CHAPTER 2

Prevalence of Hepatitis C Virus Infection in various Indian Population Groups
INTRODUCTION

Hepatitis C virus (HCV) infection is becoming a major menace and is evolving as a public health problem globally (WHO 1999). Studies on the prevalence of HCV in different countries suggest that more than 300 million people worldwide got infected (Brown and Gaglio 2003), out of which at least 20 million people reside in India (Khaja et al., 2002). It is estimated that about 0.2 to 26% of the healthy population in different countries are afflicted by chronic HCV infection (Aymard et al., 1993; Mutimer et al., 1995; Shakil et al., 1995; Panigrahi et al., 1997; WHO 1997; Murphy et al., 2000). Besides blood transfusions, several other routes appear to be responsible for the spread of HCV infection (Frank et al., 2000). These include the use of unscreened blood, and blood products, transplantation of tissues and organs, medical and dental care with contaminated or inadequately sterilized needles, syringes and equipment, sharing needles among drug abusers, sexual transmission, perinatal transmission and sharing of personal things like razor blades or tooth brushes.

Chronic Renal Failure (CRF) patients on maintenance and hemodialysis, hemophiliacs and thalassemics form a major risk group for HCV infections due to the frequent use of blood, blood products apart from multiple invasive medical procedures to which these patients are exposed (Sato et al., 1994). While the risk of transmission of HCV has been considerably reduced in developed countries owing to increased screening procedures (Bruguera et al., 1990; Baur et al., 1991; Arankalle et al., 1992), the problem is not properly addressed in developing countries like India.

Apart from other liver diseases, HCV is now shown to be associated with a number of extra-hepatic manifestations such as membranoproliferative glomerulonephritis, oral cancer and Lichen Planus (Johnson et al., 1993; Hadziyaminnis et al, 1997; Nagoa et al., 2000). While mandatory screening methods decreased the prevalence of HCV infection in the developed countries, these procedures are not routinely implemented in developing countries like India (McCullough, 1993) and therefore, an abnormally high prevalence of HCV infection has been reported in Indian patients (Baur et al., 1991; Kant and Arora, 1996).
Current estimates indicate that approximately 1.8-2.5% of Indian population is presently infected by HCV (Arankalle et al., 1992; Thyagarajan et al., 1998; Madhavi et al., 2003). The prevalence rate as well as the significance of HCV infection varies considerably from country to country, probably because of cultural factors and social habits that influence HCV transmission. The aim of the present study was to assess the prevalence of HCV infection in various groups in South India, Hyderabad. This study includes patients attending gastroenterology camps, voluntary blood donors, and various high risk groups of individuals such as those with chronic renal failure (CRF), health care workers, individuals who had tattooing and pilgrims that practice slashing of the body and patients having frequent blood transfusions. We have also included several patients with Lichen Planus. Apart from this we did a population based epidemiological study on a tribal population namely Lambada.
MATERIALS AND METHODS

Human subjects: For the present study, blood samples were collected from patients (n=704) attending gastroenterology camps, volunteers (n=2036) from various blood banks in Hyderabad, patients (n=380) with a medical history of haemodialysis / renal transplantation, and individuals attending the outpatient wing of the Owaisi Hospital and Research Center in Hyderabad. We also included individuals considered to be at high risk, such as health care workers (n=256), persons who had tattooing (n=70), and pilgrims (n=90). In addition, patients with multiple transfusions for diseases like thalassaemia (n=152) and haemophilia (n=105) and patients with Lichen Planus (n=52) were also included in this present study. Apart from this we screened a sect of Lambada tribal population (n = 890) Penumaka, Guntur (dt), Andhra Pradesh.

Statistical analysis was done by student’s ‘t’ test and chi-square method. The protocol for this study confirmed to the ethical guidelines of the 1975 Declaration of Helsinki, as reflected in a priori approval by the institutional safety committee.

Detection of HCV: Anti HCV antibody was tested by using third generation ELISA kits (Abbott Labs, Chicago, IL). The test was performed according to the manufacturers protocol.

