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RESEARCH ARTICLE

Epidemiology and Molecular characterization of Hepatitis C virus in Tamilnadu (India)during the year 2013

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Abstract

HCV infection is emerging as a serious public health problem in Tamil Nadu. Chronic hepatitis C virus infection leads to liver fibrosis, cirrhosis and hepatocellular carcinoma (HCC). The transmission of HCV is primarily through exposure to infected blood. In this study, patient's serum samples were screened for IgG antibodies by ELISA and RNA amplification during the period January to December 2013. HCV viral RNA was identified by semi nested RT-PCR using genotype specific PCR primers (using specific core region) for the determination of HCV genotypes. Of the 680 cases, 38 (5.58%) were positive for HCV IgG antibodies. Gender wise distribution revealed 59.6% were male and 40.4% were females. Among the IgG positives, 33 (86.8 %) were pediatric cases and 5(13.2 %) were adults. Six samples were detected with viral RNA among these were pediatric (83.3 %) and adult (16.7%). An age wise breakup revealed that maximum number of cases was in the age group of 6-12 years. Sequence analysis results showed 1(b) genomic subtypes as the most prevalent genotype amongst the pediatric age group in Chennai. In developing countries like India, surveillance is very important and utilizing well equipped and established laboratories for the diagnosis and management of HCV cases in emergency situations which may in turn reduce HCV related morbidity and mortality.

Introduction

Hepatitis C virus (HCV) causes acute and chronic human hepatitis infection, which is an important global health problem. In 1989, the HCV virus was first discovered in USA with more than three to four million people getting infected every year due to hepatitis. The World Health Organization has estimated that 3 percent of the 7 billion people worldwide are being chronically infected by hepatitis virus (Mavromara *et.al*., 2005). Since humans are the natural hosts of HCV, this can eventually lead to permanent liver damage and carcinoma.

HCV belongs to the *Flaviviridae* family and *Hepacivirus* genus. (Ishii *et.al*., 1999 and World Health Organization 1999). The size of the virus is about 50-60 nm and the virion contains a single-stranded positive RNA approximately 10,000 nucleotide base pairs in length and consisting of one ORF. The virus is encapsulated by an external lipid envelope and has an icosahedral capsid (Albertoni *et.al*., 2010). HCV is a heterogeneous virus, classified into 6 genotypes and it has more than 50 subtypes. Due to the high genome variability, nucleotide sequences of HCV genotypes varies by approximately 31-34%, whereas 20-23% variation among subtypes. Mixed virus of quasi species populations provide a survival advantage for the virus to create multiple variant genomes and a

high rate of generation of variants allow rapid selection of mutants. Infected blood and blood products like serum, plasma, sexual relationships with infected people and availability of injectable drugs had remarkable effects on HCV epidemiology. Thousands of people die each year from hepatitis and liver cancer caused by HCV virus infection. Approximately 80% of patients with acute hepatitis C infection progress into a chronic disease state leading to serious hepatic disorders, 10-20% of them develop chronic liver cirrhosis and hepatocellular carcinoma. HCV has an incubation period of 6-8 weeks. The infection is often asymptomatic so it is very hard to detect in the beginning stages. This makes it very difficult to treat them earlier. Because of this reason hepatitis C virus disease is called as "silent disease". Even though antibodies are produced against several HCV proteins during infection the virus mutates to escape from neutralizing antibodies produced in our body. Patients with chronic hepatitis C sometimes have symptoms such as fatigue, muscle aches, nausea and pain. Chronic HCV infection also presents with autoimmune and immune complex-mediated diseases (Suzuki *et al*., 2005).

Phylogenetic analysis of full-length or partial sequences of HCV strains isolated in various regions of the world has led to the identification of six HCV genotypes (Mavromara *et al*., 2005) and a large number of subtypes (Simmonds P.,2005;Cantaloube J.F and Attoui H.,2005). HCV genotyping is an essential tool for epidemiological studies (Mora *et al.,* 2010; Pawlotsky *et al.,* 2003). There is a wide geographical variation in genotype distribution. Genotypes I, II and III are more frequent in countries like North America, Europe and Japan. Genotype 4 is found in Egypt, Central Africa and the Middle East countries whereas genotype 5 is found in South Africa and genotype 6 is a seen south east Asian countries. These genotypes differ by 31–34% in their nucleotide sequence and by around 30% in their amino acid sequence. Analysis of sequences to by HCV genotyping can be used for predicting response to anti-viral therapy (Guindon S and Gascuel O A., 2003).

