DISCUSSION
The work presented in this dissertation was mainly aimed at establishing the diagnostic potential of HBV DNA probe and its indispensable use in identification of HBV variants. The various serological markers that are indicative of ongoing and past exposure to HBV, are anti-HBc IgM, anti-HBc IgG, anti-HBs IgG, HBsAg and HBeAg. Amongst these HBsAg is the first marker to appear in the serum after an acute HBV infection and persists till the end of the infective phase. This is the reason why HBsAg is taken as a reliable marker for large scale screening of serum samples for detection of HBV infection. The commercial kits available for detection of HBsAg, detects the protein in the range of 0.5 ng-0.1ng and requires 100-200 µl of serum. Therefore a low level expression of HBsAg would go undetected.

Molecular hybridization assay is a powerful tool to detect complementary nucleic acid sequences in biological materials and is extensively used in biological research and clinical medicine to detect DNA or RNA in tissues and serum. The detection of hepatitis B virus DNA in serum by nucleic acid hybridization provides a direct and highly sensitive marker of viral replication. A systematic analysis of various factors affecting sensitivity and specificity of DNA dot hybridization test was carried out in order to optimize the conditions of hybridization.

HBV probe used for hybridization was a recombinant plasmid carrying a tetramer of HBV and was found to be
advantageous over a monomer because of higher yield of insert DNA from the same quality of plasmid. For all the hybridizations reported in the text, a vector free insert, twice purified, was used in order to eliminate any non specific signal.

5.1 Optimization of DNA Probe Hybridization Technique

The method of processing of serum samples in order to make the viral nucleic acids available for hybridization, has an important role in determining the efficiency of hybridization. Several methods of processing of serum samples have been tried by various investigators. Theses methods involve either extraction of viral genome from Dane particles present in the sera (190), or direct spotting of serum aliquots on membrane filter (177,178). The direct method of spotting has an inherent limitation of the amount of serum which could be applied on the membrane. This limitation arises from the high amount of protein, polysaccharides and other cellular components present in the serum. With higher amounts of serum, these cellular components lead to masking of the viral DNA adsorbed on the membrane, thereby making it inaccessible for hybridization with the probe DNA. This often results in non specific hybridization. It was observed that the intensity of hybridization signal obtained was not proportional to the amount of serum, beyond 20 µl. Beyond 200 µl no hybridization signal was observed due to complete blocking of the membrane (data
not presented). So the method of direct spotting of serum can be used only under condition of high viral load and hence is not ideal for screening of apparently, healthy individuals like voluntary blood donors and contacts. **Isolation of viral DNA** from serum and its application on membrane filter, though time consuming, was found to be ideal because of lack of constraint on the amount of serum that could be used. For example in case of Fig 7B because of low virus titre in the serum no signal could be obtained in less than 1ml of serum. For large scale and routine analysis of serum samples, it is not always possible to obtain high amount of sera. The amount of sera found to be optimal was about 200 µl, though in a few cases of (Fig. 7A) carriers, even 50µl was sufficient to detect the virus.

DNA spot hybridization assay is a localized hybridization and is driven by the amount of DNA present on the membrane. Thus percentage retention of DNA spotted on the membrane is an important factor that governs the sensitivity. Direct application of serum samples on membranes under vacuum has been reported to result into losses of viral DNA sequences as judged by their presence in the subsequent layers of transfer membranes (189). The same was found to be true in case of cloned DNA as well as DNA extracted from serum (Fig 6A and 6B). **Manual spotting was thus found to be more sensitive**
In any diagnostic test that is based on radiolabelled probes, for specific detection of target sequences, the amount of radioactivity incorporated in the probe is a major governing factor in determining the limit of detection. Higher the specific activity of the probe, lower is the number of target sequences required for detection. Amongst the currently available techniques of radiolabeling such as nick translation, random priming or 5'/3' end labeling, random priming method gave a specific activity of about $10^9$ cpm/µg of DNA, requiring only 50-60 ng of DNA per reaction. On the other hand 400-500 ng of probe DNA is needed to achieve similar order of specific activity by nick translation. We were able to detect 1-3 pg of HBV DNA (1 pg = 3 x $10^5$ viral particles) in 24 hours using a probe of specific activity of $10^8$ cpm/µg of DNA. Similar results were obtained by other investigators (216-218).