RNA ISOLATION

Materials required
1. Lysis buffer
   Guanidinium thiocyanate-4M
   Sodium citrate-25 mM
   Sarcosyl-0.5%
   2-mercaptoethanol-0.1M
2. Water saturated phenol pH:4
3. Chloroform and Iso amyl alchol (24:1v/v)
4. Isopropanol
5. 80% Ethanol
6. DEPC (Diethyl Pyrocarbonate) water
Procedure

Guanidinium iso thiocyanate acid phenol chloroform method is used to isolate RNA form both tissues and plasma or serum (Chomczynski and Saachi 1987). 200μl of serum was mixed with 500μl lysis buffer, 50μl of 3M Sodium Acetate, 500μl water-saturated phenol and 200μl chloroform: isoamyl alcohol and mixed by inversion after the addition of each reagent. The final suspension was shaken vigorously for 10 sec. and cooled on ice for 15 min. Samples were centrifuged at 10,000 g for 20 min at 4°C. After centrifugation, RNA was present in the aqueous phase whereas DNA and proteins were present in the interphase and phenol phase. The aqueous phase was transferred to a fresh tube, mixed with equal volumes of chloroform: isoamyl alcohol and centrifuged at 10,000 g for 20 min at 4°C. For precipitation of RNA, the aqueous phase was transferred to a fresh tube, mixed with 1 ml of isopropanol, and then placed at -20°C for over night. Then RNA sample in isopropanol was sedimented at 10,000g for 20 min. The RNA pellet was resuspend in 80% ethanol, sedimented, air dried (15 min), and dissolved in 20μl of DEPC treated water at 65°C for 10 min.

RT-PCR

Materials required

1. Milli-Q grade water
2. 25 pmol of both forward and reverse primers
3. 10mM of each dNTPs
4. 2.5U of Taq polymerase
5. 10X PCR Buffer
6. 1.5mM MgCl2
7. 100U of MMLV-Reverse Transcriptase
8. Template

Procedure

Prior to RT-PCR the RNA was denatured by heating at 95°C for 2 minutes, followed by rapid chilling. PCR amplification of RNA was done essentially by the method of Das et al. (1993). Briefly, the 5' NCR (non-coding region) amplification was carried with 25 Pico mole each of the primers, 10X Taq buffer (Mg²⁺ free), 1.5 mM MgCl₂,
200mM dNTPs, 25 units of ribonuclease inhibitor (RNAsin), 100 units of MMLV reverse transcriptase (Promega, Madison, USA), 2.5 units of Taq DNA polymerase and RNA template was made up to a volume of 50μL. RT-PCR step was carried out in single tube using a programmable thermocycler (MJ Research, Massachusetts, USA) as follows.

<table>
<thead>
<tr>
<th>No. of Cycle</th>
<th>Steps</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>42°C</td>
<td>60 min</td>
</tr>
<tr>
<td>1</td>
<td>2</td>
<td>95°C</td>
<td>3 min</td>
</tr>
<tr>
<td>36</td>
<td>3</td>
<td>94°C</td>
<td>30 sec</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>54°C</td>
<td>45 sec</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>72°C</td>
<td>60 sec</td>
</tr>
<tr>
<td>1</td>
<td>6</td>
<td>72°C</td>
<td>5 min</td>
</tr>
<tr>
<td>1</td>
<td>7</td>
<td>4°C</td>
<td>Infinite</td>
</tr>
</tbody>
</table>

First round PCR was done using the forward primer (5'ACTGTCTTCACGCAGAAAGCGTCTAGCCAT3') and reverse primer (5'CGAGACCTCCCCGGGCACTCGCAAGCACC 3'). 10 μl of the first round PCR product was reamplified with internal primers forward primer (5'ACGCAGAAAGCGTCTAGCC-ATGGCGTTAGT 3') and the reverse primer (5'TCCCCGGGCACTCGCA-AGCACCTATCAGG 3') for another 35 cycles under same program. A negative control, a positive control and a water blank were maintained during reverse transcription and amplification to exclude false positive results in the PCR due to cross contamination. Size of the PCR products (expected size ~276bp) were analyzed on 2% agarose gels followed by staining with ethidium bromide and visualized under a UV illuminator (Fig. 2.1).