The first generation of anti-HCV test widely used c100-3 epitopes from the non structural NS4 region. Third-generation enzyme immunoassays (EIAs) with 99% specificity including core, NS3, NS4, and NS5 regions of the HCV genome can be used to detect antibodies in plasma or serum against various HCV epitopes and the optical density (OD) ratio of the reaction showed the amount of antibodies in the serum or plasma samples (Alhusain *et al*.,2009;Najib U Khan *et al*.,2011).

The HCV RNA in the plasma in active infection can be detected following post exposure of one to three weeks. Qualitative assays are based on target amplification using either polymerase chain reaction (PCR), real-time PCR or transcription mediated amplification known as TMA. In polymerase chain reaction, first RNA extraction was done then the cDNA synthesised and double-stranded DNA copies of HCV genome were synthesized, whereas single stranded RNA copies are generated in TMA. In "real-time" PCR, the number of fluorescent signals per cycle is proportional to the amount of HCV RNA in the sample (Ohno *et al.*,1997;Harrison .,1999).

2. Experimental Section

2.1 Study Population:

Blood samples were collected from 680 patients suspected to be suffering from HCV infection referred to King Institute of Preventive Medicine and Research, Chennai, from different Government hospitals of Chennai during the period from January to December 2013.The study subjects were high risk individuals which includes multiple blood transfusion recipients, haemodialysis patients and with other miscellaneous conditions. The patients were not on antiviral therapy at the time of sample collection. The patient history was recorded in the lab request form for future references.

2.1.1 Sample Processing:

The serum and plasma samples were collected from the subjects. The serum samples were stored at -20°C and plasma samples were kept at -80°C at King Institute of Preventive Medicine and Research, Chennai. The serum samples were subjected to serological test and plasma samples were used for molecular analysis.

2.1.2 Serological investigation by ELISA

The samples were screened by ELISA for the presence of anti HCV IgG antibodies as per manufacturer's protocol (Erba Lisa ®Hepatitis C kit detection). Based on the OD values positive and negative values were calculated.

2.1.3 Molecular characterization of HCV

2.2 RNA Extraction

The HCV total viral RNA was extracted using commercially available viral RNA extraction kit QIAGEN GmBH, Hilden, Germany, out from the plasma samples .

2.2.1 cDNA synthesis

The isolated RNA processed with Random hexamers which were more efficient and primer specific to the 3'-UTR and reverse transcriptases. This was carried out using Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT; Promega) Reactions were assembled as per manufacturer's instructions employing a constant amount of HCV RNA (15 µl for a 50 µl reaction) due to high percentage of GC content in HCV. The incubation temperature 35°C -50°C were used for the conversion of cDNA synthesis

2.3 Nested PCR assay:

The cDNA of HCV was carried out for nested PCR using two sets of primers specific to Pre core and core protein gene viral regions described by Tanaka *et al*.,1996. Cycling conditions of first-round PCR consisted of 35 cycles of denaturation at 94° C for 60 sec, annealing tem at 53° C for 60 sec, and extension was at 72° C for 60 sec. The second PCR test was performed with 40 cycles , denaturation at 94° C for 45 sec, annealing at 53° C for 45 sec, and extension at 72° C for 45 sec. Carry over contamination was prevented as described by Kwok & Higuchi method. The final amplification product was mixed with bromophenol blue, loaded in gel electrophoresis using 2% agarose gel.

Sequencing of HCV

Genotyping was carried out by nested PCR of core region and the amplicons were further separately packed and submitted for the sequence analysis. The acquired sequences were submitted to GenBank and phylogenetic tree constructed using Guindon *et al*., 2003, method. The genbank ID for the submitted sequences are included in this study (seq1 KJ596532 to seq6 KJ596537). These sequences showed more than 80% similarity with the existing HCV 1b subtype genome.

