One of the least curable malignancies, hepatocellular carcinoma (HCC) is the third most frequent cause of cancer death worldwide (Parkin et al, 2005, Bruix et al, 2004), and infection with HBV acting as a major contributor. The WHO has reported HBV to be second only to tobacco as a known human carcinogen [WHO, Hepatitis B, 2002]. The incidence of HCC in HBV-related cirrhosis in East Asian countries has been reported to be 2.7% [Michielsen et al, 2005]. The annual risk of HCC is 0.5% for asymptomatic HBsAg carriers and 0.8% for patients with chronic hepatitis B [Okada et al, 1976, Liaw et al 1986], while patients with HBV-cirrhosis have 1000 times higher risk of developing HCC, compared to HBsAg negative individuals [Michielsen et al, 2005, Beasley1981]. Thus, it is likely that the probability of acquiring HCC increases with severity of underlying liver disease [Michielsen et al, 2005]. Chronic infection by HBV is by far the most important risk factor for HCC in humans. About 80% of Indian patients with HCC have HBV-associated liver disease (Dhir et al, 1998).

According to the WHO report on prevention of HBV in India [WHO 2002], HBsAg prevalence among general population ranges from 0.1% to 11.7%, being between 2% to 8% in most studies. HBsAg prevalence rate among blood donors ranged from 1% to 4.7%. With the exception of higher HBsAg positivity in some North Eastern states (~7%), no substantial geographical variation was apparent in other parts of India. Considering, on an average, HBsAg carrier rate of 5%, the total number of HBV carriers in the country was estimated to be about 50 million that forms nearly 15% of the entire pool of HBV carriers in the world and is the second largest pool of chronic HBV infections in the world [WHO, 2002].

The mechanisms of carcinogenesis in HBV infection have been extensively studied, and a major factor is chronic necro-inflammation with subsequent fibrosis and hepatocyte proliferation. However, HCC may occur in HBsAg carriers without cirrhosis. Both HBV and host hepatocytes may contribute to the final pathogenic outcomes, either individually or synergistically. Therefore, it is reasonable to consider that apart from host factors, viral factors are likely involved in HBV related hepato-carcinogenesis [Liu et al, 2007].
Several viral factors like viral genotype, viral load and e antigen status have been associated with HBV related liver disease severity and hepato-carcinogenesis [Liu et al, 2007]. India is a vast country, comprised of multiracial communities with wide variations in culture, ethnicity, food habits, lifestyle of different communities and thus infectious and chronic disease patterns [Sen et al, 2002].

The current study was designed to shed light on many important aspects of HBV molecular epidemiology that are very much important for identifying the population at risk of acquiring HBV and developing severe disease, and also pose a risk of transmission through different modes. This information are essential for determining the risk factors associated with HBV infections, to formulate necessary preventive measures to lessen the burden of new infections and spread of newly introduced genotype to other parts, from north-eastern India.

6.1 Family screening based seroprevalence of HBV in family contacts

The most recent World Health report (Jong-wook 2003) indicated a total of 714 600 new cases of HCC worldwide, with 71% among men. Liver cancer ranked 3rd for male subjects and 5th for women. In our studied cohort also, the majority of the HBV related chronic HBV and cirrhosis cases were males. Our data shows that the seroprevalence of HBV in family contacts is as high as 14.48%.

The peaking of infection rates in adulthood in Indian population suggests a close relationship of acquisition of infection in the adults [Chowdhury et al, 2005]. Intrafamilial aggregation of HBV infected persons in a family has been well documented in India [Chakravarty et al, 2005]. Household contacts of subjects with chronic HBV infection are known to be at high risk of acquiring infection through multiple modes [Maddrey 2000]. Horizontal transmission through close contact with carriers and perinatal routes was identified as an important mode of transmission of HBV in tribal communities of Andaman and Nicobar islands [Murhekar et al, 2000]. Very high endemicity of HBV infection in the tribal populations have been suggested to be due to their association with a number of socio-culture practices like endogamy, bloodletting, scarification, and tattooing and eating of orally processed food. A serological survey on 722 family members of 215 HBV infected Index cases of eastern India revealed that intra-familial horizontal transmission is more significant mode of transmission than sexual mode of transmission in later life for maintaining HBV carrier pool in this community [Chakravarty et al, 2005]. In our cohort, the number of cases who further developed chronic hepatitis B infection was 7.88% (172/2182); which is a high fraction of cases who had HBV infection as per family contacts and relations (54.43%). Majority of the patients who had chronic HBV infection based on family screening were females (59.88%), majorly wives of index patients (93.20%), which is indicative of the
sexual mode of transmission. Alternatively, inadequately sterilized needles and syringes (previously practiced) may be an important cause of transmission of hepatitis B in northeast India. Our data is contrary to other recent reports which showed that sexual transmission was not the predominant mode of transmission in some families, even when one of spouses had high levels of viremia, suggesting that sexual transmission in adult life may not be an efficient mode of transmission in this population [Datta et al, 2006].