AGAROSE GEL ELECTROPHORESIS

Reagents:

1. Agarose
2. **TBE**
   
   5X – 54 g of Tris base
   27.5 g of boric acid
   20 ml of 0.5 M EDTA (pH 8.0)

3. **6X – Gel loading buffer**
   
   0.25% bromophenol blue
   0.25% xylene cyanol
   30% glycerol in H₂O

4. **Ethidium bromide (10mg/ml)**

**Equipment**

Agarose gel electrophoresis unit
Microwave oven
Power supply device with capacity 500 V/200 mA
UV transilluminator
Gel documentation system

**Method**

1. Agarose (2g) added and dissolved in 100ml of 1X TBE by boiling in microwave for 2-3 min. To this 5μl Ethidium Bromide (EtBr) is added and mixed gently. The agarose solution is poured into gel caster with comb that creates wells for loading the samples.

2. The polymerized gel is placed in horizontal, submerged electrophoresis unit. Sufficient electrophoresis buffer is poured into the electrophoresis tank until gel gets submerged.

3. Amplified DNA samples mixed with 1x loading dye and loaded gently into the slots of the submerged gel using a micropipette. Along with the DNA samples, a DNA ladder with known markers is loaded for reference.
4. After loading the samples, the tank is closed with the lid that contains electrodes which are connected to power supply unit. Electrophoresis is performed at 120V/100 m.amps for ½ hour.

5. After the run, the gel is removed from the unit and observed under UV transilluminator for the presence of amplified DNA. The amplified DNA bands are documented by UV gel doc system and saved. After documentation the gel is disposed in biohazard bag.
### Table 2.1

Range of separation of gels containing different amounts of standard agarose

<table>
<thead>
<tr>
<th>Agarose Concentration In gel (%W/V)</th>
<th>Range of separation of Linear DNA molecules (kb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.3</td>
<td>5-60</td>
</tr>
<tr>
<td>0.6</td>
<td>1-20</td>
</tr>
<tr>
<td>0.7</td>
<td>0.8-10</td>
</tr>
<tr>
<td>0.9</td>
<td>0.5-7</td>
</tr>
<tr>
<td>1.2</td>
<td>0.4-6</td>
</tr>
<tr>
<td>1.5</td>
<td>0.2-3</td>
</tr>
<tr>
<td>2.0</td>
<td>0.1-2</td>
</tr>
</tbody>
</table>
RESULTS

The population size in the present investigation is 4735 which include 394 (8.3%) individuals positive for HCV infection as detected by RT-PCR method (Fig2.1), but the third generation ELISA detected only 304 (6.4%) positive individuals. The prevalence of HCV among selected population groups is furnished in Table 2.2. Among the 704 patients who attended the gastroenterology out patient unit, 10 (1.4%) individuals were positive for HCV by both ELISA and RT-PCR methods. Out of 2036 voluntary blood donors screened, 49 (2.4%) were positive in ELISA, while 51 (2.5%) were positive by RT-PCR method. In the risk group category total number of patients screened were 1053, out of which 207 (19.6%) and 292 (27.7%) were positive by ELISA and RT-PCR methods respectively. Among 72 patients of lichen planus screened, 21 (40%) were found to be positive by ELISA and 23 (44%) were found to be positive by RT-PCR methods. A tribal population of 890 was screened for HCV infection considering age and sex specific prevalence. In this population 17 (1.9%) are positive by ELISA and 18 (2.02%) are positive by RT-PCR. The overall prevalence of HCV is higher in patients with lichen planus and risk groups compared to others. The screening results indicate that ELISA picked up in 304 (6.4%) cases, while 394 (8.3%) cases were detected by RT-PCR with significant difference since RT-PCR proved more sensitive than ELISA.