3. Results and Discussion

Hepatitis C virus is a common etiological agent causing chronic liver disease. Elisa was carried out for 680 serum samples out of which 38 samples were positive for IgG antibodies. Age wise and gender wise positivity distribution was plotted based on the Elisa results (Figure1-4). (Table 1).The results showed that more than 78% cases had history of blood transfusion and 10% of cases had undergone haemodialysis. RNA was extracted from the plasma samples and subjected to nested PCR for the amplification of viral genome [Table 2]. The PCR products were loaded and bands were separated by electrophoresis based on the molecular weight (Fig 4). Among them, 6 samples showed clear band at the range of 343bp and this was confirmed with the standard strain. The PCR positive samples were further amplified for purity and then the amplicons were sent for the sequencing along with the primers. The BLAST results confirmed that the sequence were 1b subtype genome of HCV which is most prevalent in India. Another study was conducted by Raghuraman *et al*., in 2003 which demonstrated the preponderance of genotype 1 among South Indian population. These sequences were further compared for the similarity using clustalW. The results showed (Figure 5) that the identified sequences were similar to that of Delhi, Andhrapradesh and Vellore sequences. They showed more than 80% similarity to other HCV sequences.

3.1 Phylogenetic Analysis.

HCV 1b amplicon sequences of precore and core regions from KIPMR isolates were compared with and similar sequences were retrieved from online database - BLAST (Basic Local Alignment Search Tool) program of National center for Biotechnology information (http://www.ncbi.nlm.nih.gov/). Phylogenetic analyses was conducted using MEGA6 . The evolutionary relationship was analysed using the Neighbor-Joining method (Mavromara.,2005). We had selected 500 replicates for boot strap analysis . Maximum Composite Likelihood method was used for evolutionary distances analysis (Mavromara *et.al*., 2005) and are in the units of the number of base substitutions per site. Codon positions included were 1^{st}, + 2^{nd} +,+ 3^{rd} and non coding regions. The tree scales, length, evolutionary distances were drawn with same units to infer the phylogenetic tree (Fig 5).

The phylogenetic tree showed positive evolutionary relationship of the core protein of Chennai strains with strains from various parts of the world, submitted during different years. The three Chennai strains (gi643193047, gi643193043 and gi643193051) were grouped with strains from Spain and France which occurred in the year of 2007 and the Chennai strain (gi643193049) was grouped with Belarus (2013). The other Chennai strains (gi643193045 and gi643193053) were grouped with Bangladesh (2012) and Indonesia (2010). In this tree, the Hepatitis C virus/2014/gi37977 strain presented as an outgroup. These groups were formed based on the phylogenetic relationship. The list of different country sequence and GenBank Number used for construction of Phylogenetic tree were mentioned in Table 3.

HCV had been identified as a causative agent in most cases of post transfusion hepatitis in Chennai. In our study, a total of 680 cases were reported during the year January 2013 to December 2013, of which the 38 (5.58%) were positive for IgG antibodies. The study included symptomatic patients, who were high risk individuals mainly multiple blood transfusion recipients (leukemia, lymphoma, Thalassemia Major & Pancytopenia patients) and chronic kidney disease patients who underwent periodical haemodialysis. There was a high incidence of positivity among the pediatric population, who were Leukemia /Lymphoma patients (78.94%). Hepatitis C being a blood borne infection had maximum prevalence percentage among post transfusion recipients. In studies by Suliman Qadir Afridi *et a1* in 2014 reported that (Suliman *et al*.,2014) 4.7% of HCV prevalence has been reported in Pakistan which was similar to that in our population.

In most of the studies conducted so far, either the numbers of samples reported are too small to draw any solid conclusion or the methodological differences have made it impossible to conduct a formal Meta analysis to determine accurate prevalence estimates. Among all the published reports, 99% of the data originated from erroneous non-PCR qualitative screening methods such as Elisa, based on the detection of anti-HCV IgG antibodies. According to study of Mavromara et al., 2005, 875 samples were screened for HCV-specific antibodies, where only 2 samples showed equivocal results(Albertoni *et al*., 2010).

