in USA for more than a decade, USFDA has not yet made the test mandatory. This is because of the fact, that there is no clear cut evidence to prove, that NAT implementation can reduce significant HBV transmission by blood transfusion (AABB 17th Ed). Studies have not been able to clearly state avoided HBV related morbidity and mortality (Velati et al., 2008).

On the other hand, it has commented on theoretical increased possibility of finding NAT reactive blood donors because of immunisation (AABB 17th Ed, Stramer et al., 2011). HBV vaccination can be picked up by sensitive HBsAg immunoassays as well as NAT (Dow et al., 2002, Stramer et al., 2011). After implementation of surrogate marker testing, Alanine Aminotransferase (ALT) and anti-hepatitis B virus core antigen (HBcAb) for remaining non-A, non-B hepatitis in the late 1980s decrease per unit risk of PTH from one out of 200 to about one out of 400.

Introducing the regularly improved antibody assays in the early 1990s reduced the risk of PTH due to hepatitis C virus to about one out of 0.1/million. Although some additional hepatitis virus also exists like hepatitis G virus, which appears to be less contributor to PTH, it has been almost eradicated. Early history of PTH during World War II and the immediate postwar period, the demand for blood and blood components in the US increased substantially.

However, increased new setup of blood banks, transfusion services, and other blood collection and laboratory support services, the technology for blood collection, processing, and storage of whole blood and blood components materialized speedily, could not stop transmission of HBV virus. Grady chalmers (1964) reported the results of a retrospective study of PTH in nine Boston teaching hospitals from 1952 to 1962. It was found that one of the hospitals provides 29% of the blood transfused was from paid donors as commercial sources. At the same time as in the other eight hospitals were taking blood from volunteers (Free) blood donors was transfused. They found the incidence of symptomatic or icteric Post Transfusion Hepatitis in recipients of blood products from volunteer blood donors was 0.6 cases per 1000 units compared with 2.8 cases per 1000 units in recipients of blood or blood products from a mixture of volunteer and commercial blood donors.

Walsh et al., (1970) reported the incidence of icteric and anicteric hepatitis in patients undergoing open-heart surgery in those patients who were given blood from either commercial or volunteer blood donors during the surgery. They found icteric and anicteric hepatitis developed in about 51% of the recipients of transfused blood from commercial donors, whereas no hepatitis was found in patients who received blood from volunteer donors. They have estimated the hepatitis carrier rate for commercial blood donors to be 6.3% and for volunteer donors to be, 0.6%.

After 1971, some studies found an increased risk of post transfusion hepatitis from blood transfused by paid blood donor. More so, there were no clinical experts at that time and no controlling authorities as well. Role of hepatitis B virus (HBV) in PTH was long suspected. Australia antigen was the first marker that could be identified in the serum by Blumberg et al. (1965). Inflammation of the liver called hepatitis, through HBV infection has been demonstrated conclusively to be serious complication, sometimes fatal to blood transfusions when it was transferred on a large scale in the blood and plasma unscreened during World War II.

Even though this clinical recognition and little knowledge of the causative agent until the 1960s when the discovery was expected for antigen to be present in the serum of the indigenous population in Australia (Australia antigen) ultimately led to the relationship of this antigen to human hepatitis *(Blumberg et al. 1965).*

Australia antigen is a surface protein of the viral particles that were presented at a later time to be a virion hepatitis B. The name was changed to Australia antigen introduced on the Hepatitis Surface Antigen (HBsAg) and tests for the detection of this antigen in blood donors in the United States in 1970. In this antigen addition to the universal adoption of the system of volunteers from all donors, led to nearly 70% reduction in post transfusion hepatitis and reduced the total to more than 85% in transfusion-related hepatitis B infection *(Alter et el. 1997).*

After the development of tests for HBsAg detection, it became clear that the virus was not the main factor causing the disease from blood transfusion-associated hepatitis (TAH), and in addition to it, also accounted for at least 75% of *cases (Dienstag et al., 1977).*

Some other study observed that, non-B might be caused by the hepatitis A virus (HAV) and was considered so but later on, hepatitis B virus was the causative equal. Still, the HAV was discovered as also the causative agent when tracking cases of hepatitis-B in the future were tested in retrospect for seroconversion of antibodies to hepatitis A, it was confirmed that it had no concern with hepatitis B. Thus, it became known that there was another non- A non-B (NANB) also has been responsible for PTH. *Feinstone SM, et al. (1973).*

Choo et al. (1989) cloned the main causiative agent for hepatitis non A non B, disclosed the sequence of molecular and expressed epitopes that serve as a basis now for a very specific serological test usually used for screening blood donors. The term non A and non B and hepatitis C virus (HCV) was found responsible for at least 90% of cases of Transfusion Associated Hepatitis (Alter et al., 1995).

Hepatitis B virus carry on in about 5 to 10% of immunocompromized adults, and as many as 90% of infants are infected prenatally. Indomitable presence of hepatitis B, means the presence of hepatitis B surface antigen (HBsAg) in the serum for more than six months, they have been estimated to affect about 350 million people globally. The hepatitis is mediated by the cellular immune response of the host to the infected liver cells.

