Chapter 5

Discussion
DISCUSSION

Infection with HCV is the cause of most of the chronic liver disease cases in various parts of the world. The death toll due to HCV, from end-stage liver disease and its complications including hepatocellular carcinoma is increasing continuously over the years (Lavanchy et al, 2000; Okuda K, 1997). As a consequence, hepatitis C infection is both a devastating problem for the individual patient and also a huge financial burden on the society with limited resources to provide its treatment in developing countries. Moreover, there is a paucity of information on HCV prevalence from the developing countries of the world.

Recently, information on HCV prevalence in the developing countries, especially in the Asia and Pacific regions, where the vast majority of human population resides, are increasing significantly. It is necessary to have detailed data on the prevailing HCV genotypes in these regions. In these parts of the world, HCV epidemiological data had been derived mostly from blood donors and small segments of liver disease patients (Issar et al, 1995; Sarin et al, 1996; Panigrahi et al, 1997; Sawant et al, 1999; Sood et al, 1999; Amarapurkar et al, 2001; Mathai et al, 2002; Das et al, 2002; Raghuraman et al, 2003).

Infection with hepatitis C virus (HCV) is a major cause of transfusion-associated hepatitis, cirrhosis and hepatocellular carcinoma. HCV infection, a global public health problem is quite prevalent in India.

Data on HCV prevalence in large metropolitan cities like New Delhi, showed a significant seropositivity (1.85%) among voluntary and
replacement blood donors which is supposed to be a reflection of the general population (Panigrahi et al, 1997). The aforesaid study was conducted among healthy blood donors at the All India Institute of Medical Sciences (AIIMS), New Delhi, India to screen for anti-HCV antibody. Two hundred and ninety-five individuals (1.85%) among a large number of healthy voluntary blood donors (15,898), were HCV positive. Among patients with liver disease, 13.83% were anti-HCV antibody positive, and HCV was associated with 9% of the acute cases. (Panigrahi et al, 1997). No significant difference was observed between the HCV seropositivity rate of males and females. The age distribution of anti-HCV positive subjects showed a maximum prevalence rate of 1.8% in the age group of 20-29 years. Since this group comprises the majority of healthy blood donors, it shows that the prevalence of anti-HCV antibodies in such healthy voluntary blood donors of New Delhi, India is considerably higher than similar studies conducted in majority of the industrialized nations and this represents a large reservoir of infection capable of inflicting significant disease burden on the society.

Contrary to the above observations, a low prevalence of HCV seropositivity was reported from semi-urban areas of South India, 0.8% (Chandrashekharan et al, 2000), and from Western India, 0.3% (Garg et al, 2001) and to a moderate rate (1.8%) from Central India (Jaiswal et al, 1996).

Data on prevalence of HCV marker in the general population of the city of Kolkata is not available. However, the only study conducted among multiple transfusion recipients and multiple needle stick injury cases in Kolkata, during February-July 1996, showed high HCV seropositivity (8.8%)
among the latter group (Neogi et al, 1997). A total of 153 samples of different age groups and of both sexes were screened by ELISA for detection of Anti-HCV antibody. Anti-HCV was found in 13% of multi-transfused cases and in 8.8% cases with multiple needle-stick injury. In the above study a very high seropositivity (20%) was observed amongst males between the 31-40 years age group. HCV seroprevalence was noted more in males (13%) than in females (8.2%) and an increasing trend had also been observed amongst the multiple blood transfusion cases in Calcutta. (Neogi et al, 1997).

A recent study among the intravenous drug users of the city of Kolkata showed an extremely high seroprevalence (80%) of HCV infection (Sarkar et al, 2003). On the contrary to above information, a collaborative work of the present study showed a considerably low prevalence (0.87%) of HCV infection in the rural asymptomatic population of West Bengal (Chowdhury et al, 2003). Similar observation was made in a previous study conducted in rural Rajasthan, North Western India, where the rate of prevalence was 0.1% (Chadha et al, 1999). But invariably, the urban population of India are exposed more to risk factors for blood borne diseases than rural population.

The overall prevalence of active HCV infection among liver disease patients in this study was significantly high (15.9%). Such high prevalence of HCV among liver disease patients were also reported from Africa (21%-33%) (Tsega et al, 1995; Soni et al, 1996), Korea (15.4%) (Kim et al, 1993) and Indonesia (11.8%) (Sulaiman et al, 1991) and also from Saudi Arabia (Ayoola et al, 1992) and Yemen (el Guneid et al, 1993).
In the present study, the aetiological factors of hepatitis in the anti-HCV and HCV RNA negative patients varied. In acute hepatitis, hepatitis E, hepatitis A, hepatitis B viruses were the predominant causes in that order. In chronic hepatitis, hepatitis B virus was the most common cause, followed by autoimmune hepatitis and cryptogenic liver disease. In other high risk populations in the present study, the prevalence of HCV infection was quite high, 42.4% among IDUs, 10.3% among haemophiliacs and 42.8% among thalassemics.