Gender wise distribution revealed 405(59.6%) were males and 275 (40.4%) was females. Among the IgG positives, 33 (86.8 %) were pediatric cases and 5(13.2%) were adults. The samples were collected among the age group range of 0-81 years. An age wise breakup revealed that maximum number of cases (4.12%) was in the age group of 6 to 12 years. According to Najib *et al*., 2011, blood donors from various districts of the KPK province and the federally administered tribal area of Pakistan were tested for anti-HCV antibodies by ELISA and RT-PCR. Out of the 224 (3.13%) blood donors, 135 (1.89%) were positives by ELISA out of this 118 (1.65%) blood donors had active HCV infection which was detected by RT-PCR. This study suggests that ELISA should be used for anti-HCV diagnosis in public sector hospitals and health care units(Najib *et al*.,2011).

The symptomatic patients had fever (100%), myalgia (100%), hepatomegaly (94.7%), splenomegaly (73.6%), abdominal pain (42.1%), abdominal distension (36.8%), vomiting (31.8%), jaundice (68.4%) and ascites (28.9%). Icteric symptoms such as yellow discolouration of skin and sclera as well as high colored urine were observed in 34.2% of the population. There was a significant rise in the bilirubin content among the infectious individuals (94.7%). Elevated levels of liver enzymes like ALT were observed in 97.4% of patients. One of the patients (2.6%) had co-existence of HbsAg and anti HCV.

In the present study, reverse transcription-PCR in HCV genotyping system, RNA was extracted from plasma using HELINI™ Pure fast Viral Nucleic acid Mini spin prep kit. The samples were subjected to RT PCR using group specific primers and 6 samples were detected with viral RNA. Among these, 83.3% were in pediatric group and 16.7% in adults. According to experiments performed by Ohno O *et al*., 1997,new genotyping system based on PCR of the core region with genotype-specific PCR primers for the determination of HCV genotypes 1 to 6 was developed. The outer primers and the second round PCR primers for sequencing were designed on the basis of the conserved nature of these sequences. Sc2 and Ac2 were the sense and antisense primers for the core region, respectively. S7 and A5 were the sense and antisense primers, respectively, for the inner region. Harrison *et al*., 1999 (Tanaka *et al*.,1996), reported that HCV infection acquired at birth or in early childhood usually progress slowly, but these infections are not always mild.

In this study, by using in house primer [Sc2, Ac2 (first round PCR) and S7, A5 (second round PCR)] 6 samples were confirmed positive samples and were subjected for sequencing (seq1 KJ596532 to seq6 KJ596537). Out of which 86.8% were pediatric cases while 13.2% were adults. According to sequence analysis report, 1(a) and 1(b) genomic subtypes were the most prevalent genotype amongst the age group 6-12 from 2013-2014 in Chennai. The reports of Jean-Michel Pawlotsky *et al*., 1998 suggested that there is a linkage between interferon resistance and genotype 1b.The significance of genotyping the specific gene segment of genoype1b , which is commonly associated with interferon resistance could throw insights in identifying newer drug regimen and modifying the drug regimen..

Tables

Table 1: Percentage of HCV IgG antibodies distribution in different groups.

Blood transfusion recipients	Haemodialysis patients	Miscellaneous group	Unknown etiology
30(78.94%)	**4(10.52%)**	**2(5.26%)**	**3(7.38%)**

Table 2: Oligonucleotide primers used for the first and second round of Nested PCR for HCV detection

Table: 2 Oligonucleotide primers used for nested- PCR

Primer	PCR round	Sequence (59–39)	Nucleotide position
Sc2	1F	GGGAGGTCTCGTAGACCGTGCACCATG	3 to24
Ac2	1R	GAG(AC)GG(GT)AT(AG)TACCCCATGAG(AG)TCGGC	417-391
S7	2F	AGACCGTGCACCATGAGCAC	212–8
A5	2R	TACGCCGGGGGTCA(TG)T(GA)GGGCCCCA	343–319