In chronic condition continuing virus replication may lead to progression to cirrhosis and hepatocellular carcinoma. In the first phase of persistent chronicity of hepatitis B virus, virus replication continues in the liver, and further replication of the viral genome may be detected in DNA extracted from the liver biopsies. Markers of virus replication in serum include HBV DNA, the S1 proteins (HBsAg) and a soluble antigen, hepatitis B *e* antigen (HBeAg) which is secreted by infected liver cells (hepatocytes).

In those cases infected at a very early, this phase may persist for life but some time virus levels decline usually. Ultimately, in most individuals, there is immune clearance of infected liver cells associated with seroconversion from HBeAg to anti-HBe. During the period of replication, the viral genome may join together into the

chromosomal DNA of some liver cells and these cells may persist and expand clonally.

1.2 Early recognition of viral hepatitis

The infectious nature of the disease had already been recognized in the early days of medical microbiology, in 1885 by Lürmann during an “icterus epidemic” which occurred after a small pox vaccination campaign. The vaccine had been made from human “lymph” (probably obtained from the vaccine-induced lesions in other vacci- nated persons) Lürmann, (1885).

Furthermore, addition of human serum to vaccines was not unusual at that time and considered necessary to stabilize yellow fever vaccine. Several out-breaks of hepatitis were observed in recipients of yellow fever vaccine, the largest in 1942 among U.S. American Army personnel with 50,000 clinical cases MacCallum, (1972).

Seeff et al., (1987) has reported that the epidemic was caused by HBV with probably 280,000 additional unrecognized infections. Epidemiological observations in the first half of the last century pointed to at least two types of sub-cellular pathogens: Type A mainly affected children, was spread at often epidemic levels via food or drinking water contaminated with feces and was never chronic. Type B was often transmitted through medical interventions in which human blood or serum was intentionally or, due to a lack of hygiene, accidentally injected or inoculated from one person to the next MacCallum, (1972). The most serious problem of blood transfusion was that even a blood donor in appar- ently perfect health, who had never had jaundice, could transmit the disease to the recipient and that the recipients often developed severe acute or chronic hepatitis.

1.3 Discovery of Australia antigen

The first hint came from an unexpected source. American physician and geneticist Baruch Blumberg wanted to study genetic markers for susceptibility to certain illnesses, especially cancer, and had gathered serum samples world-wide from a wide variety of ethnic sources in the 1950′s and 60′s.

The possibilities for recognizing genetic differences with laboratory methods were very limited at that time. Blumberg used an immunological approach. He postulated that people, who had received blood products from a large number of donors, e.g. due to hemophilia, would have developed antibodies against "polymorphic" serum proteins. These are proteins that show small genetic differences from person to person in amino acid sequence, and may be recognized as foreign to the body of the recipient after a transfusion (Gerlich, et al. 2007).

Blumberg's co-worker, Harvey Alter, did indeed discover a new antigen in several samples of the huge serum collection, quite often in Australian aborigines, for whom the Australia Antigen (AuAg) was named (Gerlich, 2013).

1.4 Early Detection of Hepatitis B Virus

In the 1960s, virology was still a new science, primarily dedicated to basic research. Its clinical relevance was limited. Whereas scientists had been successful in spreading viruses of many major infections in cell cultures, these techniques were suboptimal for diagnosis of most viral diseases. It usually took a long time for the cytopathic effect of virus growth to become evident in cell culture.

Many virus strains were only able to grow in the artificial host cell systems after a prolonged adaptation. In addition, there were many viruses that did not generate any cytopathic effect and could, therefore, not be recognized even if virus replication did occur. Rarely, observing the viral material with an electron microscope or using certain biological methods, e.g. hemagglutination, were helpful in such cases. These methods of virus detection enabled the development of

vaccines, but for clinical diagnostics on an individual basis they were too time-consuming, too difficult or unsuitable.

Detection of antiviral antibodies as an alternative approach, detection of antibodies which the patients had produced against the antigens of the disease-causing agents was adopted as a diagnostic method. However, this approach was challenging as it depended upon detection of the reaction of the patient's antibodies with the viral antigen.

The method most often used was the quite difficult complement fixation reaction (CFR), which was initially developed for diagnosis of syphilis. In addition to the human patient serum, and the viral antigen (e.g. from infected chicken embryos or tissue cultures), one needs sheep erythrocytes as indicator cells, rabbit antibodies against the sheep erythrocytes for producing an immune complex on the erythrocyte membrane and, finally, complement (usually from guinea pigs) for the CFR.

Complement is a multi-protein complex in animal sera that binds to immune complexes. When assembled and activated on cell membranes, holes are punched in the cells by the complement, which leads to lysis of the cells. If the patient serum does not contain antibodies, the complement lyses the erythrocytes, and the non-transparent red reaction mix becomes a transparent red. If immune complexes were formed previously in the mix of patient serum and viral antigen, these bind the complement away, such that it can no longer lyse the erythrocytes. CFR requires four complex biological component mixtures from four different animal species and these mixtures must all be standardized quantitatively by the individual lab.

One qualitative CFR result was usually not sufficient, since the antibodies detected could have come from a previous, unrecognized infection and not from the current illness. For diagnosis of highly dominant acute infections one had to demonstrate an increase in antibody titers. To achieve this a first sample was required, which should have been taken as soon as possible after the start of the illness, followed by a second serum sample from the patient, at least one week

later that had to contain a significantly higher amount of antibodies against the corresponding virus.