Most of the HCV seropositive subjects in our study were viraemic (83.5%), indicating active and possibly recent infection among liver disease patients. Such high prevalence of viraemia was also observed in other studies conducted in several countries of the world (Garner et al., 1997; Ahmed et al., 1998; Madhava et al, 2002; Strickland et al, 2002). About 33-34% of the seropositive subjects in this group had elevated ALT levels suggesting the presence of necro-inflammatory active liver disease and viral replication. A significant proportion of the chronic liver disease patients (67.2%) had normal serum ALT levels, probably reflecting the fluctuations of ALT levels in chronic HCV cases. These findings are in agreement with the findings of other studies (Dincer et al., 2001).

There are only a few community based studies on HCV prevalence world wide, which have been reported mainly from the industrialized countries of Europe and America, like USA (Alter et al.,1999), Italy (Guadagnino et al.,1997; Maio et al, 2000), France (Dubois et al.,1997), and also from Egypt (Habib et al, 2001). However, blood donors as an epidemiologic database, represent a skewed population sample because of the
lack of participation by children and senior citizens and under-representation of women (Heintges et al, 1997). Therefore, the available data do not effectively represent the general population. Two previous community based studies from India on HCV prevalence involved population samples that were arbitrarily chosen for convenience, without adhering to rigorous standard sampling procedures that eliminate bias in sample selection (Arankalle et al, 1995; Chadha et al, 1999). Because 70% of the individuals reported in these studies were children or adolescents, a low HCV prevalence (0.09%-0.12%) was observed in above studies.

A collaborative work (Chowdhury et al, 2003) which is a part of the present study conducted among asymptomatic general population is a population based epidemiology study of HCV infection in rural West Bengal based on a sample representative of the rural community. The strength of that investigation was the inclusion of large representative of cohorts using a standardized systematic sampling procedure. The sampling approach was designed to eliminate bias introduced by any unidentified local social or cultural factors that could potentially influence the transmission and prevalence of HCV in a specific geographic area.

The molecular detection of HCV infection among the aforesaid population (Chowdhury et al, 2003) and characterization of the strains were performed in the Division of Virology of this Institute as a part of the present study. The overall prevalence of HCV infection in the general population in this study was low (0.87%) than that reported from the United States, Italy, Egypt, Japan and Australia (Tanaka et al, 1992; Farrell et al, 1993). The increase in HCV prevalence with age, suggests a steady cumulative increase
of incidence of infection. This may be caused by sporadic transmission of
infection persisting in the community over the years. This is in contrast to the
abrupt and unpredictable changes of incidence, as reported from Italy, Japan,
and Egypt (Tanaka et al, 1992; Guadagnino et al, 1997; Abdel-Aziz et al,
2000).

In the developed world hepatitis C virus infection is predominantly
associated with sharing contaminated equipment between injecting drug
users. On the other hand, in developing countries inadequately sterilized
medical equipment, transmission of infected blood and cultural practices have
been implicated for transmission of HCV infection. Therefore, accurate risk
factor assessment is essential for education with an aim of risk reduction in
culturally diverse populations. Risk factors associated with HCV infection
among Caucasians are IDU (89%), body piercing (47%) and tattooing (32%)
(Dev et al, 2004). Risk factors in South East Asian patients are injection
therapy (89%), dental therapy (70%) and surgery (38%). Usually most
Caucasian patients (94%) correctly identify their mode of acquisition
compared with 33% of South East Asian patients (P < 0.0001). Accurate risk
factor documentation in medical records is more common in Caucasians (96
vs. 32%; P < 0.0001). The majority of patients identified blood-to-blood and
sexual/vertical transmission as important modes of acquisition. Ethnicity
influences perception and knowledge of risk factors. (Dev et al, 2004).

The low prevalence of HCV in the rural study population precluded
extinctive analysis of the risk factors associated with HCV infection.
Univariate analysis revealed that history of medical injection using reusable
glass syringes correlated with HCV infection. Therefore, health education to
the people regarding these modes of transmission of this virus may prove to be useful preventive intervention in this country. Improved assessment of risk factors in high-risk groups is needed and education should be culturally appropriate and address the concerns of all populations with HCV.