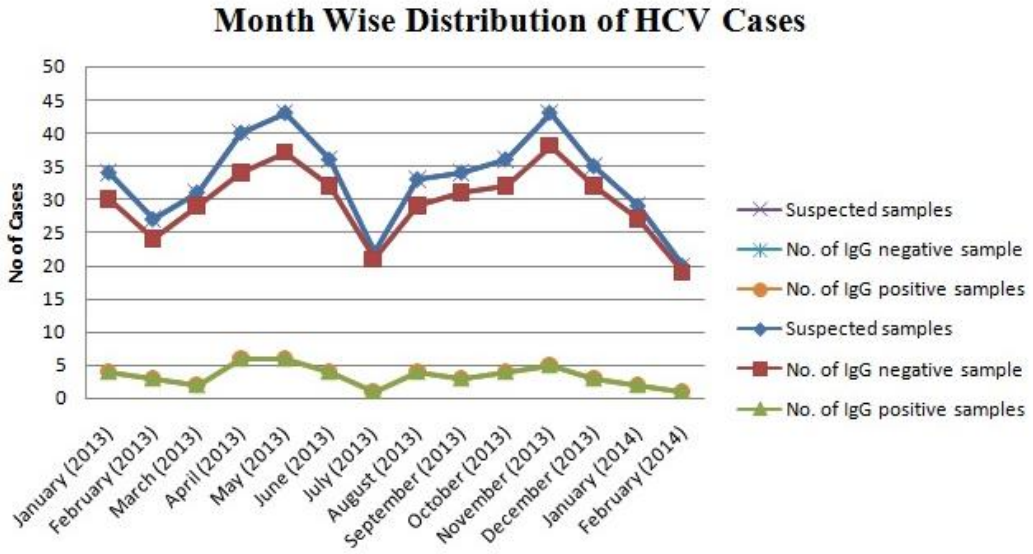

Fig 1

The distribution of samples shows that of a total of 463 suspected cases, 415 were IgG negative and 48 were IgG positive

Fig1: The month wise distribution of total samples of suspected cases and IgG positive/negative cases.

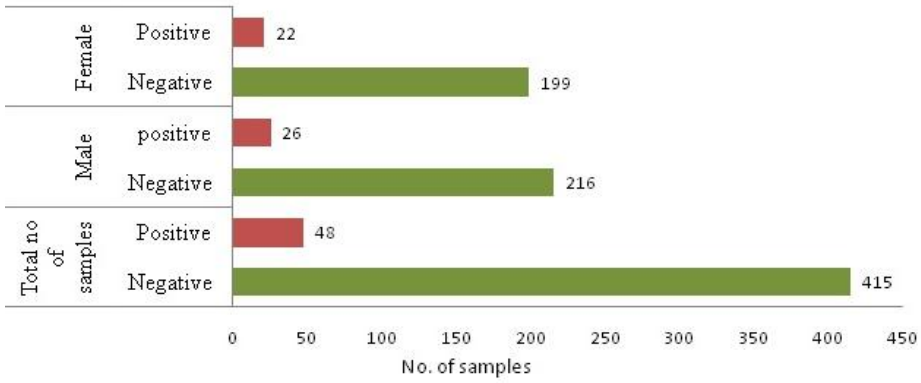

FIG 2-GENDER WISE DISTRIBUTION OF IgG POSITIVE

The gender wise distribution depicts that among the 463 samples, 242 samples were male (216- IgG negative, 26- IgG positive) and 221samples were female (199- IgG negative, 22- IgG positive).

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Fig2: Gender wise distribution of HCV samples. Total number of male and female positive / negative distribution of the suspected cases.

Fig 3: Age wise distribution of HCV cases. Suspected cases were grouped based on different age groups.