Quantification of CFRs and similar biological reactions was only possible by diluting patient sera in sequence in steps of two and determining the highest dilution that had just given a positive result. Only a titer increase of at least a factor of four could be rated as significant. Early virus diagnostics therefore had to use complicated methods which are almost forgotten today. However, the scientists of the 1960's would have been happy to have these methods available for diagnosing viral hepatitis.

Retrospective analysis showed that the outbreak was caused by hepatitis B virus (HBV) with probably 280,000 additional unrec- ognized infections (Seeff LB, 1987).

1.5 History of Hepatitis B Virus

Hepatitis virus transmittence through blood transfusion was first reported in *1943 (Beeson PB, 1943).* Subsequent investigations, including experimental human transmission studies, initially pointed to the existence of two viruses with different modes of transmission, incubation periods, severity, and outcome *(Krugman, 1967).* Hepatitis B virus was responsible for all cases of hepatitis through blood transfusion called Serum Hepatitis or post transfusion hepatitis.

The outbreak of percutaneously transmitted hepatitis was described more than a century ago, when 15% of German dockyard workers developed jaundice after smallpox vaccination with lymph derived from convalescent persons *(Lurman A and Eine, 1985).*

While HCV cannot be excluded, the most likely responsible agent was HBV. However, HBV infection may have afflicted the human race since ancient times, judging by the surprising prevalence of the virus in Asia and Africa, where it has been continued by perinatal and percutaneous transmission.

Feinman et al., (1988) reported that in the third and fourth decades of the last century, with the broad use of syringes, the percutaneous spread of viral hepatitis regained opprobrium. Because the disease seemed to be associated with the sharing of poorly sterilized needles, it became known as “post inoculation jaundice.”

Additional, support for a link between needle exposure and infection came from reports of severe hepatitis among children and British Army groups vaccinated with convalescent measles, mumps sera and along with recipients of yellow fever vaccine *(Beensson, 1943).*

The present terminology, hepatitis B, was proposed in the late 1940s and adopted by the World Health Organization (WHO) in the mid-1970s. As a proof that HBsAg was associated with a transmissible infectious agent came from the earliest studies, giving an unresponsive immunodiffusion test *a*bout 75% of recipients of HBsAg-positive donor blood were found to have either hepatitis or serologic markers of HBV infection. Whereas hepatitis also was prominent in recipients of HBsAg-negative donor blood *(Gocke, 1970).*

Identification for HBsAg evolved from agar gel diffusion to counter electrophoresis, to radioimmunoassay and finally, to enzyme immunoassay (EIA). Screening of HBsAg was made mandatory by federal regulation in July 1972 and third-generation HBsAg testing was mandated in 1975 for all blood donations. Other studies of transfusion associated hepatitis conducted in the Far and Middle East, Europe, Canada, and Australia during 1980s and 1990s revealed that even the hepatitis frequency ranged from 3% to 19% only a few cases could have been attributed to HBV.

In the majority of studies reported occult hepatitis were identified, and hepatitis B accounted for 7% or less of total cases in following studies *(Feinman et al., 1988).* Popular and broadly use of more sensitive tests for HBsAg and adoption of all-volunteer blood donor programs in developed countries has virtually eliminated transfusion-associated hepatitis B virus.

1.6 Structure of Australia antigen

Blumberg, (2002) was observed under the electron microscope (EM), purified AuAg appeared as small round particles which, unlike viruses, were of variable sizes between 17–25 nm. Most importantly, Blumberg and his team came to the conclusion that these particles did not contain any nucleic acid. Their experimental approach was a bit questionable from today's ethical standards, but not comparable to Krugman's experiments. They injected 1 millicurie radioactive 32_P-phosphate as a biochemical precursor for newly synthesized DNA to a terminally ill HBsAg carrier, obtained 250 ml HBsAg containing plasma after one day, purified the HBsAg and followed the distribution of 32_P in a density gradient. The purified HBsAg particles contained a small amount of 32_P, but with phenol extraction it did not remain in the aqueous phase as did nucleic acids but went into the protein/lipid containing phenol phase (Millman, 1970).

In retrospect it is apparent that the conclusion was correct but the experiment was inconclusive, because the nucleic acid of HBV is covalently linked to a protein and is therefore extracted in the phenol phase. Independently, the author showed that the ultraviolet-spectrum of purified HBsAg was that of pure protein and not that of a nucleoprotein (Gerlich, 1973).

These findings were incompatible with the nature of viruses. Blumberg was at that time, however, so convinced that AuAg was an infectious hepatitis pathogen that he postulated for a while a novel nucleic acid-free infection principle that he named ICRON after the Institute for Cancer Research in Philadelphia where he worked at that time (Blumberg, 2002, p109). Griffith, (1967) formulated that the pathogen of a spongiform encephalopathy (scrapie in sheep) was infectious but would not have any nucleic acid, which Stanley Prusiner later proved with his prion theory.