6.2 HBeAg status

Three phases of chronic HBV infection are recognized: phase 1 patients are HBeAg positive with high levels of virus in the serum and minimal hepatic inflammation; phase 2 patients have intermittent or continuous hepatitis of varying degrees of severity; phase 3 is the inactive phase during which viral concentrations are low and there is minimal inflammatory activity in the liver. In general, patients who clear HBeAg have a better prognosis than patients who remain HBeAg-positive for prolonged periods of time. The outcome after anti-HBe seroconversion depends on the degree of pre-existing liver damage and any subsequent HBV reactivation. HBeAg may play an important role in the interaction of the virus with the immune system. Secreted HBeAg has been proposed to have an immunoregulatory function in uterus by establishing T-cell tolerance to HBeAg and HBcAg that may predispose neonates born to HBV-infected mothers to develop persistent HBV infection (Milich et al, 1990).

In our studied cohort comprising of patients from northeast India, HBeAg was found in a high fraction of chronic HBV (74%) and cirrhosis cases (65%). Taking into consideration a 10% sero-conversion rate per year, HBeAg positivity is significantly higher in the northeast Indian patients compared to reports from other parts of India such as North India [21% (Dixit VK et al, 2007)] and 55% (Chauhan et al, 2006)], South India [19.56% (Chandra et al, 2003)] and western India [53.41% (Patel et al, 2012)]. Our data also shows that the HBeAg positivity status is still higher compare to only report published from Northeast India which showed HBeAg positivity of 42.1% in children, 32.7% in adolescents and adults (Biswa D et al, 2007).

Comparatively the HBeAg positive status in Northeast India is significantly higher compared to reports from adjoining countries like China [22% (Fung et al, 2011) and 35.66% (Zhang et al, 2011)] and Taiwan [34.06% (Yang et al, 2012)] and definitely higher compared to southeast Asian countries like [21% (Merican et al, 2000)] and Western countries [23.96 from Belgium (Deltenre et al, 2012) and 37% from Germany (Fischer]
et al., 2012). The remarkable high HBeAg positive cases in cirrhosis cases (65%) in our cohort is indicative of significance of HBeAg status in determining liver disease severity in a major fraction of chronic HBV related liver disease cases. Moreover, it was found that high viral load (an independent viral risk factor for liver disease severity) was significantly associated with e antigen positive status (p<0.001) in both the chronic HBV and cirrhosis groups. When correlated with the biochemical profile, HBeAg positivity (p=0.016) and high viral load was associated with higher SGPT levels in chronic HBV cases. A prospective cohort study with 11 years of follow-up assessed the relationship between HBV viral load and mortality. Viral load was found to be associated with increased mortality from HCC and chronic liver disease in HBV-infected subjects. The relative risk (RR) for HCC mortality in patients with viral load < \(10^5\) copies/mL was 1.7 (95% CI, 0.5-5.7), whereas it was 11.2 (95% CI, 3.6-35.0) in patients with viral load > \(10^5\) copies/mL. Viral load may thus be a useful prognostic tool in HBV infection.

Normally, the hepatitis B virus (HBV) needs three proteins (also called antigens) in order to manufacture more HBV. These are called the core, surface, and “e” or HBeAg antigens. But some HBV, with certain mutations, are able to reproduce without the “e” antigen. This is called HBeAg-negative hepatitis B. This infection can be harder to treat. HBeAg-negative chronic hepatitis B is a phase of chronic HBV infection during which a mutation arises [Precore mutation (G1896A), core promoter mutations (e.g. A1762T/G1764A)] resulting in the inability of the virus to produce HBeAg. Such patients tend to have more severe liver disease and run a more rapidly progressive course. The annual probability of developing cirrhosis varies from 0.1 to 1.0% depending on the duration of HBV replication, the severity of disease and the presence of concomitant infections or drugs (Chauhan et al., 2006).