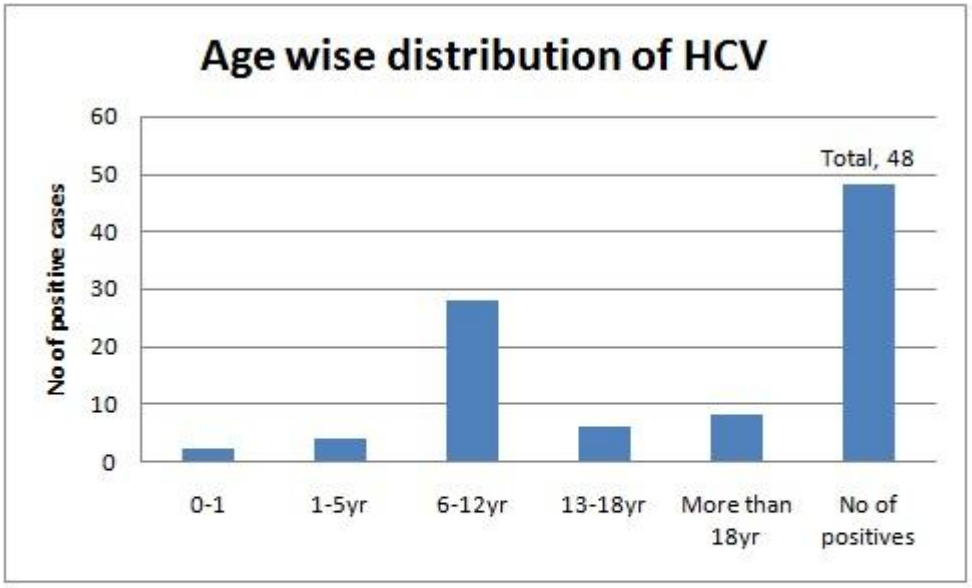

FIG 3 –AGE WISE DISTRIBUTION OF HCV POSITIVES

An age wise breakup revealed that maximum number of cases 28 (62.5%) was in the age group of 6-12.

Fig4: Distribution of positive percentage of HCV IgG cases in pediatrics and adults.

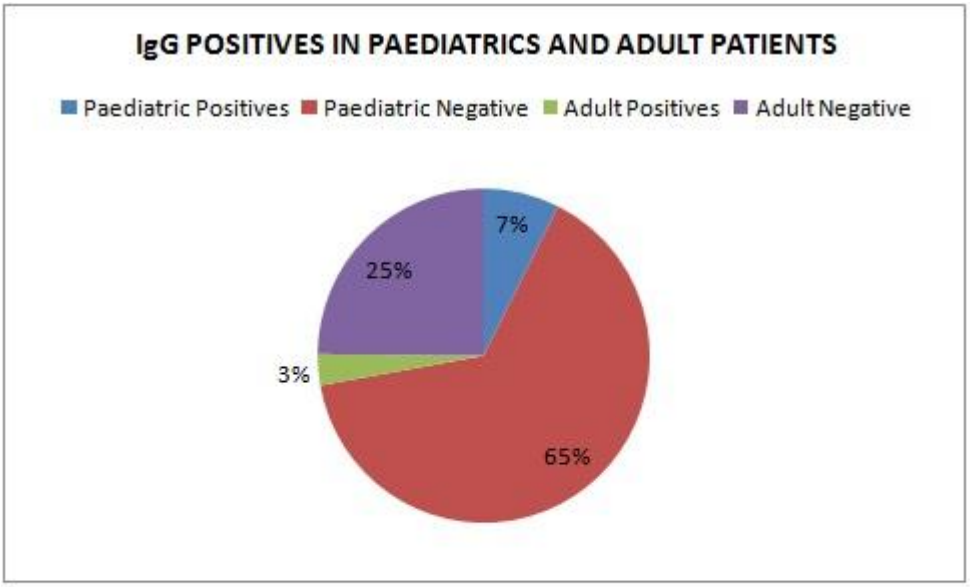

Fig 4- IgG POSITIVES IN PAEDIATRICS AND ADULT PATIENTS

The above representation shows that among the 48 IgG positives, 14 (29.1%) were adults and 34(70.8%) were paediatric cases.