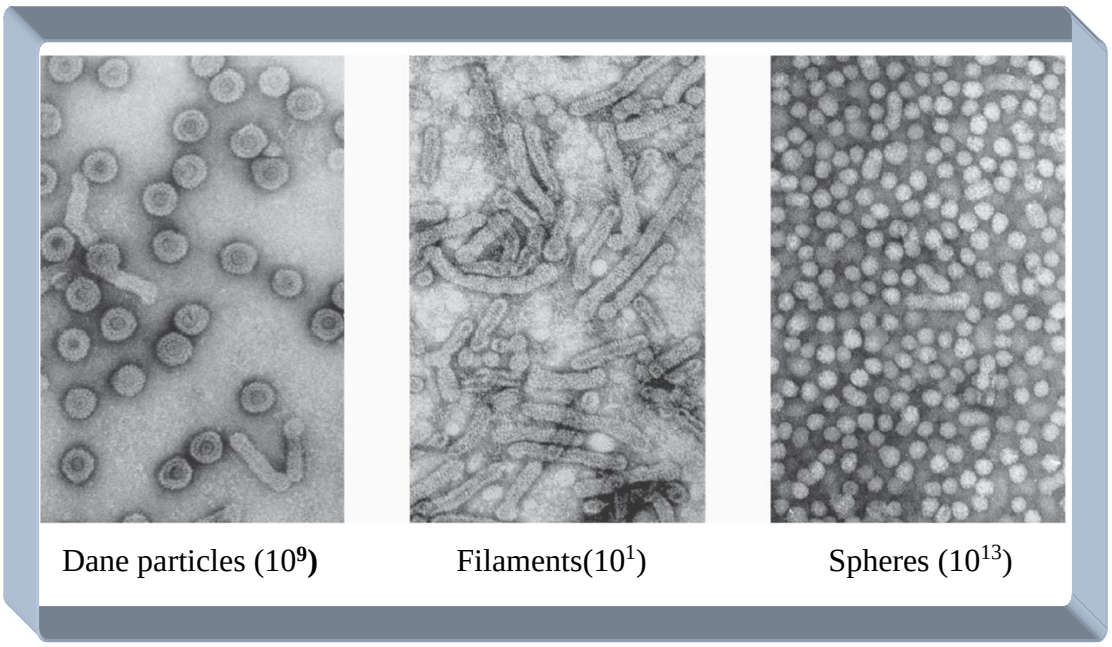

Figure 1.1 HBV associated particles under Electron microscopy.
Source: Wolfram H Gerlich, (2013)

1.6.1 Dane particle of HBV

Dane, et al., (1970) discovered that Australia antigen appared not only on the small pleomorphic particles, but also on larger, virus-like objects 42 nm in size with a clearly visible inner core. Subsequently, in 1971, his British colleague June Almeida was able to release the core particles from the so-called "Dane particles" by treatment with mild detergent, and showed by immune EM that hepatitis B (HB) patients formed antibodies (anti-HBc) against this core antigen (Almeida, Rubenstein and Stott, 1971).

This suggested that the Dane particles were the actual virus causing hepatitis B. Australia antigen was obviously the surface antigen of the virus envelope, and was named HBsAg (s for surface) thereafter. The infected hepatocyte forms the HBsAg protein in large surplus and secretes it, in addition to the complete virus, as round or filamentous non infectious particles of about 20 nm diameter into the blood leading to an approximately three thousand fold excess of these subviral particles (Figures 1.1 and 1.2). This was the reason that the Dane

particles could not be recognized in AuAg preparations purified by ultracentrifugation or size chromatography.

1.6.2 Discovery of HBV DNA

Since the HBV still could not be grown in cell cultures or in practical laboratory animals, and patient sera contained no more than few nanograms (ng) of Dane particles/ml at most, a direct biochemical detection of the nucleic acid within HBV was not possible at that time. Several re- searchers tried to identify the HBV genome by indirect methods.

Purified Austrelia Ag preparations would contain a reverse transcriptase like retroviruses, i.e. a DNA polymerase which accepts exogenously added RNA templates. Al- though this exciting publication could not be confirmed, it opened the route to the detection of the HBV genome.

In 1973 William S. Robinson was trying to reproduce that report, and was certainly able to detect an endogenous DNA polymerase activity within HBV and, in 1974, he identified the product of that activity