The prevalence of HCV among high risk group was detailed in Table2.3. The populations included in this category are persons more frequently exposed to contaminated blood and blood products, health care workers (Nursing staff, diagnostic staff and people who handle biohazard waste), Chronic renal failure patients on hemodialysis, thalassaemias and haemophiliacs, persons having tattoos and people practice religious procedures. The table depicts the total number of patients screened in each group and the result checked by both commercially available ELISA and in house RT-PCR method. Statistical analysis revealed a significant difference among chronic renal failure patients in whom the ELISA method picked 30% positive while RT-PCR picked 51.5% positive cases. By both the methods, HCV positive cases have been
1) Positive sample; 2) Positive sample; 3) Negative sample; 4) Positive sample; 5) 100 bp DNA marker (from bottom to top 100, 200, 300, 400, 500, 600, 700…… 1500bp); 6) Negative sample; 7) Positive control

Fig 2.1 Agarose gel electrophoresis showing 276 bp of 5' UTR PCR amplification of HCV RNA
Table 2.2

Prevalence of HCV in various categories of patients

<table>
<thead>
<tr>
<th>Category</th>
<th>Number</th>
<th>Screening by Anti HCV ELISA</th>
<th>Screening by RT-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients attending Gastroenterology camps</td>
<td>704</td>
<td>10 (1.4)</td>
<td>10 (1.4)</td>
</tr>
<tr>
<td>Voluntary Blood Donors</td>
<td>2036</td>
<td>49 (2.4)</td>
<td>51 (2.5)</td>
</tr>
<tr>
<td>Risk Groups*</td>
<td>1053</td>
<td>207 (19.6)</td>
<td>292 (27.7)</td>
</tr>
<tr>
<td>Patients with Lichen Planus</td>
<td>52</td>
<td>21 (40)</td>
<td>23 (44)</td>
</tr>
<tr>
<td>Lambada Tribe*</td>
<td>890</td>
<td>17 (1.9)</td>
<td>18 (2.02)</td>
</tr>
<tr>
<td>Total</td>
<td>4735</td>
<td>304 (6.4)</td>
<td>394 (8.3)</td>
</tr>
</tbody>
</table>

*For break up see table II

*For age and gender specific break up see table III

Number in parenthesis indicates percentage
detected up to 2.7% in health care workers, 5.5% in pilgrims and 2.8% in persons having
tattoos. It was observed that among thalassaemics and haemophiles RT-PCR was more
sensitive identifying 31% and 33% positive cases respectively in comparison to 29.6% and
31.4% patients respectively by ELISA.

Among Lambada tribal population, age and sex specific prevalence of HCV
infection was observed. The results of this screening were furnished in table 2.4, Fig 2.2.
Out of the 890 individuals (526 males and 324 females) screened, 17 (1.9%) are positive
by ELISA. They include 10 males and 7 females. By RT-PCR method, 18 (2.02%)
individuals are positive for HCV with 11 males and 7 females. All the subjects were
clinically asymptomatic.
Table 2.4
Age and sex specific prevalence of HCV in the Lambada tribe

<table>
<thead>
<tr>
<th>Age groups (years)</th>
<th>MEN</th>
<th>WOMEN</th>
<th>TOTAL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Anti HCV No. positive (%)</td>
<td>Anti HCV No. positive (%)</td>
<td>Anti HCV No. positive %</td>
</tr>
<tr>
<td>&gt; 10</td>
<td>0/16 (0.00%)</td>
<td>0/11 (0.00%)</td>
<td>0/27 (0.00%)</td>
</tr>
<tr>
<td>11-20</td>
<td>1/118 (0.84%)</td>
<td>1/87 (1.10%)</td>
<td>2/205 (0.91%)</td>
</tr>
<tr>
<td>21-30</td>
<td>2/110 (1.8%)</td>
<td>1/60 (1.6)</td>
<td>3/170 (1.76%)</td>
</tr>
<tr>
<td>31-40</td>
<td>3/97 (3.0%)</td>
<td>3/81 (3.7%)</td>
<td>6/178 (3.37%)</td>
</tr>
<tr>
<td>41-50</td>
<td>2/72 (2.10%)</td>
<td>1/55 (1.8%)</td>
<td>3/127 (2.3%)</td>
</tr>
<tr>
<td>51-60</td>
<td>1/66 (1.5%)</td>
<td>1/47 (2.1%)</td>
<td>2/113 (1.76%)</td>
</tr>
<tr>
<td>≥ 60</td>
<td>1/47 (2.00%)</td>
<td>0/23 (0.00 %)</td>
<td>1/70 (1.40%)</td>
</tr>
<tr>
<td>Total</td>
<td>10/526 (1.90%)</td>
<td>7/364 (1.92%)</td>
<td>17/890 (1.90%)</td>
</tr>
</tbody>
</table>
Fig 2.2