Very little information on the prevalence and molecular epidemiology of HBeAg negative chronic infections is available from India. In Mediterranean populations, genotype D has been shown to present an extremely high prevalence of HBeAg negative chronic HBV infection. The annual rate of progression to cirrhosis is 8–10% in HBeAg negative patients compared with 2–5% in HBeAg positive patients (Hadziyannis et al., 2001). Long-term prognosis is poorer among HBeAg-negative individuals compared with their HBeAg-positive counterparts (Hadziyannis et al., 2001). In our cohort, the development of chronic HBV (10%) related liver disease and cirrhosis (3%) was found in a high percentage of e- and anti-HBe –ve cases, which is of clinical significance.
6.3 Hepatitis B virus genotyping

There has been an explosion of knowledge with respect to HBV genotypes and their association with activity of liver disease, and treatment response. HBV Genotype A(X70185) is mainly found in North Western Europe, North America, Philippines, Hong Kong and south and eastern Africa. Genotype B (D00331) and C(X01587) strains belong in the indigenous population of Southeast Asia. Genotype D(X72702) is most widely distributed genotype and found universally including India. Genotype E(X75664) is found in West and South Africa, whereas Genotype F(X75663) is found in South and Central America. Genotype G (FR1) and H have been reported from isolated places of Germany, France and USA. Migration and behavioral patterns may change the prevailing genotype in a given region. Moreover, because of the spontaneous error rate of viral reverse transcriptase, the HBV genome evolves with an estimated rate of nucleotide substitution at 1.4–3.2x $10^5$/site/year (Okamoto H et al, 1987; Orito E et al, 1989). Currently, eight genotypes (A–H) of HBV are defined by divergence in the entire HBV genomic sequence >8% (Okamoto H et al, 1988; Norder H et al, 1994; Arauz-Ruiz P et al, 2002; Bartholomeusz A et al, 2004). HBV genotypes have distinct geographical distributions (Miyakawa Y et al, 2003).

Geographical location of India is between West and Central Asian countries and East Asian countries, having different HBV genotype distributions. Gene flow from these neighbouring countries, due to anthropological migration in the past has contributed to considerable genetic, geographic and socio-cultural diversity of the Indian population [Basu et al, 2003, Sahoo et al, 2006]. This multiethnic origin of the Indian populations is also reflected in the HBV genotype distribution in different parts of the country. Moreover, recent increase in trade, trafficking and use of illicit drugs and frequent visits to and from different countries have also considerably influenced the epidemiology of HBV and other parenteral infections in India and specially in the eastern and north eastern parts of India [Beyrer et al, 2000, Vivekanandan et al, 2004, Chaudhuri et al, 2005]. It is reported that HBV genotype affects clinical outcome and treatment responses. For example, in Asia, genotype C is found to be commonly associated with more severe liver disease, cirrhosis and the development of HCC, compared to genotype B [Liu and Kao 2007, Arbuthnot et al, 2000, Wu et al, 2006, Chen et al, 2006, Iloeje et al, 2006, Chen et al, 2007 Hsieh et al, 2004, Yu MW et al 2005] whereas in Western Europe and North America, genotype D is more associated with severe liver disease and a higher incidence of HCC, than genotype A [Liu and Kao 2007, Moriya et al, 1998].
We investigated the HBV genotypes prevalent in Northeastern India and their relation with the development of chronic HBV and cirrhosis. Our results show that HBV isolates from chronic HBV and cirrhosis patients from northeast India could be categorized into two major categories; one in which resembles a single genotype, either genotype A, C or D; and the second, where isolates have 'mixed' genotypes by virtue of multiplex PCR analysis and showing homology with at least two genotypes (A+D, C+D or B+C) over the analyzed regions of the HBV genome. HBV genotype D was the most prevalent genotype (62.30%) in our studied cohort. Most importantly, there is a high prevalence of HBV genotype C (13.96%) in our studied cohort and especially in the states of Manipur and Arunachal Pradesh, which is distinctly higher than any other corner of India. Moreover, HBV genotype C was found to be associated with higher viral load and increased cirrhosis risk compared to chronic HBV cases [OR=1.670 (0.940-2.964) at 95% CI]. In HBeAg positive cases (which itself is a risk factor associated with HBV related liver disease severity), it was found that presence of HBV genotype A or C doubled the risk of cirrhosis development compared to chronic HBV.