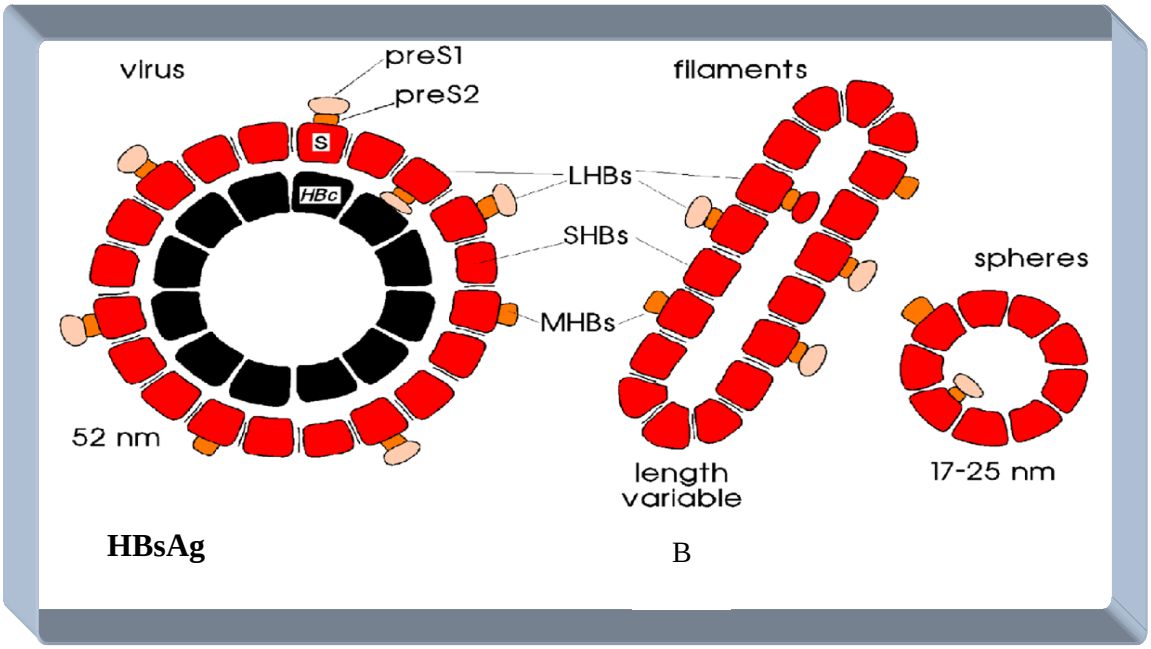

Figure 1.2 Model of the hepatitis B virus (Dane particle) and the filamentous or spherical HBsAg particles.
Source: Seitz S, et al., (2007)

Dane particle size in 42 nm but in negative staining the outer preS1 and preS2 domains were not visible. 52 nm is the hydrodynamic and also measured by cryo-EM as the outer diameter (Seitz et al., 2007). (Figure 1.2) HBsAg protein comes in three forms: large (L-) HBs protein with the preS1, preS2 and S domain, middle protein (M-) without preS1 and SHBs without preS1 and preS2 itself the viral DNA (Robinson, et al., 1974). One feature of HBV helped with the recognition of its DNA even without the techniques of molecular biology available today.

The DNA within the core particle is, in principle, double-stranded. However, the viral DNA polymerase does not finish synthesizing one strand before the virus is released into the bloodstream. There, it lacks the nucleotide triphosphates which are the building blocks for further DNA synthesis, such that there remains a single-stranded gap in the viral DNA (Figure 1.3). If one provides those building blocks in the test tube, the synthesis restarts in the form of an endogenous DNA polymerase reaction (i.e. without externally added nucleic acid template), and if the nucleotide triphosphates are radioactively labeled this process can not only be detected, but the viral DNA could also be characterized as a small open circular DNA with ca. 3200 bases.

In view of these findings, it was generally believed that Dane particles were viruses, but Robinson himself insisted that it would be first necessary to prove that the DNA would encode the viral proteins and infectious HBV. He planned to clone the small amounts of available viral DNA in E. coli with the then newly developed methods in molecular biology. But he was not allowed to do this because of safety concerns about genetically altered organisms which had been raised at the famous conference of Asilomar (Berg et al., 1975).

1.6.3 Cloning of HBV DNA

Cloning and sequencing of the HBV DNA was reported almost simultaneously by three other pioneers in molecular biology and their teams who had a biosafety laboratory, (William and Valenzuela, 1979) The cloned DNA was shown to be indeed circular and to encode the genes for HBsAg, HBcAg, the putative endogenous DNA polymerase and an unexpected X gene.

Thus, the cloned DNA was most likely a true copy of the virus genome. The cloning opened the way to manufacturing HBV DNA, HBsAg and HBcAg in almost unlimited amounts using gene technology, instead of painstakingly extracting it from the scarce and highly-infectious patient material. This was important, since these three materials would be used soon in large amounts for diagnostics and vaccine development. Will H et al., (1982) published in nature as final proof that the Dane particle is the HBV in the sense of Robert Koch's postulates came from an elegant animal experiment. While it was still not possible to reliably infect cell cultures with HBV, experimental injection of cloned HBV DNA into livers of chimpanzees initiated highly efficient replication of HBV and even acute hepatitis B.

1.6.4 Serological diagnosis of HBV infections

The introduction of Ausria-125 was the beginning of an impressive development in virus diagnostics. However the test had one major disadvantage: the radioactivity caused significant difficulties in the normal diagnostic laboratory. It was therefore a big step forward when it became possible to label the antibodies used with enzymes, and later with chemiluminescence-generating groups. The test principle of the solid-phase sandwich immuno- assays has been maintained to the present. However, for assay of numerous antigens and antibodies in many fields of biomedicine, even if the forms of the solid-phase and the signal generation have changed.

The assay of HBsAg was soon complemented by the detection of antibodies against HBsAg (anti-HBs) and HBcAg (anti-HBc). Typically, anti-HBc appears with the onset of acute hepatitis or after an unnoticed clinically silent HBV infection event. If HBsAg is found without anti-HBc, the patient can still develop hepatitis. If the HBV infection is completely under immune control, the HBsAg disappears.