Anti HCV positively in different age groups of Lambada Tribes

Anti HCV positivity in different age groups
DISCUSSION

Hepatitis C virus infection is a global health problem including India where it has been estimated that more than 20 million people are already infected (Khaja et al., 2002). We report here the prevalence of HCV in the general population and in selected groups of patients. There are only a few community based studies of HCV prevalence worldwide, which has been reported mainly from the industrialized countries of Europe and America (Franc et al., 2000). There is a paucity of information on HCV prevalence in developing countries, especially in Asia and Pacific regions, where the largest segment of population resides (Debonine et al., 1999; Sun et al., 1999; Poovorawan et al., 2002). Therefore, the data in various population groups with respect to HCV infection, characterization of prevailing HCV genotypes, are likely to provide new insight in the causation of liver disease in India. In the present investigation, the overall prevalence of HCV infection in general population estimated as 1.4%. The general prevalence in India is 1.5 to 1.8% which is slightly more than that observed in our study. Another study published by Chowdhury et al. (2003) from eastern India shows a prevalence of 0.87%, which is less than that, observed in our study.

The over all prevalence of HCV infection in blood donors was observed as 2.4%. But the work published on North Indian population by Panigrahi et al. (1997) shows that a prevalence of 1.85% which is slightly less than that observed in our study. There is relatively low prevalence of 1% HCV infection in blood donors from developed countries like USA, UK, Europe, while in developing countries like Nigeria, Ukraine and in the Central African countries, as well as Egypt the prevalence ranging from 6 to 28% was observed. (Tanaka et al., 1992; Farrell et al., 1993; Delaporte et al., 1993; Kowo et al., 1995; Halim et al., 2000). This clearly shows the necessity for screening of HCV and avoiding of donors with risk factors to improve the awareness in general population.

Chronic renal failure (CRF) patients on maintenance by haemodialysis, multiple transfused hemophiliacs and thalassaemics are able to form a major risk group for HCV infection owing to the frequent use of blood, blood products apart from multiple invasive medical procedures to which these patients are exposed. It is now well documented that the prevalence of HCV in CRF patients averages between 10 – 40 % (Sato et al., 1994).
Several studies have shown that 10-30% of patients on haemodialysis are infected with HCV prior to kidney transplantation (Triolo et al., 1992; Umlanif et al., 1992) while around 10% of the uninfected patients become infected after transplantation due to contaminated blood or hospital equipment (Vuitton et al., 1996). However, data on the prevalence rates of HCV infection in Indian patients with respect to CRF is lacking. In the present study, out of 124 chronic renal failure patients, 78 (63%) were positive for HCV - RNA, whereas 48 (39%) were positive for ELISA. The low prevalence of anti-HCV antibodies in these patients may be attributed to the administration of immunosuppressive drugs or acute cases. Our study noticed a higher prevalence of HCV infection when compared to the other reported data. This situation demands screening of HCV among CRF's, thalassaeemics and haemophilics. Our study also clearly showed that among risk group patients, RT-PCR is a more sensitive and reliable method of diagnosis compared to ELISA. This observation was also made by David-Neto et al, (1997) and Madhavi, et al., (2004) who emphasize on the importance of RT-PCR for HCV screening.

The other risk groups studied include Pilgrims and persons having tattoos, a prevalence of 5.5% and 2.8% was observed in these 2 groups respectively. Tattoos have been involved in HCV transmission (Holsen et al., 1993; Sherman et al., 1995). In a Taiwanese study, 12.6% of 87 tattooed healthy young men without other risk factors were found to be anti-HCV-positive as compared with 2.4% of 126 matched control subjects. Tattooing has been linked to HCV infection in Australia (Kaldor et al., 1992). Not exceptionally, tattoos are surrogate indicators of unconfessed intravenous drug abuse. Health education and awareness to the people regarding these modes of HCV transmission may prove to be useful preventive intervention in the developing countries.