Most HCV seropositive test subjects among asymptomatic cases and voluntary blood donors in this study had measurable viraemia (80.76%), which is consistent with findings in studies from France (80.6%), Italy (75.9%), and the United States (73.9%) (Alter et al, 1994; Guadagnino et al, 1997; Dubois et al, 1997) although a lower incidence has been reported from Egypt (65.5%) (Abdel-Aziz et al, 2000). In the present study, only one third of anti-HCV seropositive asymptomatic general population had increased serum ALT levels. The normalcy of ALT levels in two thirds of anti-HCV positive subjects underscores the relative insensitivity of ALT determination in identifying active HCV infection.

HCV and other RNA viruses exhibit a high frequency of nucleotide substitution during replication, resulting in significant genetic heterogeneity among the virus strains. Among various methods of genotyping, we adopted the RT-PCR method described by Ohno et al (1997). The concordance between Ohno's genotyping assay and nucleotide sequencing, for genotypes 1 and 3, was 75%. Ohno's type-specific primer based genotyping assay may be used for distinguishing between HCV genotype 1 and non-1 HCV genotypes in laboratories that do not possess nucleotide sequencing facilities. (Raghuraman, 2003). In this study also the concordance of Ohno's PCR typing data and sequencing data was almost 94%, and only 23 samples had discordant PCR and sequencing data. Among them, 18 were untypeable by
PCR but after sequencing all of them showed specificity to known genotypes like 1a, 3a or 3b. One strain (NB51) showing genotype 3b specificity in PCR was resolved as 3a by sequencing. These discordant results were due to significant changes in the type-specific primer regions which have been elaborated in the results section. Hence, in spite of the high reliability of Ohno’s genotyping method, the frequently changing pattern of HCV quasispecies necessitates confirmation of the results by sequencing.

HCV genotypes are important predictors of response to interferon therapy. Moreover, there are geographical differences in the distribution of genotypes of HCV. It has been suggested that genotypic distribution of HCV is likely to provide information regarding the introduction, spread, and evolution of the virus in a defined human population and may be informative of human population migration (Zein, 2000; Smith et al., 1997; Ray et al., 2000). Information on the distribution of HCV genotypes in the Indian subcontinent has been derived mostly from studies on selected population groups. Despite some inter-study differences in frequencies of various genotypes, in general, genotype 3 predominates in northern India, Pakistan, and Nepal, whereas one report from southern India indicated a higher prevalence of genotype 1 (Valliammai et al., 1995).

According to recent studies, HCV genotype 3 is prevalent in Thailand and other South East Asian countries with the exception of Indonesia (Dev et al., 2002) whereas in Japan, China and Indonesia, 1b and 2a are predominant HCV genotypes (Kim et al., 1993; Hotta et al., 1997; Dev et al., 2002).

The genotypic prevalence data in the present study showed predominance of genotype 3, which is concordant with earlier reports from
North India (Panigrahi et al, 1996) and South India (Valliammai et al, 1995; Amarapurkar et al, 2001), as they also reported high prevalence of genotype 3 in their study population. The distinctive shift in the genotypic prevalence clearly indicates the fast changing evolutionary scenario of HCV and the ensuing danger caused by the virus in this geographical region. In this study among asymptomatic general population and voluntary blood donors, genotype 3 was the predominant genotype, comprising over 60% of the isolates, confirming the previously reported results from different parts of the subcontinent. On the other hand, genotype 1b was predominant among cirrhosis and carcinoma patients indicating the propensity of this genotype to enhance disease progression.

In the current study, the frequency for HCV genotypes in different age groups was compared for the two study population, i.e., acute and chronic liver disease cases. There were significant correlation between HCV genotype and age of patients. The frequency of genotype 1b was found to increase in older individuals in both the groups. On the other hand, genotype 3b was found to be more prevalent among young age group. Genotype 3a showed a similar pattern of distribution with 3b. Since we could not detect other subtypes of type 1 (other than a subtype 1a detected after sequencing), it is not possible to assess the age preferences of different subtypes of genotype 1 from currently available data. The association of type 1b with older age may indicate that 1b was the former main subtype in this region and subsequently there was a shift in predominating subtype of HCV from 1b to 3a and 3b. Recent studies from some part of the world indicate the increased rate of intravenous drug use to be the cause of the shift in genotypic prevalence from
subtype 1b to 3a (Kalinina et al, 2001; Bourliere et al, 2002). But in the present study population the subjects were not exposed to intravenous drug abuse, whereas most of the exposures were due to improper sterilization of glass syringes and needles for reuse in injections during treatment of ailments. Therefore, it is difficult to hypothesize any possible reason for the genotypic shift in our study population.