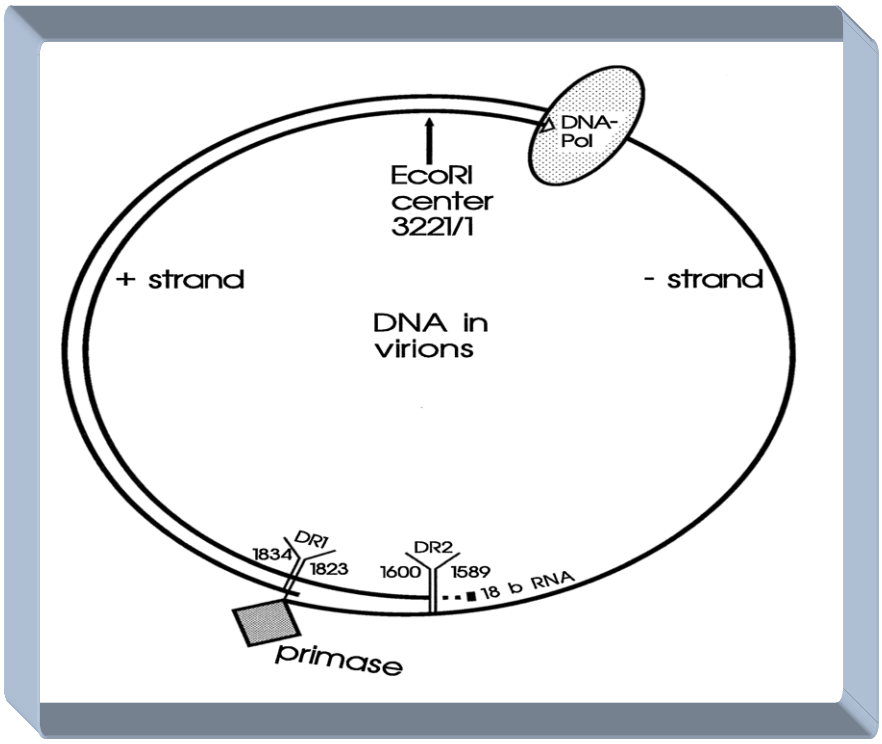

Figure 1.3: Biochemical structure of HBV DNA

Source: Wolfram H Gerlich, (2013)

The molecule is open circular. The minus-strand has full length within the core particle and even a redundancy of 9 bases at the ends around the nick. The plus-strand is incomplete leaving a large single-stranded gap. The 5′ end of the minus-strand is covalently linked to the primase domain of the DNA polymerase which is present with its active center of the reverse transcriptase domain at the 3′ end of the plus-strand. The 5′ end of the plus-strand still contains its primer which is - in this case- derived from the 18 capped 5′ terminal bases of the degraded pregenomic HBV mRNA but the anti-HBc remains and anti-HBs usually appears as a sign of immunity. If the infection becomes chronic, HBsAg and anti-HBc remain positive (Figure 1.5). With these three HBV markers, it was possible to establish the infection or immunity status of a person in routine diagnostics since the early 1980s.

1.7 Significance of anti-HBs

The very high anti-HBs concentration necessary for a positive reaction with HBsAg in the Ouchterlony technique (as used by Blumberg) was an exception

occurring only after repeated exposure to HBsAg. This happened before 1972 in frequently transfused persons as in the case of hemophiliacs.

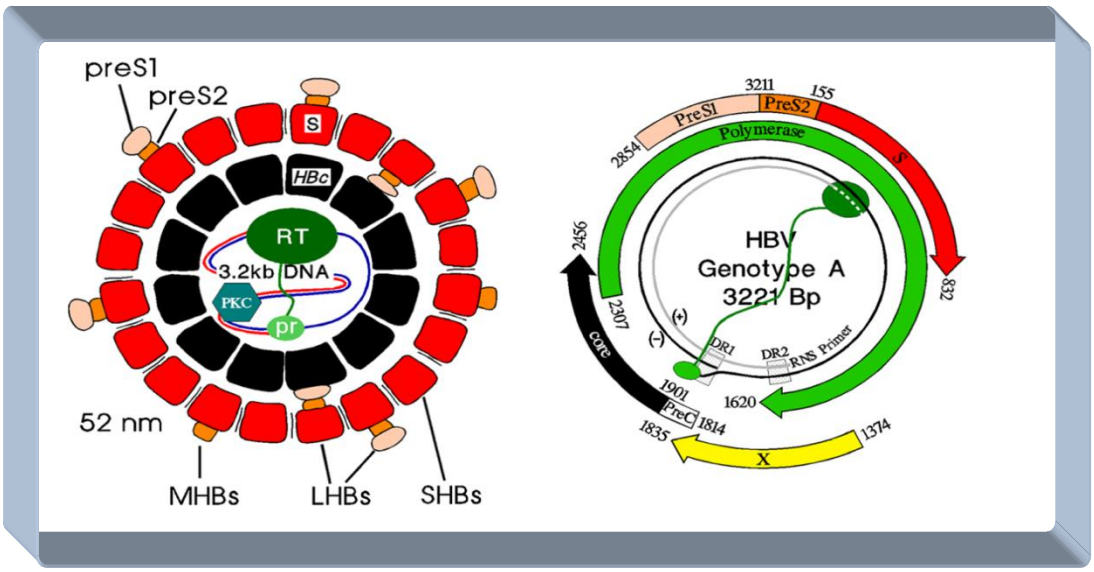

Figure 1.4 Structural components of HBV (left) and open reading frames (ORF) for encoding proteins in the covalently closed form of HBV DNA
Source: Wittkop L, et al., (2010)

The HBV core contains besides the HBV genome the HBV polymerase with the primase (pr) and the reverse transcriptase (RT) domain and the cellular protein kinase C alpha (PKC) (Wittkop, et al., 2010).