The sensitivity of hybridization can be further improved by two ways. One is amplification of the hybridization signal and second is amplification of the target molecules. Amplification of hybridization signal has been reported by several investigators (213) by converting a single step hybridization to a two step or a multiple sandwich hybridization. Most of these procedures involve the use of two adjacent oligonucleotide fragments which are not homologous to each other but are present of the target sequence. While one of the two oligonucleotides is
immobilized on a solid support, the other one is radiolabelled. In the first step the fragment immobilized on the solid support is hybridized to the target sequence present in the solution. The presence of the target sequence is then revealed by the second oligonucleotide, which is radiolabelled. Two step hybridization process although eliminates the necessity of immobilizing the target DNA sequence on a solid support, does not offer increase in sensitivity.

A single stranded bacteriophage M13 based two step hybridization, the principle of which has been described in Fig.11, offered at least 4-6 fold increase in sensitivity (Fig13A). In addition, M13 DNA offers itself as a universal probe. Inspite of partial sequence homology M13 DNA has with human mini-satellite DNA (282 bp), the technique works perfectly well with serum samples, as no false positive signal was obtained with serum negative for HBV (Fig 13B). For eventual use of M13 based sandwich hybridization, one can use M13 DNA devoid of the 282 bp sequence of protein 3, homologous to human DNA.

Amplification of the target molecules can be achieved by employing the recently developed technique of polymerase chain reaction which has revolutionized the field of nucleic acid based diagnostics. Data presented in fig.18 clearly demonstrated the need for use of PCR in detection of HBV, where samples negative for surface antigen as
well as negative by conventional dot blot techniques is diagnosed as positive.

Our results clearly indicate that with the help of PCR, one can detect as low as \(3-10\) viral particles (fig14A), which is in line with the observations made by other investigators (224).

To study the relationship between HBV and human liver disease, it is important to know the state of HBV in liver and serum. The presence of \(42\) nm infectious Dane particles can be revealed by DNA polymerase activity which is an integral part of the viral core, while presence of HBeAg is normally used as a monitor of actively replicating viral DNA in hepatocytes. Another way of detecting the presence of HBV in serum and liver is to use a cloned HBV DNA as a specific probe. The technique not only detects the presence of Dane particles in the serum, but also can determine the state of viral DNA in the hepatocytes i.e. free or integrated into the hepatocyte chromosome. Detection of free viral DNA in liver and serum of some HBeAg negative patients suggests that hybridization test is a much more sensitive and reliable test for serum infectivity (177).

5.2 HBV DNA: An Essential Diagnostic Marker Of Infection

The marker that is more frequently used to detect HBV infection is the surface antigen, which is usually present in huge excess (1000 fold) as compared to number of
Dane particles (184). When the surface antigen detection test was used in combination with DNA probe based spot blot hybridization test, we came across cases that were negative for the presence of HBsAg by Abbot ELISA but positive for the presence of HBV DNA. The results of analysis of the blood samples obtained from voluntary blood donors from two different sources (Red Cross Blood Bank and G.B.Pant, New Delhi) indicate that around 5-7% of the samples are missed for the diagnosis of HBV infection based on surface antigen detection alone (fig 15, fig 18). The presence of HBV DNA was also evident in some of the HBsAg negative contacts obtained from another source (Delhi Cantonment) (fig.17). Such observations have been also reported by other investigators (177), where HBV DNA has been detected in sera negative for HBsAg as well as in sera negative for HBeAg. However one cannot rule out the possibility that disappearance of HBeAg could precede a decrease in viral replication. Also there are instances when no HBV DNA is found in the presence of HBeAg (177), probably indicating that these markers are expressed independent of one another and appear at various stages of viral replication. Our observations as well as the studies carried out by others clearly indicate the necessity of DNA probe assay for unambiguous diagnosis of HBV infection. The merit of the DNA probe based diagnostic assay lies in its actual detection of infectious virions in the blood, as demonstrated by the presence of 42nm Dane
particles in a HBsAg negative, DNA positive serum sample (fig 16C). Cases like this are easily mis-diagnosed if the analysis is based only on serological observations.