HBV genotypes A and D have been well documented from different parts of mainland India [Thakur et al, 2002, Gandhe et al 2003, Kumar et al.2005, Banerjee A et al, 2006, Banerjee et al 2006, Chattopadhyay et al, 2006 and Chattopadhyay et al, 2006]. HBV genotype D to be predominant with a low frequency of genotype A in northern Indian HBV infected patients which was comparable to the HBV genotype distribution documented from western and southern parts of India [Vivekanandan et al,2004, Gandhe et al, 2003]. In sharp contrast to rest of the parts of India, the eastern part of India presents an interesting epidemiology of three different HBV genotypes (genotypes A, C and D) in comparable proportions [Vivekanandan et al,2004, Banerjee A et al, 2006, Banerjee et al, 2006]. Higher prevalence of HBV genotype C in our cohort is of clinical significance, as genotype C was associated with more severe liver disease and increased risk of HCC compared with other HBV genotypes (Kao JH et al, 2000; Orito E et al, 2001a). In Bangladesh, the predominant genotypes are D and C. Overall distribution of HBV genotype was found to be 50% genotype D,37.5% genotype C 37.5%, mixed C+D 7.5% and A and B each 2.5% (Mahtab et al, 2006). In the earlier study from NE India which included patients from Arunachal Pradesh, the predominant genotype was genotype A (41.6%) followed by genotypes C (27.8%) and D (11.1%) (Borkakoty et al, 2008).

Our results also suggests occurrence of events of mixed genotypes and possible recombination between HBV genotypes, especially genotype A+D (9.21%) in chronic HBV and cirrhosis patients from northeast India. We report it as mixed genotype and not a recombinant one, as we haven’t completed the whole genome sequencing and its analysis by tools such as ‘Bootstrap plots’ or ‘SimPlot program’ (Ray, 1999) which are seldom used for recombinant HBV genotype identification and analysis. Bearing in mind the extreme compactness of HBV genome and the
strategy of replication, with a single, particle-associated RNA genome converting into a partially double stranded DNA virus, the probability of a template switch appears very low (Georgi-Geisberger et al, 1992). However, it is most likely that, whatever the original mechanism, mixed infection in a host arises either from simultaneous transmission of several genotypes or from sequential infections with different genotypes.

Thakur et al, prospectively studied the prevalence and clinical significance of HBV genotypes A and D in 130 histologically proven chronic HBV-infected Indian patients and showed that HBV genotype D is associated with more severe liver disease, and may predict the occurrence of HCC in young Indian patients. Whether the mixed genotypes have an advantage for replication and persistence in hosts over either of the genotype is not known. But it is evident from earlier study (Thakur et al, 2002) and also from some studies by other groups that genotype D is more responsible for worsening of the liver disease compared to genotype A. This may be due to some viral mutations harmful to the host are genotype D associated.

Knowledge of HBV genotype enables clinicians to identify those patients at increased risk of disease progression whilst aiding the selection of appropriate antiviral therapy. (Tanwar et al, 2012). Accumulating evidences clearly indicate that HBV genotypes can significantly influence HBeAg seroconversion rates, viremia levels, mutational patterns that could significantly influence the heterogeneity in clinical manifestations and even response to antiviral therapy [Osiowy et al, 2006; Schaefer et al, 2005; Echevarria et al, 2006]. The simultaneous presence of different genotypes in the ethnically distinct population of northeastern India is unique, providing opportunity to directly compare the clinical significance of HBV genotypes in disease manifestations in the north-eastern population. The comparison of clinical and virological characteristics between HBV genotypes A, C and D revealed the higher potentials of genotypes A and C in causing disease severity in this part of India, as they were associated with prolonged HBeAg positivity, higher ALT levels and higher viremia. Our data is complimentary to earlier data published by Datta et al, 2008 from eastern India.