The two start codons in the PreC/core ORF and the three start codons in the HBs ORF. For detection of naturally acquired anti-HBs after HBV infection, the Ouchterlony or other antibody tests such as CFR were not sufficiently sensitive, and a feasible HBV neutralization test was not available. Abbott put the first adequate test for anti-HBs on the market in 1975, which was still a solid phase RIA with Iodine 125-labeled-HBsAg as a reagent. The test was hugely important for the development and licensing of the hepatitis B vaccine, the origins of which went back to a patent of Blumberg in 1970 (Blumberg, 2002).

According to his idea, the first generation of HB vaccines contained purified HBsAg 20 nm-particles from HBV carriers. Successful immunization was, and is, proven by the detectable presence of anti-HBs. Furthermore, complete recovery from acute or chronic hepatitis B is best demon strated by a positive anti-HBs result.

1.8 Significance of anti-HBc

The landmark result of Almeida on the development of anti-HBc during acute hepatitis B was soon confirmed by others, in particular by Jay Hoofnagle in 1973. The first experimental tests in the 1970s, including CFR, used HBcAg from infected liver or from Dane particles as antigen, but later "recombinant" HBcAg produced in E. coli or yeast was used. In this experience, the natural HBcAg yielded highly specific results, but the results obtained with the more readily available recombinant HBcAg from E. coli suffered from a certain degree of non-specificity which remains a problem to the present (Pasek, et al., 1979).

In 1982, the first commercial anti-HBc test was released as an enzyme immunoassay. Anti-HBc had, in years following, transiently gained significance as a surrogate test in blood donors for hepatitis C infection which could not yet be diagnosed, and also transiently for HIV, because of the partially overlapping transmission pathways of HBV, HCV and HIV. Since anti-HBc neither proves active infection nor immunity, it could be considered clinically unnecessary except for epidemiological studies or confirmatory testing. However, anti-HBc not only provides evidence of prior infections, but also of an ongoing, occult HBV

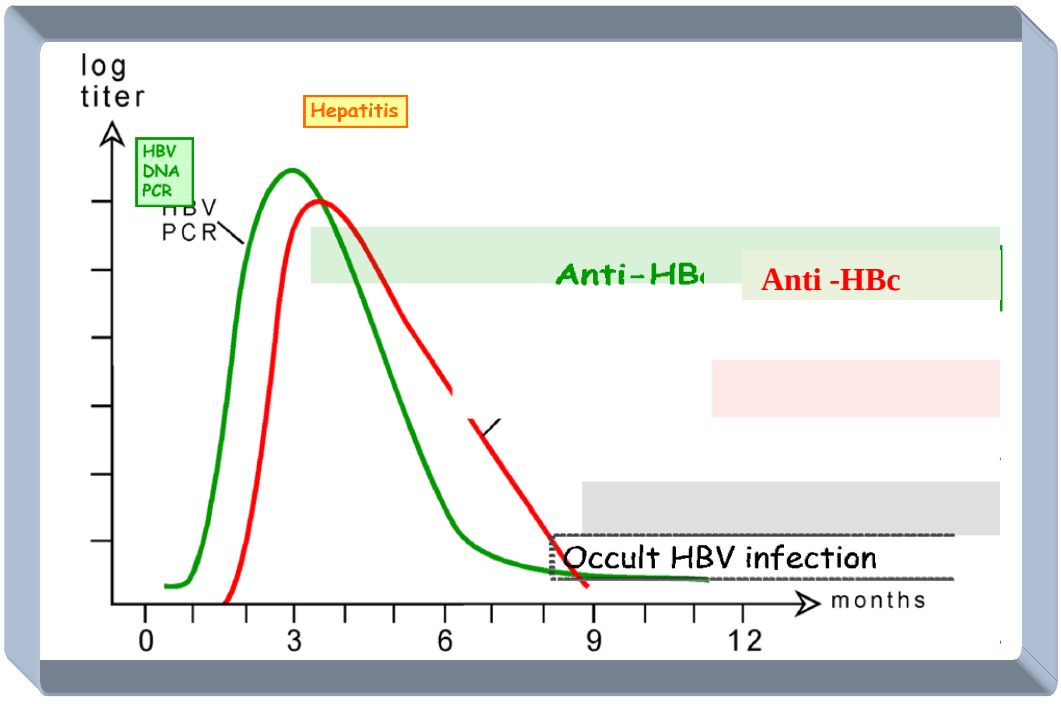

Figure 1.5 Schematic representation of the course of acute HBV infections with resolution.
Source: Wolfram H Gerlich, (2013)

After the infecting event (time 0) follows a lag phase of several weeks without detectable markers. Thereafter HBV DNA (within the virus) and HBsAg increase exponentially in the serum. HBV DNA is detected earlier because its assay is much more sensitive. The peak of HBV DNA and HBsAg is reached before outbreak of the acute disease and both decrease after the onset of clinical symptoms. Initially, HBV DNA decreases faster because it has a shorter half life time in serum than HBsAg.

HBsAg finally disappears whereas HBV DNA may remain detectable in traces. Antibodies against the HBV core antigen (anti-HBc) appear with the onset of symptoms while the protective antibody against HBsAg (anti-HBs) appears very late, usually several weeks or months after disappearance of HBsAg. Disappearance of HBsAg is considered to be a sign of resolution but the virus often remains in occult form in the liver infection, whereby the word "occult" refers to the apparent lack of HBsAg, which is present in an undetectable amount in such cases.