5.3 Identification Of Variants Of HBV:
Antigen-antibody systems often used as a criteria to screen blood donors for HBV infection are not adequate since like Hepatitis B, non A non B type of hepatitis is also more often transmitted from blood of professional blood donors. Non A non B hepatitis appears to be transmitted more effectively by percutaneous route. In hospital settings, blood transfusion to haemodialysis patients, to haemophiliacs and cancer patients who receive either clotting factor concentrates produced from large pools of human plasma or pools of human platelets, are the route cause of transmission of non A non B hepatitis (259). Occupational contacts with patients and health care personnel also fall in high risk group. Another category of people who are more prone to transfusion related non A non B hepatitis are the drug abusers. Evidence has accrued to suggest, that some cases of non A non B hepatitis might actually represent cryptic forms of HBV infections. This stems from the observation of hepatitis B like particles in HBsAg negative sera and detection of HBV DNA in liver biopsies and serum of hepatitis patients who had no HBV markers in serum and hence were diagnosed as having nonA non B hepatitis (117,226-229). This suggests that, in the
absence of any serological markers of HBV infection, HBV DNA probe can pick up cases of cryptic HBV which could either be mutants or variants of HBV.

In order to explore the possibility of existence of such variants, some of the HBsAg negative but DNA positive voluntary blood donor samples were further screened for the presence of other serological markers of HBV infection and were found to be negative (table 1), suggesting the presence of some variant forms of HBV in them. Similar observations were made by Lai et al (196). Out of 1411 HBsAg negative voluntary blood donor samples analyzed, 71 were found to be positive for serum HBV DNA. 31% of the 71 cases were anti-HBc positive, while 69% were negative for all markers of HBV infections. 68 out of the 71 subjects exhibited abnormal level of amino transferases (ALT). Presence of variant forms of HBV was indicated. Detection of HBV DNA in seronegative patients of liver cirrhosis as well as in patients categorized under NANB (table 3 and table 4) raises the possibility of involvement of genetic variants of HBV in diseases.

5.3.1 Characterization Of Variant:
Location of variations or mutations on the genome requires cloning of the viral DNA sequences present in these subjects and its subsequent analysis by DNA sequencing. Major impediment in direct cloning of variant sequences present in the serum, was the limiting amount of DNA and
also unavailability of large amount of serum. This problem was circumvented by making use of the recently introduced technique of DNA amplification by polymerase chain reaction. The primers designed for the analysis by PCR were such that they could amplify specific sub-regions of HBV genome. Amplification of the genome in short stretches had the advantage of detecting any major changes in the genome, although small changes in form of point mutations would go unnoticed.

The region of the HBV genome scanned by PCR was between nucleotide 637 bp and 2001 bp with respect to ayw serotype, using primer combinations; S1-S2, X1-X2, S1-X2 and C1-X1 (fig 22 A,B ). The size of the fragment expected from an unaltered HBV DNA for all serotypes is shown in table 2. Regions S1-S2, X1-X2 and C1-X1 didn't show any difference in size of hybridizable band as judged by Southern blot analysis (fig 25, fig 27, fig 30) in any of the four serum sample analyzed. Interestingly, using primer combination S1-X2 an additional hybridizable fragment, about 200 bp smaller than expected was obtained in three out of four samples (fig.27). This observation strongly suggests the presence of a gross deletion of about 200 bp in the region analyzed in some of the viral particles present in the serum. Similar observations were made in a patient suffering from cirrhosis (Fig.31) where an additional band hybridizable with HBV was detected for the primer combination S1-X2, which was also around 200 bp
smaller than expected. This patient was also seronegative for HBV markers.