Fig-5 : HCV Nested PCR of Positive Samples

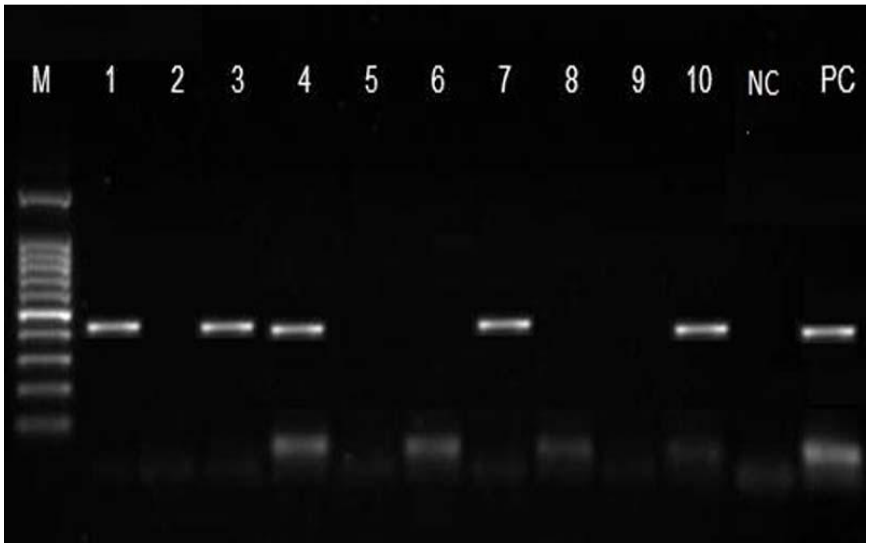

Fig5: HCV RNA amplification by nested PCR showed positives band at 343 base pairs in agarosegel electrophoresis.

Fig6: Sequences were compared for the similarity using ClustalW. Phylogetic tree showed the more related sequences of various groups with new sequences.

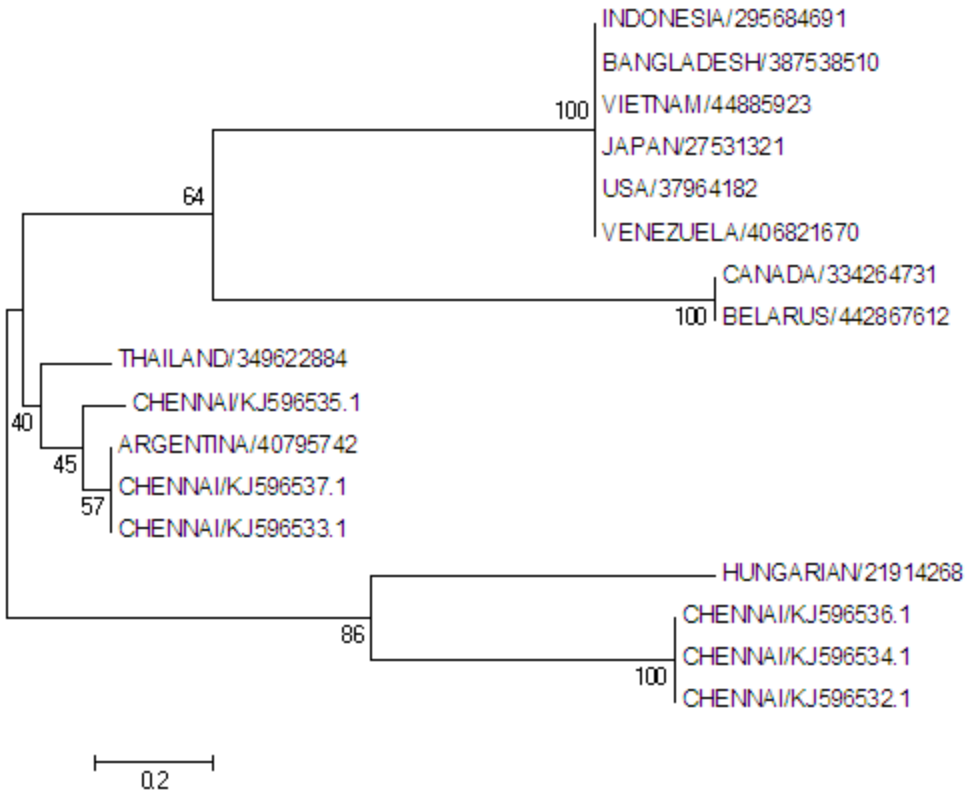

Fig6: Phylogetic tree for HCV

4. Conclusions

The sequence analysis of HCV showed genotype 1b as the predominant subtype circulating in Chennai. As persistent hepatitis C virus infection can progress to liver cirrhosis and hepatocellular carcinoma and since interferon resistance among subtype I and II are common, the predominance of this serotype among the population indicates difficulties to be faced by the clinicians in treating HCV positives in future.

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Conflicts of Interest

The authors declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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