In blood transfusion, up to 200 mL blood plasma is transferred, and the smallest traces of HBV can lead to infection in the recipient. Anti-HBc can be a marker for occult infection, as was recognized by Hoofnagle already in the 1970s. For a long time the small residual risk caused by occult infected blood donors was tolerated. But with todays increased safety demands, blood donors have to be tested for both HBsAg and anti-HBc in many countries, e.g. in Germany, since 2006 (Hoofnagle, et al., 1978)

An anti-HBc determination is also important before medically induced immunosuppression, since an occult HBV infection can be reactivated under these circumstances with severe or even fatal consequences. This was already recognized in 1975 by Arie Zuckerman and colleagues but even today not all hematologists are aware of this problem. Reactivation can be suppressed with preemptive antiviral therapy if the problem is recognized in advance (Galbraith et al., 1975).

1.9 Significance of IgM antibodies to HBcAg

Anti-HBc total antibody (IgG, IgM) assays were useful to determine whether a patient ever had contact to HBV but this marker could not distinguish whether the infection was acute or persistent or resolved. It had been long known that the early immune response against an infectious agent induced antibodies of the immunoglobulin class M (IgM, M for macroglobulin), whereas weeks or months later the antibodies belonged mainly to immunoglobulin class G (IgG, G for Gamma). It was, therefore, only necessary to determine the immunoglobulin class M of the antibodies to distinguish between a fresh and an old infection. This used to be costly and laborious, as biophysical methods (like ultracentrifugation, ion exchange or gel chromatography) were needed to separate IgG and IgM.

After sometime very simple test principle was developed for IgM Anti-HAV by Bertram Flehmig and independently by the author of the present paper for IgM Anti-HBc (Gerlich, 1986), covering the solid phase with an antibody against IgM (anti-μ chain) and consequently using it to capture the IgM from the sample. The HAV-Ag or HBcAg is then added and, if bound by specific IgM, detected with a labeled antibody. Today, this test principle is standard for assay of most antiviral IgM antibodies.

1.10 Quantitation of IgM anti-HBc

Differentiating between an acute and chronic hepatitis B infection was often difficult. Large clinical studies initiated by Reiner Thomssen in the 1970s showed that the interpretation of the IgM anti-HBc results obtained by the author were not as straightforward as most clinical virologists had expected. Since the anti-μ capture assay is very sensitive, some patients who resolved acute HBV remained positive for years and even patients who had chronic HBV without known acute phase were positive. Only an accurate well standardized quantitation allowed distinction of clinically apparent acute from chronic HBV (Gerlich, 1986).

Not all clinical virologists liked this differentiated evaluation and made the test artificially insensitive leading to the problem that mild acute infections were no

longer recognized. One reason why IgM anti-HBc remains moderately positive in chronic HBV infections was detected by David Milich (La Jolla, California). HBcAg is an unusual T cell- independent antigen which can activate B cells to produce IgM anti-HBc (Milich and McLachlan, 1986).

The strong B cell immunogenicity of HBcAg may be part of the immune evasion strategy of HBV because it may interfere with the activation of HBcAg specific cytotoxic T cell reactions. The IgG anti-HBc found in resolved or inactive cases is induced by the normal T cell-dependent class switch. The increased prevalence of HBV in Indian subcontinent and genetic differences, can contribute to the fact that a sample can be NAT reactive but immunoassay non-reactive. Therefore, history of HBV vaccination should be reviewed while evaluating the NAT results.

The screening for anti-HBcore antibodies (HBcAb) is employed by various blood banks in india to to decrease the transmission of HBV and enhance blood safety. However, In India, HBcAb is not a mandatory test as per Drug and Cosmetic Act, 1940.

Nowadays, Individual donor Nucleic Acid Test (ID-NAT) has been adopted for routine screening of blood for HIV, HBV and HCV in some blood Banks. However, the adding together of such tests leads to increase in the cost of transfused blood. Therefore, this study was carried out to look into the agreement between the hepatitis B core antibody with HBsAg and ID-NAT with HBsAg for enhancing the blood safety without compramisinig the quality.

1.11 Statement of the problem

1.11.1 In India, Director General of Health Services (DGHS) mandates testing of HIV, HBsAg, HCV, malaria and Syphilis by law .

1.11.2 Detection of HBcore antibodies is not mandatory for screening of hepatitis B virus infection in India till date.

1.11.3 Majority of blood banks do not screen for this marker. This could increase risk in transmission of HBV to patients during blood transfusion.

1.12 Aims and Objective

1.12.1 To find out the reactivity of HBsAg in the donor population

1.12.2 To find out the reactivity of isolated HBcAb in blood donors

1.12.3 To study the significance of isolated HBcAb in blood donors by confirming viral DNA (ID-NAT).

1.12.4 To find out the relativity between hepatitis B core antibodies and/or ID-NAT test for safe blood transfusion.

1.13 Purpose of the Study

1.13.1 Enhancing blood safety by introducing an additional test – Hepatitis B Core Antibodies

1.14 Research Hypothesis

1.14.1 HBCoreAb test confers additional safety to the blood supply chain.

1.14.2 Should antibody testing be performed along with Nucleic Acid testing blood to confer additional blood safety?

1.15 Significance of the study

1.15.1 If the hypothesis is proved right, it will build a case for making HBCoreAb test mandatory along with ID- NAT testing to enhance safe blood transfusion.