These findings clearly demonstrate co-existence of two kinds of virus populations in voluntary blood donors as well as in patients that were investigated, one having a size similar to HBV while other having a deletion in the region between S gene and X gene.

During the past few years, several putative variants of hepatitis B virus have been reported. Patients infected with these variants are found to have no markers of HBV infection except for presence of HBV DNA in serum or liver and certain epitopes of HBsAg, detectable only by high affinity monoclonal antibody based RIA (260). Molecular cloning and subsequent nucleotide sequence analysis of some of these variant genomes has been recently reported by different investigators. The regions that have been found to be hotspots for mutations are the pre S-S region, precore-core region. Those mutations occurring in immunodominant epitopes of HBV antigen(s) could be of significance as it would effect the antibody response to HBV. Mutations affecting the protein translation and folding lead to absence or altered antigenic expression.

Thiers et al (215) identified a point mutation in the S gene of some HBsAg negative forms of liver disease. Recently a replication defective mutant of HBV (adw), has been reported where the defect has been shown to reside at nucleotide position 2798, which is found to be a missence
mutation in the 5' position of the polymerase gene. The serum of this patient was found to be negative for the presence of all serological markers of HBV infection. As the virus was incapable of replicating the, viral DNA could only be detected in the liver (248). Presence of detectable HBV DNA in serum in the cases that we have analyzed, suggests the ability of the variant to replicate. The state of HBV DNA in hepatocytes could not be ascertained due to lack of liver biopsy.

Absence of serological markers of HBV infection could be attributed to such variations at the DNA level, that would change the amino acid composition, giving rise to altered antigenic properties. In the samples that we analyzed we found the existence of two kinds of viral population. One of them had a deletion in the regulatory region involving the enhancer sequences and promoter of X which can effect the expression of core and surface antigen. The other kind of viral population was of normal size and the absence of serological markers could either be due to lack of host immune response or fine point mutations, giving rise to altered antigenic expression.

A deletion of around 200 bp has also been reported by Prof. F. Baralle (personal communication) in the core region in a HBsAg positive individual. Recently Gerken et al, (261) have reported a variant of HBV having a 183bp deletion in the preS1 (2995-3177 with respect to adr
subtype). Out of 12 clones sequenced, 9 showed this deletion and 3 out of 9 showed numerous point mutations in preS1 and preS2. The rest of the three clones had small deletions and insertion in the same region of preS1. The viral DNA in the peripheral blood mononuclear cells indicated the existence of the 183bp deletion solely on the basis of PCR. Inspite of all these changes the patient was positive HBsAg indicating that the protein structure remained unaltered. The presence of heterogeneous population of HBV DNA molecules in the same individual have been reported by others also (264, 265, 266).

A part (248bp) of the X gene was cloned from the normal size viral particles and sequenced. The sequence data shows the existence of three point mutations at nucleotide number 1402, 1438 and 1450 of ayw serotype. Mutations in the X region has been reported by Blum et al (248), in the nucleotide position of 1388 (G-C), 1439 (G-A), 1481 (G-A), 1514 (G-A), 1632 (A-G) of adr serotype variant, that was isolated from a 73 year old white male having a metastasizing hepatocellular carcinoma. The significance of mutations in the X gene is unknown because this region is less conserved among all HBV subtypes and codes for a protein whose structure and function are still largely unknown.

Liang et al (241) have reported another variant isolated from a patient suffering from chronic liver disease, whose blood caused hepatitis in chimpanzees. This variant
had a total of 105 nucleotide substitutions representing a 4% sequence heterogeneity. Apart from X gene the mutations were observed in preC/C region, preS and S region. 34 amino acid substitutions were noted. The patient was negative for all serological markers.

A frequently encountered mutation has been observed in the pre C region where a G → A mutation causes formation of a translational stop codon, which makes the virus incapable of making HBeAg (233-240). This is because the precore protein has a signal sequence which is essential for the synthesis and secretion of a correct precursor polypeptide composed of the precore and core sequences. HBeAg is expressed after proteolytic cleavage of the composite polypeptide at both its amino and carboxy terminal sequences. The antibody to HBeAg is usually detectable in these patients as these are mainly directed against the entire precore protein. Variants harboring this precore defect has been seen to cause more severe hepatitis.