Among the liver disease cases, sequencing data of the untypeable isolates showed that eight of them had maximum homology with type 3a reference strains. But the multiplex RT-PCR for genotyping did not yield any product with 3a type-specific primers from these isolates. When these sequences were aligned with 3a reference sequences, significant changes in the 3a specific primer binding region were detected, but overall, the sequences maintained 98% homology with reference strains. Sequence analysis of another five isolates that showed maximum homology with 3b reference strains also yielded similar results. In this study two strains showed maximum homology with one Indian (IND308) and one Nepalese (NE137) strain, which were designated as type 3b by phylogenetic analysis (Tokita et al, 1994). One isolate showed maximum homology with a type 1a strain, but could not be amplified by 1a-specific primer in genotyping PCR. This isolate was the only 1a strain we could detect during this study. On the other hand an isolate showed maximum sequence homology with a Thai strain (Th580) typed as 6b. Since the genotyping method used here does not encompass 6b, this isolate was not typeable by multiplex PCR. Although this is the second report of detection of type 6 in this country, the previous one being from Pune, a city in Western India (Lole et al, 2003), it is difficult to find out any
explanation for the spread of a Thai strain over to this subcontinent. But geographical proximity and frequent flux of travellers between India and Thailand suggest that HCV might have spread along with HIV, since strain similarity among these populations have already been reported for HIV infection in India (Naik et al, 1991, Chakrabarti et al, 2000). It also indicates that such strains may be circulating all over South Asia, but due to the lack of intensive surveillance system many of them could not be detected earlier. Recent studies conducted on South-East Asian population support the concept that race and ethnicity are important determinants of treatment outcome in HCV infected patients (Dev et al, 2002). Therefore, further knowledge of molecular epidemiology of HCV infection is essential in designing treatment modalities for control of HCV infection in this part of the world.

Molecular epidemiological studies on HCV subtypes within a major specific genotype often provides useful information. Among the asymptomatic population in this study, determination of nucleotide sequence of three of the five untypeable strains revealed that they belonged to genotype 3 and had a 98-99% homology with subtype 3e, described only from Nepal (Tokita et al, 1994). The finding of genotype 3e in eastern India, which is geographically close to Nepal, warrants the analysis of a larger cohort from this geographical region to test the hypothesis that this genotype may represent a region specific type that might have spread via population migration. In Western countries, genotype 3 had been linked to injection drug abuse (Kalinina et al, 2001). However, in the population that we have studied, intravenous drug abuse was nearly non existent, but because of the high frequency of injections by improperly sterilized syringe and needles used for
medical treatment genotype 3 might have spread through injections in this community as well.

The high incidence of chronic hepatitis indicates that most individuals are incapable of spontaneously mounting an immune response that will clear the virus (Liang et al, 2000). In addition, diverse HCV genotypes, subtypes, and quasi-species increase the potential for viral escape from immuno surveillance (Liang et al, 2000). Infection with human hepatitis C virus is a result of a bilateral process of host-virus interactions. There are factors on both sides that contribute to clearance and to chronicity. Virus strategy to survive is built on several basic features. Among which the most important feature is rapid error-prone replication that leads to accumulation within one host of multiple virus variants (quasispecies). Viral heterogeneity could be multiplied by recombination of HCV genomic / subgenomic RNA molecules (Isaguliants et al, 2003). Quasispecies nature gives virus an advantage in adaptation to varying host environment.

Therefore, the identification of the immunological and virological correlates that determine resolution verses chronic evolution of HCV infection and the development of vaccines and immuno therapy are challenging endeavors.

In the present study, it was observed that genetic heterogeneity of the quasispecies in the chronic cases is greater in genotype 1b strains than in genotype 3a or 3b strains. This observation underscores the putative greater capability of 1b strains of HCV to evolve into chronicity.

Genetic variation in responders and nonresponders was characterized by complexity and diversity. The complexity, the distribution of variants in
the population, was estimated by calculating the Shannon entropy. Diversity was measured as the mean genetic distance calculated for all pairs of sequences.

Present data showed no significant difference between responders and nonresponders for either measure of core-HVR-1 genetic heterogeneity. A similar observation was also reported by Polyak et al (1997). On the other hand, other studies based on cloning and sequencing of HCV strains have reported that patients with heterogeneous virus population are less responsive than patients with homogeneous virus population (Chayama et al, 1995; Kanazawa et al, 1994).

In this study, the quantitative parameters for all mutations assessing genetic heterogeneity of HCV quasispecies did not differ significantly among responders and non-responders. But, nonsynonymous mutations were significantly more frequent ($P<0.05$) than synonymous mutations in responders.