Transmission of HCV from infected patients to healthcare workers has been documented, and molecular evolutionary analysis has confirmed this mode of transmission (Seeff 1991; Suzuki et al., 1994). Hollow-bore needle-stick exposures are the main cause of HCV transmission from patient to health care workers (Thomas 2000). Prospective studies have shown that the average risk of infection after a needle-stick injury involving HCV-positive blood may be as high as 3% with a range between 0.013% and 10% in different studies (Puro et al., 1995; Grande et al., 2001; Sermoneta et al., 2001; Ross et al.,
2002). On the other hand, mucous membrane or skin contamination have not been associated with an increased risk of HCV infection. Prevalence among health-care workers in the present study is 2.7%, in UK it is between 0.3% and 0.7% (Thomas et al., 1993), 0.8% in Germany (Weber et al., 1995), 1.8% in Spain (Perez et al., 1992), 1.97-2% in Italy (Sulotto et al., 2002), and 2.7% in Hungary (Mihaly et al., 2001).

In the past few years, lichen planus has been linked to HCV infection, with studies demonstrating a higher prevalence of anti-HCV antibodies in patients with cutaneous and oral lichen planus, compared with general population (Bellman et al., 1995; Nagao et al., 1995; Sanchez-Perez et al., 1996; Imhof et al., 1997; Mignogna et al., 1998; Chuang et al., 1999). The reported rates of association are variable, probably because of differences in study design, and geography. A study in Indianapolis, USA reported this region has a low endemic prevalence of HCV, but a relatively high prevalence of (3.5%) anti HCV antibody in patients with lichen planus (Chuang et al., 1999). In Miami, USA 23% patients of lichen planus are positive for anti HCV antibodies. Similarly in Japan, patients with lichen planus positive for HCV antibody are very high i.e. 60% (Bellman et al., 1995; Nagao et al., 1995). These reports clearly indicate that HCV infection predisposes patients to the development of lichen planus. Some speculate that long-term infection may lead to an aberrant immunologic response (Sanchez-Perez et al., 1996). In our study the prevalence of HCV among lichen planus patients is 44% that suggest that it is appropriate to screen all patients with lichen planus for HCV infection.

An assessment of tribal population exposure to HCV infection becomes imperative on account of various customs they follow. These customs include rituals (e.g. scarification), traditional medicine (e.g. blood letting), tattooing, ear-or body – piercing, having single barber for the whole community etc (Tumminelli et al., 1995; Sun et al., 1996). These are all the potential sources through which infection can spread with in the community. Out of 18 subjects, one subject who was negative for ELISA had a history of renal transplant for chronic renal failure. This patient was under immunosupression therapy and probably failed to elicit a detectable antibody response against the viral infection. No significant gender difference was observed in the prevalence of HCV in males and females. The youngest positive subject was 19 years old and the oldest positive subject was 62 years
old. In the present study children up to 10 years are not exposed to HCV. The age related prevalence of HCV was relatively higher in the age group of 21-40 years. In rural India, the majority of primary healthcare providers in the villages do not have optimal infrastructure for sterilization. It may be worth mentioning at this stage that many of the under-qualified medical personnel in rural areas believe that the injection of medicines (IV or IM or SC) works faster and better than oral medication. Creating awareness among the populace regarding these modes of transmission of the virus may prove to be a useful to prevent HCV infection in rural and tribal areas. A prevalence of 5.5% HCV infection was reported in the Bagia tribe from central India, while the corresponding figure for other tribes from western India is 0.5% (Reddy et al., 1995; Arankalle et al., 1999). A relatively high prevalence of 7.89% was reported from the Lisu community of Arunachal Pradesh (north-eastern India) (Pukhan et al., 2001), whereas in our study the prevalence rate is 1.9%. The variation in the prevalence of HCV infection among the various tribes in India makes further studies in this direction imperative.

The population groups included for the present work are showing different frequencies of HCV infection, chronic renal failure (CRF) patients showing highest frequency and patients attending gastroenterology camp showing lowest frequency. The study is significant as it fills the lacunae of data from South India.