A few scattered point mutations have also been reported clustered in the amino terminal of core gene of a variant isolated from a 43 year old white homosexual man (239). Along with these point mutations a 36 nucleotide insertion was also observed at the amino terminal resulting in the addition of 12 extra amino acids to the core protein.

Existence of HBV-like sequences in patients suffering from acute non A non B hepatitis is evident from fig 32 A where
specific HBV sequences of expected size from S and X region could be detected after amplification and hybridization. Detailed analysis of one of the serum samples by PCR using the same primer combination as used for voluntary blood donor, indicated a major alteration in the region between by S and X region and also in the region covering part of X and core (fig. 32B). Unlike the variants found in VBD and cirrhotic patients only one band of smaller size was detectable for the primer combination S1-X2 and C1-X1. The nature of the variation again appears to be that of a deletion in this case in two regions one covering enhancer sequence and second in the core region.

In the recent years evidence has gathered for the association of HBV with non A non B hepatitis. HBV DNA sequences could be detected in 8% of post-transfusional non A non B hepatitis patients in Taiwan and amount of HBV DNA detected was in the range of 1 - 5 pg/ml of serum. This suggested that a substantial number of cases were misdiagnosed as non A non B (262, Abs 86).

Our studies suggest that around 6% of patients categorized under non A non B have an association with HBV related viruses. PCR analysis of one possible variant detected in a non A non B patient, yielded fragments that were of same size as classical HBV using primer combinations S1-S2 and X1-X2 but showed a 200bp deletion
between region S to X and X to C. Though involvement of HBV variants in nonA nonB has been indicated by other groups, no characterization at the molecular level has so far been reported.

We have thus been able to identify a new kind of variant of hepatitis B which has a deletion of 200bp in the region encompassing the enhancer and the promotor of X gene. This virus coexists with another virus having point mutations in this region instead of a deletion. Its detection in three out of four cases analyzed, indicates its apparently high frequency of occurrence in healthy 'carriers'.

Detection of this kind of variant in a patient suffering from non alcoholic cirrhosis of liver and existence of a variant having deletions of 200bp in two regions of the viral genome (the enhancer and the core), in a patient clinically categorized under non A non B suggests the possible infectious nature of some of these variants. This is further substantiated by the detection of 42nm viral particles under electron microscope. Rearrangements at the genetic level might have lead to changes in the immunogenecity of the viral particles and thus affected their clearance by the immune system.

How is a HBV variant acquired? Is it transmitted as a distinct strain present from the beginning of infection? Is its appearance a consequence of mutations which occurs during the course of infection with wild type HBV?
Experimental transmission studies and the follow up analysis of chronic HBV carriers should allow us to address these issues. Recently in one such study, a chronic HBV carrier was followed up for 6 years. It was found that a gradual emergence and takeover of mutated forms of HBV occurred (263).

What are the factors that favour the selection of the variant? HBV has a mutation rate 10,000 times greater than that of other DNA viruses and 100 times less than that of retro viruses (267). Such a high frequency is presumably the result of genome replication through an RNA intermediate using reverse transcriptase which does not have a proof reading capacity.

Finally, what is the need for understanding more about variants? This is answered by detection of some mutants of HBV that are capable of causing disease in individuals who have been previously vaccinated against HBV with sufficient titres of circulating neutralizing antibodies (242). Zuckerman, 1990 (unpublished observation) found a mutation at position 587 (S region) in 44/1500 vaccinated cases who became HBsAg positive. These observations emphasises the need to redesign the present vaccines so as to also have protection against variants of HBV.

Development of diagnostic methods for detection of variants is essential in order to reduce the rate of post transfusional hepatitis especially in cases which are negative for all serological markers of HBV infection.
Understanding more about these variants would also help in developing improved therapeutic measures, as it has already been seen that some variants are capable of causing more severe form of chronic hepatitis, but respond poorly to interferon therapy.