This important finding of the present study, that pretreatment nonsynonymous substitutions in core-HVR-1 were significantly more frequent than synonymous substitutions in patients who had cleared their HCV after interferon therapy, suggested a stronger selective pressure for changes in amino acids in these patients. It has been demonstrated previously that the 27-amino-acid segment located in the N-terminal portion of the HCV envelope protein, HVR-1, contains linear neutralizing B-cell epitopes (Farci et al, 1994; Shimizu et al, 1994; Shimizu et al, 1996; Zibert et al, 1995).

Recent evidence suggested that HVR-1 is also a helper T-cell recognition site (Shirai et al, 1999). Thus, the selection pressure driving the
genetic variations of the virus may come from neutralizing antibodies whose production depends on T cells specific for this region. Other studies demonstrated that IFN therapy was associated with an increased rate of fixation of mutations in the HVR-1 region compared to the same region in untreated patients, supporting the idea that IFN acts partially via immunomodulation (Pawlotsky et al, 1999; Polyak et al, 1998).

From the observations in this study, one may hypothesize that in addition to genotype, the pretreatment viral quasispecies status could influence IFN-stimulated immune responses and ultimately virus eradication.

In the present study, which included a majority of HCV genotype 1 and 3-infected patients, the complexity and diversity of core-HVR-1 were not associated per se with virus eradication after IFN therapy. In contrast, the higher proportion of nonsynonymous substitutions found in responders suggests that the HCV-specific immune response is involved in the clearing of HCV by IFN. Pretreatment viral quasispecies status, and the intensity and quality of the anti-HCV immune responses of individuals, could be putative determinants of HCV RNA clearance.

During the time of infection it was observed that specific nonsynonymous changes in CTL epitopes and accelerated change in the coding sequence of core-HVR1 were evident in those who had become chronic carriers, and this corroborates to the observations of other research groups (Cantaloube et al, 2003; Sheridan et al, 2004).

The mechanisms that determine viral clearance or viral persistence in chronic viral hepatitis have yet to be identified. Recent advances in molecular
genetics have permitted the detection of variations in immune response, often associated with polymorphism in the human genome (de Andrade et al., 2004). Differences in host susceptibility to infectious disease and disease severity cannot be attributed solely to the virulence of microbial agents.

Two research groups independently reported on HCC-associated mutations of the core protein, suggesting a possible role for the mutations in the development of HCC (Horie et al, 1996; Horie et al, 1999; Ruster et al, 1996; Shimizu et al, 1997). There were, however, some discrepancies between their observations; one group identified point mutations in a hydrophilic region between amino acids (aa) 39 and 76 (Shimizu et al, 1997), while the other group reported deletion mutations near the N terminus of the core protein that caused a frame shift, giving rise to a truncation at the C-terminus (Ruster et al, 1996). Apart from the HCC-associated mutations in the core protein, Xu et al (2001) recently identified a novel HCV protein, the F protein, that was synthesized from the initiation codon of the core protein followed by a ribosomal frame shift into the +2/-1 reading frame at or near codon 11. It was previously reported that mutations were clustered in a region between aa 39 and 76 of the core proteins of HCV isolates from patients with HCC, and therefore, this region was referred to as a clustering variable region (Horie et al, 1996; Shimizu et al, 1997).

However, the present study showed that there were 0.9 ± 0.6 and 1.4 ± 0.8 mutations in the region from aa 39 to 76 in isolates from patients with and without HCC, respectively, and the difference was not statistically significant ($P = 0.14$). It was also observed that codon 45 (Gly) was completely conserved among all isolates analyzed. Hence, the present observation does
not agree with the information in previous reports by Horie *et al* (1996, 1999), who showed that codon 45 was Gly in isolates from cancerous tissues of patients with HCC, while it was Ser in isolates from noncancerous tissues and sera of patients without HCC. However, any deletion or insertion in the core protein-coding sequences of the isolates resulting in frameshift mutation and production of truncated core protein, was not encountered during this study. The nonsynonymous mutations in carcinoma strains detected in this study resulted in distinct change in hydropathic index of the core and E1/E2 proteins especially in the second hydrophilic region.

In a large country like India, it is necessary to have population based data from different parts of the country. Even with a prevalence of HCV below 1%, there is a large reservoir of over 8 million HCV- infected persons in India. However, despite the magnitude and projections, chronic viral Hepatitis is yet to be perceived as a public health priority in India. It is hoped that the descriptive epidemiologic data presented here should underscore the significance of this major threat to public health.